The Expression of ST2 Gene in Helper T Cells and the Binding of ST2 Protein to Myeloma-Derived RPMI8226 Cells[1]

Ken Yanagisawa,[*,2] Yoshiyuki Naito,[†] Kenji Kuroiwa,[*,‡] Takao Arai,[‡] Yusuke Furukawa,[§] Hiroshi Tomizuka,[||] Yasusada Miura,[||] Tadashi Kasahara,[¶] Tsunao Tetsuka,[*] and Shin-ichi Tominaga[*]

*Department of Biochemistry II, §Division of Hemopoiesis, Institute of Hematology, and ||Division of Hematology, Department of Medicine, Jichi Medical School, 3311-1 Yakushiji, Minamikawachi-machi, Kawachi-gun, Tochigi 329-04; ‡Department of Applied Biological Science, Faculty of Science and Technology, Science University of Tokyo, Noda, Chiba 278; ¶Kyoritsu College of Pharmacy, Minato-ku, Tokyo 105; and †Department of Cell Signaling, DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA 94304, USA

Received for publication, September 2, 1996

The ST2 gene, which is specifically induced by growth stimulation, encodes interleukin-1 receptor-related proteins. Using the RT-PCR method, we found that the ST2 gene was broadly expressed in hematopoietic cell lines. It was also expressed specifically in helper T cell lines among lymphocytic cell lines. We analyzed the expression of ST2 in mouse helper T cell subsets with Northern blotting analysis. Mouse Th1 cell lines so far studied did not express ST2 mRNAs. On the other hand, one of the Th2 cell lines, D10, expressed ST2L (transmembrane form) without stimulation, while co-stimulation by PMA and A23187 induced ST2 (soluble form) mRNA. These results suggest that the ST2 gene is involved in the regulation of the immune system. IL-1α, IL-1β, and receptor antagonist did not bind to ST2L protein, which prompted us to search for the specific ligand of ST2. The recombinant human ST2 protein was purified and labeled with FITC. The labeled human ST2 protein bound with myeloma-derived RPMI8226 cells among the various B-cell lines, indicating possible involvement of ST2 in T-cell/B-cell interaction.

Key words: helper T-lymphocytes, interleukin-1 receptor family, ligand of ST2, ST2 gene product, Th1 and Th2 cells.

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 2, 2012

Many ligand-receptor systems, especially a number of cytokine systems, are involved in cell-cell interactions, such as cell growth regulation, immune reaction, and inflammation. The IL-1 and IL-1 receptor system is one of the best-studied cytokine-receptor systems, but its signal transducing mechanisms are still not clearly understood when compared with those of other cytokines (*1*).

The ST2 gene was originally found to be one of the primary response genes in the G_0/G_1 transitional state of BALB/c-3T3 cells (*2*), and a Ha-*ras* oncogene-responsive gene (*3*). Subsequently, we cloned ST2L cDNA, encoding a membrane-bound protein whose extracellular domain is almost identical to the ST2 protein, and which shows a striking overall similarity to IL-1R type I, based on its amino acid sequence (*4*). The ST2 gene products were clarified to consist of two forms, a soluble secreted form (ST2) and a transmembrane form (ST2L), and both are members of the IL-1R family, which in turn belongs to the immunoglobulin superfamily (*5*).

Recently, the presence of an IL-1R accessory protein (IL-1R AcP), which forms a high-affinity IL-1 binding complex with IL-1R type I (IL-1R I), has been reported (*6*). IL-1R AcP is also one of the IL-1R family members, possessing three immunoglobulin-like domains. The similarities between muIL-1R I and muIL-1R AcP, muIL-1R I and muST2L, and muIL-1R AcP and muST2L are 26.7, 28.3, and 25.7%, respectively. Furthermore, the murine ST2 gene was mapped on chromosome 1 (*7*), and the human ST2 gene was mapped on chromosome 2 (*8*). In both cases, the *St2* loci were very tightly linked to *Il-1rI* and *II* loci. This evidence taken together suggests close functional relationships among these members of the IL-1R superfamily.

The ST2 protein was reported not to bind with IL-1s (*9, 10*) or to bind only slightly with IL-1β (*11*); therefore, ST2L is thought to react with distinct ligand(s) other than IL-1s. Eventually, candidates for ligands of ST2 protein were purified or cloned and appeared to be different from IL-1s (*9, 10*).

In this study, we investigated the expression pattern of the ST2 gene in hematopoietic cell lines at the messenger and protein levels in order to understand the physiological function of ST2 and ST2L proteins. The expression of the ST2 gene was observed in a variety of hematopoietic cell

[1] This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan.

[2] To whom correspondence should be addressed. Phone: +81-285-44-2111 (Ext. 3143), Fax: +81-285-44-2158, E-mail: kenyanag@jichi.ac.jp

Abbreviations: Bt_2 cAMP, $N^6,O^{2'}$-dibutyryl cyclic AMP; dNTPs, deoxynucleotide triphosphates; FITC, fluorescein isothiocyanate; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1R, interleukin 1 receptor; IL-1ra, interleukin 1 receptor antagonist; IL-1R AcP, interleukin 1 receptor accessory protein; mAb, monoclonal antibody; mu, murine; PBMC, peripheral blood mononuclear cells; PMA, phorbol myristate acetate; RT-PCR, reverse transcription-polymerase chain reaction.

lines other than lymphocytic cells with RT-PCR, and specifically in Th2 cells of lymphocytic cell lines. In our hands, the ST2L protein also did not bind with IL-1s, and the cloned candidate for the ligand of ST2 protein was reported to be unable to initiate signal transduction (*10*); therefore, we searched for the membrane-bound ligand(s) of ST2 proteins on B-cell lines. FITC-labeled recombinant human ST2 protein appeared to bind specifically with myeloma-derived RPMI8226 cells. These observations suggest the possible implication of the ST2 and ST2L proteins in the immune system.

MATERIALS AND METHODS

Cell Preparation and Culture—PBMC was isolated by sedimentation on Ficoll-Hypaque density gradients as described previously (*12*). Monocytes and lymphocytes were further purified from the PBMC by centrifugal elutriation in a Hitachi SRP6Y rotor (Hitachi, Tokyo) (*13*).

HPB-ALL, HPB-MLT, MOLT-3, CCRF-CEM, NALM-1, NALM-6, BALL-1, Daudi, Raji, U-266, RPMI8226, JOSK-I, KCL-22, U-937, THP-1, KG-1, ML-1, HL-60, KU812, K562, and HEL cells were kindly provided by Drs. M. Ohta and M. Saito (Cancer Institute, Hokkaido University, School of Medicine). UT-7 cells were gift from Dr. N. Komatsu (Division of Hematology, Department of Internal Medicine, Jichi Medical School), and TF-1 and EL-4 cells were from Drs. R. Tsuruta, S. Watanabe, and T. Yokota (Department of Molecular Biology, The Institute of Medical Science, The University of Tokyo). Cells were basically cultured with RPMI 1640 plus 10% FCS. The UT-7 cells were cultured as described (*14*). COS7 cells were a gift from Dr. S. Nagata (Osaka Bioscience Institute).

RT-PCR—Total RNA was extracted from cells by the acid guanidinium thiocyanate method (*15*). Otherwise, total RNAs from 2F1, 5C10, SP-B21, and TA23 were gifts from Drs. Y. Harada and T. Yokota (Department of Molecular Biology, The Institute of Medical Science, The University of Tokyo) (*16*).

To obtain cDNA, 10 μg of total RNA was denatured at 70°C for 10 min and immediately chilled on ice. Then first-strand cDNA was synthesized with the denatured total RNA in a total volume of 20 μl containing 100 pmol of random hexamer oligodeoxynucleotides (Takara Shuzo, Shiga), 1.25 mM dNTPs, 1mM dithiothreitol, 20 U of RNase inhibitor (Toyobo, Osaka), 200 U of Moloney murine leukemia virus reverse transcriptase (BRL) in the reaction buffer, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, and 1 mg/ml BSA. After incubation for 60 min at 37°C, the enzyme was denatured at 70°C for 10 min.

PCR was carried out in a final volume of 50 μl containing 1.25 U of Taq polymerase (Takara Shuzo), 200 μM dNTPs, and 1 μM each of the forward and reverse primers, in a buffer consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 2 μl of the reverse-transcribed total RNA mentioned above. The primers used to amplify a 393-bp fragment of the human ST2 gene, corresponding to the nucleotide numbers from -17 to 376 of the human ST2 cDNA (*16*), were 5′-CTTGATTGATAAACAGAATG-3′ (forward) and 5′-CTGATCCAGATACTGTTGAA-3′ (reverse). The samples were denatured initially at 94°C for 2 min and amplification was performed for 40 cycles with denaturation at 94°C for 1 min, annealing at 50°C for 2 min, and extension at 72°C for 3 min. Ten microliters each of the PCR samples were analyzed by 5% polyacrylamide gel electrophoresis (PAGE).

Northern Blotting Analysis—Mouse helper T cell clones were incubated with or without stimulation for the indicated period. Depending on the cell line, 10 or 50 ng/ml of PMA (Calbiochem, La Jolla, CA), 0.5 or 1 μM A23187 (Calbiochem), 10 μg/ml of cyclosporin A (CsA), 10 μM prostaglandin E_2 (PGE_2), and 1 mM Bt_2 cAMP were used for stimulation. After the incubation, the total RNA was extracted from the cell lines as described above, and in some experiments, poly(A)RNA was isolated using the Fast Track™ mRNA Isolation Kit (Invitrogen, San Diego, CA).

Northern blotting was performed as described in Ref. *17* using the following cDNA probes: mouse ST2 [a 1.6-kb *Hinc*II fragment of mouse cDNA (*2*)], and mouse IL-5 [a 1.5-kb *Bam*HI fragment of pcDSRαmoIL-5 (*18*)].

Immunoprecipitation—The cell lysate in 1 ml of the lysis buffer [10 mM Tris-HCl (pH 7.8 at 20°C), 0.15 M NaCl, 1 mM EDTA, and 1% (w/v) Nonidet P-40] was precleared by adding 50 μl of a 50% (v/v) suspension of Protein A-conjugated Sepharose to the lysis buffer, and the tube was rotated for 1 h at 4°C. 1B2 mAb against human ST2 was added to the supernatant (*14*), and the tube was rotated at 4°C for 1 h. Then 30 μl of a 50% (v/v) suspension of Protein A-conjugated Sepharose was added to the tube and the tube was rotated for another hour at 4°C. After centrifugation, the pellet was washed three times with the lysis buffer. The pellet was suspended in 150 μl of a 2-fold concentrated sample buffer for SDS-PAGE, and heat-treated at 95°C for 10 min, then the tube was centrifuged. The resultant supernatant (50 μl) was processed for SDS-PAGE, and subsequently for immunoblotting.

Immunoblotting—Immunoblotting was performed as described by Takagi *et al.* (*19*) with some modifications. The proteins separated by SDS-PAGE were electrophoretically transferred to an Immobilon P membrane filter (Millipore) in 0.1 M Trizma base/0.192 M glycine by a semidry blotting system (Nihon Eidou, Tokyo). The filter was blocked in 10 mg/ml of skim milk in Tris-buffered saline containing 0.05% (v/v) Tween-20 (TTBS). The immunoblotting was performed with a mAb G7 (IgM class) against the human ST2 gene product (*14*) in the blocking solution. After 1 h at room temperature, the filter was washed three times with TTBS, then kept for 1 h at room temperature with peroxidase-conjugated goat anti-mouse IgM antibody (Kirkegaard and Perry Lab., Gaithersburg, MD). After washing as above, the protein bands were detected by chemiluminescence using the ECL system of Amersham.

IL-1 Binding Assay—A 1.7-kb *Eco*RI-*Bgl*II fragment of mouse IL-1R type I cDNA (gift from Drs. K. Kuno and K. Matsushima, Cancer Research Institute, Kanazawa University), and a 1.7-kb *Hinc*II-*Apa*I fragment of mouse ST2L cDNA were inserted into the *Xba*I site of the pEF-BOS plasmid (provided by Dr. S. Nagata, Osaka Bioscience Institute) with *Xba*I adaptor. They were introduced into the COS7 cells as described in Ref. *19*. After transfection, the COS7 cells were incubated for 2 days at 37°C, then the binding of the radiolabeled IL-1s to the cells was studied in duplicate in a reaction mixture of 200 μl for 1 h at room temperature, and the bound IL-1s were measured after centrifugation through phthalate oil as

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 2, 2012

described previously (*20*). Murine ^{125}I-labeled IL-1β was purchased from Du Pont-New England Nuclear, while murine IL-1α and human IL-1ra were from Genzyme and R&D, respectively. IL-1α and IL-1ra (5 μg) were each radiolabeled with [^{35}S]Sulphur labeling reagent (Amersham) according to the manufacturer's protocol.

Production and Purification of Recombinant Human ST2 Protein—The expression vector (pEF-BOS-ST2H) containing the entire coding region of human ST2 (hST2) cDNA was constructed and transfected into COS7 cells as described in Ref. *14*. After 24 h, the medium was replaced with serum-free Dulbecco's modified Eagle's medium, and cultured for another 24 h. Then the culture fluid was collected and processed for purification. Two hundred milliliters of the conditioned medium from 20 dishes (diameter 10 cm) was used as a starting material. First, 2 volumes of 20 mM Tris-HCl (pH 7.5 at 20°C) were added to decrease the salt concentration of the conditioned medium, and the sample was applied to a heparin-agarose column (bed volume 10 ml) equilibrated with buffer A (20 mM Tris-HCl (pH 7.5 at 20°C), 50 mM NaCl). The loading flow rate was 5 ml/h. Then the column was washed with 50 ml of buffer B [20 mM Tris-HCl (pH 7.5 at 20°C), 0.1 M NaCl] at a flow rate of 10 ml/h. hST2 protein was eluted with a gradient of 0.1 to 0.5 M NaCl in 100 ml of 20 mM Tris-HCl (pH 7.5 at 20°C) at a flow rate of 4 ml/h, and fractions of 1 ml were collected (fractions 1-90). The presence of hST2 protein was monitored with immunoblotting using the G7 mAb against hST2 protein (*14*). The fractions containing hST2 protein (fractions 60-72; 13 ml) were combined, diluted 10-fold with buffer C (20 mM triethanolamine, pH 7.5), and applied to a Mono Q HR 5/5 column (Pharmacia/LKB Biotechnology) equilibrated with buffer C. The column was washed with 5 ml of buffer C, and then hST2 protein was eluted with a linear gradient of 0 to 1 M NaCl in 20 ml of buffer C. Fractions of 1 ml were collected. The protein concentrations were determined by the method of Smith *et al.* (*21*).

SDS-PAGE and immunoblotting were performed as described previously (*14*). Silver staining analysis was carried out according to the protocol of the manufacturer using 2D-Silver Stain-II kit (Daiichi Pure Chemical, Tokyo). The samples were applied under non-reducing conditions, unless otherwise mentioned.

FITC-Labeling of Human ST2 Protein and Its Binding to B Cell Lines—The purified hST2 protein was labeled with FITC (Molecular Probes, Eugene, OR) according to the manufacturer's protocol. For flow cytometry, cells were washed with PBS containing 0.1% (w/v) BSA (PBS/BSA), resuspended with 20 μl of FITC-labeled hST2 protein (7 μg/ml) in PBS/BSA, and then incubated at room temperature for 15 min. The cells were washed with PBS/BSA twice and analyzed by flow cytometry with a FACScan (Becton-Dickinson).

RESULTS

RT-PCR Analysis of the Expression of ST2 and ST2L mRNAs in Hematopoietic Cell Lines—The expression of the ST2 gene in hematopoietic cell lines was investigated with RT-PCR. A typical result of RT-PCR analyzed with PAGE is shown in Fig. 1. The anticipated 393-bp fragment, which corresponds to the common portion of the ST2 and ST2L cDNAs, was amplified with RNAs not only from the various hematopoietic cell lines, but also from human PBMC. The results of RT-PCR are summarized in Table I. The ST2 gene was expressed in the helper T cell lines among the T cell lineage, but not in the lines of B cell lineage thus far examined. Interestingly, the Th0 cell line, SP-B21 was positive but TA23, a Th1 cell line, was negative. Other hematopoietic cell lines, including monoblastic, myeloblastic, basoblastic, erythroblastic, and megakaryoblastic cell lines, expressed the ST2 gene widely, as shown in Table I.

Expression of ST2L Protein in PBMC—To examine which population of PBMC expresses the ST2L protein, we

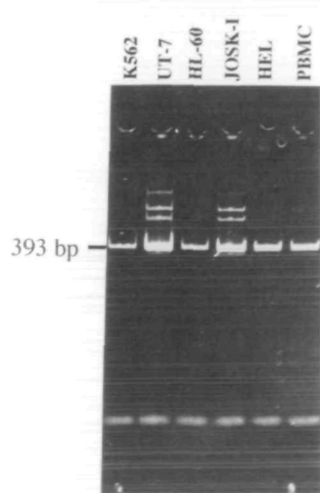

Fig. 1. **PAGE analysis of the RT-PCR products with hematopoietic cells.** RT-PCR was performed and the PCR products were analyzed as described in "MATERIALS AND METHODS." The anticipated PCR product, 393-bp in size, is marked on the left side. The source of the RNAs is indicated above the gel photograph.

TABLE I. **Expression of ST2/ST2L mRNA in various human hematopoietic cell lines.**

Lineage	Clone	Cell type	ST2/ST2L expression
T lineage	HPB-ALL	ALL-derived thymic T	−
	HPB-MLT	ALL-derived thymic T	−
	MOLT-3	ALL-derived thymic T	−
	CCRF-CEM	ALL-derived thymic T	−
	2F1	Helper T	+
	5C10	Helper T	+
	SP-B21	Helper Th0	+
	TA23	Helper Th1	−
B lineage	NALM-6	Pre-preB/preB	−
	BALL-1	Immature B	−
	Daudi	Burkitt lymphoma-derived	−
	Raji	Burkitt lymphoma-derived	−
Monocyte	JOSK-I	Monoblastic	+
	KCL-22	Monoblastic	+
	U-937	Monoblastic	+
	THP-1	Monoblastic	−
Myelocyte	TF-1	Myeloblastic	+
	KG-1	Myeloblastic	+
	ML-1	Myeloblastic	+
	HL-60	Myeloblastic	+
	KU812	Basoblastic	+
Others	K562	Erythroblastic	+
	HEL	Erythroblastic	+
	UT-7	Megakaryoblastic	+

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 2, 2012

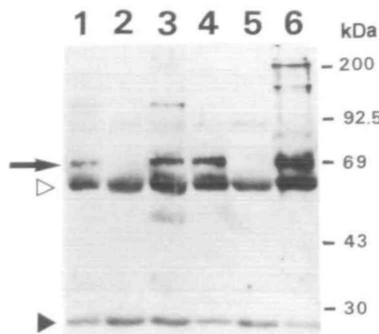

Fig. 2. **Immunoprecipitation and immunoblotting analysis for the ST2L expression in PBMC and hematopoietic cell lines.** Cells (5×10^6) were subjected to immunoprecipitation and immunoblotting analysis with anti-human ST2 mAb as described in "MATERIALS AND METHODS"; the lymphocytic fraction by elutriation (lane 1), the monocytic fraction by elutriation (lane 2), the whole PBMC (lane 3), JOSK-I cells (lane 4), and UT-7 cells (lane 6), or no cells (lane 5). The open and filled arrowheads show heavy and light chains of the immunoglobulin used in the immunoprecipitation, respectively. The arrow indicates the position of the ST2L protein.

used a mAb against the human ST2 protein, which also recognizes human ST2L protein (*14*). The expression of the ST2L protein was analyzed with immunoprecipitation and immunoblotting. As shown in Fig. 2, a band of 69 kDa was detected specifically with anti-hST2 mAb from the immunoprecipitated lysates of JOSK-I and UT-7 as positive controls (lanes 4 and 6), which were shown to express the ST2 gene with RT-PCR (Table I). The same band was also observed with the whole PBMC lysate (Fig. 2, lane 3). To obtain more information, PBMC was further divided into lymphocytic and monocytic fractions with an elutriator as described in "MATERIALS AND METHODS," and these fractions were examined separately as shown in lanes 1 and 2 of Fig. 2, respectively. The specific band which reacted with

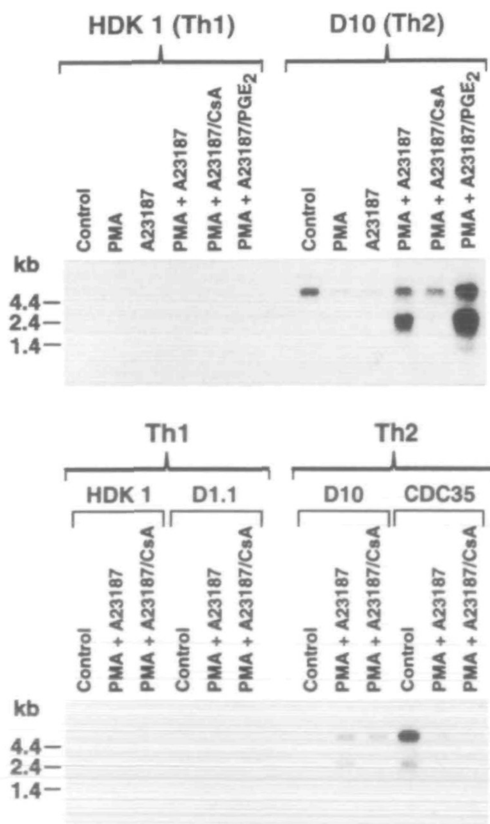

Fig. 3. **Expression of ST2 and ST2L mRNAs in mouse Th1 and Th2 cell lines.** The expression of ST2 and ST2L mRNAs was examined in mouse helper T cell lines. The indicated cell lines were cultured in the presence or absence of the described components for 6 h. PMA and A23187 were used at 50 ng/ml and 0.5 μM, respectively, and the concentrations of the other reagents were described in "MATERIALS AND METHODS." Poly(A) RNA (0.5 μg) was applied to each lane and processed for Northern hybridization using the mouse ST2 cDNA probe as described in "MATERIALS AND METHODS."

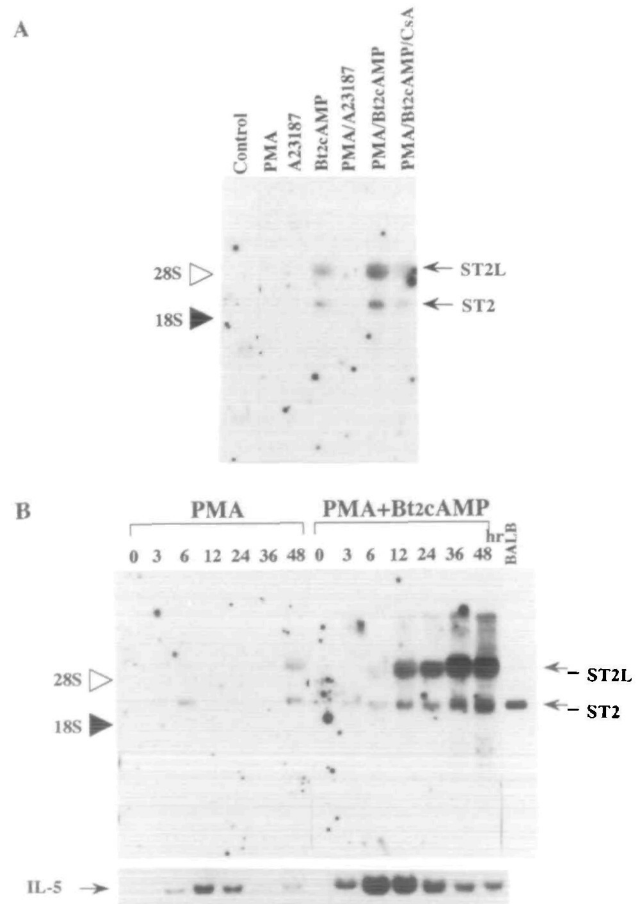

Fig. 4. **Expression of ST2 and ST2L mRNAs in the mouse thymoma cell line, EL-4.** Panel A: EL-4 cells were incubated with or without stimuli as indicated (10 ng/ml of PMA and 1 μM A23187 were used; the concentrations of other reagents were as described in "MATERIALS AND METHODS") for 12 h. Then the total RNA was extracted from the cells, and 15 μg of total RNA was applied in each lane and analyzed by Northern hybridization using the mouse ST2 cDNA fragment as a probe. Panel B: The time course of the expression of ST2 and ST2L mRNAs in the presence of 10 ng/ml of PMA or PMA plus 1 mM Bt_2 cAMP was analyzed by Northern blotting as described in Panel A. Total cellular RNA was extracted at the indicated hours after the stimuli, and hybridized with mouse ST2 cDNA fragment (upper) or mouse IL-5 cDNA fragment (lower). One hundred nanograms of poly(A) RNA prepared from serum-stimulated BALB/c-3T3 cells was applied in the lane designated BALB. Open and filled triangles correspond to the positions of 28 S and 18 S ribosomal RNA, respectively.

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 2, 2012

the anti-ST2 mAb was detected in the lysate of the lymphocytic fraction, but not in that of the monocytic fraction.

Expression of ST2 and ST2L mRNAs in Mouse Helper T Cell Lines—We observed the expression of the ST2 gene in human helper T cell lines (Table I), and of the ST2L protein in a fraction of lymphocytic cells of PBMC (Fig. 2). In order to investigate the expression mechanism of the ST2 and ST2L mRNAs in helper T cell subsets, we examined mouse helper T cell lines by Northern hybridization analysis. The poly(A)RNAs extracted and purified from mouse Th1 cell lines, HDK 1 and D1.1, and Th2 cell lines, D10 and CDC35, in the presence or absence of various stimulants, were analyzed with Northern blotting (Fig. 3) as described in Ref. *22*. The internal control, using G3PDH cDNA as a probe, presented no problem (data not shown). ST2 and ST2L mRNAs were not detected in the Th1 cell lines even when they were stimulated. On the other hand, ST2L mRNA (upper band in Fig. 3) was expressed in unstimulated Th2 cell lines, both D10 and CDC35, but ST2 mRNA (lower band in Fig. 3) was scarcely detected in unstimulated CDC35 cells and was not detected in unstimulated D10 cells. When D10 cells were stimulated with either PMA or A23187, the signals for ST2L decreased; however, with co-stimulation by PMA and A23187, ST2 mRNA was extensively induced, while the signal of ST2L mRNA was as intense as it was without stimulation. This induction was canceled by the addition of CsA, and greatly enhanced by the addition of PGE_2. In contrast to D10, the induction of ST2 mRNA was not observed upon co-stimulation by PMA and A23187 in CDC35 cells.

For a more detailed study of this Th2 cell-specific expression of ST2 and ST2L mRNAs, the mouse thymoma cell line, EL-4, was stimulated with various reagents, and analyzed by Northern hybridization. EL-4 cells were reported to express IL-2 mRNA, whose expression is specific to Th1 cells, when stimulated with PMA, whereas upon co-stimulation by PMA and Bt_2 cAMP, EL-4 cells expressed IL-5 mRNA, whose expression is specific to Th2 cells (*23*). ST2 and ST2L mRNAs were induced by stimulation with Bt_2 cAMP alone, and barely induced by PMA (Fig. 4A). When both PMA and Bt_2 cAMP were added, ST2 and ST2L mRNAs were highly induced, and this induction was inhibited by the addition of CsA, as in the case of IL-5 mRNA, indicating that the ST2 gene expression is of Th2 type. As shown in Panel B of Fig. 4, the expression of ST2 and ST2L mRNAs reached a maximal level 36 h after the stimulation, while IL-5 mRNA reached a maximal level at 6 h. Interestingly, the expression of ST2L mRNA is much greater than that of ST2 mRNA in EL-4 cells, contrary to the expression pattern in BALB/c-3T3 cells (*24*).

Binding Study of IL-1s to the ST2L Protein—Mouse IL-1R I and ST2L were transiently expressed on COS7 cells as described in "MATERIALS AND METHODS." The expression of IL-1R I and ST2L proteins on the COS7 cell surface was confirmed with immunofluorescence microscopy (data not shown). The binding activity, if any, to mouse ^{35}S-labeled IL-1α, mouse ^{125}I-labeled IL-1β, and human ^{35}S-labeled IL-1ra was examined with COS7 cells that were expressing ST2L protein. IL-1s bound only to the COS7 cells that expressed IL-1R I, but not to the cells expressing ST2L protein or to the cells that were transfect-

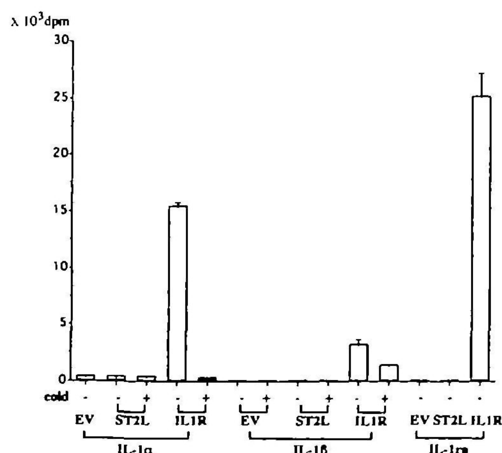

Fig. 5. **IL-1 binding assay to ST2L expressed on COS7 cells.** COS7 cells (5 ×10⁵) transfected with pEF-BOS empty vector (EV), pEF-BOS containing mouse IL-1R type I cDNA (IL1R), or pEF-BOS containing mouse ST2L cDNA (ST2L) were incubated with ^{35}S-labeled IL-1α, ^{125}I-labeled IL-1β, or ^{35}S-labeled IL-1ra with or without a 100-fold excess of the corresponding unlabeled IL-1 (cold). Binding was determined by the phthalate oil centrifugation method (*20*). The bound radioactivity is shown.

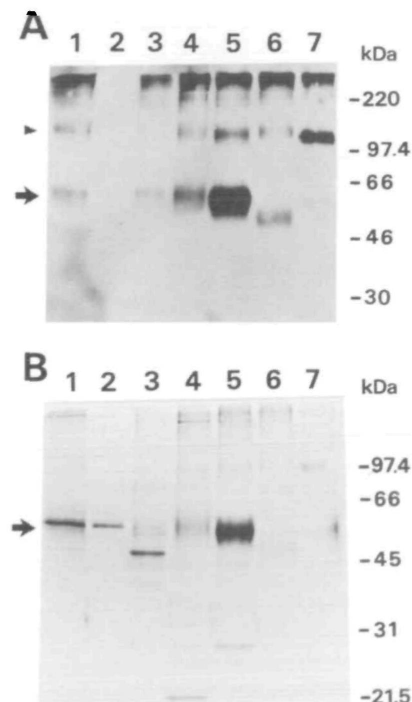

Fig. 6. **Purification of the hST2 protein; SDS-PAGE analysis of the fractions from heparin-agarose column chromatography.** Panel A: Samples were separated by 10% SDS-PAGE and processed for immunoblotting using the G7 mAb against hST2 as described in Ref. *14*. Ten microliter aliquots of the conditioned media from COS7 cells transfected with pEF-BOS-ST2H plasmid (lane 1), of the flow-through fraction from the heparin-agarose column (lane 2), and of fraction numbers 48 (lane 3), 57 (lane 4), 66 (lane 5), 75 (lane 6), and 84 (lane 7) out of 90 fractions eluted, were analyzed under the non-reducing condition. The arrow indicates the position of the glycosylated hST2 protein and the arrowhead indicates the position corresponding to the possible dimer of hST2. Panel B: The same samples as in Panel A were analyzed by silver staining as described in "MATERIALS AND METHODS."

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 2, 2012

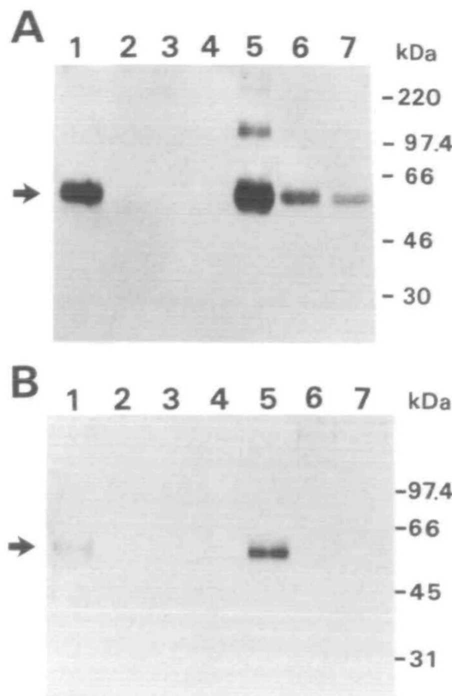

Fig. 7. **Purification of the hST2 protein; SDS-PAGE analysis of the fractions from Mono Q column chromatography.** Panel A: Aliquots of 10 μl each of the applied sample (combined fraction numbers 60 to 72 of the heparin-agarose column) (lane 1), of the flow-through fraction (lane 2), and of the samples from fraction numbers 9 to 13 (corresponding to lanes 3 to 7, respectively) out of 20 fractions eluted with a linear gradient from the Mono Q column were analyzed by immunoblotting as in Fig. 6. Panel B: The same samples as in Panel A were analyzed by silver staining.

ed with the empty vector (Fig. 5).

Expression and Purification of the hST2 Protein—The result described above and previous reports (*9, 10, 25*) suggest the presence of unknown ligand(s) for ST2 proteins. In order to search for the ligand(s), we decided to purify the recombinant hST2 protein without the artificial fused polypeptide expressed transiently in COS7 cells.

The medium containing secreted hST2 protein was collected 48 h after transfection. Employing heparin-agarose column chromatography as the first step, we could obtain the recombinant hST2 protein as a major component in SDS-PAGE and silver staining analysis (Fig. 6, panel B, lane 5). The bands of lower mobility in the immunoblotting analysis, corresponding to oligomers, disappeared and converged with the 60 kDa band in the presence of dithiothreitol, suggesting the presence of oligomeric forms of ST2 protein (data not shown). The fractions of the heparin-agarose column eluate containing the hST2 protein were then applied to a Mono Q FPLC column, and the hST2 protein was purified to apparent homogeneity (Fig. 7, panel B, lane 5). Starting with 200 ml of the culture medium, containing 200 mg of protein, we finally obtained 120 μg of the purified protein.

Binding Study of hST2 to B Cell Lines—Th2 cells have been reported to play a role in humoral immunity (*26*); therefore, Th2-specific expression of the ST2 gene suggests that B-cell lines might be candidates for cells expressing ligand(s) for the ST2 proteins.

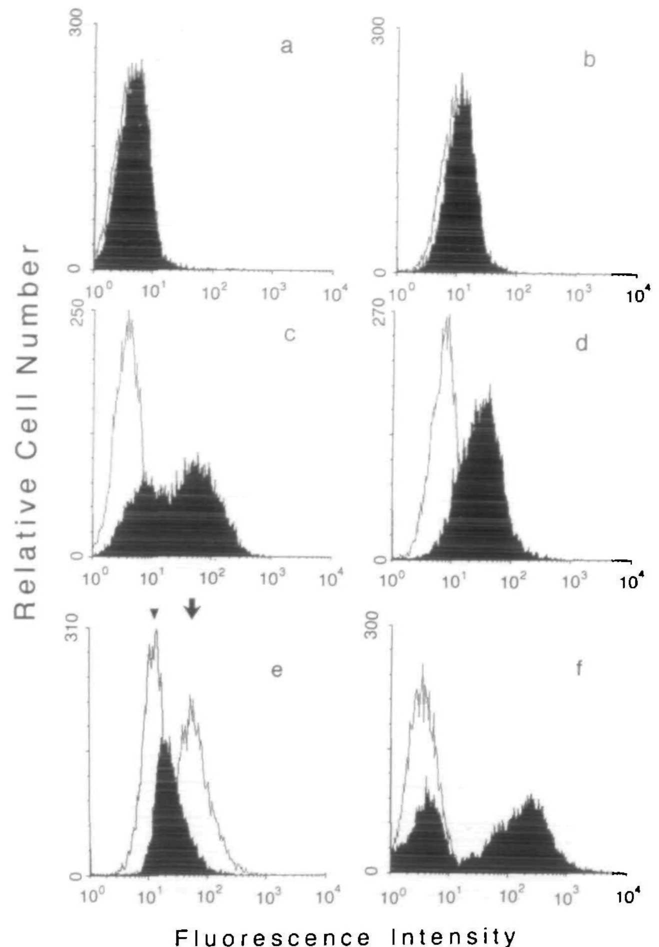

Fig. 8. **Flow cytometry of various cells with FITC-hST2.** Flow cytometric analysis was carried out on Daudi (a), Sultan (b), NALM-1 (c), RPMI8226 (d and e) cell lines, or PBMC from a myeloma patient (f). The cells were treated with FITC-hST2 (filled area) or left untreated (open area) except for Panel e. In Panel e, the arrow and the arrowhead indicate the positions of the cells with or without FITC-hST2 treatment, respectively. The flow cytometric profile of the cells treated with FITC-hST2 in the presence of a 4.3-fold excess of unlabeled hST2 is also shown (filled area in Panel e).

The purified hST2 protein was labeled with FITC and used as a probe for flow cytometric analysis in order to search for the ligand(s) of ST2 and ST2L proteins. Then, the available B-cell lines were examined by flow cytometry for the binding of FITC-labeled hST2 protein (FITC-hST2). The result is shown in Fig. 8. Daudi (Burkitt lymphoma-derived lymphoblast-like) and Sultan (multiple myeloma-derived) cells were negative for binding (Fig. 8, a and b), and Raji (Burkitt lymphoma-derived lymphoblast-like), BALL-1 (immature B), NALM-6 (ALL-derived pre-preB/preB), and U-266 (multiple myeloma-derived) cells were also negative (data not shown). On the other hand, NALM-1 (CML-blastic crisis-derived pre-preB) and RPMI8226 (multiple myeloma-derived) cells showed a positive shift in the flow cytometric profiles (Fig. 8, c and d). The reason for the heterogeneity in the case of NALM-1 cells is unclear at present, and requires further investigation. Significantly, the binding of FITC-hST2 to RPMI8226 cells was inhibited by the addition of a 4.3-fold excess of unlabeled hST2 protein (Fig. 8, e).

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 2, 2012

The PBMC of a patient suffering from multiple myeloma was examined with FITC-hST2, after his informed consent had been obtained. As shown in Panel f of Fig. 8, about 60% of the cells were highly positive for binding of the hST2 protein, while the PBMC from normal volunteers showed no such shift in the profile of flow cytometry (data not shown). This patient had become leukemic with myeloma cells, and the ratio of leukemic cells in the PBMC was 60%, as judged by May-Giemsa's staining (data not shown).

DISCUSSION

To investigate the physiological functions of the ST2 gene products, we tried to identify which cells in the hematopoietic system express this gene. Initially, ST2 mRNA was found in mouse fibroblasts (*2, 3*); then human ST2 mRNA was found to be expressed in peripheral lymphocytes, helper T cells, and a myelomonocytic cell line, KG1a, and the human ST2 cDNA was cloned from a helper T cell line, 5C10 (*16*). Other groups also reported the expression of ST2 and ST2L mRNAs in mouse hematopoietic tissues and cell lines (*25, 27*). So we decided to focus on the hematopoietic system to investigate ST2 and ST2L expression.

Among lymphocytic cell lines thus far examined, only helper T cell lines expressed ST2 and ST2L mRNAs as judged from RT-PCR analysis, whereas ST2 and ST2L were expressed widely among the other hematopoietic cell lines (Table I). Using mAbs against the human ST2 protein, we then tried to clarify what kind of cells express the ST2 and ST2L proteins in PBMC. PBMC were fractionated with an elutriator, and the ST2L protein was detected only in the lymphocytic fraction, but not in the monocytic fraction, by means of immunoprecipitation and immunoblotting analysis (Fig. 2). Microscopic observation with the immunofluorescence technique also showed that relatively large lymphocytic cells were stained with anti-ST2 mAb (data not shown). Therefore, we conclude that at least one fraction of lymphocytes in PBMC expresses ST2L protein on its surface. We also analyzed the NK cell fraction by immunoprecipitation, but ST2 and ST2L proteins were never detected in it (Yanagisawa, K. and Tominaga, S., unpublished data).

Based on these observations, we next analyzed the expression of ST2 and ST2L mRNAs in mouse helper T cell lines. Two mouse helper T cell subsets were characterized. One is Th1 cells that produce IL-2, IFN-γ, and lymphotoxin, and are thought to mediate cellular immunity, and the other is Th2 cells that produce IL-4, IL-5, and IL-10 and are proposed to be involved in humoral immunity (*26*). We found Th2-specific expression of ST2 and ST2L mRNAs by Northern hybridization analysis (Fig. 3). ST2L mRNA appeared to be expressed in the Th2 cell lines, D10 and CDC35, without stimulation. The message decreased when the cells were stimulated with PMA or A23187 alone, whereas ST2L mRNA was not decreased and ST2 mRNA was induced markedly upon co-stimulation by PMA and A23187 in D10 cells. This synergistic stimulation was not observed in CDC35 cells; on the contrary, the ST2L mRNA decreased upon co-stimulation by PMA and A23187 in these cells. According to Mosmann *et al.* (*28*), there are individual differences between clones, especially in the Th2 group. Therefore, this diversity may be due to the difference between the cells, but this remains to be clarified. The induction of soluble ST2 mRNA with PMA and A23187 was suppressed by CsA and superinduced by PGE_2, suggesting that the activation of ST2 promoter is regulated *via* the Ca^{2+} pathway involving calcineurin and the protein kinase A pathway (*29, 30*).

We then examined the expression of ST2 and ST2L mRNAs in the mouse thymoma cell line, EL-4, which was reported to express either Th1 type or Th2 type cytokines, depending on the stimuli (*23*). Our results showed that the mRNA of IL-5, one of the Th2 type cytokines, was induced slightly with PMA alone, but was markedly induced upon co-stimulation by PMA and Bt_2 cAMP, which confirmed the previous report (*23*). Like IL-5 mRNA, ST2 and ST2L mRNAs were induced with PMA and Bt_2 cAMP, but were barely induced with PMA alone (Fig. 4). These data suggest that the expression of ST2 and ST2L is similar to that of Th2 type cytokines, and that the ST2 gene products play a role in the immune system and may be involved in T-cell/B-cell interaction.

The expression of IL-5 mRNA reached a maximal level 6 h after the stimulation in EL-4 cells, whereas those of ST2 and ST2L mRNAs reached a maximal level at 36 h (Fig. 4B). This may be related to the fact that ST2 and ST2L mRNAs were also induced later than typical immediate early genes in serum-stimulated BALB/c-3T3 cells, although it has been suggested that the ST2 gene is a primary response gene (*24*). In EL-4 cells, ST2 and ST2L mRNAs were expressed simultaneously with the stimulation; however, the expressions of ST2 and ST2L are differentially regulated in D10 cells, as is shown in Fig. 3. The alternative promoter usage of Fit-1S and Fit-1M (rat homologues of ST2 and ST2L, respectively) was reported previously (*27*), and this may explain the different modes of ST2 and ST2L expression in D10 cells.

ST2 and ST2L are members of the IL-1 receptor family, and the latest member, IL-1R AcP (*6*), also shows a similar homology to IL-1R I and ST2L. IL-1R AcP was cloned as a second subunit of the IL-1R complex (*6*), but the involvement, if any, of ST2 and ST2L proteins in the IL-1R system remains unknown.

Previous papers have indicated that IL-1s do not bind with soluble ST2 protein (*9, 10, 25*), and our result, using a different assay system with the membrane bound type ST2L protein (Fig. 5), also suggests that ST2 proteins have specific ligand(s) distinct from IL-1 proteins. Recently, Kumar *et al.* reported the purification of proteins binding to ST2 protein from the conditioned media of BALB/c-3T3 cells and human umbilical vein endothelial cells (*9*), and Gayle *et al.* also reported the cloning of a candidate for a membrane bound type ligand for ST2 protein, whose ability to initiate signal transduction by ST2 protein is not clear (*10*).

Th2-specific expression of the ST2 gene led us to postulate that B-cell lines are good candidates to be screened for the expression of ligand(s) for ST2 and ST2L proteins. We highly purified the recombinant hST2 protein as shown in Figs. 6 and 7, and then labeled it with FITC to search for ligand(s) of ST2 and ST2L proteins on B cell lines. Using flow cytometry, RPMI8226 (multiple myeloma-derived) cells showed distinct ST2 binding activity among the B-cell lines, but other multiple myeloma-derived cell lines (Sultan and U-266) did not show detectable binding activity. The PBMC derived from a multiple myeloma patient also

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 2, 2012

showed a significant shift in flow cytometry, but we have not had a chance thus far to examine the blood of other leukemic multiple myeloma patients. These results suggest that some of the highly differentiated B-lymphocytes, such as plasma cells, express ligands for ST2 and ST2L proteins. Another cell line that bound with FITC-hST2 was a pre-pre B cell line, NALM-1, but other immature B-cell lines thus far examined did not bind with FITC-hST2. It remains to be elucidated whether the ligand(s) for ST2 and ST2L proteins is (are) expressed in B cells in the course of differentiation. In view of the evidence that ST2 and ST2L are expressed in Th2 cells, there is a possibility that this ligand-receptor binding might be involved in T-cell/B-cell interactions to convey immune responses.

The presence of functional membrane-bound ligands, such as the Fas ligand, has been reported in other cytokine receptor families (*31*). We are working to identify the ligand expressed on RPMI8226 cells by expression cloning.

We thank Drs. Atsushi Miyajima (Institute of Molecular and Cellular Biosciences, The University of Tokyo), Sumiko Watanabe (The Institute of Medical Science, The University of Tokyo), Hans Yssel, Naoko Arai (DNAX Research Institute of Molecular and Cellular Biology), and Shigekazu Nagata (Osaka Bioscience Institute) for valuable discussions.

REFERENCES

1. Dinarello, C.A. (1991) Interleukin-1 and interleukin-1 antagonism. *Blood* **77**, 1627-1652
2. Tominaga, S. (1989) A putative protein of a growth specific cDNA from BALB/c-3T3 cells is highly similar to the extracellular portion of mouse interleukin 1 receptor. *FEBS Lett.* **258**, 301-304
3. Werenskiold, A.K., Hoffmann, S., and Klemenz, R. (1989) Induction of a mitogen-responsive gene after expression of the Ha-*ras* oncogene in NIH 3T3 fibroblasts. *Mol. Cell. Biol.* **9**, 5207-5214
4. Yanagisawa, K., Takagi, T., Tsukamoto, T., Tetsuka, T., and Tominaga, S. (1993) Presence of a novel primary response gene ST2L, encoding a product highly similar to the interleukin 1 receptor type I. *FEBS Lett.* **318**, 83-87
5. Sims, J.E. and Dower, S.K. (1994) Interleukin-1 receptors. *Eur. Cytokine Netw.* **5**, 539-546
6. Greenfeder, S.A., Nunes, P., Kwee, L., Labow, M., Chizzonite, R.A., and Ju, G. (1995) Molecular cloning and characterization of a second subunit of the interleukin 1 receptor complex. *J. Biol. Chem.* **270**, 13757-13765
7. Tominaga, S., Jenkins, N.A., Gilbert, D.J., Copeland, N.G., and Tetsuka, T. (1991) Molecular cloning of the murine ST2 gene. Characterization and chromosomal mapping. *Biochim. Biophys. Acta* **1090**, 1-8
8. Tominaga, S., Inazawa, J., and Tsuji, S. (1996) Assignment of human ST2 gene to chromosome 2 at q11.2. *Hum. Genet.* **97**, 561-563
9. Kumar, S., Minnich, M.D., and Young, P.R. (1995) ST2/T1 protein functionally binds to two secreted proteins from Balb/c 3T3 and human umbilical vein endothelial cells but does not bind interleukin 1. *J. Biol. Chem.* **270**, 27905-27913
10. Gayle, M.A., Slack, J.L., Bonnert, T.P., Renshaw, B.R., Sonoda, G., Taguchi, T., Testa, J.R., Dower, S.K., and Sims, J.E. (1996) Cloning of a putative ligand for the T1/ST2 receptor. *J. Biol. Chem.* **271**, 5784-5789
11. Reikerstorfer, A., Holz, H., Stunnenberg, H.G., and Busslinger, M. (1995) Low affinity binding of interleukin-1β and intracellular signaling via NF-κB identify Fit-1 as a distant member of the interleukin-1 receptor family. *J. Biol. Chem.* **270**, 17645-17648
12. Furukawa, Y., DeCaprio, J.A., Freedman, A., Kanakura, Y., Nakamura, M., Ernst, T.J., Livingston, D.M., and Griffin, J.D. (1990) Expression and state of phosphorylation of the retinoblastoma susceptibility gene product in cycling and noncycling human hematopoietic cells. *Proc. Natl. Acad. Sci. USA* **87**, 2770-2774
13. Yuo, A., Kitagawa, S., Motoyoshi, K., Azuma, E., Saito, M., and Takaku, F. (1992) Rapid priming of human monocytes by human hematopoietic growth factors: Granulocyte-macrophage colony-stimulating factor (CSF), macrophage-CSF, and interleukin-3 selectively enhance superoxide release triggered by receptor-mediated agonists. *Blood* **79**, 1553-1557
14. Yoshida, K., Arai, T., Yokota, T., Komatsu, N., Miura, Y., Yanagisawa, K., Tetsuka, T., and Tominaga, S. (1995) Studies on natural ST2 gene products in the human leukemic cell line UT-7 using monoclonal antihuman ST2 antibodies. *Hybridoma* **14**, 419-427
15. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159
16. Tominaga, S., Yokota, T., Yanagisawa, K., Tsukamoto, T., Takagi, T., and Tetsuka, T. (1992) Nucleotide sequence of a complementary DNA for human ST2. *Biochim. Biophys. Acta* **1171**, 215-218
17. Tominaga, S. (1988) Murine mRNA for the β-subunit of integrin is increased in BALB/c-3T3 cells entering the G_1 phase from the G_0 state. *FEBS Lett.* **238**, 315-319
18. Yokota, T., Coffman, R.L., Hagiwara, H., Rennick, D.M., Takebe, Y., Yokota, K., Gemmell, L., Shrader, B., Yang, G., Meyerson, P., Luh, J., Hoy, P., Pène, J., Brière, F., Spits, H., Banchereau, J., de Vries, J., Lee, F.D., Arai, N., and Arai, K. (1987) Isolation and characterization of lymphokine cDNA clones encoding mouse and human IgA-enhancing factor and eosinophil colony-stimulating factor activities: Relationship to interleukin 5. *Proc. Natl. Acad. Sci. USA* **84**, 7388-7392
19. Takagi, T., Yanagisawa, K., Tsukamoto, T., Tetsuka, T., Nagata, S., and Tominaga, S. (1993) Identification of the product of the murine ST2 gene. *Biochim. Biophys. Acta* **1178**, 194-200
20. Dower, S.K., Kronheim, S.R., March, C.J., Conlon, P.J., Hopp, T.P., Gillis, S., and Urdal, D.L. (1985) Detection and characterization of high affinity plasma membrane receptors for human interleukin 1. *J. Exp. Med.* **162**, 501-515
21. Smith, P.K., Krohn, R. I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76-85
22. Naito, Y., Endo, H., Arai, K., Coffman, R.L., and Arai, N. (1996) Signal transduction in Th clones: Target of differential modulation by PGE_2 may reside downstream of the PKC-dependent pathway. *Cytokine* **8**, 346-356
23. Lee, H.J., Koyano-Nakagawa, N., Naito, Y., Nishida, J., Arai, N., Arai, K., and Yokota, T. (1993) cAMP activates the IL-5 promoter synergistically with phorbol ester through the signaling pathway involving protein kinase A in mouse thymoma line EL-4. *J. Immunol.* **151**, 6135-6142
24. Yanagisawa, K., Tsukamoto, T., Takagi, T., and Tominaga, S. (1992) Murine ST2 gene is a member of the primary response gene family induced by growth factors. *FEBS Lett.* **302**, 51-53
25. Rößler, U., Thomassen, E., Hültner, L., Baier, S., Danescu, J., and Werenskiold, A.K. (1995) Secreted and membrane-bound isoforms of T1, an orphan receptor related to IL-1-binding proteins, are differently expressed *in vivo*. *Dev. Biol.* **168**, 86-97
26. Mosmann, T.R. and Coffman, R.L. (1989) TH1 and TH2 cells: Different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* **7**, 145-173
27. Bergers, G., Reikerstorfer, A., Braselmann, S., Graninger, P., and Busslinger, M. (1994) Alternative promoter usage of the Fos-responsive gene *Fit-1* generates mRNA isoforms coding for either secreted or membrane-bound proteins related to the IL-1 receptor. *EMBO J.* **13**, 1176-1188
28. Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A., and Coffman, R.L. (1986) Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* **136**, 2348-2357

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 2, 2012

29. Liu, J., Farmer, Jr., J.D., Lane, W.S., Friedman, J., Weissman, I., and Schreiber, S.L. (1991) Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* **66**, 807-815
30. Betz, M. and Fox, B.S. (1991) Prostaglandin E_2 inhibits production of Th1 lymphokines but not of Th2 lymphokines. *J. Immunol.* **146**, 108-113
31. Suda, T., Takahashi, T., Golstein, P., and Nagata, S. (1993) Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell* **75**, 1169-1178

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 2, 2012