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Impaired TCR-Mediated Apoptosis and Bcl-XL Expression in T Cells Lacking the Stress Kinase Activator SEK1/MKK4

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The dual specificity kinase SEK1 (MKK4) is a direct activator of stress-activated protein kinases (SAPK/JNK) in response to environmental stresses or mitogenic factors. We show in Sek1−/−Rag2−/− chimeric mice that a Sek1 null mutation augments the susceptibility of peripheral T cells to TCR/CD3 religation-induced apoptosis. Sek1−/− T cells failed to induce expression of the death suppressor Bcl- XL in response to Ag receptor activation. The Sek1 mutation did not alter the induction of apoptosis in response to etoposide, cisplatinum, Adriamycin, and γ-irradiation. Moreover, we show that CD3ε activation alone leads to SEK1 activation in Sek1+/+ T cells. These results suggest that SEK1 transduces cellular survival signals during T cell stimulation. The Journal of Immunology, 1998, 161: 3416–3420.

Materials and Methods

Mice

The generation of ES cells homozygous for the Sek1 mutation and Sek1+/− and Sek1−/− somatic chimeras using Rag2−/− blastocyst complementation has been described (16). T cells from Sek1+/−Rag2−/− mice were tested for the Sek1 mutation using PCR (12). If not otherwise stated, all mice used for experiments were between 6 and 10 wk of age. Mice were kept in accordance with guidelines of Canadian Medical Research Council.

TCR religation on primary activated T cells

Splenic T cells (4 × 10⁷) were cultured in 24-well Costar plates (Fisher Scientific) in 2 ml αMEM medium (supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 15 mM HEPES, 10−⁵ M β-mercaptoethanol, and 10% FCS) for 48 h at 37°C. Cells were activated with soluble anti-CD3ε plus exogenous murine rIL-2 (clone 145-2C11; 3 μg/ml) and exogenous murine rIL-2 (25 U/ml; Genzyme, Cambridge, MA) (17). After 48 h, activated T cells were harvested using Lympholyte-M and washed, and the remaining B cells were removed using anti-IgG magnetic beads (10:1 bead:cell ratio; Cedarlane Laboratories, Hornby, Ontario, Canada). Purified activated T cells (0.5 × 10⁷/ml) were cultured for 4 h in αMEM containing 50 U/ml of murine rIL-2 (Genzyme), and repleted in 96-well plates containing 3 μg/ml plate-bound anti-CD3ε (clone 145-2C11) or plate-bound anti-TCRβ (clone H57.597) and rIL-2 (50 U/ml) in the presence or absence of anti-CD28 (1 μg/ml; clone 37.51, hamster IgG). Cell viability and apoptosis were assessed 48 h after religation using eosin staining to determine total live and dead cell numbers, the chromogende 7-amino-actinomycin D (7AAD) (1 μg/ml; 4°C, 15 min) (18), and propidium iodide (PI) staining (50 μg/ml PI in 0.1% sodium citrate plus 0.1% Triton X-100; overnight incubation at 4°C) (19), as described. 7AAD and PI staining were analyzed by flow cytometry using a FACScalibur (Becton Dickinson, Mountain View, CA).

Apoptosis in response to environmental stress

For induction of apoptosis by environmental stresses, splenic T cells were activated with soluble anti-CD3ε (3 μg/ml; clone 145-2C11) plus murine rIL-2 (25 U/ml) in Iscove’s modified Dulbecco’s medium (IMDM) (10% FCS, 10−⁵ M β-mercaptoethanol), as above. Activated cells were harvested using Lympholyte-M. Triplicate samples of activated splenic T cells (1 × 10⁸) were cultured in 24-well plates (IMDM; 10% FCS, 10−⁵ M β-mercaptoethanol; 2 ml final volume) and activated for 16, 24, or 48 h using the following stimuli: 1) γ-irradiation (100 or 300 rad); 2) Adriamycin (0.1 or 0.2 μg/ml); 3) cisplatinum (1 or 5 μg/ml); 4) etoposide (2.5 μM); and 5) heat shock (39°C, 41°C, and 43°C for 1 h). T cells were then

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harvested, and triple stained with anti-CD4 (phycoerythrin), anti-CD8 (FITC), and 7AAD. Cell viability was determined by flow cytometry. Optimal time courses, concentrations, and dosage regimens for induction of cell death (LD50) were determined in pilot experiments.

**Cytometry**

To control for activation and to test for the expression of cell surface and activation markers, splenic T cells were stimulated as above (anti-CD3ε (3 μg/ml; clone 145-2C11) plus murine rIL-2 (25 U/ml) in IMDM medium (10% FCS, 10% 2B2 medium) plus murine rIL-2 (25 U/ml)). Activated cells were harvested; incubated with anti-CTLA4, anti-CD69, anti-IL-2Rα/CD25, anti-CD4, anti-CD8, anti-CD44, anti-ICAM-1, anti-TCRαβ, anti-CD3ε, or anti-CD95 Abs (all from PharMingen, San Diego, CA); and analyzed for the expression of cell surface markers using a FACScalibur (Becton Dickinson).

**Western blotting and SAPK phosphorylation assay**

Purified lymph node T cells (2 × 10⁶) were activated with anti-CD3ε (10 μg/ml) and/or anti-CD28 (1 μg/ml), as described (17, 20, 21). Cells were lysed in ice-cold lysis buffer (10 mM NaCl, 20 mM PIPES, pH 7, 0.5% Nonidet P-40, 5 mM EDTA, 0.05 mM β-mercaptoethanol, 100 μM NaVO₄, 50 mM NaF, 20 μg/ml leupeptin, and 1 mM benzamidine). Cleared lysates were adjusted to equal protein concentrations (Bio-Rad (Richmond, CA) protein assay). Proteins were separated by SDS-PAGE and probed for actin, Bcl-2, and Bcl-XL (both from PharMingen) Abs. Activated SAPKs and SEK1 were detected using phospho-SAPK- and phospho-SEK1-specific Abs indicative of activation (New England Biolabs, Beverly, MA).

**Results and Discussion**

**Enhanced TCR/CD3ε religation-induced apoptosis of Sek1⁻/⁻ T cells**

We have reported previously that Sek1⁻/⁻/Rag⁻/⁻ chimeric mice have normal numbers and ratios of CD4⁺ and CD8⁺ T cells in lymph nodes and spleen (16). However, the thymi of Sek1⁻/⁻ chimeric mice were 4 to 5 times smaller than those of age-matched 129/J mice or Sek1⁺/⁺ chimeras due to a decrease in the population of double-positive thymocytes. A key result was that Sek1⁻/⁻ thymocytes were more susceptible to apoptosis in response to the physiologic stimuli CD3/TCR and CD95 (16).

Religation of TCR/CD3 molecules on activated T cells leads to the induction of apoptosis in these lymphocytes (17, 22, 23). TCR/CD3 religation-mediated cell death depends on a functional TCR/CD3 signal and can be inhibited by CD28 costimulation through a physiologic stimuli CD3/TCR and CD95 (16).

**FIGURE 1.** Enhanced CD3ε-mediated apoptosis in Sek1⁻/⁻ T cells. A, CD3ε religation-induced apoptosis. For CD3ε religation-induced cell death, splenic T cells from one Sek1⁺/⁺ and two different Sek1⁻/⁻ chimeric mice were activated for 2 days with anti-CD3ε (3 μg/ml) plus rIL-2 (25 U/ml), and subsequently religated for 48 h with plate-bound anti-CD3ε. It should be noted that exogenous rIL-2 was added to all activation and religation cultures to exclude a potential effect of IL-2 in these assays (12). Percentages of viable cells were determined in triplicate (±SD) by eosin staining of viable cells and 7AAD staining. The differences in cell viability of Sek1⁻/⁻ vs Sek1⁺/⁺ T cells were statistically significant (p < 0.01; Student’s t test). Background apoptosis of nonreligated T cells was comparable among Sek1⁺/⁺ and Sek1⁻/⁻ T cells (<1%). One result representative of three experiments is shown. B, PI profile for anti-CD3ε religation-induced apoptosis. The subdiploid (<G₁) peak represents apoptotic cells. Different cell cycle phases (G₁, S, G₂/M) are indicated. Controls were T cells cultured in the absence of CD3ε religation. One result representative of three experiments is shown.

TCR/CD3-mediated apoptosis of Sek1⁻/⁻ T cells might have a physiologic role following in vivo challenge with other viruses and/or bacteria.
controls were T cells cultured in the absence of CD3
e (25 U/ml), as described in Figure 1. After 24 h of CD3
Sek1 SAPK/JNK in Sek1
B
). Actin is shown to control for protein levels on the gel. The prolif-
lower panel
middle panel
upper panel
expression of Bcl-XL (3
m
). Total cell lysates were Western blotted for ex-
rIL-2 (3
m
), T cells were harvested and lysed, as described in
right panels
left panels
FIGURE 2. Defect in CD3
3418 SEK1 IN T CELL APOPTOSIS
C
A
B
Impaired CD3-mediated Bcl-XL up-regulation in Sek1
T cells
Expression of Bcl-XL is the principal mechanism that protects peripheral T cells from apoptosis (21, 24). Moreover, it has been shown that Bcl-XL, but not Bcl-2, can rescue T cells from religation-mediated cell death (20). Thus, we analyzed Bcl-XL and Bcl-2 expression in T cells following CD3e activation (Fig. 2A). Interestingly, whereas CD3e stimulation triggered Bcl-XL but not Bcl-2 expression in activated Sek1
T cells, Sek1
T lymphocytes failed to up-regulate Bcl-XL expression.
SEK1 is a direct activator of SAPKs/JNKs in response to CD28 costimulation (11, 12). Moreover, CD28 costimulation can rescue T lymphocytes from TCR/CD3e religation-induced apoptosis via potent Bcl-XL induction (20). Interestingly, in both Sek1
and Sek1
chimeric mice, CD28 costimulation led to comparable induction of Bcl-XL (Fig. 2A) and rescued T cells from TCR/CD3e religation-induced apoptosis (Fig. 3). These results indicate that CD28-mediated survival signals are still operational in Sek1
T cells. Intriguingly, our results indicate that SEK1 links TCR/CD3 signaling, but not CD28 costimulation to the induction of Bcl-XL expression. The biochemical branch points for these differences are not known. Surprisingly, whereas CD3e plus CD28 stimulation, but not CD3e stimulation alone, induces activation of SAPK/JNK in Sek1
lymph node T cells (Fig. 2B), CD3e plus CD28 stimulation and CD3e stimulation alone can induce SEK1 activation (Fig. 2C).
Since TCR/CD3 activation of Sek1
T cells leads to SEK1 activation, but TCR/CD3 activation of Sek1
and Sek1
thymocytes and peripheral T cells does not induce SAPK/JNK activity (data not shown and Ref. 11), these results suggest that SEK1-mediated survival signals might be independent of SAPK/JNK activation. Thus, SEK1 might function in a novel and distinct

FIGURE 2. Defect in CD3e-mediated Bcl-XL expression in Sek1
peripheral T cells. A, T cells were isolated from the spleens of Sek1
and Sek1
chimeric mice and activated with anti-CD3e (3 µg/ml) and rIL-2 (25 U/ml), as described in Figure 1. After 24 h of CD3e religation (plus 50 U/ml rIL-2) (left panels) or anti-CD3e plus CD28 religation (plus 50 U/ml rIL-2) (right panels), T cells were harvested and lysed, as described in Materials and Methods. Total cell lysates were Western blotted for expression of Bcl-XL (upper panel), Bcl-2 (middle panel), and actin (lower panel). Actin is shown to control for protein levels on the gel. The proliferative responses of Sek1
and Sek1
T cells were similar under the indicated stimulation regimen following to CD3e cross-linking (12). Controls were T cells cultured in the absence of CD3e religation. One result representative of three experiments is shown. B, Normal induction of SAPK/JNK in Sek1
T cells. Sek1
and Sek1
lymph node T cells were not activated (control) or activated with soluble anti-CD3e (10 µg/ml) plus soluble anti-CD28 (1 µg/ml) Abs for 5 min (37°C) (anti-CD3/CD28). Cells were lysed, and proteins were transferred on a membrane and probed for phospho-SAPK indicative of activated SAPK/JNK and total SAPK/JNK protein by Western blotting. Similar results were obtained in kinase assays using GST-c-Jun as an in vitro substrate (not shown). It should be noted that we failed to detect SAPK/JNK activation (<2 times above background) in Sek1
and Sek1
T cells in response to CD3e alone (not shown and Ref. 11). C, Induction of SEK1 following CD3e stimulation. Sek1
lymph node T cells were activated with soluble anti-CD3e (10 µg/ml) Ab alone (CD3e) or anti-CD3e (10 µg/ml) plus soluble anti-CD28 (1 µg/ml) Abs (CD3e/CD28) for 0, 5, and 20 min (37°C). Cells were lysed, and proteins were transferred on a membrane and probed for phospho-SEK1 indicative of activated SEK1 and total SEK1 protein (45 kDa) by Western blotting.

FIGURE 3. CD28-mediated survival signals are still operational in Sek1
T cells. Splenic T cells from Sek1
and Sek1
chimeric mice were activated for 2 days with anti-CD3e (3 µg/ml) plus rIL-2 (25 U/ml), and subsequently untreated for 48 h (control), religated for 48 h with plate-bound anti-CD3e alone (CD3e), or religated for 48 h with plate-bound anti-CD3e plus anti-CD28 (CD3e + CD28), as described in Materials and Methods. Viable cells were determined in triplicate (±SD) by eosin and 7AAD staining, and percentages of viable cells were calculated relative to control values (100%). Background apoptosis of control nonreligated T cells was comparable among Sek1
and Sek1
T cells (<10%). Note enhanced apoptosis of Sek1
T cells following CD3e religation alone. Differences in cell viability of CD3e-religated Sek1
vs Sek1
T cells were statistically significant (p < 0.01; Student’s t test). One result representative of three experiments is shown.
signaling pathway. Alternatively, we cannot exclude the possibility that the Sek1 mutation effects thymocyte maturation and that the population of peripheral T cells in these chimeric mice may be biologically different from peripheral T cells in normal mice. In addition to impaired Bcl-XL expression, alternate possibilities, including down-regulation of Bax or Bad, failure to activate Nur77, underexpression of caspases, or overexpression of IAPs, might account for impaired apoptosis in Sek1/MKK4-deficient T cells. Nevertheless, our results clearly indicate that Sek1 expression is important for Bcl-XL expression and to provide survival signals in CD3-activated peripheral T cells.

**T cell death in response to metabolic poisons**

It has been proposed from transfection studies with dominant negative signaling mutants that the Sek1->SAPK/JNK signaling cascade may be a common intracellular pathway required for the induction of apoptosis in response to many types of cellular stresses (7–10). However, we could not observe any significant differences between freshly isolated (not shown) or activated (rIL-2 and anti-CD3e) Sek1⁺/⁺ and Sek1⁻/⁻ peripheral T cells in either the extent or kinetics of cell death in response to cisplatinum, Adriamycin, γ-irradiation, and etoposide (Fig. 4), heat shock, or UV irradiation (not shown). It should be noted that heat shock and UV irradiation do not induce SAPK/JNK activity in Sek1⁺/⁺ cells, whereas all other indicated poisons can still activate SAPK/JNK presumably via activation of SEK2/MKK7 (16, 25, 26). Thus, our results do not support the hypothesis that SEK1-mediated activation of SAPKs/JNKs is required for the induction of T cell death in response to UV irradiation and heat shock. These results imply that enhanced TCR/CD3-mediated apoptosis of Sek1⁻/⁻ T cells is not due to increased susceptibility to cell death per se, but appears to be specific for TCR/CD3-triggered death signals. SEK1-independent signaling cascades are operative during the induction of apoptosis in response to metabolic poisons.

**Conclusion**

We report in Sek1⁻/⁻/RAG⁻/⁻ chimeric mice that a null mutation of the dual specificity stress kinase SEK1/MKK4 facilitates TCR/CD3e religation-induced cell death of activated peripheral T cells. Sek1⁻/⁻ T cells failed to up-regulate the death suppressor Bcl-XL following CD3e activation. However, CD28 costimulation induced normal Bcl-XL expression in Sek1⁻/⁻ T cells and rescued activated Sek1⁻/⁻ T cells from TCR/CD3e religation-mediated apoptosis. Sek1⁻/⁻ T cells displayed normal susceptibility to apoptosis in response to various environmental stresses. We also report that TCR/CD3 activation alone induces SEK1 phosphorylation. Our data in Sek1 gene-targeted T cells indicate that SEK1 mediates survival signals required by T cells to resist premature apoptosis during activation. These results provide a mechanistic explanation for enhanced cell death of Sek1⁻/⁻ T lymphocytes in response to TCR/CD3e religation.

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**References**


