

Characterization of Signaling Pathways Activated by the Interleukin 1 (IL-1) Receptor Homologue T1/ST2

A ROLE FOR JUN N-TERMINAL KINASE IN IL-4 INDUCTION*

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T1/ST2 is a member of the interleukin (IL)-1 receptor superfamily, possessing three immunoglobulin domains extracellularly and a Toll/IL1R (TIR) domain intracellularly. The ligand for T1/ST2 is not known. T1/ST2 is expressed on Type 2 T helper (Th2) cells, and its role appears to be in the regulation of Th2 cell function. Here, we have investigated T1/ST2 signal transduction, using either transient overexpression of T1/ST2 or a cross-linking monoclonal antibody to activate cells. We demonstrate that T1/ST2 does not activate the transcription factor NF- κ B when overexpressed in murine thymoma EL4 cells, or in the mast cell line P815 treated with the anti-T1/ST2 antibody. However, a chimera comprising the extracellular domain of the type 1 IL-1 receptor and the intracellular domain of T1/ST2 activates NF- κ B both by overexpression and in response to IL-1. This artificial activation requires the IL1RacP recruited via the extracellular portion (IL1R1) of the chimera. T1/ST2 is, however, able to activate the transcription factor activator protein-1 (AP-1), increase phosphorylation of c-Jun, and activate the MAP kinases c-Jun N-terminal kinase (JNK), p42/p44 and p38. Anti-T1/ST2 also induces the selective expression of IL-4 but not IFN- γ in naive T cells. Importantly, this effect is blocked by prior treatment with the JNK inhibitor SP600125 confirming that JNK as a key effector in T1/ST2 signaling. The lack of effect on NF- κ B when T1/ST2 is homodimerized identifies T1/ST2 as the first member of the IL-1 receptor superfamily so far studied that is apparently unable to activate NF- κ B, consistent with evidence indicating the lack of a role for NF- κ B in Th2 cell function.

T1/ST2 was originally identified in murine fibroblasts as a late response gene induced by either serum or by overexpression of the *v-mos* or *Ha-ras* oncogenes (1, 2). By alternative 3' processing of a primary transcript the T1/ST2 gene encodes two mRNAs: an abundant short secreted glycoprotein (sST2) and a rare longer transmembrane form (termed T1/ST2) (3–5). T1/ST2 has been shown to be expressed on mast cells (6) and on

Th2 cells but not on Th1 cells (7, 8). In addition to being a stable cell marker on Th2 cells, T1/ST2 has been shown to be important in Th2 effector function since treatment of mice with a monoclonal antibody against T1/ST2 inhibited allergic airway inflammation in response to both allergen provocation and viral antigen (9, 10). Studies on T1/ST2-deficient mice, however, have yielded conflicting evidence as to the functional role of T1/ST2 (11–13).

T1/ST2 is a member of the Toll/IL-1 receptor superfamily, which shows ~29% homology to the Type 1 IL-1 receptor (IL1R1) (3). The superfamily can be broadly divided into two subfamilies. All have a homologous Toll/IL-1 receptor (TIR) domain in their cytosolic portions, responsible for signal transduction. The two subfamilies differ extracellularly, with the IL1R subgroup having immunoglobulin domains and the Toll-like receptor subgroup having leucine-rich repeats. T1/ST2 belongs to the IL1R subgroup (14). The gene for T1/ST2 is tightly linked to the genes encoding other receptors in the IL1R subgroup on both mouse (3) and human chromosomes (15). However, T1/ST2 does not bind IL-1 α , IL-1 β or the IL-1 receptor antagonist (16). As yet there is no functional ligand identified for T1/ST2, although two binding proteins have been identified (17, 18).

The signaling pathway for the IL-1 receptor has now been well characterized, and several other members of the family such as the IL18R and TLR2, -4, -5, and -9 share many of the intracellular signaling components with IL1RI (19). Upon ligand binding both IL1R1 and IL18R require recruitment of their respective accessory proteins to signal (20, 21). The adaptor protein MyD88, which also possesses a TIR domain, is then recruited to the active complex and interacts with the receptors through homotypic TIR domain interactions. The IL1R-associated kinases IRAK, IRAK2 and the recently identified IRAK4 (22, 23) are then recruited to the complex. IRAK and IRAK2 have been shown to interact with tumor necrosis factor receptor-associated factor-6 (TRAF-6) (24). TRAF-6, in a preassembled complex with TAK-1 binding protein (TAB)-2, can then cause activation of the transcription factor NF- κ B through the kinase TAK-1 and the I κ B kinase complex (25). In addition it has been shown that these receptors can cause activation of p42/p44 and p38 MAP kinases and c-Jun N-terminal kinase (JNK). Activation of these kinases also occurs through MyD88

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¹ The abbreviations used are: IL, interleukin; MAP, mitogen activated protein; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; AP, activator protein; IL1R1, type I interleukin 1 receptor; IL1RacP, interleukin 1 receptor accessory protein; MAPK, mitogen activated protein kinase; mAb, monoclonal antibodies; EV, empty vector; PE, phycoerythrin; TLR, Toll-like receptor.

and IRAK, and recently a role for the low molecular weight G protein Ras in p38 activation has been demonstrated (26).

Little is known about how T1/ST2 signals. It was demonstrated that treatment of cells expressing a chimeric receptor comprising the extracellular domain of IL1R1 and the intracellular domain of T1/ST2 with IL-1 activated NF- κ B (27) and p38 MAP kinase (17), implying that T1/ST2 might signal in a similar way to IL1R1. The ability of the T1/ST2 chimera to activate NF- κ B is somewhat inconsistent with data indicating that NF- κ B activation is more associated with Th1 type responses. Furthermore, the putative unpurified ligand for T1/ST2 described by Kumar *et al.* (17) was unable to activate NF- κ B but was shown to activate p38 MAP kinase. Here we have examined T1/ST2 signal transduction in detail, using either overexpression of T1/ST2 or an anti-T1/ST2 cross-linking antibody. We have found that T1/ST2 can activate JNK, p38, p42/p44 MAP kinase, and the transcription factor activator protein-1 (AP-1). Interestingly we can find no evidence for NF- κ B activation and demonstrate that the effect of the IL1R1-T1/ST2 chimera on NF- κ B occurs via the IL-1 receptor accessory protein (IL1RAcP). Finally, we have found that anti-T1/ST2 induces IL-4 production from naïve T cells, and importantly that the specific JNK inhibitor SP600125 blocks this effect. Our study is therefore the first to characterize T1/ST2 signaling and demonstrates that T1/ST2 is a TIR domain-containing receptor, which cannot activate NF- κ B when homodimerized, consistent with its role in promoting Th2 responses.

EXPERIMENTAL PROCEDURES

Animals—Female Balb/c mice (6–8 weeks of age) were obtained from Harlan (Bicester, Oxon, UK). Mice were maintained in individually ventilated pathogen free cages.

Materials—Human embryonic kidney 293 and EL4 murine thymoma cells were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). Cell culture media and serum were obtained from Invitrogen. The murine mastocytoma cell line P815 was a gift from Dr. T. Kamradt (Deutsches Rheumaforschungszentrum, Berlin, Germany). The IL1RAcP-deficient cell line EL4 D6/76 was a kind gift from Prof. Werner Falk (Universität Regensburg, Regensburg, Germany). Human recombinant IL-1 α was a gift from the NCI, National Institutes of Health, Biological Resources Branch (Rockville, MD). The 22-bp oligonucleotide, 5'-AGT TGA GGG GAC TTT CCC AGG C-3', containing the NF- κ B consensus sequence (*underlined*), and the T4 polynucleotide kinase were obtained from Promega (Madison, WI). PhosphoPlus™ SAPK/JNK (Thr-183/Tyr-185) and p42/p44 MAP kinase (Thr-202/Tyr-204) antibody kits and the phospho-c-Jun (Ser-63) II and c-Jun antibodies were obtained from New England Biolabs, Ltd. (Hitchin, UK). The monoclonal anti-T1/ST2 antibody 3E10 has been described previously (9). The anti-FLAG M2 antibody was obtained from Sigma. The I κ B antibody was a kind gift from Prof. R. Hay (University of St. Andrews, St. Andrews, UK). The JNK inhibitor SP600125 was purchased from Calbiochem (CN Biosciences, Notts, UK).

Expression Vectors—The cloning of T1/ST2 into the expression vector pCDNA3.1 has already been described (9). Using this as template, T1/ST2 was subcloned into pCMV-Flag (IBI Kodak, New Haven, CT) using oligonucleotide primers of sequence 5'-GTCAGATCTCAGTA-AATCGTCTCT-3, 5'CTGGTCGACTCAAAAGTGTTCAG-3'. The components for the PathDetect™ CHOP, Elk-1, and c-Jun *trans*-reporting system (pFA-CHOP, pFA-Elk-1, pFA-c-Jun, pFc2-dbd, pFR-Luc, pFc-MEK3, pFc-MEKK, and pFc-MEK1) were purchased from Stratagene (La Jolla, CA). The plasmids encoding the IL1R1 and IL1RAcP were gifts from Werner Falk (Universität Regensburg). The pG13-5 κ B-luc plasmid was a kind gift from Dr. R Hofmeister (Universität Regensburg). The AP-1-luciferase plasmid was obtained from Stratagene (La Jolla, CA). The plasmids encoding MyD88 constructs were gifts from M. Muzio (Mario Negri Institute, Milan, Italy). The chimeric IL1R-T1/ST2 construct was a kind gift from Prof. J. Sims (Immunex Corp., Seattle, WA).

Cell Culture—HEK293 and EL4 cell lines were cultured in Dulbecco's modified Eagle's medium while the P815 and EL4 D6/76 cell lines were cultured in RPMI 1640 medium. All medium contained 10% (v/v) fetal calf serum, 100 units/ml gentamicin, and 2 mM L-glutamine. Cells

were maintained at 37 °C in a humidified atmosphere of 5% CO₂. For use in transfection assays, HEK293 were typically seeded at 2 × 10⁴ ml⁻¹ in 96-well plates 24 h prior to transfection, whereas EL4 and EL4 D6/76 cells were seeded at 5 × 10⁵ ml⁻¹ 16 h prior to being used. P815 cells were seeded in 6-well plates at 2 × 10⁶ ml⁻¹ for cross-linking and immunoblot analysis.

Transient Transfection and Reporter Gene Assays—EL4 and EL4D6/76 cells were transfected with plasmids as indicated in the figure legends in a final volume of 0.6 ml using DEAE-dextran. Following 16–18 h of recovery, cells were seeded at a density of 10⁶ ml⁻¹ viable cells (as determined by trypan blue exclusion) prior to stimulation. HEK293 cells were transfected with plasmid concentrations as indicated in the figure legends with FuGENE 6 (Roche Molecular Biochemicals) according to manufacturer's recommendations. Transfection efficiency was normalized in all experiments by transfection of cells with a plasmid encoding 40 ng of *Renilla* luciferase. In all cases the amount of DNA transfected was kept constant by the addition of various amounts of the appropriate empty vector plasmid. Cells were either left untreated or stimulated with IL-1 (10 ng/ml) for 6 h as indicated following a period of recovery (16–18 h). To assay firefly and *Renilla* luciferase activity, cells were lysed using passive lysis buffer (Promega, Southampton, UK), and luciferase activity was determined by standard protocols.

Detection of Surface T1/ST2 by Flow Cytometry—For surface staining, an anti-T1/ST2 rat anti-mouse monoclonal antibody 3E10 was used. For P815 cells biotinylated 3E10 was detected with streptavidin-PE (BD Pharmingen, San Diego, CA). Staining of 3E10 was blocked by preincubating the cells with a 100-fold excess of unconjugated 3E10. Gates were set on viable cells according to forward and side scatter and exclusion of propidium iodide (0.3 μ g/ml). Samples were analyzed on a FACSCalibur using CellQuest software (Becton Dickinson, Mountain View, CA).

Cross-linking of T1/ST2—6-well plates were coated with 20 μ g/ml of either mAb 3E10 (rat anti-mouse T1/ST2) or rat IgG (PharMingen) for 16 h at 4 °C. Wells were washed three times with phosphate-buffered saline and 2 ml of P815 cells were added at a density of 2 × 10⁶ ml⁻¹ for 3 h at 37 °C. Control cells were stimulated with IL-1 (10 ng/ml) for 15 min. Cells were harvested samples prepared for either immunoblot analysis or electrophoretic mobility shift assay. For cross-linking studies on naive spleen cells 24-well plates were coated with 3E10 or control IgG under the same conditions prior to the addition of cells at a density of 1 × 10⁶ ml⁻¹.

Immunoblot Analysis—Treatment of cells was terminated by the addition of 1.5 ml of ice-cold phosphate buffered saline. Total cell lysate from each sample was extracted in ice-cold radioimmune precipitation buffer, RIPA: (phosphate-buffered saline buffer containing 1% Nonidet P40, 0.5% w/v sodium deoxycholate, 0.1% SDS, 10 μ g/ml phenylmethylsulfonyl fluoride, 30 μ l/ml aprotinin, and 10 μ l/ml sodium orthovanadate). Protein estimations of cell extracts were determined by the dye binding assay of Bradford. Equal amounts of protein (4–8 μ g) were subjected to 10% SDS-PAGE and Western blotting according to the method of Laemmli (28). The antibodies recognizing FLAG, phosphorylated p42/p44, JNK, c-Jun, and ATF-2 were used at a dilution of 1:1000 in 1% fat-free milk in Tris-buffered saline/0.1% Tween. The I κ B antibody was used at a dilution of 1:50 in 5% fat-free milk.

p38-MAPK Immunoprecipitation-Kinase Assay—HEK293 cells were transfected with FLAG-tagged p38 and other plasmids as described in the figure legend and allowed to recover for 16 h. Prior to harvesting cells were treated with IL-1 (10 ng/ml) for 15 min. Cells were lysed with 500 μ l of 1 \times lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM Na₃VO₄, 1 μ g/ml leupeptin), and FLAG-p38 was immunoprecipitated with 2–5 μ g of anti-FLAG M2 antibody (Kodak). Beads were then washed three times with lysis buffer and then with kinase buffer (25 mM Tris, pH 7.5, 5 mM β -glycerolphosphate, 2 mM dithiothreitol, 0.1 mM Na₃VO₄, 10 mM MgCl₂). Pellets were resuspended with 50 μ l of 1 \times kinase buffer supplemented with 200 μ M ATP and 2 μ g of ATF-2 fusion protein (Cell Signaling Technologies, Beverly, MA) and incubated for 30 min at 30 °C. The reaction was terminated by addition of 25 μ l of 3 \times SDS sample buffer. Samples were boiled and then analyzed by SDS-PAGE and Western blotted for phosphorylated ATF-2 or FLAG-p38.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared from P815 cells following cross-linking as described previously (29). P815 cells were collected by centrifugation before preparation of nuclear extracts as above. Nuclear extracts were assessed for NF- κ B binding activity as described previously (30).

Intracellular Cytokine Analysis—Intracellular detection of IL-4 and

IFN- γ in CD4⁺ T cells was performed essentially as described (31). All intracellular antibodies and reagents were from Caltag (Burlingame, CA). Cells from the spleens of naïve Balb/c mice were either left in medium for 1 h or were pretreated with the JNK inhibitor SP600125 for 1 h. Cells were then added to plates precoated with 20 μ g/ml 3E10 mAb or isotype-matched control rat IgG for 5 h. After 1 h of incubation 10 μ g/ml of Brefeldin A (Sigma) was added. Cells were washed and surface-stained with Tricolor-conjugated anti-CD4 mAb. Cells were permeabilized and intracellular cytokines detected with PE-conjugated anti-IL-4 or fluorescein isothiocyanate-conjugated anti-IFN- γ . Lymphocytes were gated on CD4-positive cells, and quadrants set using PE- or fluorescein isothiocyanate-conjugated isotype control mAbs. The frequencies of IL-4- or IFN- γ -positive cells were determined, and data are presented as the mean percentages of IL-4- or IFN- γ -stained CD4⁺ cells.

RESULTS

T1/ST2 Does Not Activate the Transcription Factor NF- κ B but Can Activate AP-1—We first investigated the effect of T1/ST2 on the transcription factor NF- κ B. As shown in Fig. 1A we were unable to observe any activation of an NF- κ B luciferase reporter construct upon overexpression of T1/ST2 in EL4 cells over a range of plasmid concentrations. This lack of effect was not due to an inability of these cells to respond to activators of NF- κ B since overexpression of the IL1R1 (Fig. 1A, left panel) and treatment with IL-1 (Fig. 1A, right panel) both activated NF- κ B. To ensure that this lack of effect was not cell-specific, several other cell lines were tested including HEK293 cells and HeLa cells. Similarly, no activation of NF- κ B by T1/ST2 was observed in any of the cell lines tested (not shown).

To confirm this result we employed a different approach involving an anti T1/ST2 cross-linking monoclonal antibody on P815 cells. These cells express T1/ST2 as shown by fluorescence-activated cell sorter analysis (Fig. 1B), and this antibody has been shown to induce IL-4 and IL-5 production (32). Following cross-linking, no effect on NF- κ B was observed above an isotope-matched control IgG when measured using electrophoretic mobility shift assay, nor was any degradation of I κ B observed (Fig. 1C). Incubation time of the cells with anti-T1/ST2 mAb was 3 h (Fig. 1C, lane 3). Earlier incubation times of the cells with antibody showed no responses (not shown). Treatment of the cells with IL-1 for 15 min activated NF- κ B and caused I κ B degradation (Fig. 1C, lane 2).

We next investigated the ability of T1/ST2 to activate another transcription factor, AP-1. It has been reported that IL-1, IL-18, and several of the known TLR ligands can also activate AP-1 through their respective receptors (33). T1/ST2 was able to induce an AP-1 luciferase reporter construct in EL4 cells up to 4-fold above control levels (Fig. 1D). Treatment of the cells with IL-1 also activated AP-1. In addition cross-linking of P815 cells with anti-T1/ST2 antibody caused phosphorylation of c-Jun at contact times of 1 and 3 h (Fig. 1E, lanes 2 and 4). These results implied that T1/ST2 is able to activate AP-1 but not NF- κ B.

An IL1R1-T1/ST2 Chimera Activates NF- κ B via Recruitment of the IL1RACp—It has previously been reported that a chimera consisting of the extracellular portion of the IL1R1 and the intracellular portion of T1/ST2 could activate both NF- κ B (27) and p38 MAP kinase (17) in COS7 and Jurkat cells both by overexpression and in response to IL-1 treatment. As this was in contrast to our results observed with overexpression of full-length T1/ST2, we investigated the effect of the chimera in our system. In Fig. 2A it can be seen that overexpression of a chimera, comprising the IL1R1 extracellular and transmembrane regions and the cytosolic domain from T1/ST2 (IL1R_{extm}-T1/ST2_{cyto}), was capable of activating NF- κ B in EL4 cells. This effect was seen by overexpression of the plasmid (Fig. 2A) and was promoted following IL-1 treatment (not shown). The effect of the chimera on NF- κ B activation was

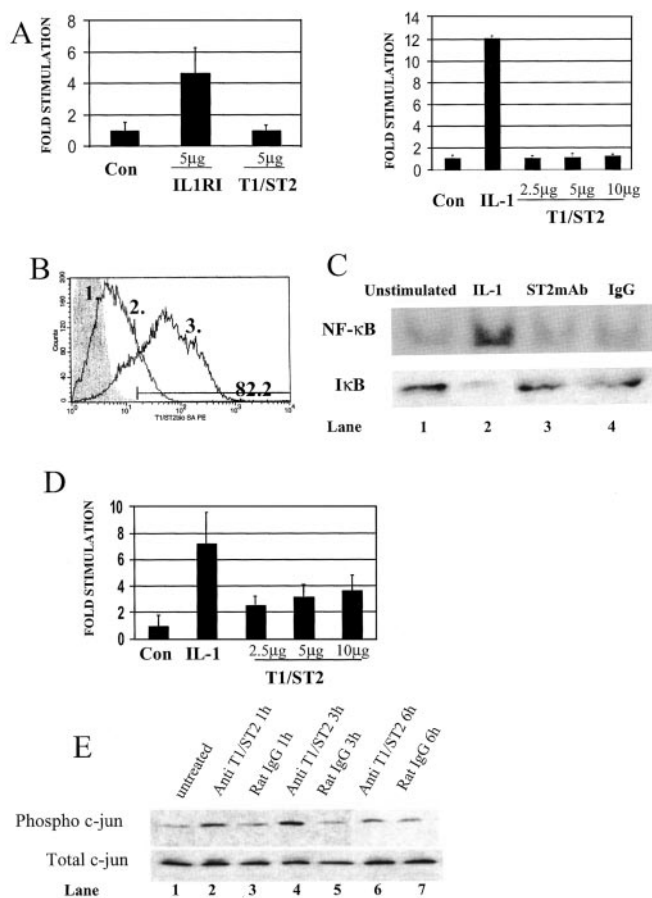


FIG. 1. T1/ST2 does not activate NF- κ B but does activate AP-1. EL4 cells (7×10^6) were transiently transfected with plasmids encoding FLAG-tagged T1/ST2 or IL1R1 as indicated and (A) 5 μ g NF- κ B luciferase plasmid or (D) 5 μ g AP-1 luciferase plasmid, both along with 1 μ g of thymidine kinase *Renilla* luciferase for 16–18 h. The empty vector (EV) control plasmid was pCMV-Flag. IL-1 effects were measured after 6 h of incubation with 10 ng/ml IL-1. Extracts were prepared and measured for luciferase activity. Results are normalized for *Renilla* luciferase activity and represented as fold stimulation over the non-stimulated EV control. Results are expressed as mean \pm S.D. from three separate experiments, each carried out in triplicate. B, T1/ST2 was detected on P815 cells by fluorescence-activated cell sorter analysis. Aliquots of cells were either unstained (1), blocked with excess unlabelled anti-T1/ST2 antibody 3E10 (2), or stained with biotinylated 3E10 (3). 82.2% of cells were T1/ST2⁺. C, P815 cells (2×10^6 per ml) were added to 6-well plates and treated with IL-1 (10 ng/ml, 15 min) (lane 2) or added to plates coated with either anti T1/ST2 for 3 h (lane 3) or isotype-matched control antibody (lane 4). Cells were harvested and assayed for NF- κ B by electrophoretic mobility shift assay (upper panel) and I κ B by immunoblotting (lower panel). E, P815 cells (2×10^6 per ml) were added to 6-well plates either untreated (lane 1) or coated with anti-T1/ST2 (lanes 2, 4, 6) or isotype-matched control antibody (lanes 3, 5, 7) for the incubation times shown. Cells were harvested and assayed for phospho-c-Jun by immunoblotting of cell lysates. The upper panel shows phosphorylated c-Jun, while the lower panel shows total c-Jun, indicating equal loading of samples. All results shown are representative of three separate experiments.

inhibited by overexpression of the isolated TIR domain of MyD88, which acts as a dominant negative form of MyD88 (Fig. 2B) (24).

It was possible that the IL1R-T1/ST2 chimera was acting via the IL1RACp. We investigated this possibility by transfecting this chimera into the IL1RACp-deficient cell line EL4 D6/76. It is known that this cell line is unresponsive to IL-1 due to the absence of IL1RACp. As shown in Fig. 2C, we confirmed this and then demonstrated that the responsiveness of EL4 D6/76 cells to IL-1 can be restored upon co-transfection of IL1RACp (20). We observed that overexpression of the chimera IL1R_{extm}-

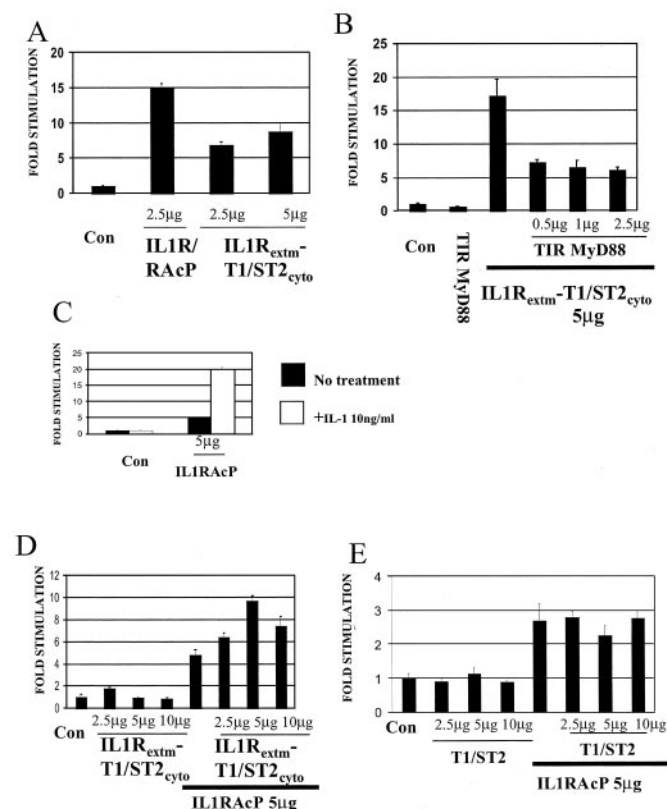


FIG. 2. Activation of NF- κ B by an IL-1 receptor-T1/ST2 chimeric construct is dependant on MyD88 and IL1RAcP. A and B, EL4 cells (7×10^6) were transiently transfected with plasmids encoding 2.5 μ g each of IL1R/IL1RAcP or the IL1R_{extm}-T1/ST2_{cyto} chimera at the indicated amounts (A) or 5 μ g of chimera and increasing amounts of plasmid encoding the isolated TIR domain of MyD88 as shown (B). C, D, and E, EL4 D6/76 cells deficient in IL1RAcP (7×10^6) were transiently transfected with 5 μ g of plasmid encoding IL1RAcP and treated with medium alone or IL-1 (10 ng/ml) for 6 h (C), the indicated amounts of chimera alone or chimera with 5 μ g of IL1RAcP (D), or the indicated amounts of plasmid encoding T1/ST2 or T1/ST2 with 5 μ g of IL1RAcP (E). Each cell population was also transfected with 5 μ g of NF- κ B luciferase plasmid and 1 μ g thymidine kinase *Renilla* luciferase for 16–18 h. Extracts were prepared and measured for luciferase activity. Results are normalized for *Renilla* luciferase activity and represented as fold stimulation over the non-stimulated EV control. Results are expressed as mean \pm S.D. from three separate experiments, each carried out in triplicate.

T1/ST2_{cyto} was unable to activate NF- κ B in these cells (Fig. 2D) implying a requirement for IL1RAcP. This was further suggested when IL1RAcP was transfected into the cells since the chimera potentiated the response to IL1RAcP alone by up to 2-fold at an IL1RAcP plasmid concentration of 5 μ g/ml (Fig. 2D). Conversely, Fig. 2E shows that co-transfection of T1/ST2 and IL1RAcP had no effect on the level of activation of NF- κ B by IL1RAcP (right side). T1/ST2 alone also has no effect on these cells (left side). Taken together, these results imply that the IL1R1-T1/ST2 chimera activates NF- κ B because it recruits IL1RAcP, which cannot be recruited by full-length T1/ST2.

Activation of AP-1 by T1/ST2 Occurs via JNK—As we had observed the ability of T1/ST2 to activate AP-1 we next investigated further upstream signals involved in this activation. JNK is a well characterized upstream activator of the AP-1 transcription factor complex. To investigate this, a reporter assay using a *trans*-reporter system of a c-Jun-GAL4 construct and Gal-luciferase, which serves as a readout for JNK activation, was used. Fig. 3A demonstrates that in a similar fashion to the combined effect of IL1R1/IL1RAcP, overexpression of T1/ST2 can activate JNK in both HEK293 (left panel) and EL4

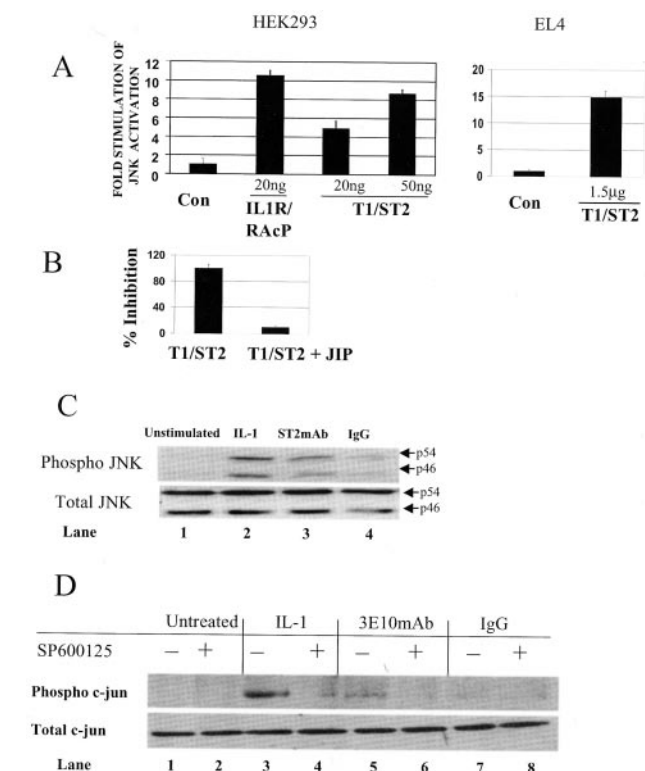


FIG. 3. T1/ST2 activates AP-1 via c-Jun N-terminal kinase. A and B, HEK 293 cells (2×10^4) or EL4 (7×10^6) were transfected with the components of GAL4-c-Jun⁽¹⁻²²³⁾ system. For HEK293 cells, 2 ng of each fusion *trans*-activator plasmid was used with 80 ng of GAL4-luciferase. For EL4 cells, 1.25 μ g of each fusion *trans*-activator plasmid was used with 5 μ g of GAL4-luciferase. Cells were also transfected with the indicated amounts of plasmid encoding FLAG-tagged T1/ST2 or B, 20 ng of JNK inhibitory protein (*JIP*). A plasmid encoding *Renilla* luciferase was also transfected (40 ng in the case of HEK293 or 5 μ g in the case of EL4). Results are normalized for *Renilla* luciferase activity and represented as fold stimulation over the nonstimulated EV control. Results are expressed as mean \pm S.D. from three separate experiments, each carried out in triplicate. C and D, P815 cells were added to 6-well plates coated with anti-T1/ST2 mAb 3E10 or control IgG for 3 h or were treated with IL-1 (10 ng/ml) for 15 min. Lysates were assayed by immunoblotting for phospho-JNK (C) or phospho-c-Jun (D). The lower panels in C and D indicate the respective levels of total JNK or c-Jun in each sample. For inhibition of JNK, cells were pretreated with SP600125 at 25 μ M for 1 h prior to addition of the cells to the plate-bound antibody or treatment with IL-1 as shown.

cells (right panel). This effect was inhibited when cells were transfected with a plasmid encoding JNK inhibitory protein-1 (*JIP*-1), implying that the effect of T1/ST2 is specific for JNK (Fig. 3B). This effect was unlikely to be due to nonspecific activation of JNK by an overexpressed protein since other overexpressed proteins such as IKK-2 and truncated forms of MyD88 had no effect (not shown). In addition, cross-linking of P815 cells also caused phosphorylation of JNK with activation occurring at 3 h (Fig. 3C, lane 3). Phosphorylation of c-Jun, as observed by cross-linking, was inhibited by the specific JNK inhibitor SP600125 (Fig. 3D, compare lanes 5 and 6). IL-1 was also found to increase JNK and c-Jun phosphorylation on P815 cells (Fig. 3C, lane 2 and 3D, lane 3).

T1/ST2 Causes Activation of p42/44 and p38 MAP Kinases—We next investigated the ability of T1/ST2 to activate p42/p44 MAP kinase using an Elk-1 *trans*-reporter system consisting of an Elk-1-GAL4 construct and Gal-luciferase, which serves as a readout for p42/44 activation. As shown in Fig. 4A, overexpression of T1/ST2 in HEK293 cells (left panel) or EL4 (right panel) activated p42/p44. 20 or 50 ng of a plasmid encoding T1/ST2 was required for this effect in HEK293 cells, while 2.5 μ g of

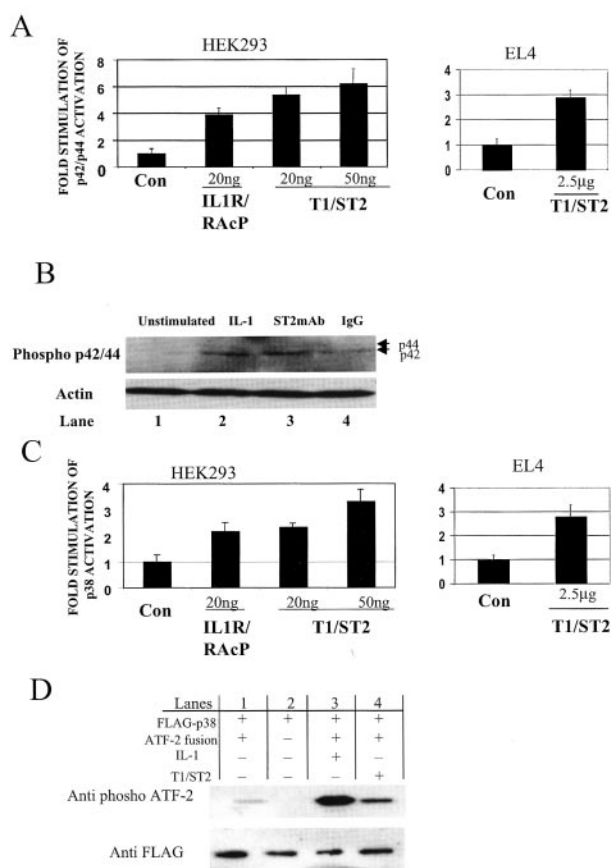


FIG. 4. T1/ST2 activates MAP kinases p42/44 and p38. HEK 293 cells (2×10^4) or EL4 (7×10^6) were transfected with the components of GAL4-Elk1⁽³⁰⁷⁻⁴²⁸⁾ (A) or GAL4-CHOP⁽¹⁻¹⁰¹⁾ (C) systems that serve as a readout for p42/44 and p38, respectively. For HEK293 cells, 2 ng of each fusion *trans*-activator plasmid was used with 80 ng of GAL4-luciferase. For EL4 cells, 1.25 µg of each fusion *trans*-activator plasmid was used with 5 µg of GAL4-luciferase. Cells were also transfected with 20 ng each of plasmid encoding IL1RI and IL1RAcP (HEK293 cells) or the indicated amounts of plasmid encoding FLAG-tagged T1/ST2 (HEK293 and EL4). A plasmid encoding *Renilla* luciferase was also transfected (40 ng in the case of HEK293 or 5 µg in the case of EL4). Results are normalized for *Renilla* luciferase activity and represented as mean \pm S.D. from three separate experiments, each carried out in triplicate. B, P815 cells were added to 6-well plates coated with anti-T1/ST2 mAb 3E10 or control IgG for 3 h or were treated with IL-1 (10 ng/ml) for 15 min, and lysates were assayed for phosphorylated p42/44 or for β -actin, which served as a loading control. D, HEK293 cells (1×10^5 /ml), were transfected with 4 µg of FLAG-tagged p38 and 4 µg of T1/ST2 (lane 4) as indicated. 16–18 h posttransfection, cells were harvested and the kinase assay performed as described under “Experimental Procedures.” For the sample in lane 3, cells were treated with IL-1 (10 ng/ml) for 15 min prior to harvesting. Samples were assayed for phosphorylated ATF-2 or FLAG-tagged p38 by immunoblotting. Identical results were obtained in an additional experiment.

plasmid was required for EL4 cells. 20 ng each of IL1RI and IL1RAcP were able to activate p42/44 in HEK293 cells (*left panel*). As shown in Fig. 4B cross-linking of P815 cells for 3 h also activated p42/44 MAP kinase (*lane 3*) as indicated by increased phosphorylation when compared with unstimulated cells (*lane 1*) or cells plated on control IgG (*lane 4*).

We also measured the effect of T1/ST2 on p38 MAP kinase. The first assay used was an overexpression system using plasmids encoding CHOP-Gal4 and Gal-luciferase, which serve as a readout for p38 activation. As can be seen in Fig. 4C, overexpression of a plasmid encoding T1/ST2 activated p38 MAP kinase at 20 and 50 ng in HEK293 (*left panel*) and at 2.5 µg in EL4 cells (*right panel*). This effect was confirmed in another

TABLE I
Effect of anti-T1/ST2 mAb IL-4 and IFN- γ production in naive CD4⁺ cells

Splenocytes from naive BALB/c mice were isolated and incubated in medium alone or medium containing the JNK inhibitor SP600125 (25 µM) for 1 h at 37°C. 1-ml aliquots containing 1×10^6 cells were then added to 24-well plates coated with anti-T1/ST2 mAb 3E10 or control IgG for 1 h. Brefeldin A was then added to each well for 4 h, after which intracellular IL-4 or IFN- γ was determined in CD4⁺ cells as described under “Experimental Procedures.” Data are presented as the mean percentage of cells that had detectable IL-4 or IFN- γ . Values are representative of data obtained in four separate experiments.

| Treatment of cells | % IL-4 producing cells | % IFN- γ producing cells |
|------------------------|------------------------|---------------------------------|
| Anti T1/ST2 | 9.34 | 1.07 |
| Anti T1/ST2 + SP600125 | 1.58 | 1.29 |
| IgG | 0.75 | 1.17 |
| IgG + SP600125 | 0.9 | 1.30 |

assay for p38, involving immunoprecipitation of a FLAG-tagged p38 construct from T1/ST2 transfected cells, the immunoprecipitate being assayed for its ability to phosphorylate ATF-2. Phosphorylation of ATF-2 was measured by immunoblotting samples using a phospho-ATF-2 antibody. As can be seen in Fig. 4D, phosphorylation of ATF-2 was enhanced in anti-FLAG immunoprecipitates from cells co-transfected with T1/ST2 and FLAG-p38, when compared with anti-FLAG immunoprecipitates from cells transfected with FLAG-p38 alone (compare *lane 4* to *lane 1*). *Lane 2* is a specificity control since there is no substrate in the assay, and *lane 3* shows that IL-1, a known activator of p38, is able to induce ATF-2 phosphorylation in this assay. The level of p38 in all samples is equivalent (*lower panel*).

Induction of IL-4 by Cross-linking T1/ST2 in Naive T Cells Is Mediated by JNK—Using four separate assays (transfection-based assays for JNK and AP-1 and phosphorylation of JNK and c-Jun) we had demonstrated JNK to be a key effector in T1/ST2 signaling. We therefore tested the functional significance of JNK activation by T1/ST2. As all T1/ST2 signaling studies performed above were on cell lines we investigated whether cross-linking stimulated IL-4 (Th2-inducing) or IFN- γ (Th1-inducing) production in naive CD4⁺ cells. It has previously been reported that cross-linking of T1/ST2 in Th2 cells results in increased production of the Th2 cytokines IL-4 and IL-5 (32). As shown in Table I, T1/ST2 cross-linking induced a 9-fold increase in the frequency of IL-4-producing cells compared with control IgG-treated cells. Consistent with the known propensity of T1/ST2 to induce Th2 responses (32) there was no increase in IFN- γ -producing Th1 cells following T1/ST2 cross-linking. The increase in frequency of IL-4-producing cells elicited by T1/ST2 cross-linking was almost abolished when cells were pretreated for 1 h with the JNK inhibitor SP600125 (Table I). Reduced IL-4 in cells treated with SP600125 was not due to cell cytotoxicity, as negligible cell death (as assessed by trypan blue exclusion) was seen in all groups. T1/ST2 cross-linking of naive spleen cells therefore selectively up-regulates a Th2, but not a Th1, response via a JNK-mediated pathway.

DISCUSSION

In this study we have examined T1/ST2 signal transduction using either heterologous overexpression of T1/ST2 or an activating T1/ST2 antibody. Overexpression of IL1RI and IL1RAcP has been shown by us and others to activate IL-1 type signals indicating the usefulness of the overexpression approach in the absence of a ligand (34, 35). This approach has also been used for characterizing signaling pathways of the novel TLRs whose ligands are unknown, although the approach differed slightly as CD4 fusions were made of TLRs 7, 8, and 9 prior to overexpression (36). Using these approaches we have

found that T1/ST2 is a functional member of the Toll/IL1R superfamily capable of activating signaling pathways. Moreover, we found a difference between T1/ST2 and other members of this family in that although T1/ST2 can activate AP-1, JNK, p42/p44, and p38 MAP kinase, it apparently does not activate NF- κ B. This is consistent with a role for T1/ST2 in Th2 cell function since NF- κ B has been more strongly implicated in Th1 responses rather than Th2 and also concurs with conclusions drawn by Kumar *et al.* (17) on an unidentified T1/ST2 ligand that activates p38 but not NF- κ B. Our data provide functional relevance for the observed JNK activation since the selective induction of IL-4 in CD4⁺ T cells following T1/ST2 cross-linking was blocked by the specific JNK inhibitor SP600125.

Members of the Toll/IL-1 receptor superfamily are structurally related and are primarily categorized on the basis of a conserved intracellular region called the TIR domain. It previously has been shown in the case of the IL1R1, IL18R, and several of the TLRs that this TIR domain is essential in mediating transcriptional activation via these receptors. Signaling pathways activated by these receptors overlap in all cases so far investigated and include the transcription factors NF- κ B and AP-1 and MAP kinases (14). Previously it has been shown that a chimeric receptor of the extracellular and transmembrane domain of the IL1R1 and the cytoplasmic domain of T1/ST2 was able to signal NF- κ B activation (27). However, our data suggests that this effect is dependent on IL1RAcP. It is known that upon binding IL1, IL1R1 and IL1RAcP interact via their extracellular domains, so the signal transmitted via this chimera is likely to be due to interaction of the IL1R1 extracellular portion of the chimera with the IL1RAcP (37). Our evidence for this is the lack of effect of the chimera on NF- κ B in a strain of EL4, which lacks IL1RAcP. Transfection of the cells with a plasmid encoding IL1RAcP, however, renders the cells sensitive to the chimera. Overexpression of T1/ST2 or cross-linking of T1/ST2 is unlikely to recruit IL1RAcP. Our findings are in agreement with those reported by Born *et al.* (38) who have shown that the IL1RAcP can act as an accessory protein for the IL1R_{extm}-T1/ST2_{cyto} chimera following addition of IL-1 in S49.1 cells.

In the case of IL-1, it is known that activation of JNK and AP-1 by IL1R1 requires IL1RAcP similar to the activation of NF- κ B by this receptor (39, 40). It may be that the cells tested here do not express the receptor accessory protein (as yet unknown) required by T1/ST2 for NF- κ B activation or that the overexpression and antibody approach is unable to allow accessory protein recruitment. Overexpression of T1/ST2, however, can clearly drive other signals in the cells, notably activation of AP-1, JNK, p38, and p42/p44 MAP kinases. Furthermore, cross-linking of T1/ST2 can drive these signals and lead to IL-4 but not IFN- γ production. This cross-linking would result in homodimerization of T1/ST2, which may induce different signals from those induced by a heterodimeric complex. It therefore remains a possibility that formation of such a heterodimer is required for NF- κ B activation but not activation of MAP kinases. However, given that the activation of MAP kinases requires both IL1R1 and IL1RAcP in the case of the IL-1 system, it is also a possibility that the T1/ST2 accessory protein is recruited upon overexpression or cross-linking of T1/ST2, but does not activate NF- κ B. It is possible that this putative accessory protein might have already been identified and classed as one of the other orphan receptors of the IL1R family. Born *et al.* (38) have attempted to classify IL1R superfamily members into receptors or accessory proteins based on chimera studies. Only the two known accessory proteins (IL1RAcP and IL18RAcP) were categorized as accessory proteins. In addition, two novel members of the family, TIGGIR1 and IL1RAPL seem to fall

into neither group and might therefore constitute a new subgroup of the family. It would be interesting to investigate whether the full-length version of either of these can interact with T1/ST2.

The lack of effect of T1/ST2 on NF- κ B suggests that not all TIR domain-containing receptors signal in a similar way. This has also been shown for TLRs, where TLR2 and TLR4 differ in the sets of genes their ligands induce (33) and also in their ability to differentially activate dendritic cells to favor either Th1 or Th2 development (41). Furthermore, TLR4 but not IL-1, IL-18, or CpG DNA (acting via TLR9) can activate NF- κ B in the absence of MyD88 using the novel adapter protein Mal (MyD88 adapter like) (42, 43). Our data therefore concurs with the concept that TIR domain-containing receptors, although capable of activating similar signals, may activate (or fail to activate) other divergent signals. The basis for this difference may lie in each TIR domain, which although similar in amino acid sequence, are not identical. In fact, although the structures of the TIR domain in TLR1 and TLR2 are broadly similar there are clear differences (44). A fuller understanding of the TIR domain will await further structural determination.

Our signaling data with T1/ST2 is an important confirmation of a previous report on T1/ST2 signaling that identified a putative ligand. Using a partially purified ligand Kumar *et al.* (17) demonstrated a lack of effect on NF- κ B but were able to observe activation of p38 MAP kinase. This provides additional evidence that T1/ST2 will not be able to affect NF- κ B.

Given the probable role of T1/ST2 in Th2 effector function, the lack of effect of T1/ST2 on NF- κ B is consistent with evidence from transgenic mice whose NF- κ B/Rel-signaling pathway is inhibited in T cells (45). NF- κ B induction was specifically inhibited in T cells by a mutated form of I κ B α . These mice exhibited impaired Th1 responses but normal Th2 responses indicative of the lack of a role for NF- κ B in Th2 function. It has also recently been reported, however, that Gata-3, a Th2-specific transcription factor whose expression is essential for IL-5 production, is dependant on NF- κ B (46). The precise role of NF- κ B in Th1 and/or Th2 cell regulation is therefore, yet to be clarified.

With respect to JNK and p38, although both of these kinases have been implicated in Th1 function (47), there is also evidence that they regulate the expression of genes in Th2 cells, such as IL-4 activation that requires JunB (48) and IL-5 that is induced by cAMP via p38 (49). p42/p44 has also been shown to play a role in Th2 cell function since TCR-mediated activation of p42/p44 is required for Th2 cell differentiation (50). We tested a role for JNK in a physiologically relevant response to T1/ST2 activation, namely the induction of IL-4. Cross-linking of T1/ST2 in Th2 cells had been shown to induce IL-4 (32). We have shown here that this selective induction of Th2 but not Th1 responses can also be observed in naive T cells, and importantly we have demonstrated that this response required JNK activation since it was blocked by a specific JNK inhibitor SP600125. Activation of JNK and probably the other MAP kinases that we have studied is therefore likely to be important for the role played by T1/ST2 in T cell function. Furthermore, the observation that anti-T1/ST2 induces IL-4 in naive T cells implies that T1/ST2 may drive a Th2-polarising signal in T cells.

In conclusion, T1/ST2 is a TIR domain-containing receptor, which appears to selectively activate MAP kinases without affecting NF- κ B, consistent with its role in Th2 cell regulation. The ligand for T1/ST2, which has yet to be identified, would be strongly predicted to target the same signals.

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