Differential requirements for JAK2 and TYK2 in T cell proliferation and IFN-γ production induced by IL-12 alone or together with IL-18

Naotoshi Sugimoto[1], Masakiyo Nakahira[1], Hyun-Jong Ahn[2], Mark Micallef[3], Toshiyuki Hamaoka[1], Masashi Kurimoto[3] and Hiromi Fujiwara[1]

[1] Department of Oncology, Osaka University Graduate School of Medicine, Osaka, Japan
[2] Department of Microbiology, Kyun-Hee University Medical School, Seoul, Korea
[3] Fujisaki Institute, Hayashibara Biochemical Laboratories, Okayama, Japan

IL-12 activates TYK2 and Janus kinase (JAK)-2 to induce the phosphorylation of various signal transducers and activators of transcription (STAT) proteins. However, little is known regarding how these JAK exhibit the distinct biological effects of IL-12. Using two JAK inhibitors, tyrphostin A1 (A1) for TYK2 and tyrphostin B42 (B42) for JAK2, we investigated the involvement of JAK2 and TYK2 in IL-12-induced T cell proliferation and IFN-γ production. B42, but not A1, inhibited T cell proliferation along with down-regulation of IL-12-induced c-Myc expression and STAT5 phosphorylation. In contrast, A1 but not B42 inhibited STAT4/STAT3 phosphorylation and IFN-γ production. IL-18, but not IL-12, induced activator protein-1 (AP-1) responsible for high levels of IFN-γ promoter activation. However, this IL-18 effect depended on the interaction of AP-1 with STAT4. A1 prevented AP-1 binding by inhibiting STAT4 involvement and down-regulated synergistic IFN-γ promoter activation. These results indicate that JAK2 activation is required for IL-12-mediated T cell growth, whereas the TYK2-STAT4 signaling pathway is critical for IFN-γ expression that is mediated by IL-12 alone and enhanced synergistically by combination with IL-18.

Received	31/5/02
Revised	23/10/02
Accepted	19/11/02

Key words: T lymphocyte / Cytokine / Transcription factor / Signal transduction

1 Introduction

A variety of cytokines activates distinct members of the Janus family of protein tyrosine kinases (Janus kinases, JAK) through ligation of the corresponding cytokine receptors [1]. Activation of JAK leads to tyrosine phosphorylation of a family of signal transducers and activators of transcription (STAT), which are important in the regulation of gene expression by cytokine receptors [2, 3]. Stimulation of the IL-12 receptor (IL-12R) with IL-12 results in the activation of two JAK family members, JAK2 and TYK2, and then induces tyrosine phosphorylation of STAT4 and STAT3 [2, 3].

IL-12 plays a central role in immune and inflammatory responses by activating this JAK-STAT pathway [4]. Modulation of signal transduction pathways by targeting protein tyrosine kinases has been regarded as a novel strategy for the treatment of infectious diseases, cancer and autoimmune diseases [5–7]. While simultaneous activation of TYK2 and JAK2 is induced in IL-12R signaling [2–4], the relative requirements for these two JAK in the expression of various IL-12 bioactivities remain unclear. Tyrphostins, a group of compounds derived from benzylidenemalononitrile nucleus, are potent inhibitors of protein tyrosine kinases [8]. A number of recent studies reported the selective inhibition of protein tyrosine kinases such as JAK2 [9, 10]. Such selective JAK inhibitors would be a powerful tool in examining how each JAK member contributes to mediating a given IL-12 bioactivity.

In this study, we utilized two tyrphostins designated A1 and B42 capable of inhibiting TYK2 and JAK2 activation, respectively. The results show that inhibition of JAK2 activation by B42 down-regulates T cell proliferation along with inhibition of c-Myc expression and STAT5 phosphorylation. In contrast, A1 but not B42 inhibited STAT4/STAT3 phosphorylation and IFN-γ production following IL-12 stimulation. Synergistic IFN-γ expression by IL-12 and IL-18 costimulation depended on the role of STAT4 in up-regulating the promoter binding of activator

[I 23274]

Abbreviations: JAK: Janus kinase **A1:** Tyrphostin A1 **B42:** Tyrphostin B42 **STAT:** Signal transducers and activators of transcription **AP-1:** Activator protein-1 **JNK:** c-Jun N-terminal kinase **EMSA:** Electrophoretic mobility shift assay

 0014-2980/03/0101-243$17.50+.50/0

protein-1 (AP-1), which is induced by IL-18 and functions as a potent IFN-γ transcription factor. A1 inhibited synergistic IFN-γ expression through canceling the enhancing effect of STAT4. These results indicate different roles for TYK2 and JAK2 in the mediation of distinct IL-12 bioactivities and define a particular condition in which an IL-12-involving inflammatory response is modulated without influence on T cell growth.

2 Results

2.1 Effects of A1 and B42 on the phosphorylation of TYK2, JAK2, STAT4, STAT5 and STAT3 following IL-12 stimulation

A1 and B42 selectively inhibit IL-12-induced phosphorylation of TYK2 and JAK2, respectively [10]. We confirmed this in the 2D6 T cell clone (Fig. 1A). Our previous study [11] showed that IL-12 stimulation induces high levels of STAT4/STAT3 and STAT5 phosphorylation in 2D6 cells. We examined the effects of A1 and B42 on IL-12-induced STAT phosphorylation (Fig. 1B). STAT4 phosphorylation was inhibited selectively by A1. In contrast, STAT5 phosphorylation was preferentially prevented by B42, and STAT3 phosphorylation was down-regulated by A1 as well as B42. We also examined the effects of A1 and B42 on STAT4, STAT3 and STAT5 activation in primary activated T cells prepared by stimulation with anti-CD3 plus anti-CD28 (Fig. 1C). Similar patterns of inhibitory effects of the two inhibitors on STAT4 and STAT5 phosphorylation were observed, but STAT3 activation was down-regulated strongly by A1 and only slightly by B42. Although there were differences in the amounts of STAT proteins between the five groups of nuclear protein samples (Fig. 1B, C), this could be explained by considering that nuclear translocation of STAT proteins occurs depending on their phosphorylation.

2.2 IL-12-induced T cell proliferation is selectively inhibited by B42

Cells from the 2D6 clone were stimulated with IL-12 in the presence of A1 and/or B42. Fig. 2A shows that A1 did not affect IL-12-driven 2D6 proliferation, whereas B42 induced a dose-dependent inhibition of the proliferation. Addition of both B42 and A1 resulted in only a slight augmentation of the B42-mediated inhibition. In light of the observed correlation between the expression of c-Myc and the induction of cell proliferation [12], we examined whether IL-12 signaling activates c-myc in 2D6 cells and, if so, whether c-myc induction is modulated by A1 and/or B42. The c-myc gene was activated and peaked 3 h after IL-12 stimulation (Fig. 2B), and c-

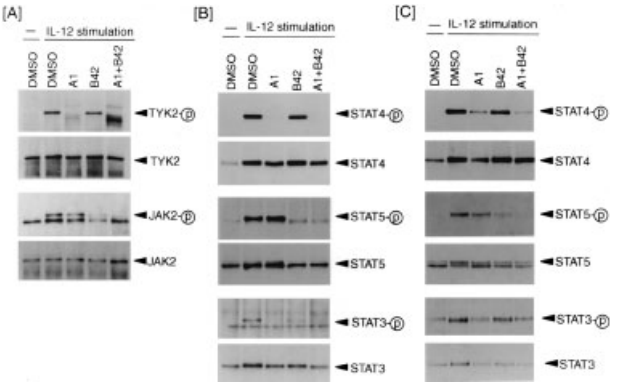

Fig. 1. Effects of A1 and B42 on TYK2, JAK2, STAT4, STAT5 and STAT3 phosphorylation. Cells from the 2D6 clone (A, B) or anti-CD3/anti-CD28-activated lymph node T cells (C) were stimulated with 1,000 pg/ml rIL-12 for 10 min in the presence of 50 μM A1 and/or B42. Cell lysates were immunoprecipitated with anti-TYK2, anti-JAK2 (cytoplasmic extracts), anti-STAT4, anti-STAT5 or anti-STAT3 Ab (nuclear extracts), immunoblotted with anti-phosphotyrosine mAb (each upper panel) and then reblotted with the relevant Ab (each lower panel). Data are representative of five (A, B) and two (C) similar experiments.

Myc protein was detected (Fig. 2C). This c-myc induction was down-regulated by B42 but not by A1 (Fig. 2D). Thus, the JAK2 inhibitor inhibits 2D6 cell proliferation along with down-regulation of IL-12-mediated STAT5 activation and c-myc induction.

2.3 IFN-γ production induced by IL-12 alone or together with IL-18 is selectively inhibited by A1

IL-12 and IL-18 stimulate IFN-γ production [4, 13]. Moreover, these two cytokines exert a synergistic effect on IFN-γ production [14, 15, 16]. We examined the effects of A1 and B42 on IFN-γ production stimulated with IL-12. Addition of A1 to IL-12-stimulated 2D6 cell cultures induced a dose-dependent inhibition of IFN-γ production. In contrast, B42 failed to produce any inhibitory effect (Fig. 3A). Similar effects were observed in primary activated T cells (Fig. 3B).

Cells from the 2D6 clone were starved of IL-12 for 30 h to eliminate IL-12 signals retained during IL-12 culture. IL-12-starved 2D6 cells were stimulated with IL-12 and/or IL-18. IL-18 alone induced low levels of IFN-γ production, but the level was markedly enhanced by combined stimulation with IL-12 and IL-18 (Fig. 4A). Such synergistic IFN-γ expression in 2D6 cells was inhibited selectively by A1 (Fig. 4B). Synergistic IFN-γ production by IL-12 plus IL-18 and A1-selective inhibition of the

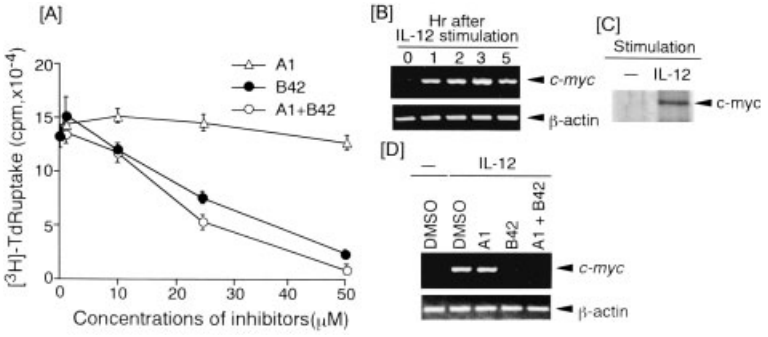

Fig. 2. B42 inhibits IL-12-induced T cell proliferation and c-myc induction. (A) Cells from the 2D6 clone were stimulated with 250 pg/ml rIL-12 for 24 h in the presence of A1 and/or B42. (B, D) Cells from the 2D6 clone were stimulated with rIL-12 for indicated h (B) or for 3 h (D) in the presence or absence of 50 μM A1 and/or B42. Total RNA was isolated and examined for c-myc expression. (C) Cells from the 2D6 clone were stimulated with 1,000 pg/ml rIL-12 for 12 h. Cell lysates were immunoblotted with anti-c-Myc Ab. Data are representative of three (A), five (B, D) and two (C) similar experiments.

synergy were also observed when primary activated T cells were exposed to IL-12 and used after 6-h IL-12 starvation (Fig. 5).

2.4 A1 inhibits IL-12-activated STAT4 from up-regulating the binding activity of IL-18-induced AP-1 and IFN-γ promoter activation

To investigate the mechanism by which A1 inhibits synergistic IFN-γ expression by IL-12 plus IL-18, we first examined whether A1 modulates IL-18 signaling. Because IL-18, but not IL-12, was shown to induce c-Jun N-terminal kinase (JNK) activation in 2D6 cells [16], the effect of A1 and/or B42 on IL-18-mediated JNK activation was determined. JNK phosphorylation was not influenced by either tyrphostin (Fig. 4C).

Our previous study [16] demonstrated that while IL-18 elicits high levels of IFN-γ promoter activation by inducing a potent transcription factor AP-1 [17], the binding of AP-1 to the IFN-γ promoter requires its interaction with STAT4. Namely, IL-12-activated STAT4 contributes to IFN-γ promoter activation by up-regulating the binding activity of IL-18-induced AP-1. This was confirmed as shown in Fig. 6A: the binding of IL-18-induced AP-1, as determined by electrophoretic mobility shift assay (EMSA), was enhanced when cells were stimulated simultaneously with IL-12.

To show that STAT4 forms a complex with AP-1 to up-regulate AP-1 binding, we took advantage of oligo DNA precipitation techniques. Nuclear extracts from 2D6 cells stimulated with IL-12 and/or IL-18 were allowed to interact with agarose beads coupled to the AP-1-binding oligonucleotide sequence. The bound proteins were analyzed by immunoblotting with anti-phosphoserine c-Jun/anti-c-Jun or with anti-STAT4 (Fig. 6B). A much larger amount of c-Jun bound to the AP-1 sequence in the nuclear extract from IL-12/IL-18-stimulated than from IL-18-stimulated cells. c-Jun in the former group was serine-phosphorylated. Importantly, a large amount of STAT4 was recovered from AP-1 oligo DNA precipitation in this group, supporting the notion that STAT4 interacts with c-Jun/AP-1 to facilitate c-Jun/AP-1 binding to the

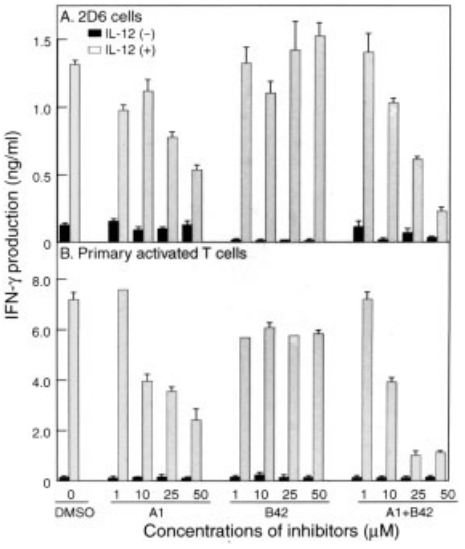

Fig. 3. IL-12-induced IFN-γ production is inhibited by A1 but not by B42. Cells from the 2D6 clone (A) or anti-CD3/anti-CD28-activated lymph node T cells (B) were stimulated with 250 (A) or 1,000 (B) pg/ml rIL-12 for 12 h in the presence or absence of A1 and/or B42. IFN-γ concentrations in culture supernatants were assayed by ELISA. Data are representative of five (A) and three (B) similar experiments.

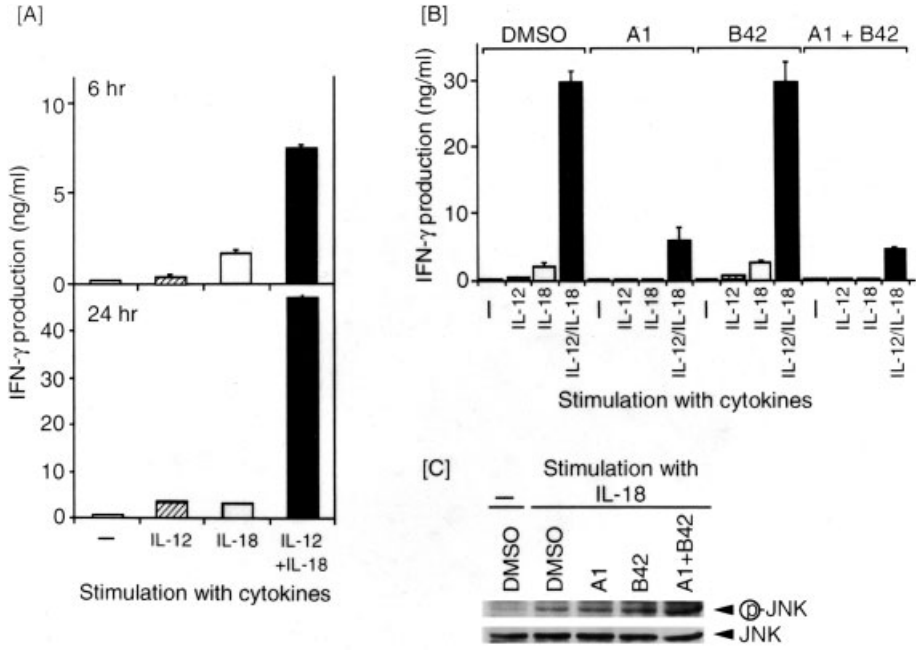

Fig. 4. A1 inhibits synergistic IFN-γ production induced by simultaneous stimulation with IL-12 plus IL-18. (A, B) Cells from the 2D6 clone were harvested from 30-h cultures deprived of IL-12 and stimulated with 250 pg/ml rIL-12 and/or 100 ng/ml rIL-18 for 6 or 24 h (A) or for 10 h (B) in the presence or absence of 50 μM A1 and/or B42. (C) IL-12-starved 2D6 cells were stimulated with 100 ng/ml rIL-18 for 10 min in the presence of 50 μM A1 and/or B42. Cell lysates were immunoblotted with anti-phosphoserine JNK Ab and reblotted with anti-JNK Ab. Data are representative of five (A), three (B) and two (C) similar experiments.

AP-1-related sequence [16]. Compared to STAT4, STAT3 was only slightly recovered (Fig. 6C). Based on these observations, we examined whether A1-mediated inhibition of STAT4/STAT3 activation down-regulates the binding activity of IL-18-induced AP-1. As shown in Fig. 6D, AP-1 binding activity induced by IL-12 plus IL-18 was potently and slightly down-regulated by A1 and B42, respectively, and almost completely by A1 plus B42. Similar observations to those in Fig 6A–D were made using primary activated T cells instead of 2D6 cells (Fig. 7), except for a moderate inhibition in the induction of AP-1 binding activity by B42 (Fig. 7D).

2.5 A1 inhibits IL-12-activated STAT4 from up-regulating IFN-γ promoter activation

We finally investigated the effect of tyrphostins on IFN-γ promoter activation induced by synergy between IL-12 and IL-18 (Fig. 8). For this, 2D6 cells were transiently transfected with the luciferase reporter plasmid carrying the IFN-γ promoter fragment which contains the AP-1-binding sequence [16]. High levels of IFN-γ promoter activation were induced by stimulation with IL-12 and IL-18. This synergistic IFN-γ promoter activation was

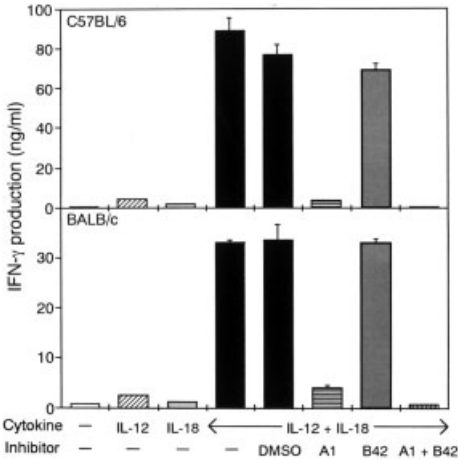

Fig. 5. A1 inhibits IL-12/IL-18-induced IFN-γ production by primary activated T cells. Activated C57BL/6 or BALB/c lymph node T cells were exposed to 1,000 pg/ml rIL-12 for 48 h to allow the induction of IL-18R. These T cells were rested for 6 h in cultures without IL-12 and then stimulated with 250 pg/ml IL-12 and/or 100 ng/ml IL-18 for 12 h in the presence or absence of 50 μM A1 and/or B42. Data are representative of two similar experiments.

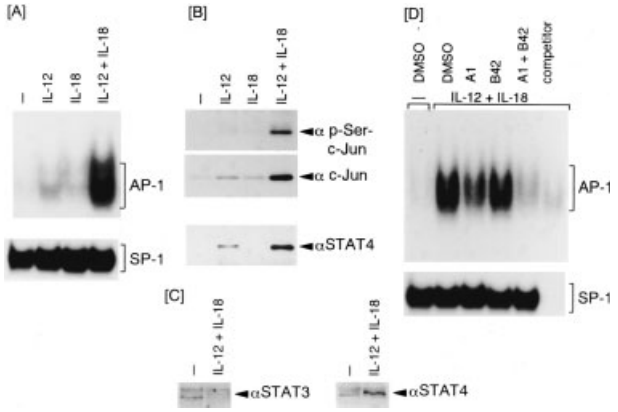

Fig. 6. A1 inhibits IL-12-activated STAT4 from up-regulating the binding activity of IL-18-induced AP-1. (A and D) IL-12-starved 2D6 cells were stimulated with 1,000 pg/ml IL-12 and/or 100 ng/ml IL-18 for 3 h in the presence or absence of 50 μM A1 and/or B42. Nuclear extracts were examined in EMSA for binding to an oligonucleotide probe corresponding to a consensus AP-1 binding sequence. Portions of the same samples as used for AP-1 binding (upper panels) were examined for SP-1 binding (a loading control; lower panels). In one group in (D), EMSA was performed in the presence of a 100-fold molar excess of unlabeled oligonucleotide competitor which was incubated with the extracts prior to addition of labeled probe. (B and C) Nuclear extracts from cytokine-stimulated 2D6 cells were incubated with agarose beads coupled to AP-1 consensus oligonucleotide for 45 min. The bound proteins were separated by SDS-PAGE, transferred to PVDF membranes, and blotted with anti-phosphoserine c-Jun followed by reblotting with anti-c-Jun or blotted with anti-STAT4 or anti-STAT3 Ab. Data are representative of five (A), three (B), two (C) and three (D) similar experiments.

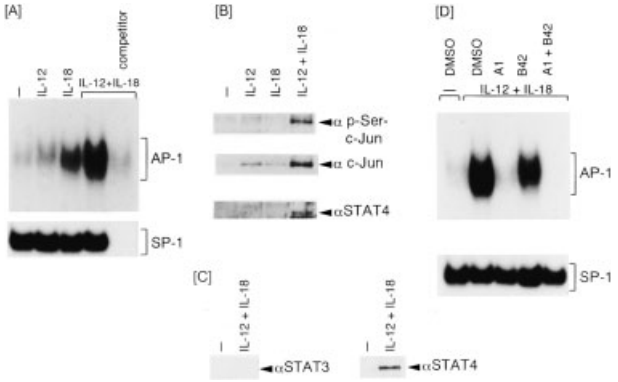

Fig. 7. A1 inhibits the induction of AP-1 binding by IL-12/IL-18 stimulation in primary activated T cells. Activated BALB/c lymph node T cells were exposed to 1,000 pg/ml IL-12 for 48 h. These T cells were rested for 6 h in the absence of IL-12 and then stimulated with 1,000 pg/ml IL-12 and/or 100 ng/ml IL-18 for 3 h. The similar protocols of experiments as in Fig. 6 [(A, D): EMSA; (B, C): oligo DNA precipitation] were performed using primary activated T cells instead of 2D6 cells. Data are representative of five (A), three (B), two (C) and three (D) similar experiments.

almost completely canceled by treatment with A1. Taken together, A1 inhibits the promoter-binding activity of IL-18-induced AP-1 and IFN-γ promoter activation by down-regulating STAT4/STAT3 activation following IL-12 stimulation.

3 Discussion

The present results demonstrate that the JAK2 inhibitor B42 inhibits IL-12-mediated T cell proliferation whereas the TYK2 inhibitor A1 does not. In contrast, the contribution of IL-12 alone or together with IL-18 to IFN-γ production is selectively down-regulated by the TYK2 inhibitor A1. The results also show that B42-mediated inhibition of T cell proliferation correlates with down-regulation of c-myc induction and STAT5 phosphorylation, while A1-mediated inhibition of IFN-γ expression is associated with a decrease in STAT4 phosphorylation. IFN-γ expression was induced much more potently by simultaneous stimulation with IL-12 and IL-18 than by stimulation with IL-12 alone. While synergy was achieved through the capacity of IL-12-activated STAT4 to up-regulate the binding activity of IL-18-induced AP-1 to the IFN-γ promoter, A1 was shown to inhibit synergistic IFN-γ expression by down-regulating the activation of STAT4 and canceling its enhancing effect on AP-1 binding.

Distinct roles of JAK2 and TYK2 in the mediation of IL-12 bioactivities should be addressed based on selective inhibition of these bioactivities by their respective inhibitors. Regarding the specificity of targets for A1 and B42, earlier studies [10] showed that A1 and B42 inhibit TYK2 and JAK2/JAK3 activation, respectively, following IL-12 stimulation. It is also possible that one or both of these tyrphostins have kinase targets other than JAK. In fact, a recent study of Du et al. [18] showed that A1 inhibits CD40-mediated signaling pathway following CD40 ligand stimulation. Even though A1 may also act on (a) kinase(s) other than JAK in these cases, A1 is considered to inhibit TYK2 in IL-12R-mediated signaling.

Our previous study [11] showed that 2D6 cell proliferation correlates with JAK2 rather than with TYK2 activation; IL-12 induces STAT5 phosphorylation without JAK3 activation; and STAT5 physically interacts with JAK2, although functional interactions between JAK2 and STAT5 have not yet been demonstrated. The present study defined such an interaction by demonstrating that the JAK2 inhibitor reduces IL-12-induced STAT5 phos-

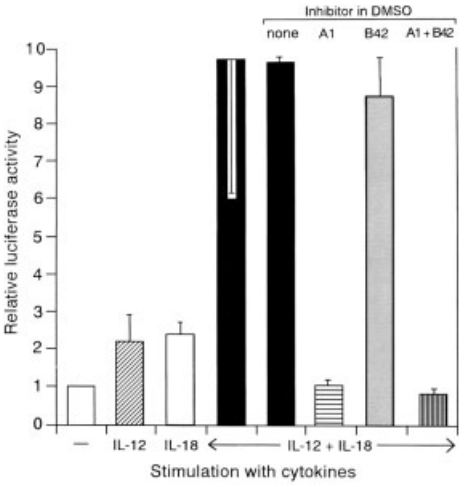

Fig. 8. A1 inhibits IL-12/IL-18-induced IFN-γ promoter activation. IL-12-starved 2D6 cells were transfected with 25 μg of pGL3-IFN-γ and 2 μg of pRL-TK plasmids and immediately stimulated with 1,000 pg/ml IL-12 and/or 100 ng/ml IL-18 in the presence or absence of 50 μM A1 and/or B42. After 10 h, 2D6 cell lysates were subjected to the luciferase reporter gene assay. Luciferase activity was normalized for transfection efficiency against Renilla Luciferase activity (pRL-TK plasmid). Relative luciferase activities were expressed as ratios of each cytokine-stimulated group to a control (cytokine-stimulation-negative) group. Data are representative of three similar experiments.

phorylation and confirmed a role for JAK2 in IL-12 induction of T cell proliferation [10]. In addition, c-myc as a representative growth-related gene was found to be induced following IL-12 stimulation, consistent with a previous report [19]. This c-myc induction was dependent on JAK2 activation. While a role of STAT5 has not been defined for c-myc regulation, these results suggest the involvement of JAK2 and STAT5 in c-myc-associated mitogenic signaling.

In contrast to T cell proliferation, it appears that IFN-γ production more closely correlates with TYK2 phosphorylation than does JAK2 activation. This is accordant with a study using TYK2-deficient mice [20]. However, the study of Bright et al. [10] using A1 and B42 inhibitors showed results fundamentally different from ours regarding the effect of the inhibitors on IFN-γ production. In their study, both A1 and B42 down-regulated IL-12-stimulated IFN-γ production, and the inhibitory effect was stronger in the latter compared to the former. The discrepancy in the effect of B42 on IFN-γ production between their study and ours may be reconciled by considering the exposure times of the responding cells. Their study examined the effect of B42 by culturing Th1 cells with B42 for 48 h [10]. Because B42 induces inhibition of T cell growth, the B42-mediated inhibitory effect on IFN-γ production may involve a reduction in IFN-γ production due to a decrease in the number of viable cells. In fact, we found that more than 24-h culture in the presence of B42 decreases the number of viable cells along with an increase in the number of dead cells in our 2D6 Th1 cell model (unpublished observations). Therefore, we defined the culture period during which the numbers of viable cells do not greatly differ between the inhibitor-exposed and control groups. We found that B42 did not produce any inhibitory effect on IFN-γ production during the defined culture period.

A critical role for STAT4 in IL-12-stimulated IFN-γ production has been demonstrated by establishing STAT4-deficient mice [21, 22]. However, it is obvious that IL-18 is a much stronger inducer of IFN-γ production than IL-12 [15–17]. It has also been established that the expression of potent IL-18 activity depends on simultaneous stimulation with IL-12 [15–17]. Recent studies have indicated that IL-12 and IL-18 utilize different signaling pathways for IFN-γ production [14, 17] and that a mechanism of their synergy stems from their collaboration at the transcriptional level of IFN-γ gene expression [16, 17]. An interesting aspect of this study concerns the down-regulatory effect of the TYK2 inhibitor A1 on IL-12/IL-18-induced IFN-γ production. Potent inhibition of IL-12/IL-18 synergy by A1 may raise the following possibilities: A1 inhibits IL-18 signaling as well or almost completely abrogates the enhancing effect of IL-12 on IL-18 signaling. IL-18 is known to induce NF-κB [14] and JNK activation [14, 23], both of which are potentially involved in IFN-γ gene expression. Stimulation of 2D6 cells with IL-18 induced only marginal levels of NF-κB (unpublished observations), but IL-18 induced JNK activation ([16] and this study). The present study showed that A1 does not inhibit IL-18-induced JNK activation, making it unlikely that A1 modulates this pathway of IL-18 signaling.

An important set of transcription factor-binding sites resides between positions –280 and –180 on the IFN-γ promoter [24], including STAT-common binding motif- and AP-1-binding sites. These were shown to be responsible for IL-12/IL-18-mediated activation of the human IFN-γ promoter [17]. However, our previous study [16] demonstrated that mouse STAT4 does not bind to the STAT-common binding site; the synergistic activation of the promoter fragment lacking this site can also be induced by IL-12/IL-18 stimulation. STAT4 up-regulated IFN-γ promoter activation not by direct binding to a given promoter region but by enhancing the transactivation of IL-18-induced AP-1 through the formation of the STAT4-AP-1 complex [16]. Because the interaction of STAT4 with AP-1 is an essential mechanism of synergy for IFN-γ production, this mechanism could explain the present

results that A1 capable of down-regulating the TYK2-STAT4 signaling pathway can markedly inhibit IL-12/IL-18-mediated synergistic IFN-γ expression. The present study also supports the mechanism of STAT4-mediated up-regulation of IFN-γ promoter activation that was shown in the previous study [16].

IL-12 itself failed to induce JNK activation [16] but was capable of inducing the accumulation c-Jun in the nuclear compartment along with the recruitment of STAT4 [16]. Therefore, stimulation with IL-12 alone resulted in the formation of the STAT4-c-Jun/AP-1 complex [16]. However, without serine phosphorylation of c-Jun/AP-1, binding of this complex to the AP-1-binding sequence in the IFN-γ promoter was quite low. In fact, the recovery of c-Jun/AP-1 from the AP-1 sequence was very poor compared to that from IL-12/IL-18-stimulated cells ([16] and this study). This suggests that only low levels of IFN-γ expression are mediated by IL-12-activated STAT4 alone. It is also possible that IFN-γ expression mediated by serine-unphosphorylated c-Jun/AP-1 still occurs in the presence of A1 during IL-12 stimulation.

The present study illustrates a mechanism by which an inhibitor of a particular JAK regulates IL-12/IL-18-mediated production of a representative inflammatory cytokine IFN-γ. Such a regulatory effect is considered biologically relevant because strikingly high levels of IFN-γ production elicited by two IFN-γ-inducing cytokines are controlled. It should also be noted that this regulation is induced under conditions in which T cell growth is not affected. Thus, our results could provide significant implications for the development of drugs to regulate Th1 inflammatory responses involving excess IFN-γ expression.

4 Materials and methods

4.1 Cell line

The 2D6 mouse T cell clone capable of responding to IL-12 and IL-18 was used [15, 16, 25].

4.2 Reagents

A1 ($C_{11}H_8N_2O$) and B42 ($C_{17}H_{14}N_2O_3$) were obtained from LC Laboratories (Woburn, MA). Mouse rIL-12 and rIL-18 were provided by Genetics Institute Inc. (Cambridge, MA) and Hayashibara Biochemical Laboratories (Okayama, Japan), respectively. The following polyclonal rabbit antisera and monoclonal antibodies (mAb) were purchased: polyclonal anti-JAK2, anti-TYK2, anti-STAT4, anti-STAT5, anti-STAT3, anti-c-Jun and anti-c-Myc Ab were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-phosphotyrosine mAb (PY20) was from Transduction Laboratories (Lexington, KY); anti-JNK, anti-phospho-JNK and anti-phosphoserine-c-Jun Ab were from New England Biolabs (Beverly, MA).

4.3 Preparation of primary activated T cells

BALB/c and C57BL/6 mice were purchased from Shizuoka Experimental Animal Center (Hamamatsu, Japan). T cell populations were prepared from lymph node cells, and primary activated T cells were prepared as described [16]. Briefly, lymph node T cells were stimulated with 5 μg/ml immobilized anti-CD3 and 2 μg/ml co-immobilized anti-CD28 mAb for 2 days and then recultured in the presence of 1,000 pg/ml rIL-12 for 2 days to induce IL-18R.

4.4 Proliferation of 2D6 cells

Cells from the 2D6 clone (2×10^4/well) were cultured with rIL-12 for 24 h in 96-well microculture plates (Corning 25860, Corning Glass Works, Corning, NY). Cells were harvested after a final 6-h pulse with 20 kBq/well of [^{3}H]thymidine. Results were calculated from [^{3}H]thymidine uptake and expressed as mean cpm (± SE) of triplicate cultures.

4.5 IFN-γ production by T cells and measurement of IFN-γ concentration

Cells from the 2D6 clone (2×10^5/well) or primary activated T cells (4×10^5/well) were cultured with rIL-12 (250 pg/ml), rIL-18 (100 ng/ml) or a combination of these in 24-well culture plates (Corning 25820). After 24 h, IFN-γ concentrations in supernatants were measured using mouse IFN-γ ELISA kits (Genzyme, Cambridge, MA).

4.6 Preparation of cytoplasmic and nuclear extracts

Cells were harvested by centrifugation, washed with phosphate-buffered saline, and lysed in cell lysis buffer A [20 mM Hepes-KOH pH 7.0, 10 mM KCl, 1 mM $MgCl_2$, 50 mM NaF, 1 mM Na_3VO_4, 0.1% NP40, 10% glycerol, 0.5 mM dithiothreitol, 0.25 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml aprotinin, 2 μg/ml leupeptin and 0.1 mM Pefablock (Boehringer Mannheim)]. Nuclei were separated from cytoplasmic extracts by centrifugation and washed in buffer A. For immunoprecipitation/immunoblotting, nuclear extracts were prepared in buffer B (buffer A containing 0.3 M NaCl without detergent). For EMSA, nuclear extracts were prepared in buffer C (20 mM Hepes-NaOH pH 7.9, 20 mM NaF, 1 mM Na_3VO_4, 1 mM EDTA, 0.1 mM EGTA, 0.42 M NaCl, 1 mM dithiothreitol, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 0.1 mM Pefablock).

4.7 Immunoprecipitation and immunoblotting

Lysates were immunoprecipitated with anti-JAK2, -TYK2, -STAT4, -STAT3 or -STAT5 antisera conjugated to protein A-coupled Sepharose beads. The immunoprecipitates were resolved on 7.5% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, MA). For immunoblotting with anti-phosphotyrosine mAb, membranes were blocked in Tris-buffered saline (TBS) containing 1% fish gelatin, 5% BSA, and 0.1% Tween 20 and sequentially incubated with anti-phosphotyrosine mAb and horseradish peroxidase-conjugated sheep anti-mouse IgG F(ab')2 (Amersham, Arlington Height, IL). Detection was performed by enhanced chemiluminescence (ECL; Amersham). For immunoblotting with Ab to JAK family or STAT proteins, membranes were blocked in TBS containing 0.1% Tween 20 and 5% BSA, incubated sequentially with primary Ab and horseradish peroxidase-conjugated donkey anti-rabbit IgG F(ab')2 (Amersham), and detected with ECL. When a membrane was reprobed, it was first treated in reducing SDS buffer (100 mM 2-ME, 2% SDS, 62.5 mM Tris-HCl pH 6.7).

4.8 Electrophoretic mobility shift assay

The binding reaction was performed in a total volume of 20 μl in the following buffer: 10 mM Hepes-NaOH (pH 7.9), 1 mM EDTA, 30 mM NaCl, 0.1% NP40, 1 mM dithiothreitol, 1 mg/ml BSA and 5% glycerol. Each reaction, also containing 3 μg poly-(dI-dC) and ^{32}P-end-labeled probe, was initiated by the addition of ~9 μg nuclear extract and allowed to incubate at room temperature for 30 min prior to electrophoretic analysis on a 4.5% polyacrylamide gel in 0.25× TBE buffer. The AP-1 consensus oligonucleotide probe 5'-CGCTTGATGACTCAGCCGGAA-3' was purchased from Santa Cruz Biotechnology Inc. The SP-1 oligonucleotide probe 5'-ATTCGATCGGGGCGGGGCGAGC-3' was from Sigma Genosys Japan (Ishikari, Japan).

4.9 Reporter gene assay

The luciferase reporter plasmid containing a fragment of the mouse IFN-γ promoter sequence (positions –436 to +113) was constructed [16]. Luciferase reporter plasmid (25 μg) and 2 μg of pRL-TK reporter plasmid were cotransfected into 2D6 cells as previously described [16]. After transfection, the cells were stimulated with IL-12 (1,000 pg/ml) and/or IL-18 (100 ng/ml) for 10 h. A reporter gene (luciferase) assay was performed according to the procedure recommended by Promega. Data were normalized for transfection efficiency against Renilla luciferase activity of the pRL-TK reporter plasmid (Promega). The relative luciferase activity of each cytokine-stimulated sample was expressed as a ratio to the control (cytokine-unstimulated) sample.

4.10 Oligo DNA precipitation

The procedure was essentially the same as that previously described [26]. Nuclear extracts were incubated with agarose beads coupled to an AP-1 consensus oligonucleotide (TGACTCA; Santa Cruz Biotechnology). The binding reaction was performed for 45 min at 4°C in a binding buffer containing 100 mM NaCl, 10 mM Tris-HCl pH 7.5, 0.1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 0.1% NP40, 0.2 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Na_3VO_4, 50 mM NaF, 1 mg/ml BSA and 30 μg/ml poly-(dI-dC). The agarose beads were washed five times with binding buffer. The bound proteins were released with SDS loading buffer, separated by 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF membrane, and visualized with the relevant Ab.

4.11 Reverse transcription polymerase chain reaction

The procedure was essentially the same as that previously described [27]. The following oligonucleotides were used: c-myc sense primer 5'-CAGGACTGTATGTGGAGCGGTTTC-TCAGCC-3'; c-myc anti-sense primer 5'-TGCTCTTCT-TCAGAGTCGCTGCTGGTGGTG-3'; β-actin sense primer 5'-AGAAGAGCTATGAGCTGCCTGACG-3'; and β-actin anti-sense primer 5'-CTTCTGCATCCTGTCAGCAATGCC-3'. Cycle parameters were: annealing for 1 min at 60°C (c-myc) or 55°C (β-actin), elongation for 2 min at 72°C, and denaturation for 1 min at 94°C. The resulting PCR products were separated in 2% agarose gels (TaKaRa, Otsu, Japan) and visualized by ethidium bromide staining. Sequences of the c-myc and β-actin (for standardization) were amplified out of each cDNA batch with 25 and 23 amplification cycles, respectively.

Acknowledgements: We thank Mrs. M. Yasuda and Mrs. C. Fukuda for secretarial assistance. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

References

1 **Ihle, J. N.,** Cytokine receptor signalling. *Nature* 1995. **377:** 591–594.

2 **Schindler, C. and Darnell, J., Jr.,** Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. *Annu. Rev. Biochem.* 1995. **64:** 621–651.

3 **Ihle, J. N., Witthuhn, B. A., Quelle, F. W., Yamamoto, K. and Silvennoinen, O.,** Signaling through the hematopoietic cytokine receptors. *Annu. Rev. Immunol.* 1995. **13:** 369–398.

4 **Trinchieri, G.,** Interleukin-12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes. *Blood* 1994. **84:** 4008–4027.

5 **Spencer, D. M., Wandless, T. J., Schreiber, S. L. and Crabtree, G. R.,** Controlling signal transduction with synthetic ligands. *Science* 1993. **262:** 1019–1024.

6 **Levitzki, A. and Gazit, A.,** Tyrosine kinase inhibition: an approach to drug development. *Science* 1995. **267:** 1782–1788.

7 **Ito, T. and May, W. S.,** Drug development train gathering steam. *Nat. Med.* 1996. **2:** 403–404.

8 **Levitzki, A.,** Tyrphostins: tyrosine kinase blockers as novel antiproliferative agents and dissectors of signal transduction. *FASEB J.* 1992. **6:** 3275–3282.

9 **Nielsen, M., Kaltoft, K., Nordahl, M., Ropke, C., Geisler, C., Mustelin, T., Dobson, P., Svejgaard, A. and Odum, N.,** Constitutive activation of a slowly migrating isoform of Stat3 in mycosis fungoides: tyrphostin AG490 inhibits Stat3 activation and growth of mycosis fungoides tumor cell lines. *Proc. Natl. Acad. Sci. USA* 1997. **94:** 6764–6769.

10 **Bright, J. J., Du, C. and Sriram, S.,** Tyrphostin B42 inhibits IL-12-induced tyrosine phosphorylation and activation of Janus kinase-2 and prevents experimental allergic encephalomyelitis. *J. Immunol.* 1999. **162:** 6255–6262.

11 **Ahn, H. J., Tomura, M., Yu, W. G., Iwasaki, M., Park, W. R., Hamaoka, T. and Fujiwara, H.,** Requirement for distinct Janus kinases and STAT proteins in T cell proliferation versus IFN-γ production following IL-12 stimulation. *J. Immunol.* 1998. **161:** 5893–5900.

12 **Shibuya, H., Yoneyama, M., Ninomiya-Tsuji, J., Matsumoto, K. and Taniguchi, T.,** IL-2 and EGF receptors stimulate the hematopoietic cell cycle via different signaling pathways: demonstration of a novel role for c-myc. *Cell* 1992. **70:** 57–67.

13 **Okamura, H., Kashiwamura, S., Tsutsui, H., Yoshimoto, T. and Nakanishi, K.,** Regulation of interferon-γ production by IL-12 and IL-18. *Curr. Opin. Immunol.* 1998. **10:** 259–264.

14 **Robinson, D., Shibuya, K., Mui, A., Zonin, F., Murphy, E., Sana, T., Hartley, S. B., Menon, S., Kastelein, R., Bazan, F. and O'Garra, A.,** IGIF does not drive Th1 development but synergizes with IL-12 for interferon-γ production and activates IRAK and NFκB. *Immunity* 1997. **7:** 571–581.

15 **Ahn, H. J., Maruo, S., Tomura, M., Mu, J., Hamaoka, T., Nakanishi, K., Clark, S., Kurimoto, M., Okamura, H. and Fujiwara, H.,** A mechanism underlying synergy between IL-12 and IFN-γ-inducing factor in enhanced production of IFN-γ. *J. Immunol.* 1997. **159:** 2125–2131.

16 **Nakahira, M., Ahn, H.-J., Park, W.-R., Gao, P., Tomura, M., Park, C.-S., Hamaoka, T., Ohta, T., Kurimoto, M. and Fujiwara, H.,** Synergy of IL-12 and IL-18 for IFN-γ gene expression: IL-12-induced STAT4 contributes to IFN-γ promoter activation by upregulating the binding activity of IL-18-induced AP-1. *J. Immunol.* 2002. **168:** 1146–1153.

17 **Barbulescu, K., Becker, C., Schlaak, J. F., Schmitt, E., Meyerzum-Buschenfelde, K. H. and Neurath, M. F.,** IL-12 and IL-18 differentially regulate the transcriptional activity of the human IFN-γ promoter in primary $CD4^+$ T lymphocytes. *J. Immunol.* 1998. **160:** 3642–3647.

18 **Du, C., Bright, J. J. and Sriram, S.,** Inhibition of CD40 signaling pathway by tyrphostin A1 reduces secretion of IL-12 in macrophage, Th1 cell development and experimental allergic encephalomyelitis in SJL/J mice. *J. Neuroimmunol.* 2001. **114:** 69–79.

19 **Azzoni, L., Kanakaraj, P., Zatsepina, O. and Perussia, B.,** IL-12-induced activation of NK and T cells occurs in the absence of immediate-early activation gene expression. *J. Immunol.* 1996. **157:** 3235–3241.

20 **Karaghiosoff, M., Neubauer, H., Lassnig, C., Kovarik, P., Schindler, H., Pircher, H., McCoy, B., Bogdan, C., Decker, T., Brem, G., Pfeffer, K. and Muller, M.,** Partial impairment of cytokine responses in Tyk2-deficient mice. *Immunity* 2000. **13:** 549–560.

21 **Kaplan, M. H., Sun, Y. L., Hoey, T. and Grusby, M. J.,** Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature* 1996. **382:** 174–177.

22 **Thierfelder, W. E., van-Deursen, J. M., Yamamoto, K., Tripp, R. A., Sarawar, S. R., Carson, R. T., Sangster, M. Y., Vignali, D. A., Doherty, P. C., Grosveld, G. C. and Ihle, J. N.,** Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature* 1996. **382:** 171–174.

23 **Adachi, O., Kawai, T., Takeda, K., Matsumoto, M., Tsutsui, H., Sakagami, M., Nakanishi, K. and Akira, S.,** Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity* 1998. **9:** 143–150.

24 **Penix, L., Weaver, W. M., Pang, Y., Young, H. A. and Wilson, C. B.,** Two essential regulatory elements in the human interferon γ promoter confer activation specific expression in T cells. *J. Exp. Med.* 1993. **178:** 1483–1496.

25 **Maruo, S., Ahn, H. J., Yu, W. G., Tomura, M., Wysocka, M., Yamamoto, N., Kobayashi, M., Hamaoka, T., Trinchieri, G. and Fuijiwara, H.,** Establishment of an IL-12-responsive T cell clone: its characterization and utilization in the quantitation of IL-12 activity. *J. Leukoc. Biol.* 1997. **61:** 346–352.

26 **Gouilleux, F., Moritz, D., Humar, M., Moriggl, R., Berchtold, S. and Groner, B.,** Prolactin and interleukin-2 receptors in T lymphocytes signal through a MGF-STAT5-like transcription factor. *Endocrinology* 1995. **136:** 5700–5708.

27 **Nakahira, M., Tomura, M., Iwasaki, M., Ahn, H. J., Bian, Y., Hamaoka, T., Ohta, T., Kurimoto, M. and Fujiwara, H.,** An absolute requirement for STAT4 and a role for IFN-γ as an amplifying factor in IL-12 induction of the functional IL-18 receptor complex. *J. Immunol.* 2001. **167:** 1306–1312.

Correspondence: Hiromi Fujiwara, Department of Oncology (C6), Osaka University Graduate School of Medicine, 2–2, Yamada-oka, Suita, Osaka 565–0871, Japan
Fax: +81-6-6879-3989
e-mail: hf@ongene.med.osaka-u.ac.jp