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Role of the Stress Kinase Pathway in Signaling Via the T Cell Costimulatory Receptor 4-1BB

Jennifer L. Cannons,* Klaus P. Hoeflich,† James R. Woodgett,† and Tania H. Watts‡

4-1BB is a member of the TNFR superfamily expressed on activated CD4+ and CD8+ T cells. 4-1BB can costimulate IL-2 production by resting primary T cells independently of CD28 ligation. In this study, we report signaling events following 4-1BB receptor aggregation using an A+–restricted costimulation-dependent T cell hybridoma, C8.A3. Aggregation of 4-1BB on the surface of C8.A3 cells induces TNFR-associated factor 2 recruitment, which in turn recruits and activates apoptosis signal-regulating kinase-1, leading to downstream activation of c-Jun N-terminal/stress-activated protein kinases (JNK/SAPK). 4-1BB ligation also enhances anti-CD3-induced JNK/SAPK activation in primary T cells. Overexpression of a catalytically inactive form of apoptosis signal-regulating kinase-1 in C8.A3 T cells interferes with activation of the SAPK cascade and with IL-2 secretion, consistent with a critical role for JNK/SAPK activation in 4-1BB-dependent IL-2 production. Given the ability of both CD28 and 4-1BB to induce JNK/SAPK activation, we asked whether hyperosmotic shock, another inducer of this cascade, could function to provide a costimulatory signal to T cells. Osmotic shock of resting primary T cells in conjunction with anti-CD3 treatment was found to costimulate IL-2 production by the T cells, consistent with a pivotal role for JNK/SAPK in T cell costimulation.


T

cell activation requires two signals: a signal provided through the binding of Ag/MHC to the TCR as well as additional costimulatory signals. Engagement of the TCR in the absence of costimulatory molecules is insufficient to induce high levels of IL-2 production or survival of primary T cells and can render T cell clones unresponsive to further antigenic challenge (1, 2). The CD28 glycoprotein, expressed on resting T cells, is widely considered to be the primary receptor for delivery of costimulatory signals to resting T cells (3, 4). However, CD28–/− T cells are not defective in all responses, suggesting the existence of alternate costimulatory pathways (5–8). Recently, the TNFR family member 4-1BB has been shown to costimulate IL-2 production by resting primary T cells independently of CD28 signaling (9–11), suggesting that it may function as an alternate costimulatory receptor for T cell activation.

4-1BB (CD137) is expressed on activated CD4+ and CD8+ T cells (12). Its ligand, 4-1BB ligand, is expressed on APC, including activated B cells, macrophages (12), and mature dendritic cells (9). Several studies have demonstrated a role for 4-1BB in T cell activation using either a transfected ligand or anti-4-1BB Abs, or using blocking experiments with a soluble form of the 4-1BB receptor (12). Recent studies have shown that anti-4-1BB Abs induce higher levels of proliferation of CD8 T cells over CD4 T cells and that anti-4-1BB Abs can promote CTL responses and anti-tumor responses in vivo (13, 14). This has led to the suggestion that CD28 and 4-1BB act reciprocally to promote CD4 and CD8 costimulation, respectively. However, 4-1BB/4-1BB ligand can also play a role in the development of Th1 (15) and Th2 responses (10). Furthermore, 4-1BB can perpetuate a CD4 T cell response after CD28 has been down-modulated (15). The extracellular domain of 4-1BB ligand, when immobilized together with anti-CD3, is a potent activator of resting T cells from both CD28+ and CD28− mice, resulting in proliferation and IL-2 secretion (11). When signals through the TCR are high, 4-1BB can replace CD28 in the costimulation of resting T cell responses. However, in the presence of a strong CD28 signal, blocking of 4-1BB/4-1BB ligand interaction has little effect on the T cell response (10, 11). Thus, the emerging data suggest that 4-1BB may be important in sustaining T cell responses under situations in which CD28 signaling becomes limiting (16).

The molecular details by which signals from 4-1BB can replace the CD28 signal in induction of IL-2 are not yet known. Members of the TNFR gene family signal via the TNF receptor-associated factor (TRAF) family of molecules. To date, six members of the TRAF family have been identified (17–23). TRAF proteins serve as adapters to link TNFR family members to downstream signaling pathways. These include activation of the stress-activated protein kinase (SAPK) cascade, activation of NF-κB, and recruitment of the cellular inhibitors of apoptosis proteins (24). Activation of NF-κB and SAPK (also known as c-Jun N-terminal kinase, JNK) can be induced by overexpression of either TRAF2, TRAF5, or TRAF6 (22, 23, 25–30). However, activation of JNK/SAPK, but not NF-κB, in response to CD40L or TNF-α treatment of lymphocytes requires a functional TRAF2 molecule (27, 28). Recently, a new MAP kinase kinase, apoptosis signal-regulating kinase-1 (ASK-1), has been identified. ASK-1 can activate the SEK1 (also known as MKK4) and MKK3/6 pathways, resulting in

† Address correspondence and reprint requests to Dr. Tania H. Watts, Department of Immunology, University of Toronto, ON M5S 1A8, Canada. E-mail address: tania.watts@utoronto.ca

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3 Abbreviations used in this paper: TRAF, TNF receptor-associated factor; AP, alkaline phosphatase; ASK-1, apoptosis signal-regulating kinase 1; ERK, extracellular signal-regulated kinase; HA, hemagglutinin; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; MEKK1, mitogen-activated protein/extracellular signal-related kinase; MKK7, MAP kinase 7; s4-1BBL, soluble 4-1BB ligand; SAPK, stress-activated protein kinase; SEK1, SAPK/ERK kinase.
**Materials and Methods**

**Mice, cell lines, Abs, and reagents**

CD28−/− mice backcrossed onto the C57BL/6 (H-2b) background (n = 10) were obtained from Dr. Tak Mak (Amgen Institute, Toronto, Ontario, Canada) (5). C57BL/6 mice were obtained from Charles River Laboratories (St. Constant, Quebec, Canada). CD28−/− or C57BL/6 mice were used at 8–10 wk of age.

The autoreactive T cell hybrid, C8.A3, was originally obtained from Dr. Laurie Glimcher (Harvard Medical School, Boston, MA). This T cell hybridoma responds to Aβ complexed to an unidentified peptide. Although this T hybridoma can respond to anti-CD3 alone, the response of C8.A3 T cells to MHC II/peptide requires costimulation (42, 43). The BALB/c B cell hybridoma, K46J (44), expresses 4-1BB ligand constitutively, unless stimulated to produce Th1 cytokines. The K46J73.35 was obtained from Dr. Jeff Bluestone (University of Chicago, Chicago, IL). This T cell hybridoma as well as on anti-CD3-treated primary T cells induces JNK/SAPK activation. We also show that 4-1BB aggregation can induce TRAF2-mediated ASK-1 recruitment and activation. Furthermore, overexpression of a dominant-negative form of ASK-1 interferes with 4-1BB-dependent costimulation of IL-2 production, but has no effect on costimulation-independent IL-2 production, in response to a strong signal through the TCR. Finally, we show that the costimulatory signal for IL-2 production by primary T cells is induced by hyperosmotic shock, another activator of the JNK/SAPK pathway.

**Immunoprecipitation and Western blotting**

C8.A3 cells were incubated in low-serum-containing medium (2% FCS) overnight before performing JNK kinase assays. C8.A3 cells were treated with either TNF-α or sorbitol at 37°C. C8.A3 cells were incubated with anti-4-1BB (1AH2) or anti-ICAM-1 (YN-1) Abs for 5 min at 4°C, and the receptors were aggregated with anti-β1 Ig at 37°C. Cells were lysed with ice-cold lysis buffer (10 mM NaCl, 20 mM PIPES, pH 7, 0.5% Nonidet P-40, 5 mM EDTA, 0.05% 2-ME, 0.5% Triton X-100). Proteins were size-excluded on Superose 12 Sepharose beads and eluted with 10 mM reduced glutathione, 50 mM Tris, pH 8. Eluted proteins were dialyzed against a buffer containing 50 mM Tris, pH 8, 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 0.1% 2-ME, and 50% glycerol.

**Kinase assays**

C8.A3 T cells were incubated in low-serum-containing medium (2% FCS) overnight before performing JNK kinase assays. C8.A3 cells were treated with either TNF-α or sorbitol at 37°C. C8.A3 cells were incubated with anti-4-1BB (1AH2) or anti-ICAM-1 (YN-1) Abs for 5 min at 4°C, and the receptors were aggregated with anti-β1 Ig at 37°C. Cells were lysed with ice-cold lysis buffer (10 mM NaCl, 20 mM PIPES, pH 7, 0.5% Nonidet P-40, 5 mM EDTA, 0.05% 2-ME, 0.5% Triton X-100). Proteins were size-excluded on Superose 12 Sepharose beads and eluted with 10 mM reduced glutathione, 50 mM Tris, pH 8. Eluted proteins were dialyzed against a buffer containing 50 mM Tris, pH 8, 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 0.1% 2-ME, and 50% glycerol.

**Verification of endogenous expression of ASK-1**

C8.A3 cells were incubated in low-serum-containing medium (2% FCS) overnight before activation and lysis, which were conducted as described above for the kinase assays. Epitope-tagged proteins were immunoprecipitated by incubating cell lysates with 2 µg anti-HA Ab (12CA5). Endogenous TRAF2 and JNK were immunoprecipitated by incubating with 2 µg of anti-TRAF2 Ab or anti-JNK Ab, respectively, and harvested with 30 µl of protein A-Sepharose. The immune complexes were washed four times with PBS. Precipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Bedford, MA). For analysis of protein-protein interactions, immune complexes were analyzed by Western blot with the indicated Abs. Bound Abs were detected with either goat anti-rabbit Ig HRP, goat anti-mouse Ig HRP, and sorbitol were purchased from Sigma.

**Expression vectors and transfections**

HA epitope-tagged expression plasmids for ASK-1 and catalytically inactive ASK-1 K709E have been previously described (31). C8.A3 (2 × 10⁷) T cells in PBS/135 mM sucrose were transiently transfected with 50 µg of plasmid DNA at 500 V, 25 µF, 13 ohms, and 3 pulses using the BTX Electrocell Manipulator 600 (BTX, San Diego, CA). Plasmids encoding GST-SEK1 K129R (51) and GST-c-Jun (5-89) (52) bacterial fusion proteins were previously described. Fusion proteins were produced in the pLysS (BL21DE3) strain of Escherichia coli using the pGEX expression system. Proteins were affinity purified on glutathione-Sepharose beads and eluted with 10 mM reduced glutathione, 50 mM Tris, pH 8. Eluted proteins were dialyzed against a buffer containing 50 mM Tris, pH 8, 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 0.1% 2-ME, and 50% glycerol.

**A-Sepharose**

Pharmacia Biotech (Piscataway, NJ), according to the manufacturer’s instructions. 3T3 cells secreting 4-1BB linked to alkaline phosphatase (AP) were provided by Dr. Byoung Kwon (Indiana University, Indianapolis, IN). 4-1BB AP was purified on anti-AP-Sepharose, as previously described (50). AP from human placenta was obtained from Sigma (St. Louis, MO). The generation and purification of recombinant soluble 4-1BB ligand (s4-1BB) from baculovirus-infected insect cells were previously described (11).

**TNF-α and monoclonal anti-4-1BB (1AH2, a rat IgG) were purchased from Pharmigen (San Diego, CA).** The anti-TRAF2 Ab and the anti-JNK1 Ab (used in JNK/SAPK in vitro kinase assay) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-JNK/SAPK and anti-phospho-JNK/SAPK Thr185/Tyr185 were purchased from New England Biolabs (Beverly, MA). Goat anti-rabbit Ig HRP, goat anti-mouse Ig HRP, and sorbitol were purchased from Sigma.

**Verification of endogenous expression of ASK-1**

RNA was extracted from C8.A3 T cells using QIAGEN RNeasy Kit (Qiagen, Santa Clarita, CA). Single-stranded cDNA was synthesized from 3 µg of total RNA using the First Strand cDNA Synthesis Kit (Pharmacia Biotech). PCR was performed with the primers 5'-CG GGA GCC TTC ATG AGC A-Sepharose (Pharmacia Biotech, Piscataway, NJ), according to the manufacturer’s instructions. 3T3 cells secreting 4-1BB linked to alkaline phosphatase (AP) were provided by Dr. Byoung Kwon (Indiana University, Indianapolis, IN), 4-1BB AP was purified on anti-AP-Sepharose, as previously described (50). AP from human placenta was obtained from Sigma (St. Louis, MO). The generation and purification of recombinant soluble 4-1BB ligand (s4-1BB) from baculovirus-infected insect cells were previously described (11).
ACG GAG GCG GAC GAA GGC AT-3' and 5'-CC ATC GAT GTA ACA TAG TAG AGA ACA TTC-3' synthesized by Life Technologies (31) and 1 μg of template cDNA, using an initial 5-min denaturation at 94°C, followed by 25 cycles of each of the following: 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a 72°C hold for 7 min.

Primary T cell isolation

APCs were depleted from spleen cell suspensions in HBSS (Life Technologies)/2.5% FCS/50 μM 2-ME, with a mixture of Abs, anti-class II, anti-B220, anti-heat-stable Ag, (M1/69), anti-MAC-1, and anti-CD11c (N418), each at a final concentration of 10 μg/ml at 4°C for 30 min. A 1/10 dilution of baby rabbit complement (Cedarlane Labs, Hornby, Ontario, Canada) was added and the cultures were incubated at 37°C for 40 min. To remove adherent cells, the cell suspensions were passed through a Sephadex G10/nylon wool column, and then centrifuged through Percoll gradients consisting of 60, 70, and 80% Percoll layers. Small (high density) resting T cells were isolated from the 70/80% interface and used for subsequent T cell stimulation experiments. For JNK/SAPK assays, primary T cells were isolated.

T cell activation assays

For stimulation of the T cell hybrid C8.A3, monoclonal anti-CD3 (145-2C11) was immobilized on the surface of 96-well plates (Nunc, Gaithersburg, MD) by incubation in PBS overnight at 4°C. C8.A3 T cells (5 × 10^6) were incubated with immobilized anti-CD3 (0.1 μg/ml) or with 5 × 10^5 irradiated B cell lymphomas (K46J or K4673.35) in the presence or absence of 4-1BB/AP or AP (10 μg/ml) overnight. Small density T cells (1 × 10^7) from CD28+ or CD28−/− H-2^m mice were stimulated with either immobilized anti-CD3 or anti-CD28 plus anti-CD28 or plus s4-1BBL. Where indicated, anti-CD3 and costimulatory ligands were immobilized on the wells simultaneously. After 48 h of culture, supernatants were collected and assayed for IL-2 content using the IL-2-dependent line, CTLL. CTLL proliferation was measured by adding 1 μCi of [3H]thymidine (Amersham Life Science) to the wells for the last 6 h of the 24-h stimulation. The data for the IL-2 assays are expressed as mean ± SD of triplicate cultures.

Results

JNK/SAPK activation after 4-1BB receptor ligation

To assess the activation of the JNK/SAPK cascade following 4-1BB aggregation, we utilized a T cell hybrid, C8.A3, which was previously demonstrated to be responsive to 4-1BB signaling. C8.A3 T cells express low levels of 4-1BB constitutively and further up-regulate 4-1BB expression following TCR ligation (43). Resting C8.A3 T cells were treated with anti-4-1BB plus second step or, as a negative control, with anti-ICAM-1 plus second step. Previous results have shown that engagement of 4-1BB by Ab without aggregation fails to induce TRAF2 recruitment; therefore, second step aggregation was used in all experiments (11). As a positive control for JNK/SAPK activation, C8.A3 T cells were treated with either sorbitol to induce osmotic shock, or TNF-α. JNK/SAPK was immunoprecipitated from the lysates, and the immune complexes were subject to Western blot analysis with either JNK/SAPK-specific or phosho-JNK/SAPK-specific Abs. Phosphorylated JNK/SAPK was detected in C8.A3 cells stimulated with sorbitol, TNF-α, or following aggregation of 4-1BB, but not following ICAM-1 aggregation (Fig. 1A). JNK/SAPK protein was detected in all of the C8.A3 immunoprecipitates regardless of stimulation (Fig. 1B).

To verify that phosphorylated JNK/SAPK was functional, we used an in vitro kinase assay. C8.A3 T cells were stimulated with either TNF-α, sorbitol, anti-4-1BB, or anti-ICAM-1, as described above. JNK/SAPK was immunoprecipitated and protein kinase activity was determined in an immune complex kinase assay with GST-c-Jun as a substrate. Phosphorylated GST-c-Jun could be detected following treatment with sorbitol, TNF-α, as well as 4-1BB ligation, but not after anti-ICAM-1 treatment (Fig. 2A). Fig. 2B indicates that there were equivalent amounts of JNK1 present in all of the immunoprecipitates. A decline in JNK phosphorylation was not observed in Fig. 1 most likely due to the different sensitivities of the assays, a reflection of the in vitro system, and the use of different Abs (see Materials and Methods).

To verify that the results obtained in a T cell hybridoma were also valid for primary T cells, T cells were isolated from the spleen of mice, as described in Materials and Methods. The T cell hybridoma, C8.A3, expresses low levels of 4-1BB, and the anti-4-1BB Ab can induce signaling events. However, primary resting T cells do not express 4-1BB constitutively and respond poorly to this Ab, but can respond after anti-CD3 treatment to induce higher levels of receptor (15). In contrast to the results with anti-4-1BB Ab, we have found that primary resting T cells respond without prior stimulation when anti-CD3 is used in conjunction with 4-1BB ligand. Therefore, for experiments with primary T cells, we used s4-1BB ligand immobilized on plastic to signal via 4-1BB (11). Phosphorylated GST-c-Jun could be detected following T cell treatment with either sorbitol or anti-CD3, but not with s4-1BBL alone. However, the combination of anti-CD3 plus s4-1BBL resulted in greater JNK/SAPK activation than observed with anti-CD3 alone (Fig. 2C). Fig. 2D demonstrates the presence of equivalent amounts of JNK1 protein in the immunoprecipitates. This result is consistent with the observation that primary resting T cells require anti-CD3 treatment to induce 4-1BB expression (33). In contrast, C8.A3 T cells, which express a low level of 4-1BB constitutively, showed JNK/SAPK activation in response to 4-1BB ligation alone (Figs. 1 and 2A). The above data indicate that 4-1BB aggregation on the surface of a T cell hybridoma or on primary T cells leads to activation of the JNK/SAPK pathway.

Induction of ASK-1-TRAF2 association by 4-1BB receptor ligation

As discussed above, the MAP kinase kinase kinase, ASK-1, binds to TRAF2 after TNFR engagement, thereby providing the link...
between the TNFR and JNK/SAPK cascade (33, 34). To investigate the potential role of ASK-1 in 4-1BB signaling, we again used the T cell hybridoma, C8.A3. RT-PCR analysis indicated that C8.A3 T cells express ASK-1 message (data not shown). However, since Abs to ASK-1 were unavailable, we used epitope-tagged transfected ASK-1 to assess the role of ASK-1 in C8.A3 T cell activation. C8.A3 cells were transiently transfected with either vector (pcDNA3) control, HA-ASK-1, or HA-ASK-1 K709E. Thirty-eight hours following the transfection, C8.A3 T cells (3 × 10^6) were stimulated with either anti-4-1BB (5 µg/ml) or anti-ICAM-1 (5 µg/ml) Abs at 4°C for 5 min, followed by anti-rat Ig (20 µg/ml) for 5, 10, or 15 min at 37°C. C8.A3 cells were also stimulated with either TNF-α (5 ng/ml) or sorbitol (0.4 M) for 5, 10, or 15 min at 37°C. JNK/SAPK was immunoprecipitated from the lysates, and its activity was assayed in an in vitro kinase assay using 5 µg/ml GST-c-Jun (5-89) as a substrate. B, JNK/SAPK was immunoprecipitated using anti-JNK1 Ab and harvested with protein A-Sepharose. Immunoprecipitates were separated by SDS-PAGE and detected by immunoblotting with anti-JNK1. This experiment is representative of six independent experiments. C, A total of 6 × 10^6 primary T cells was stimulated with sorbitol (0.4 M) for 0 or 0.5 h as a positive control. T cells were stimulated with immobilized reagents, as follows: either anti-CD3 (1 µg/ml), anti-CD3 (1 µg/ml) plus anti-4-1BB (5 µg/ml) for 0, 0.5, 24, 48, and 70 h. JNK/SAPK was immunoprecipitated from the lysates and its activity was assayed in an in vitro kinase assay with 5 µg/ml of GST-c-Jun (5-89) as a substrate. D, JNK/SAPK was immunoprecipitated using anti-JNK1 Ab and harvested with protein A-Sepharose. Immunoprecipitates were separated by SDS-PAGE and detected by immunoblotting with anti-JNK1. This experiment is representative of three independent experiments.

**FIGURE 2.** JNK/SAPK activation following 4-1BB aggregation on T cells. A, C8.A3 T cells (8 × 10^6) were stimulated with either anti-4-1BB (5 µg/ml) or anti-ICAM-1 (5 µg/ml) Abs at 4°C for 5 min, followed by anti-rat Ig (20 µg/ml) for 5, 10, or 15 min at 37°C. C8.A3 cells were also stimulated with either TNF-α (5 ng/ml) or sorbitol (0.4 M) for 5, 10, or 15 min at 37°C. JNK/SAPK was immunoprecipitated from the lysates, and its activity was assayed in an in vitro kinase assay using 5 µg/ml GST-c-Jun (5-89) as a substrate. B, JNK/SAPK was immunoprecipitated using anti-JNK1 Ab and harvested with protein A-Sepharose. Immunoprecipitates were separated by SDS-PAGE and detected by immunoblotting with anti-JNK1. This experiment is representative of six independent experiments. C, A total of 6 × 10^6 primary T cells was stimulated with sorbitol (0.4 M) for 0 or 0.5 h as a positive control. T cells were stimulated with immobilized reagents, as follows: either anti-CD3 (1 µg/ml), anti-4-1BB (5 µg/ml), or anti-CD3 (1 µg/ml) plus anti-4-1BB (5 µg/ml) for 0, 0.5, 24, 48, and 70 h. JNK/SAPK was immunoprecipitated from the lysates and its activity was assayed in an in vitro kinase assay with 5 µg/ml of GST-c-Jun (5-89) as a substrate. D, JNK/SAPK was immunoprecipitated using anti-JNK1 Ab and harvested with protein A-Sepharose. Immunoprecipitates were separated by SDS-PAGE and detected by immunoblotting with anti-JNK1. This experiment is representative of three independent experiments.

**FIGURE 3.** Association of TRAF2 and ASK-1 following 4-1BB aggregation. C8.A3 T cells (2 × 10^6) were transiently transfected with either vector (pcDNA3) control, HA-ASK-1, or HA-ASK-1 K709E. Thirty-eight hours following the transfection, C8.A3 T cells (3 × 10^6) were stimulated with either anti-4-1BB (5 µg/ml) or anti-ICAM-1 (5 µg/ml) for 5 min at 4°C, followed by anti-rat Ig (20 µg/ml) for 15 min at 37°C. C8.A3 cells were also separately stimulated with TNF-α (5 ng/ml) for 15 min at 37°C. Lysates were subjected to immunoprecipitation with anti-HA Ab and harvested with protein A-Sepharose. A, Immunoprecipitates were separated by SDS-PAGE and detected by Western blotting with anti-TRAF2. For detection of TRAF2 and HA-ASK-1 directly in the lysates, ~1 × 10^6 cell equivalents were separated by SDS-PAGE and TRAF2 and HA-ASK-1 present in the lysates were detected by Western blot analysis with anti-TRAF2 Ab (B) or anti-HA Ab (C), respectively. Data shown are representative of five separate experiments.

**Activation of ASK-1 is induced by 4-1BB aggregation**

To determine whether ASK-1 could be activated following 4-1BB aggregation, C8.A3 T cells were transiently transfected with either vector control, HA-ASK-1, or HA-ASK-1 K709E. Following stimulation of the C8.A3 T cells, ASK-1 was immunoprecipitated with anti-HA Ab. The immune complexes were subject to an in vitro kinase assay using the kinase dead GST-SEK1 K129R as the substrate. GST-SEK1 K129R could be phosphorylated by HA-ASK-1 immunoprecipitated from C8.A3 T cells stimulated with TNF-α or anti-4-1BB treatment, but not after anti-ICAM-1 treatment (Fig. 4A). It should be noted that there are background bands in the ICAM-1 lane close to the GST-SEK1 band. However, careful examination indicates that these bands migrate at a distinct position from the GST-SEK1 band, and therefore we do not believe this indicates that ICAM activates ASK-1. As expected, C8.A3 T cells expressing the kinase dead ASK-1 variant did not show GST-SEK1 K129R phosphorylation after any of the treatment regimens (Fig. 4A). Western blot analysis indicates that each cell extract...
contained similar levels of HA-ASK-1 or HA-ASK-1 K709E (Fig. 4B).

**Dominant-negative ASK-1 interferes with 4-1BB-mediated costimulation in C8.A3 cells**

The above studies suggest that ASK-1 can be activated following 4-1BB aggregation on the surface of C8.A3 T cells. To determine the importance of the JNK/SAPK cascade in 4-1BB-mediated costimulation, we took advantage of the ability of the dominant-negative form of ASK-1 to interfere with this pathway. C8.A3 cells were transiently transfected with either vector control, HA-ASK-1, or HA-ASK-1 K709E. The C8.A3 T cell hybrid is costimulation dependent when stimulated using APC expressing the appropriate MHC/peptide complex (Ak plus an unidentified self peptide). In contrast, when stimulated with anti-CD3 immobilized on plastic, the C8.A3 T cell response is costimulation independent. The transfected C8.A3 T cells were stimulated with either vector control, HA-ASK-1, or HA-ASK-1 K709E. The C8.A3 T cell hybrid is costimulation dependent when stimulated using APC expressing the appropriate MHC/peptide complex (A\(^k\) plus an unidentified self peptide). In contrast, when stimulated with anti-CD3 immobilized on plastic, the C8.A3 T cell response is costimulation independent.

The transfected C8.A3 T cells were stimulated with either immobilized anti-CD3, or an APC (K46J73.35)-expressing transfected Ak. This APC expresses 4-1BB ligand, but little or no B7 family molecules (43). The parental cell line (K46J) lacking the transfected Ak molecule was used as a control. Following an overnight stimulation, supernatants were collected and assayed for their ability to induce proliferation of the IL-2-dependent cell line, CTLL. Mock-transfected, HA-ASK-1-transfected, and HA-ASK-1 K709E-transfected C8.A3 cells secreted similar amounts of IL-2 in response to stimulation with immobilized anti-CD3 (Fig. 5A), indicating that there was no effect of either HA-ASK-1 or HA-ASK-1 K709E transfection on costimulation-independent IL-2 production. However, when C8.A3 T cells were stimulated with APC expressing A\(^k\) and 4-1BBL, the HA-ASK-1 K709E-transfected C8.A3 T cells secreted significantly less IL-2 than vector control or HA-ASK-1-transfected C8.A3 T cells (Fig. 5B). A soluble form of the receptor, 4-1BB alkaline phosphatase (4-1BB.AP), was previously shown to specifically interfere with stimulation of C8.A3 T cells in response to Ag/MHC presented on K46J lymphomas (43). Fig. 5C confirms that 4-1BB-AP, but not the AP control, inhibits IL-2 production in this assay. These data are consistent with a role for ASK-1 in providing the link between TRAF2 and the JNK/SAPK cascade following 4-1BB aggregation, and suggest that activation of the JNK/SAPK and/or p38 MAP kinase pathways is critical to 4-1BB-mediated costimulation.

**Osmotic shock can mimic the costimulatory signal required for IL-2 production by T cells**

Hyperosmotic shock, such as caused by sorbitol treatment, results in the activation of the stress kinase cascade (54). In mouse wild-type and MEKK1\(^{−/−}\) embryonic stem cell clones, osmotic shock results in a dramatic increase in JNK/SAPK activation with only modest activation of the ERK and p38 pathways (55). Given that CD28 and 4-1BB signaling have in common their ability to activate the JNK/SAPK cascade and to costimulate IL-2 production, we reasoned that an unrelated stimulus such as hyperosmotic shock might do the same thing. For these studies, we used primary resting T cells responding to anti-CD3, plus or minus a secondary stimulus. To rule out possible effects of B7-CD28 interaction either from B7 on contaminating APC or through T cell-T cell interaction, we used CD28\(^{−/−}\) as well as CD28\(^{+/+}\) mice as a source of T cells. Fig. 6 shows IL-2 secretion by purified CD28\(^{−/−}\) or CD28\(^{+/+}\) T cells responding to immobilized anti-CD3 in the presence of either sorbitol, anti-CD28, or s4-1BBL. In a separate experiment, a concentration of 0.4 M of sorbitol was found to give an optimal costimulatory effect, with higher concentrations becoming inhibitory (data not shown). Previous experiments had shown that
costimulation with immobilized s4-1BB ligand in this system saturates at ~5 μg/ml, whereas costimulation with immobilized anti-CD28 saturates at between 5 and 10 μg/ml (data not shown). Neither anti-CD3 (Fig. 6), sorbitol, anti-CD28, nor s4-1BBL alone (data not shown) resulted in IL-2 secretion. In contrast, anti-CD3 in combination with either sorbitol, anti-CD28, or s4-1BBL leads to significant IL-2 secretion. These data suggest that sorbitol is able to partially replace the costimulatory signal for IL-2 secretion provided by CD28 or 4-1BB, most likely due to its ability to activate the JNK/SAPK pathway, demonstrating a critical role of the SAPK in T cell costimulation.

**Discussion**

The data presented in this work suggest a critical link between 4-1BB costimulation of IL-2 production and activation of the SAPK cascade. Following 4-1BB aggregation on T cells, the JNK/SAPK pathway is activated (Figs. 1 and 2). 4-1BB aggregation induces TRAF2 recruitment (11), TRAF2 association with ASK-1 (Fig. 3), and activation of ASK-1 (Fig. 4). A dominant-negative form of ASK-1 prevents 4-1BB-mediated costimulation of IL-2 production, but has no effect on IL-2 production in response to a strong signal through the TCR alone (Fig. 5). This signal can be mimicked by osmotic shock, another inducer of JNK/SAPK cascade (Fig. 6). These data are consistent with a role for ASK-1 and the JNK/SAPK cascade in 4-1BB signaling. However, it is possible that overexpression of the dominant-negative ASK-1 interferes with the activation of another endogenous MKKK. For example, in TNF-α-treated 293 cells transfected with germinal center kinase (GCK), GCK can also interact with TRAF2 and MEKK1 and serve as a link from TNFRI to the JNK/SAPK pathway (56). ASK-1 activates both the JNK/SAPK and the p38 MAP kinase pathway (32). Therefore, the blocking experiments with dominant-negative ASK-1 imply that JNK/SAPK and/or p38 activation contribute to the 4-1BB costimulatory signal.

Recent evidence has shown that the association of TRAF2 with the cytoplasmic tails of TNFR family members is upstream of ASK-1 (33, 34). Thus, based on previous experiments and the findings in the present study, the following scenario is suggested. 4-1BB aggregation induces recruitment of TRAF2, which in turn interacts with and activates ASK-1. Activated ASK-1 then phosphorylates a downstream MKK, which in turn activates JNK. Fig. 4 illustrates that following 4-1BB aggregation on the C8.A3 T cell, ASK-1 can phosphorylate GST-SEK1 K129R, a potential downstream target in the stress kinase cascade. However, the present experiments do not identify the specific MKK involved in vivo. For example, MKK7 has been shown to preferentially activate the JNK/SAPK pathway, demonstrating a critical role of the SAPK in T cell costimulation.

Previous reports have provided evidence that NF-κB is activated following 4-1BB ligation (35, 36). Using a dominant-negative IκBα construct, we have also found that NF-κB is required for IL-2 production by C8.A3 cells (unpublished observations). However, contrary to the effects of dominant-negative ASK-1, which were specific to costimulation-dependent T cell activation, blocking NF-κB activation also blocked the costimulation-independent response of the T cell hybrid to anti-CD3. Thus, in this system, one cannot distinguish whether NF-κB plays a central role in signaling from the TCR, from 4-1BB, or both.

A critical question in T cell activation is how signals from the different surface receptors are integrated to result in IL-2 secretion.

**FIGURE 5.** Role of ASK-1 in IL-2 secretion by C8.A3 T cells. C8.A3 T cells were transiently transfected with vector (pcDNA3) control, HA-ASK-1, or HA-ASK-1 K709E. Following a 38-h incubation, C8.A3 cells were stimulated with APC-expressing 4-1BB. The negative control is vector mock-transfected C8.A3 cells stimulated with APC lacking the Aβ molecule. C, Transiently transfected C8.A3 cells stimulated with APC expressing Aβ in the presence or absence of either 4-1BB.AP or AP (10 μg/ml). Note that for clarity, the scale of the y-axis scale is not the same in each panel. This experiment is representative of four independent experiments.
TCR signaling and CD28-mediated costimulation have been shown to synergize at the level of JNK/SAPK activation (37, 38). In this study, we have provided evidence that activation of the SAPK by 4-1BB plays a role in its ability to costimulate IL-2 production. In our previous studies, we found that at low concentrations of anti-CD3, an optimal dose of anti-CD28 was superior to 4-1BB ligand in costimulating IL-2 production. However, when signals through the TCR were strong, both costimulatory signals could induce comparable levels of IL-2 (11). Whether the ability of CD28 to induce a more potent costimulatory signal on resting T cells is due to its ability to induce qualitatively or quantitatively different signals compared with 4-1BB, or whether these findings reflect the differential expression of the receptors (constitutive vs inducible) is not known. CD28 signaling has a greater proliferative effect on CD4 cells over CD8 T cells (58), whereas 4-1BB has a greater effect on proliferation of CD8 over CD4 T cells (13). How the respective signaling pathways in CD4 and CD8 T cells contribute to these differences remains to be determined.

The ability of both CD28 and 4-1BB to induce JNK/SAPK activation prompted us to ask whether another activator of these kinases could replace the T cell costimulatory signal. Hyperosmotic shock results in a dramatic activation of the SAPK, with a low to modest activation of the ERK and p38 pathways (54, 55). Fig. 6 indicates that stimulation of resting T cells with anti-CD3 plus hyperosmotic shock is sufficient to induce IL-2 secretion. The production of IL-2 is comparable with that when T cells are costimulated with low doses of either anti-CD28 or s4-1BBL. Although the conditions used in this study are by no means physiological, the ability of cell stress to directly activate a costimulatory pathway in T cells may provide a means of augmenting the immune response under conditions of limiting costimulatory molecule expression in vivo. Sorbitol induces JNK/SAPK activation in primary T cells with faster kinetics than do anti-CD28 (59) or anti-4-1BB, so a stress-induced signal might function to augment T cell activation early in the T cell response, before costimulatory interactions have been fully up-regulated.

Other members of the TNFR family of signaling molecules such as CD27 and OX40 (CD134) have been shown to play a role in T cell activation by promoting expansion or by sustaining T cell responses after CD28 costimulation (60, 61). However, of these three costimulatory members of the TNFR family, only 4-1BB induces IL-2 production by resting T cells independently of CD28 signaling. Overexpression of CD27 in HEK 293 cells leads to the recruitment of TRAF2, 3, and 5 and the activation of NF-κB and JNK/SAPK (62, 63). CD27-mediated JNK/SAPK activation has also been reported in primary resting T cells (64). OX-40 expression is limited to activated T cells and is thought to play a role in promoting the immune response and in promoting a Th2 response (65–67). OX-40 also associates with TRAF2, 3, and 5, and has been shown to activate NF-κB (35, 68). OX-40 is also likely to activate the SAPK by virtue of its ability to interact with TRAF2. 4-1BB differs from OX40 and CD27 in its ability to bind TRAF1 and its lack of TRAF5 binding. Functional differences in

**FIGURE 6.** Role of the stress kinase cascade in IL-2 secretion. Resting T cells were isolated from spleens of either C57BL/6 CD28+/+ mice or CD28−/− mice, as described in Materials and Methods. T cells were incubated with immobilized anti-CD3 (1 µg/ml) in the presence or absence of sorbitol (0.4 M), immobilized anti-CD28 (1 or 10 µg/ml), or immobilized s4-1BBL (1 or 5 µg/ml), as indicated in the figures. After 48 h of culture, supernatants were analyzed for IL-2 production using CTLL cells. Note that for clarity, the scale of the y-axis scale is not the same in each panel. Similar results were obtained from three independent experiments, with two wild-type and two CD28−/− mice analyzed separately in each experiment.
the effects of these receptors on T cell activation may reflect differences in the expression of their receptors and ligands or may reflect the differential recruitment of TRAF proteins.

In summary, the results presented in this work provide evidence for a pivotal role for the JNK/SAPK cascade in 4-1BB-mediated costimulation, and provide insight into the ability of 4-1BB signaling to replace CD28 signaling under some circumstances. The ability of hyperosmotic shock to mimic the costimulatory signal for IL-2 production raises the possibility that T cells can directly respond to Ag/MHC in the absence of costimulatory signals when recognized under conditions of cell stress. This might be important in augmenting the immune response when costimulatory ligands are limiting.

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References


