Title: **Involvement of NF-κB in TGF-β-mediated suppression of IL-4 signaling**

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Running title: Suppression of IL-4 signal by TGF-β

ABSTRACT

Control of immune response requires the coordinated integration of both stimulatory and inhibitory factors. Therefore, the cross-talk of different signaling pathways is critical in providing an integrated cellular response to multiple external signals. Both interleukin-4 (IL-4) and transforming growth factor (TGF-β) are pleiotropic cytokines and play critical roles in controlling immune responses. For example, IL-4 mediates important pro-inflammatory functions in asthma including induction of the IgE isotype switch, expression of vascular cell adhesion molecules. Whereas, TGF-β is secreted from B, T and dendritic cells as well as macrophages, and negatively regulates their proliferation, differentiation and activation by other cytokines. In this study, we examined the effect of TGF-β on IL-4 signaling using B cells as well as embryonic kidney cells. TGF-β inhibited IL-4-induced IgG1 production and gene expression of germline ε transcripts in B cells. In embryonic kidney cells, TGF-β signals suppressed IL-4-induced transcription, when we monitored using germline ε promoter DNA. Furthermore, activation of NF-κB resulted in a resistance to TGF-β-mediated suppression of IL-4 signaling. These results indicate that TGF-β-mediated regulation of IL-4 signaling may act by targeting NF-κB signaling.

Key words: IL-4; TGF-β; STAT6; NF-κB

INTRODUCTION

IL-4 is a pleiotropic cytokine and known as a key mediator in the development of allergic inflammation [1]. It is associated with induction of the ε isotype switch and secretion of IgE by B cells. IL-4 also increases the expression of chemokines, other inflammatory cytokines and vascular cell adhesion molecules, promotion of eosinophil transmigration across endothelium, mucus secretion. Another critical function of IL-4 is to promote the differentiation of activated T cells into Th2 effectors [2, 3]. IL-4 transduces its signal by binding to its cell surface IL-4 receptor α chain (IL-4Rα), and either the common γ-chain or an IL-13 receptor α chain, on most cell types to cause receptor dimerization and subsequent activation of intracellularly associated Jak1 and Jak2 [4]. The Jaks then phosphorylate tyrosine residues on the cytoplasmic tail of the receptor, allowing cytoplasmic STAT6 proteins to dock via their Src homology 2 (SH2) domain. STAT6 then becomes tyrosine phosphorylated by the Jaks, dissociates, dimerizes through a SH2 domain-phosphotyrosine interaction and translocates to the nucleus to bind DNA and modulate transcription [5, 6, 7]. IL-4-dependent Th2 program of cytokine genes in activated T cells is clearly mediated by STAT6, while IL-4-independent Th2 development was promoted by expression of constitutively activated STAT6 [8, 9, 10, 11].

Transforming growth factor-β (TGF-β) family of growth factors regulates diverse biological processes. TGF-β inhibits proliferation of epithelial, endothelial and haematopoietic cells, regulates the differentiation of immune, neuronal, mesenchymal and epithelial cell types and modulates their apoptotic response [12, 13, 14]. TGF-β signaling is mediated through cell membrane transmembrane receptors located at the cell surface (TβRs) that are serine/threonine kinases, which in turn use the highly

conserved members of the Smad (Sma and MAD-related protein) family of transcription factors to transduce their signals to the nucleus [15, 16]. Perturbation of TGF-β signaling is involved in autoimmunity, inflammation and cancer. A variety of murine models provide clear evidences that eliminating TGF-β or disrupting its downstream signaling cascade leads to inflammatory disease [17, 18, 19].

In this study, we examined the effect of TGF-β on IL-4 signaling in lymphoid and non-lymphoid cells. TGF-β inhibited IL-4-induced IgG1 production and gene expression of germline ε transcripts in B cells. TGF-β signals also suppressed IL-4-induced transcription in embryonic kidney cells. Furthermore, we presented the evidence that TGF-β-mediated suppression of IL-4 signaling requires NF-κB signaling.

MATERIALS AND METHODS

Reagents and antibodies

Recombinant IL-4 was purchased from Chemicon (Temecula, CA, USA). Recombinant TGF-β was purchased from Strathmann Biotech GmbH (Hamburg, Germany). Pyrrolidine dithiocarbamate (PDTC) was purchased from Calbiochem (San Diego, CA, USA). Expression vectors, p300 [20, 21], TGF-β receptor type I (T204D) [22], CD40 [23], DN-IκB, IKKαK44M, IKKβK44M [24], IL-4Rα [25], Iε-LUC [26], C/EBPβ [27], STAT6YF [28] were kindly provided by Dr. K Miyazono (Tokyo Univ. Tokyo, Japan), Dr. J. Massagué (Memorial Sloan-Kettering Cancer Center, New York, NY, USA), Dr. H. Kikutani (Osaka Univ., Osaka, Japan), Dr. T. Fujita (Tokyo Metro. Inst. Med. Sci., Tokyo, Japan), Dr. H. Sakurai (Tanabe Seiyaku, Osaka, Japan), Dr. K. Izuhara (Saga Med, Sch., Saga, Japan), Dr. M. Aoki (Sumitomo Pharmaceuticals Co., Osaka, Japan), Dr. S. Akira (Osaka Univ., Osaka, Japan), Dr. T. Kuromitsu (Yamanouchi Pharmaceutical Co., Tsukuba, Japan), and respectively. Cε probe [29] was a kind gift of Dr. T. Watanabe (Kyushu Univ., Fukuoka, Japan). Anti-STAT6, anti-NF-κB p65 anti-phosphotyrosine antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Upstate Biotechnology (Lake Placid, NY, USA), respectively.

Cell culture, transfections, and luciferase assays

Murine splenic B cells were prepared as previously described [30]. The human EBV-negative Burkitt's lymphoma cell line DND39 was a kind gift from Fujisaki Cell Center (Okayama, Japan) and maintained in RPMI1640 medium containing 10% fetal calf serum (FCS) [29]. A DND39/Iε-LUC transformant was established by co-transfecting Iε-LUC and pSV2-bsr (Funakoshi Pharmaceutical Co., Tokyo, Japan)

and selected in the above medium containing blasticidin-S (6μg/ml) (Funakoshi Pharmaceutical Co., Tokyo, Japan). 293T cells were maintained in DMEM containing 10% FCS and transfected by the standard calcium precipitation protocol. Luciferase assay was performed as described [31].

Northern blot analysis and electrophoretic mobility shift assay (EMSA)

DND39 cells were maintained as described above. Cells ($1x10^7$) were treated with IL-4 (30 U/ml) and/or TGF-β (100U/ml) for 12, or 24 h. Total RNAs were prepared using Iso-Gen (Nippon Gene) and used in Northern analysis according to established procedures. A nylon membrane (Hybond N+, Amersham Pharmacia Biotech) and radiolabelled cDNA probes, as indicated, were used. DND39 cells were stimulated with IL-4 (30 U/ml) and/or TGF-β (100 U/ml) for 30 min. Nuclear extracts were prepared and EMSA was performed as described previously [32]. The DNA probe used for EMSA is derived from the human germline ε gene promoter (See, Fig 2A) [26] and the probe for NF-κB was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA)

Immunoprecipitation and immunoblotting

The immunoprecipitation and Western blotting were performed as described previously [31]. 293T cells were harvested and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, containing 0.5% NP-40, 1 M sodium orthovanadate, 1 M phenylmethylsulfonyl fluoride, and 10 g/ml each of aprotinin, pepstatin, and leupeptin). DND39 cells were stimulated with IL-4 (30 U/ml) and/or TGF-β (100 U/ml) for 30 min. Cell lysates were immunoprecipitated with anti-STAT6 antibody. The immunoprecipitates from cell lysates were resolved on 5-20% SDS-PAGE and

transferred to Immobilon filters (Millipore, Bedford, MA, USA). The filters were then immunoblotted with each antibody. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

RESULTS AND DISCUSSION

TGF-β suppresses IL-4-induced IgG1 production and gene expression in B cells

In order to characterize the molecular basis of the cross-talk between IL-4 and TGF-β signaling pathways, we examine the effect of TGF-β on IL-4 induced gene expression of germline ε transcripts in a human B cell line, DND39, which is responsible on IL-4. To test the expression of germline ε transcripts by IL-4, we carried out Northern blot analysis on RNA samples prepared from DND39 cells, which were treated with IL-4 and/or TGF-β. Expression of germline ε transcripts by IL-4 was detected at 12h and enhanced at 24 h in the absence of TGF-β (Fig. 1A). However, TGF-β treatment resulted in a marked reduction of germline ε transcripts expression, although TGF-β alone showed no effect on its expression. Since IL-4 also induces IgG1 production in B cells, we assess IgG1 or IgM production in murine splenic B cells by IL-4 in the absence or presence of TGF-β. As shown in Fig. 1B, IL-4 strongly induced IgG1 production in murine splenic B cells in the absence of TGF-β, whereas IL-4-induced IgG1 production was not observed in the presence of TGF-β. On the other hand, IL-4 had no effect on IgM production in the absence or presence of TGF-β. We further examined whether TGF-β has any effect on IL-4-mediated transcriptional gene activation in B cells. IL-4-mediated transcriptional responses were measured by reporter gene assay using Iε-LUC, in which the human germline ε promoter [26] drives expression of the luciferase (LUC). DND39 bearing Iε-LUC, DND39/Iε-LUC cells were treated with IL-4 and/or TGF-β and LUC activities were determined. As shown in Fig. 1C, IL-4 stimulated Iε-LUC activity in a dose-dependent manner. When cells were treated with both IL-4 and TGF-β, Iε-LUC activity was decreased by 40% compared

with the activation by IL-4 alone. These data show that TGF-β suppresses IL-4-induced germline ε transcripts expression as well as Iε-LUC transcription activity in B cells. We also examined whether TGF-β stimulation has any effect on immediate early STAT6 activation by IL-4 in DND39 cells. We first assessed changes in tyrosine-phosphorylation of STAT6, which trigger its activation, in DND39 cells. To that end, DND39 cells were either left untreated or treated with IL-4, TGF-β or IL-4 plus TGF-β, and their cell extracts were prepared and subjected to immunoprecipitation using an anti-STAT6 antibody. The immunoprecipitates were then used in Western blot analysis with an anti-phosphotyrosine antibody. As shown in Fig. 2A, STAT6 was tyrosine-phosphorylated by IL-4 even in the presence of TGF-β in DND39 cells, suggesting that TGF-β had no effect of IL-4-induced tyrosine phosphorylation of STAT6. To further confirm the effect of TGF-β on IL-4-induced DNA binding activity of STAT6 or NF-κB, EMSA was performed using nuclear extract prepared from DND39 cells treated or untreated with IL-4, TGF-β or IL-4 plus TGF-β. A specific STAT6 complex was detected in the gel in nuclear extracts from cells stimulated with IL-4 or IL-4 plus TGF-β (Fig. 2B). Although a slight enhanced NF-κB complex was detected in the gel in nuclear extracts from cells stimulated with IL-4 compared to the unstimulated nuclear extracts, no significant alteration by TGF-β stimulation was observed (Fig. 2B). The addition of anti-STAT6 or anti-NF-κB p65 antibody shifted these complexes higher in the gel, whereas control IgG had no effect (data not shown). These data show that TGF-β treatment results in no alteration of immediate early STAT6 and NF-κB activation in DND39 cells.

Reconstitution of the cross-talk of IL-4 and TGF-β signaling pathway in 293T cells

To further delineate the details of the cross-talk between IL-4 and TGF-β signaling pathways, we first carried out transient transfection experiments in 293T cells by reconstitution of IL-4 signaling pathway in 293T cells by expression of IL-4 receptor α chain (IL-4Rα) together with a germline ε promoter-luciferase construct. IL-4 activity was monitored by using Iε-LUC reporter gene activity [26]. Iε-LUC is a deletion derivative of the human germline ε promoter, which contains C/EBPβ, STAT6 and NF-κB binding motifs, drives expression of the LUC gene. 293T cells were transfected with Iε-LUC and cells were stimulated with increasing amounts of IL-4. However, IL-4 did not induce a LUC expression in 293T cells, when we transfected with Iε-LUC (Fig. 3A). Previous studies have shown that p300/CBP is involved in IL-4/STAT6-mediated transcriptional activation [33, 34]. We have also demonstrated that overexpression of p300 in 293T cells effectively enhances LIF/STAT3-mediated LUC expression. Therefore, we further expressed p300 together with IL-4Rα and Iε-LUC reporter gene in 293T cells. As shown in Fig. 3A, Iε-LUC activity was induced by IL-4 in a dose-dependent manner in the presence of p300. To assess whether these effects were mediated through mainly STAT6 or some other intermediary factors, we used a dominant negative form of STAT6, STAT6YF [28]. As expected, STAT6 YF significantly inhibited IL-4-induced Iε-LUC expression in a dose-dependent fashion (Fig. 3B).

To clarify the importance of the transcriptional activity by TGF-β signal in 293T cells, we used a constitutively active form of TGF-β receptor type I /TβR-I (T204D). As we described previously [21], in 293T cells, TβR-I (T204D) effectively stimulates p3TP-LUC, which is one of the standard reporters for assessing TGF-β activity [22].

We then assessed the effect of TGF-β signal on IL-4 signaling in 293T cells using TβR-I (T204D). 293T cells were transfected with Iε-LUC, IL-4Rα, p300 and an increasing amounts of TβR-I (T204D), and cells were stimulated with IL-4. As shown in Fig. 3C, TβR-I (T204D) suppressed IL-4-induced Iε-LUC activity in a dose-dependent fashion. This result indicates that the effect of TGF-β signal on IL-4-induced transcriptional activity can be reconstituted in 293T cells similar to those observed in murine splenic B cells or DND39 cells when we assess a LUC activity using Iε-LUC, which contains NF-κB binding motif. We next examined whether NF-κB activation has an effect on their LUC expression using CD40, which is well known as a NF-κB stimulator [35, 36]. When CD40 was expressed in 293T cells, Iε－LUC activity was increased in a dose-dependent fashion (Fig. 3D). Furthermore, overexpression of C/EBPβ had no effect on Iε-LUC activity in 293T cells (Fig. 3E).

Effect of NF-κB signaling on IL-4-induced Iε-LUC activity

To examine the effect of NF-κB signaling on IL-4-induced Iε-LUC activation, we used several inhibitors for NF-κB signaling, such as a dominant negative form of IκB, IKKα or IKKβ [24]. 293T cells were transfected with expression vectors for IL-4Rα, p300 and Iε-LUC and/or increasing amounts of a dominant negative form of IκB, IKKα or IKKβ, and the LUC activity was measured. As shown in Fig. 4A, B, C, IL-4-induced Iε-LUC activity was suppressed by any of NF-κB inhibitors. Furthermore, a chemically synthetic NF-κB inhibitor, PDTC treatment of DND39/Iε-LUC cells resulted in a mild reduction of IL-4-induced Iε-LUC activation (Fig. 5D). To further confirm the involvement of NF-κB on TGF-β effects on IL-4 signaling, we examined the effect of CD40 overexpression on TGF-β-mediated suppression of IL-4-induced Iε-LUC

activation in 293T cells. 293T cells were transfected with expression vectors for IL-4Rα, p300 and Iε-LUC and/or CD40 and/or an increasing amounts of TβR-I(T204D), after IL-4 stimulation, and then the LUC activity was measured. As shown in Fig 4E, TGF-β-mediated Iε-LUC suppression was not observed in the presence of CD40. These results indicate that the inhibitory effect of TGF-β on IL-4 signaling in 293T cells is mediated by targeting NF-κB.

Concluding remarks

We have shown here that the TGF-β suppresses IL-4 signaling in lymphoid and non-lymphoid cells. TGF-β treatment inhibited IL-4-induced IgG1 production and endogenous germline ε transcripts expression in B cells as well as IL-4-dependent reporter activity in 293T cells. We also demonstrated that activation of NF-κB resulted in a resistance to TGF-β-mediated suppression of IL-4 signaling.

Activation of the germline ε promoter in B cells triggers the recombination event leading to class switching and expression of the IgE isotype. The promoter is activated in response to multiple signals, especially IL-4 [37]. The IL-4 responsive element has previously been characterized and shown to contain binding sites for C/EBPβ, STAT6 and NF-κB [37, 38]. It has also been reported that functional synergies between them in driving IL-4-induced expression of the germline ε transcripts, although overexpression of C/EBPβ failed to activate IL-4 signaling in our system. NF-κB is a dimeric transcription factor that plays a central role in the regulation of immune functions [39]. NF-κB subunits gene-targeted knockout mice revealed that NF-κB subunits play an important in B and T cell function as well as macrophage and dendritic cell function including proliferation, antibody production and class switching. In this

study, we used CD40 expression construct to activate NF-κB signaling in 293T cells. CD40 ligation provides B cells with an important costimulatory signal that together with B cell receptor engagement and cytokine signals leads to B cell activation [35, 36], including class switching, and differentiation to antibody-secreting plasma cells. CD40 signaling results in the activation of transcription factors including NF-κB nuclear factor of activated T cells (NF-AT), and AP-1 (activator protein-1) [39, 40]. Several reports have shown that IL-4 and CD40 lignad costimulation induces germline ε transcripts in both mouse and human B cells [41, 42]. Previous studies have focused on the trans-activating roles of STAT6 and NF-κB in activation of the germline ε promoter to explain the synergism between IL-4 and CD40 ligand stimulation [43, 44]. In fact, the essential roles of STAT6 and NF-κB p50 in induction of germline ε transcripts in vivo have been also demonstrated in gene knockout experiments, in which IL-4 and CD40 lingad are unable to induce germline ε transcripts or IgE in B cells from either STAT6- or NF-κB p50-deficient mice [9, 10, 45], suggesting that both STAT6 and NF-κB plays a crucial role in IL-4 signaling, although there have been no reports describing an interaction between STAT6 and NF-κB.

Recent studies have demonstrated that both STAT6 and glucocorticoid receptor (GR) inhibit NF-κB signaling [46, 47]. STAT6 inhibits NF-κB signaling by competing for an overlapping consensus sequence within a dual NF-κB enhancer element [46] or by competition of NF-κB for the co-activator CBP (CREB-binding protein) [47]. Furthermore, the GR is shown to bind to NF-κB and present transactivation of the target genes, without alteration of the occupancy on the DNA response elements [48]. In our present study, a significant NF-κB activation by IL-4 was not observed in DND39 cells. However, a NF-κB inhibitor affected IL-4 induced transcription in DND39 cells. These

results suggest that some modification of NF-κB by TGF-β signaling may affect the formation of effective enhanceosome assemebly including co-activator CBP. More detailed time course analysis of enhanceosome occupation by these factors will be required to understand the mechanism of TGF-β-mediated suppression IL-4 signaling in B cells.

Other TGF-β-inducible genes may also involve in the suppression of IL-4-mediated expression of germline ε transcripts. A basic helix-loop-helix protein, E2A, is known to be an essential target during B-cell activation and its induction is required to promote Ig class switch recombination [49]. Recently, it has been reported that B cells lacking Id2 increased E2A activity and underwent class switch recombination to IgE at a much higher frequency than wild-type B cells. Id2 is one of TGF-β-inducible genes [50]. Therefore, Id2 was induced in wild-type B cells by TGF-β and suppressed IgE class switch [51]. At present time, we do not know whether Id2/E2A also involves in TGF-β-mediated suppression of IL-4 transcription in 293T cells and further studies are required to clarify this issue.

The present report describes an efficient system to explore the cross-talk between IL-4 and TGF-β signaling in a B cell line or an embryonic kidney cell line. Using these system, we demonstrated that TGF-β-mediated suppression of IL-4 signaling may act by targeting NF-κB signaling. Further detailed understanding of the cross-talk between IL-4 and TGF-β using these systems is therefore important as this new information may provide new therapeutic approaches for allergic diseases.

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REFERENCES

[1] W. E. Paul, Interleukin-4: a prototypic immunoregulatory lymphokine. Blood **77** (1991) pp. 1859-1870.

[2] R. A. Seder, W. E. Paul, Acquisition of lymphokine-producing phenotype by CD4+ T cells. Annu. Rev. Immunol. **12** (1994) pp. 635-673.

[3] A. K. Abbas, K. M. Murphy, A. Sher, Functional diversity of helper T lymphocytes. Nature **383** (1996) pp. 787-793.

[4] K. Nelms, A. D. Keegan, J. Zamorano, J. J. Ryan, W. E. Paul, The IL-4 receptor: signaling mechanisms and biologic functions. Annu. Rev. Immunol. **17** (1999) pp. 7073-7081.

[5] C. Schindler, J. E. Darnell, Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. Annu. Rev. Biochem. Annu. Rev. Biochem. **64** (1995) pp. 621-651.

[6] J. N. Ihle, Cytokine receptor signalling. Nature **377** (1995) pp. 591-594.

[7] J. J. O'Shea, Jaks, STATs, cytokine signal transduction, and immunoregulation: are we there yet?. Immunity **7** (1997) pp. 1-11.

[8] K. H. Kaplan, U. Schindler, S. T. Smiley, M. J. Grusby, Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. Immunity **4** (1996) pp. 313-319.

[9] K. Takeda, T. Tanaka, W. Shi, M. Matsumoto, M. Minami, S. Kashiwamura, K. Nakanishi, N. Yoshida, T. Kishimoto, S. Akira, Essential role of Stat6 in IL-4 signalling. Nature **380** (1996) pp. 627-630.

[10] K. Shimoda, J. V. Deursen, M. Y. Sangster, S. R. Sarawar, R. T. Carson, R. A. Tripp, C. Chu, F. W. Quelle, T. Nosaka, D. A. Vignali, P. C. Doherty, G. Grosveld, W.

E. Paul, J. N. Ihle, Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene. Nature **380** (1996) pp. 630-633.

[11] W. Ouyang, M. Lohning, Z. Gao, M. Assenmacher , S. Ranganath A. Radbruch, K. M. Murphy, Stat6-independent GATA-3 autoactivation directs IL-4-independent Th2 development and commitment. Immunity **12** (2000) pp. 27-37.

[12] J. J. Letterio, A. B. Roberts, Regulation of immune responses by TGF-β. Annu. Rev. Immunol. **16** (1998) pp. 137-161.

[13] T. L. Brown, S. Patil, P. H. Howe, Analysis of TGF-β-inducible apoptosis. Methods Mol. Biol. **142** (2000) pp. 149-167.

[14] N. L. McCartney-Francis, M. Frazier-Jessen, S. M. Wahl, TGF-β: a balancing act. Int. Rev. Immunol. **16** (1998) pp. 553-580.

[15] J. Massagué and D. Wotton, Transcriptional control by the TGF-β/Smad signaling system. EMBO J. **19** (2000) pp. 1745-1754.

[16] J. Massagué, TGF-β signal transduction. Ann. Rev. Biochem. **67** (1998) pp. 753-791.

[17] M. M. Shull, I. Ormsby, A. B. Kier, S. Pawlowski, R. J. Diebold, M. Yin, R. Allen, C. Sidman, G. Proetzel, D. Calvin, N. Annunziata, T. Doetschman, Targeted disruption of the mouse transforming growth factor-β1 gene results in multifocal inflammatory disease. Nature **359** (1992) pp. 693-699.

[18] L. Gorelik, R. A. Flavell, Abrogation of TGF-β signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. Immunity **12** (2000) pp. 171-181.

[19] X. Yang, J. J. Letterio, R. J. Lechleider, Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-β. EMBO J. **18** (1999) pp. 1280-1291.

[20] A. Nishihara, J. Hanai, N. Okamoto, J. Yanagisawa, S. Kato, K. Miyazono, M. Kawabata. Role of p300, a transcriptional coactivator, in signalling of TGF-β. Genes Cells **3** (1998) pp. 613-623.

[21] T. Yamamoto, T. Matsuda, A. Muraguchi, K. Miyazono, M. Kawabata , Cross-talk between IL-6 and TGF-β signaling in hepatoma cells. FEBS Lett. **492** (2001) pp. 247-253.

[22] J. Carcamo, A. Zentella and J. Massagué, Disruption of transforming growth factor β signaling by a mutation that prevents transphosphorylation within the receptor complex. Mol. Cell. Biol. **15** (1995) pp. 1573-1581.

[23] T. Yasui, M. Muraoka, Y. Takaoka-Shichijo, I. Ishida, N. Takegahara. J. Uchida, A. Kumanogoh, S. Suematsu, M. Suzuki, H. Kikutani, Dissection of B cell differentiation during primary immune responses in mice with altered CD40 signals. Int. Immunol. **14** (2002) pp. 319-329.

[24] H. Sakurai, H. Miyoshi, W. Toriumi, T. Sugita, Functional interactions of transforming growth factor β-activated kinase 1 with IκB kinases to stimulate NF-κB activation. J. Biol. Chem. **274** (1999) pp. 10641-10648.

[25] N. Harada, K. Higuchi, H. Wakao, N. Hamasaki, K. Izuhara, Identification of the critical portions of the human IL-4 receptor alpha chain for activation of STAT6. Biochem Biophys Res Commun. **246** (1998) pp. 675-680.

[26] H. Mitsuyasu, K. Izuhara K, X. Q. Mao, P. S. Gao, Y. Arinobu, T. Enomoto, M. Kawai, S. Sasaki, Y. Dake, N. Hamasaki, T. Shirakawa, J. M. Hopkin, Ile50Val variant of IL4Rα upregulates IgE synthesis and associates with atopic asthma. Nat. Genet. **19** (1998) pp. 119-120.

[27] S. Akira, H. Isshiki, T. Sugita, O. Tanabe, S. Kinoshita, Y. Nishio, T. Nakajima, T. Hirano, T. Kishimoto. A nuclear factor for IL-6 expression (NF-IL6) is a member of a

C/EBP family. EMBO J. **9** (1990) pp. 1897-1906.

[28] J. Hou, U. Schindler, W. J. Henzel, T. C. Ho, M. Brasseur, S. L. McKnight, An interleukin-4-induced transcription factor: IL-4 Stat. Science **265** (1994) pp. 1701-1706.

[29] T. Ichiki, W. Takahashi, T. Watanabe, The effect of cytokines and mitogens on the induction of Cε germline transcripts in a human Burkitt lymphoma B cell line. Int. Immunol. **4** (1992) pp. 747-754.

[30] R. L. Coffman, J. Ohara, M. W. Bond, J. Carty, A. Zlotnik, W. E. Paul, B cell stimulatory factor-1 enhances the IgE response of lipopolysaccharide-activated B cells. J. Immunol. **136** (1986) pp. 4538-4541.

[31] T. Matsuda, T. Yamamoto, H. Kishi, A. Yoshimura, A. Muraguchi, SOCS-1 can suppress CD3ζ- and Syk-mediated NF-AT activation in a non-lymphoid cell line. FEBS Lett. **472** (2000) pp. 235-240.

[32] H. Kishi, X. -C. Wei., Z, -X. Jin., Y. Fujishiro., T. Nagata., T. Matsuda, A. Muraguchi., Lineage-specific regulation of the murine RAG-2 promoter: GATA-3 in T cells and Pax-5 in B cells. Blood **95** (2000) pp. 3845-3852.

[33] C. McDonald, N. C. Reich, Cooperation of the transcriptional coactivators CBP and p300 with Stat6. J. Interferon Cytokine Res. **19** (1999) pp. 711-722.

[34] S.Gingras, J. Simard, B. Groner B, E. Pfitzner, p300/CBP is required for transcriptional induction by interleukin-4 and interacts with Stat6. Nucleic Acids Res. **27** (1999) pp. 2722-2729.

[35] I. Berberich, G. L. Shu, E. A. Clark, Cross-linking CD40 on B cells rapidly activates nuclear factor-κB. J. Immunol. **153** (1994) pp. 4357-4366.

[36] L. B. Clark, T. M. Foy, R. J. Noelle, CD40 and its ligand. Adv. Immunol. **63** (1996) pp. 43-78.

[37] S. A. Delphin, J. Stavnezer, Characterization of an interleukin 4 (IL-4) responsive

region in the immunoglobulin heavy chain germline ε promoter: regulation by NF-IL-4, a C/EBP family member and NF-κB/p50. J. Exp. Med. **181** (1995) pp. 181-192.

[38] L. Huo, T. L. Rothstein, Receptor-specific induction of individual AP-1 components in B lymphocytes. J. Immunol. **154** (1995) pp. 3300-3309.

[39] W. C. Sha, Regulation of immune responses by NF-κB/Rel transcription factor. J. Exp. Med. **187** (1998) pp. 143-146.

[40] D. A. Francis, J. G. Karras, X. Ke, R. Sen, T. L. Rothstein, Induction of the transcription factors NF-κB, AP-1 and NF-AT during B cell stimulation through the CD40 receptor. Int. Immunol. **7** (1995) pp. 151-161.

[41] H. H. Jabara, S. M. Fu, R. S. Geha, D. Vercelli, CD40 and IgE: synergism between anti-CD40 monoclonal antibody and interleukin 4 in the induction of IgE synthesis by highly purified human B cells. J. Exp. Med. **172** (1990) pp. 1861-1864.

[42] H. Gascan, J.-F. Gauchat, G. Aversa, P. van-Vlasselaer, J. E. De-Vries, Anti-CD40 monoclonal antibodies or CD4+ T cell clones and IL-4 induce IgG4 and IgE switching in purified human B cells via different signaling pathways. J. Immunol. **147** (1991) pp. 8-13.

[43] W. D. Warren, M. T. Berton, Induction of germ-line γ 1 and ε Ig gene expression in murine B cells. IL-4 and the CD40 ligand-CD40 interaction provide distinct but synergistic signals. J. Immunol. **155** (1995) pp. 5637-5646.

[44] L. A. Iciek, S. A. Delphin, J. Stavnezer, CD40 cross-linking induces Ig epsilon germline transcripts in B cells via activation of NF-κB: synergy with IL-4 induction. J. Immunol. **158** (1997) pp. 4769-4779.

[45] C.M. Snapper, P. Zelazowski, F. R. Rosas, M. R. Kehry, M. Tian, D. Baltimore, W. C. Sha, B cells from p50/NF-κB knockout mice have selective defects in proliferation, differentiation, germ-line CH transcription, and Ig class switching. J. Immunol. **156**

(1996) pp. 183-191.

[46] B. L. Bennett, R. Cruz, R. G. Lacson, A. M. Manning, Interleukin-4 suppression of tumor necrosis factor α-stimulated E-selectin gene transcription is mediated by STAT6 antagonism of NF-κB J. Biol. Chem. **272** (1997) pp 10212-10219.

[47] Y. Ohmori, T. A. Hamilton, Interleukin-4/STAT6 represses STAT1 and NF-κB-dependent transcription through distinct mechanisms. J. Biol. Chem. **275** (2000) pp. 38095-38103.

[48] R. M. Nissen, K. R. Yamamoto, The glucocorticoid receptor inhibits NFκB by interfering with Ser-2 phosphorylation of the RNA polymerase II carboxy-terminal domain. Genes Dev. **14** (2000) pp. 2314-2329

[49] N. W. Quong, D. P. Harris, S. L. Swain, C. Murre C, E2A activity is induced during B-cell activation to promote immunoglobulin class switch recombination. EMBO J. **18** (1999) pp. 6307-6318.

[50] T. Ota, M. Fujii, T. Sugizaki, M. Ishii, K. Miyazawa, H. Aburatani, K. Miyazono K, Targets of transcriptional regulation by two distinct type I receptors for transforming growth factor-β in human umbilical vein endothelial cells. J. Cell Physiol. **193** (2002) pp. 299-318.

[51] M. Sugai, H. Gonda, T. Kusunoki, T. Katakai, Y. Yokota, A. Shimizu, Essential role of Id2 in negative regulation of IgE class switching. Nat. Immunol. **4** (2003) pp. 25-30.

FIGURE LEGENDS

Fig. 1. TGF-β suppresses IL-4-induced IgG1 production and gene expression in B cells.

(A) Effect of IL-4 and/or TGF-β on germline ε transcripts (Cε) expression in DND39 cells. 20 μg of total RNA from DND39 cells treated with IL-4 (30 U/ml) and/or TGF-β (100 U/ml) for 12 or 24 h was used for Northern blot analysis. Actin mRNA is included as a loading control (lower panel).

(B) Murine splenic B cells were cultured with an increasing amounts of IL-4 in the absence or presence of TGF-β(100U/ml). After 5-day culture,, culture supernatants were harvested and isotype-specific enzymed-linked immunosorbent assays (ELISA) were performed. The error bars represent the standard deviations.

(C) DND39/Iε-LUC cells were stimulated with an increasing amounts of IL-4 in the absence or presence of TGF-β (10 or 100U/ml). After 48 h culture, cells were harvested and relative luciferase activities were measured. The error bars represent the standard deviations.

Fig2. IL-4-induced STAT6 activation in DND39 cells.

(A)IL-4-induced tyrosine phosphorylation of STAT6. DND39 cells ($1x10^7$) were stimulated with IL-4 (30 U/ml) and/or TGF-β (100U/ml) for 30 min. Cell lysates were immunoprecipitated with an anti-STAT6 antibody and immunoblotted with anti-phosphotyrosine antibody (upper panel). The blot was stripped and reprobed with an anti-STAT6 antibody (lower panel).

(B) Induction of STAT6 or NF-κB DNA binding activity by IL-4 and/or TGF-β in DND39 cells. Nuclear extracts from DND39 cells treated with IL-4 (30U/ml) and/or

TGF-β (100U/ml) for 30 min were prepared as described previously. EMSA was performed using a ^{32}P-labeled Iε or NF-κB oligonucleotide probe. The arrow shows an IL-4-induced STAT6-DNA complex or NF-κB-DNA complex.

Fig. 3. Reconstitution of the IL-4 signaling pathways in 293T cells.

(A) 293T cells were transfected with Iε-LUC (1μg), IL-4Rα (0.1μg) and/or p300 expression construct (0.1μg) as indicated. 48 h after transfection, cells were stimulated for an additional 12 h with an increasing amounts of IL-4 (0.3-30U/ml) and cells were harvested and relative luciferase activities were measured.

(B) 293T cells were transfected with Iε-LUC (1μg), IL-4Rα (0.1μg) and/or p300 expression construct (0.1μg), and/or various doses (0.1-1.0μg) of STAT6YF. 48 h after transfection, cells were stimulated for an additional 12 h with IL-4 (30U/ml) as indicated and cells were harvested and relative luciferase activities were measured.

(C) 293T cells were transfected with Iε-LUC (1μg), IL-4Rα (0.1μg) and p300 expression construct (0.1μg), and/or an increasing amounts of TβRI(T204D) (0.1-1 μg) as indicated. 48 h after transfection, cells were stimulated for an additional 12 h with IL-4 (30U/ml) and cells were harvested and relative luciferase activities were measured.

(D) 293T cells were transfected with Iε-LUC (1μg), IL-4Rα (0.1μg) and p300 expression construct (0.1μg), and/or various doses (0.1-1.0μg) of CD40. 48 h after transfection, cells were harvested and relative luciferase activities were measured.

(E) 293T cells were transfected with Iε-LUC (1μg), IL-4Rα (0.1μg) and p300 expression construct (0.1μg), and/or various doses (0.1-1.0μg) of C/EBPβ. 48 h after transfection, cells were harvested and relative luciferase activities were measured. The results are presented as fold induction of luciferase activity from triplicate experiments. The error bars represent the standard deviations.

Fig. 4. Involvement of NF-κB in Iε-LUC activation by IL-4.

(A) 293T cells were transfected with Iε-LUC (1μg), IL-4Rα (0.1μg) and p300 expression construct (0.1μg), and/or an increasing amounts of DN-IκB (0.1-1 μg) as indicated. 48 h after transfection, cells were stimulated for an additional 12 h with IL-4 (30U/ml) and cells were harvested and relative luciferase activities were measured.

(B) 293T cells were transfected with Iε-LUC (1μg), IL-4Rα (0.1μg) and p300 expression construct (0.1μg), and/or an increasing amounts of IKKαK44M (0.1-1 μg) as indicated. 48 h after transfection, cells were stimulated for an additional 12 h with IL-4 (30U/ml) and cells were harvested and relative luciferase activities were measured.

(C) 293T cells were transfected with Iε-LUC (1μg), IL-4Rα (0.1μg) and p300 expression construct (0.1μg), and/or an increasing amounts of IKKβK44M (0.1-1 μg) as indicated. 48 h after transfection, cells were stimulated for an additional 12 h with IL-4 (30U/ml) and cells were harvested and relative luciferase activities were measured.

(D) DND/Iε-LUC cells were stimulated with or without IL-4 (10U/ml) in the absence or presence of various doses (10-100 μM) of PDTC. 48 h after transfection, cells were harvested and relative luciferase activities were measured.

(E) 293T cells were transfected with Iε-LUC (1μg), IL-4Rα (0.1μg) and p300 expression construct (0.1μg), and/or CD40 (0.5μg) together with an increasing amounts of TβR-I (0.1-1.0μg). 48 h after transfection, cells were harvested and relative luciferase activities were measured. The results are presented as fold induction of luciferase activity from triplicate experiments. The error bars represent the standard deviations.

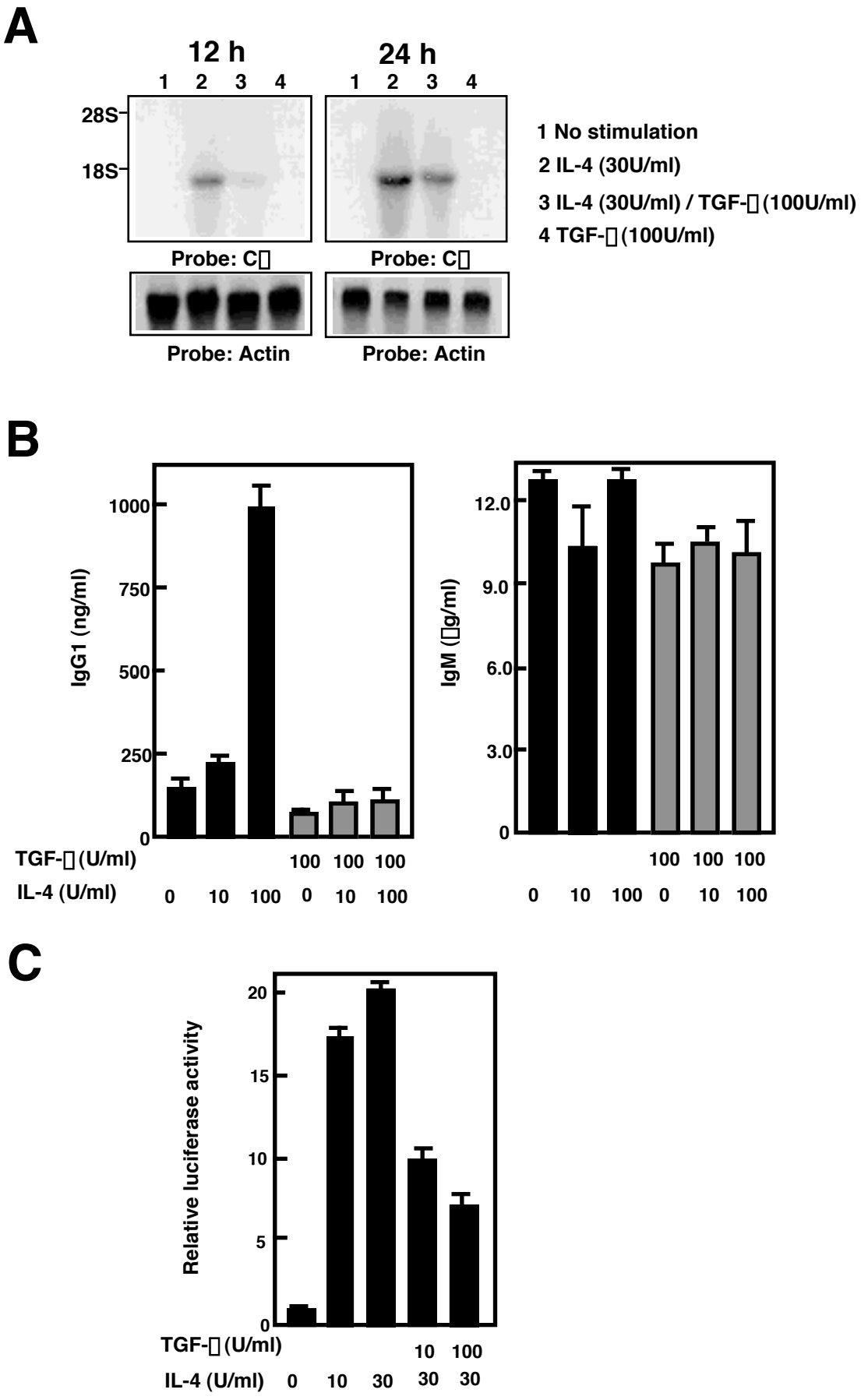
A
12 h
24 h
1 2 3 4
1 2 3 4
28S
18S
Probe: Cγ
Probe: Cγ
Probe: Actin
Probe: Actin
1 No stimulation
2 IL-4 (30U/ml)
3 IL-4 (30U/ml) / TGF-β (100U/ml)
4 TGF-β (100U/ml)
B
IgG1 (ng/ml)
1000
750
500
250
0
IgM (μg/ml)
12.0
9.0
6.0
3.0
0
TGF-β (U/ml)
100 100 100
100 100 100
IL-4 (U/ml)
0 10 100 0 10 100
0 10 100 0 10 100
C
Relative luciferase activity
20
15
10
5
0
TGF-β (U/ml)
10 100
IL-4 (U/ml)
0 10 30 30 30

Fig. 1

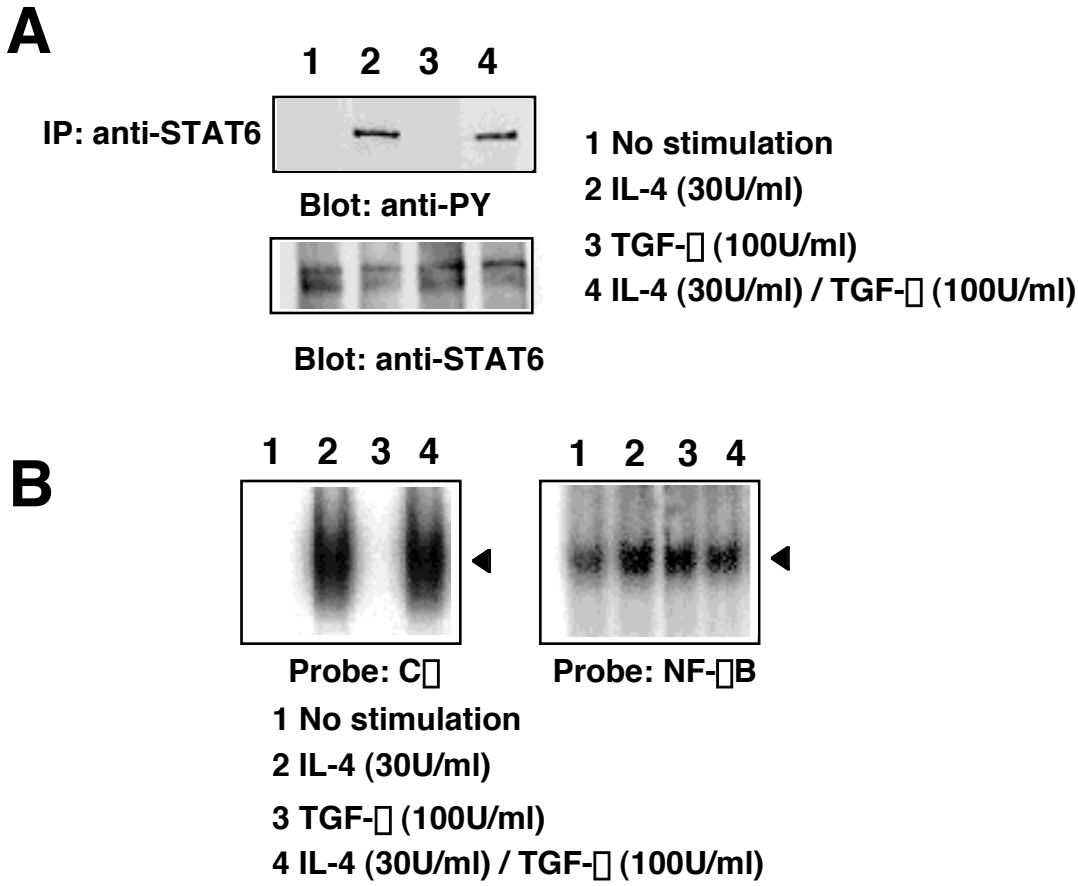
A
1 2 3 4
IP: anti-STAT6
Blot: anti-PY
Blot: anti-STAT6
1 No stimulation
2 IL-4 (30U/ml)
3 TGF-β (100U/ml)
4 IL-4 (30U/ml) / TGF-β (100U/ml)
B
1 2 3 4
1 2 3 4
Probe: Cβ
Probe: NF-βB
1 No stimulation
2 IL-4 (30U/ml)
3 TGF-β (100U/ml)
4 IL-4 (30U/ml) / TGF-β (100U/ml)

Fig. 2

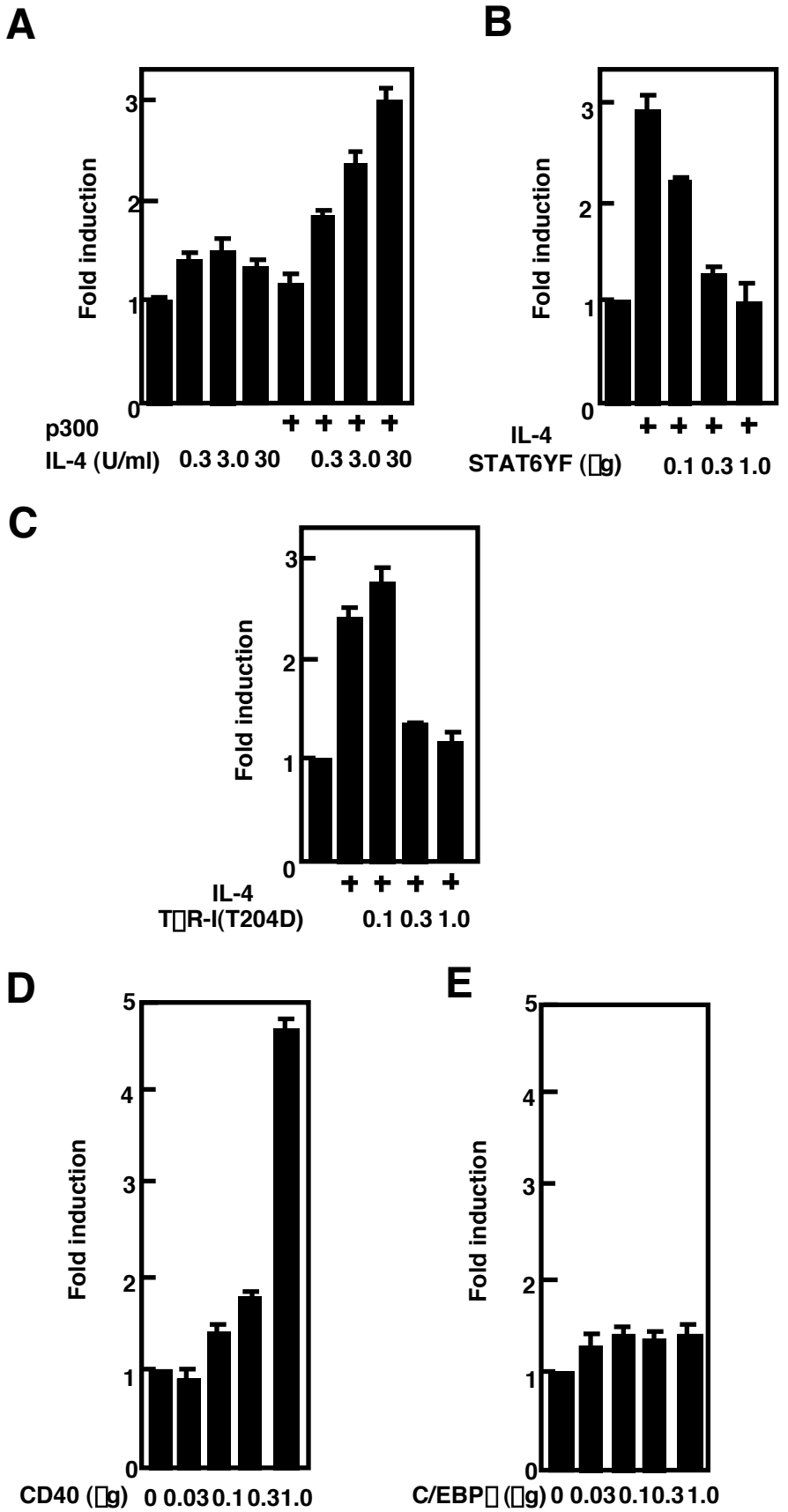
A
Fold induction
0
1
2
3
p300
+ + + +
IL-4 (U/ml) 0.3 3.0 30 0.3 3.0 30
B
Fold induction
0
1
2
3
IL-4
+ + + +
STAT6YF (□g) 0.1 0.3 1.0
C
Fold induction
0
1
2
3
IL-4
+ + + +
T□R-I(T204D) 0.1 0.3 1.0
D
Fold induction
0
1
2
3
4
5
CD40 (□g) 0 0.03 0.1 0.3 1.0
E
Fold induction
0
1
2
3
4
5
C/EBP□ (□g) 0 0.03 0.1 0.3 1.0

Fig. 3

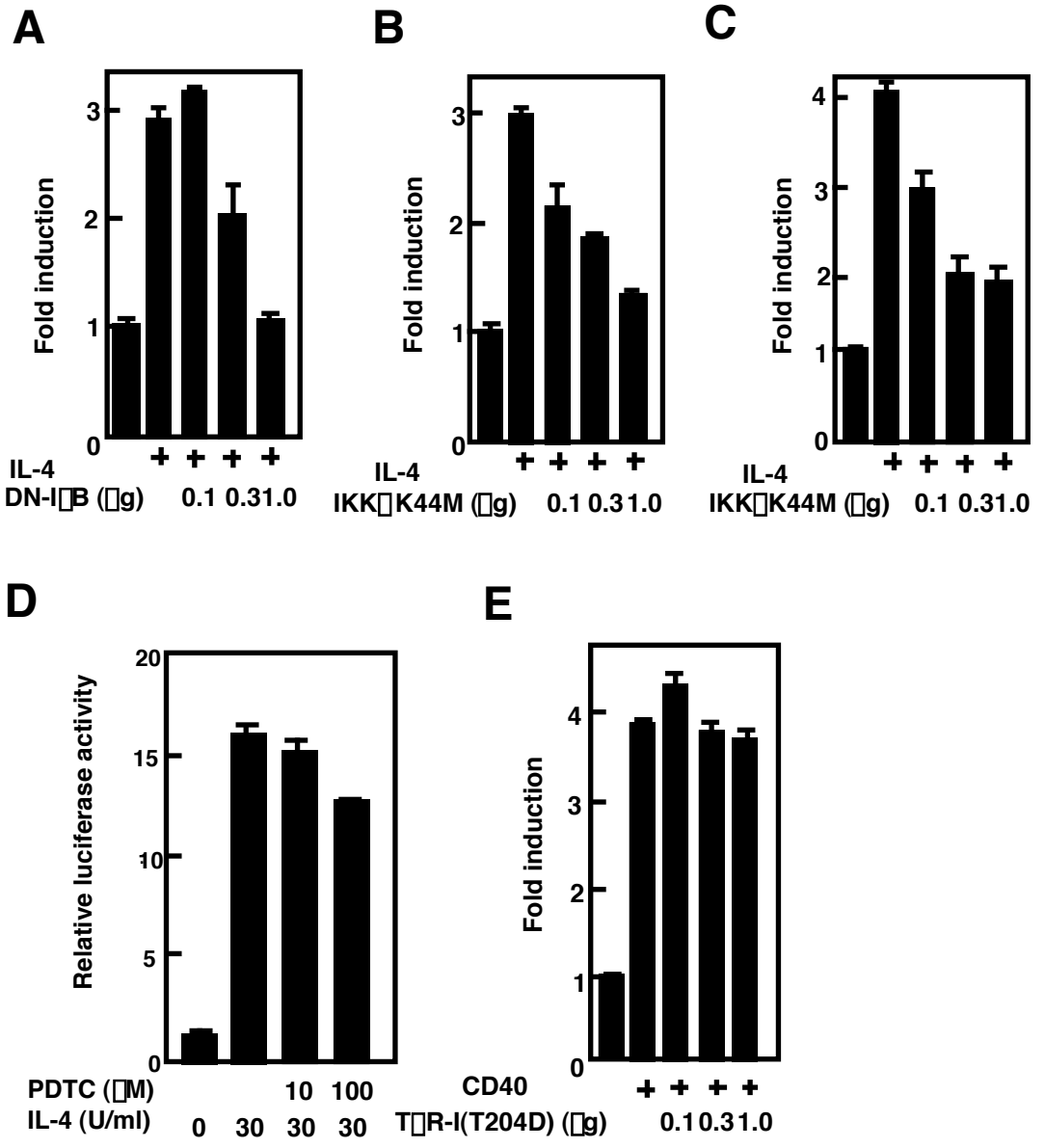
A
B
C
Fold induction
IL-4
DN-IκB (μg) 0.1 0.3 1.0
IKKβK44M (μg) 0.1 0.3 1.0
D
E
Relative luciferase activity
PDTC (μM) 10 100
IL-4 (U/ml) 0 30 30 30
CD40
TβR-I(T204D) (μg) 0.1 0.3 1.0

Fig. 4