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Interleukin-12p40 variant form reduces Interleukin-12p80 secretion

Yumi Oshikiri[a], Hidetoshi Nara[a,1], Yuji Takeda[a], Akemi Araki[a], Nobuhito Nemoto[a], Md. Yeashin Gazi[a], Shoko Saito[a], Shinichi Saitoh[a], Osamu Nakajima[b], Hironobu Asao[a,*]

[a] Department of Immunology, Yamagata University, Faculty of Medicine, Japan
[b] Research Center for Molecular Genetics, Institute for Promotion of Medical Science Research, Yamagata University, Faculty of Medicine, Japan

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ABSTRACT

IL-12 is a key cytokine for the promotion of $CD4^+$ T cells differentiation to type 1 helper T cells. IL-12 is a heterodimer (IL-12p70) consisting of p40 and p35 subunits, and is mainly secreted from activated antigen-presenting cells, such as macrophages and dendritic cells (DCs). In this study, we found that activated mouse bone marrow-derived DCs (BMDCs) produced a p40 splice variant form mRNA in addition to the conventional p40 mRNA. This p40 variant mRNA was produced by alternative splicing in exon 5, and possessed a premature stop codon. As a result, the p40 variant protein contained 157 amino acids of the N-terminal part of p40 and an additional 10 novel amino acids. When the p40 variant was expressed in HEK-293T cells, it was not secreted from the cells. To investigate the function of the p40 variant, it was co-expressed with p40 and/or p35. The p40 variant did not affect the secretion of IL-12p40 or IL-12p70, or the function of the secreted p70. In contrast, the secretion of IL-12p80, a homodimeric IL-12 with two p40 subunits, was significantly decreased when the p40 variant was expressed. This new splicing variant p40 may act to fine-tune the function of IL-12p80.

1. Introduction

Interleukin 12 (IL-12) is produced by pathogen-activated antigen-presenting cells such as dendritic cells and macrophages, and has an essential role in promoting the differentiation of peripheral naïve $CD4^+$ T cells into interferon-γ (IFN-γ)-producing type 1 helper T (Th1) cells [1]. Therefore, IL-12 is an important cytokine for linking the innate and adaptive immunity [2]. IL-12 is a heterodimeric cytokine (IL-12p70) composed of two subunits, IL-12p35 and IL-12p40 [1]. IL-12p40 also form other cytokines, such as IL-23 and IL-Y with other subunits p19 and p28, respectively [3]. IL-23 promotes the differentiation of pre-Th17 cells to Th17 cells, which are deeply involved in inflammation, tissue regeneration, and cancer development, while IL-Y appears to suppress the differentiation of Th1 and Th17 cells and to have anti-inflammatory functions [3]. IL-12p40 also forms a p40 homodimer, IL-12p80, which was initially thought to suppress the function of IL-12p70 [4–6]. However, IL-12p80 was recently found to have a unique function that has attracted much attention. IL-12p80 functions as a chemotactic factor for macrophages, activates macrophages to produce inflammatory cytokines and nitric oxide, and enhances the migratory ability of dendritic cells [7]. Thus, IL-12p40 serves as a subunit of various cytokines, and is considered to be a very important molecule in regulating immune responses.

According to the mouse gene database, splice variants exist for IL-12p40, IL-12p35, and IL-23p19. Many of them are isoforms that retain the same structure and function as their more common counterparts. One splice variant of IL-23p19 with 219 base inserts results in a deletion in the C-terminal region; however, it is unknown how this deletion translation product is involved in the function of the normal p19 molecule [8]. A splice variant form with a deletion similar to mIL-23p19 is also reported in Ebi3, which is an IL-12p40-related protein and a subunit of IL-27 [9]. To date, however, the physiological functions of these splice variant forms have not been reported.

In this study, we discovered a novel alternative splice variant form of IL-12p40, which was a C-terminal-deleted version of the original IL-12p40. We further found that this variant form had effects on IL-12p80 production and secretion.

Abbreviations: IL-12, Interleukin-12; BMDC, bone marrow-derived DC; IFN-γ, interferon-γ; LPS, lipopolysaccharide

* Corresponding author at: Department of Immunology, Yamagata University, Faculty of Medicine, 2-2-2 Iida-Nishi, Yamagata 990-9585, Japan.
E-mail address: asao-h@med.id.yamagata-u.ac.jp (H. Asao).
[1] Present address: Department of Biological Sciences, Faculty of Science and Engineering, Ishinomaki Senshu University, Ishinomaki, Japan.

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2. Material and methods

2.1. Mice

C57BL/6J mice were maintained on a 12-hour light/dark cycle under specific pathogen-free conditions, with ad libitum access to a standard diet and water, in the Research Center for Molecular Genetics of the Institute for the Promotion of Medical Science Research, Yamagata University Faculty of Medicine. All animal experiments were approved by the Animal Experiment Committee of the Yamagata University Faculty of Medicine (approval number 29099) and were conducted according to the Yamagata University animal experimental regulations.

2.2. Cell preparation and cell culture

Mouse spleen was homogenized with a glass homogenizer. The cell suspensions were passed through nylon mesh, and the red blood cells were lysed using ACK buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 10 mM EDTA). These cells were suspended in RPMI-1640 medium (Gibco) containing L-glutamine and 25 mM HEPES and supplemented with 10% heat-inactivated fetal calf serum (FCS), 50 μM 2-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin (complete RPMI-1640 medium) and cultured for 1 hour on a 100-mm culture dish. After macrophage adhesion, the cells in suspension were collected and used as splenocytes. Bone marrow-derived dendritic cells (BMDCs) were prepared as previously described [10].

Fig. 1. Comparison of the mouse *Il12b* and *Il12b variant* mRNA sequences. The *Il12b* mRNA sequence (upper) and *Il12b variant* mRNA sequence (lower) are shown. Start and stop codons are boxed. The 50-base deletion of the *Il12b variant* is indicated as a dashed line.

Briefly, bone marrow cells were isolated from the femurs and tibias of 5- to 10-week-old mice by flushing with PBS. The red blood cells were lysed using ACK buffer, and the cells were cultured in bacterial dishes in complete RPMI-1640 medium containing 10 ng/mL murine recombinant GM-CSF (PeproTech, #315-03). The medium was replaced every 3 days, and BMDCs that were cultured for 8 days were used in experiments. HEK293T cells were cultured in D-MEM (Wako) supplemented with 10% FCS. All the cells were cultured at 37 °C, in an atmosphere of 5% CO_2 in air, with humidity.

2.3. Antibodies and reagents

The antibodies (Abs) used in this study were an anti-IL-12/IL-23p40 Ab (eBioscience Technology, C17.8, #16-7123), anti-STAT4 Ab (Santa Cruz Biotechnology, H-119, sc-7959), anti-GAPDH Ab (Santa Cruz Biotechnology, V-18, sc-20357), anti-phosphoSTAT4 (Tyr693) Ab (Cell

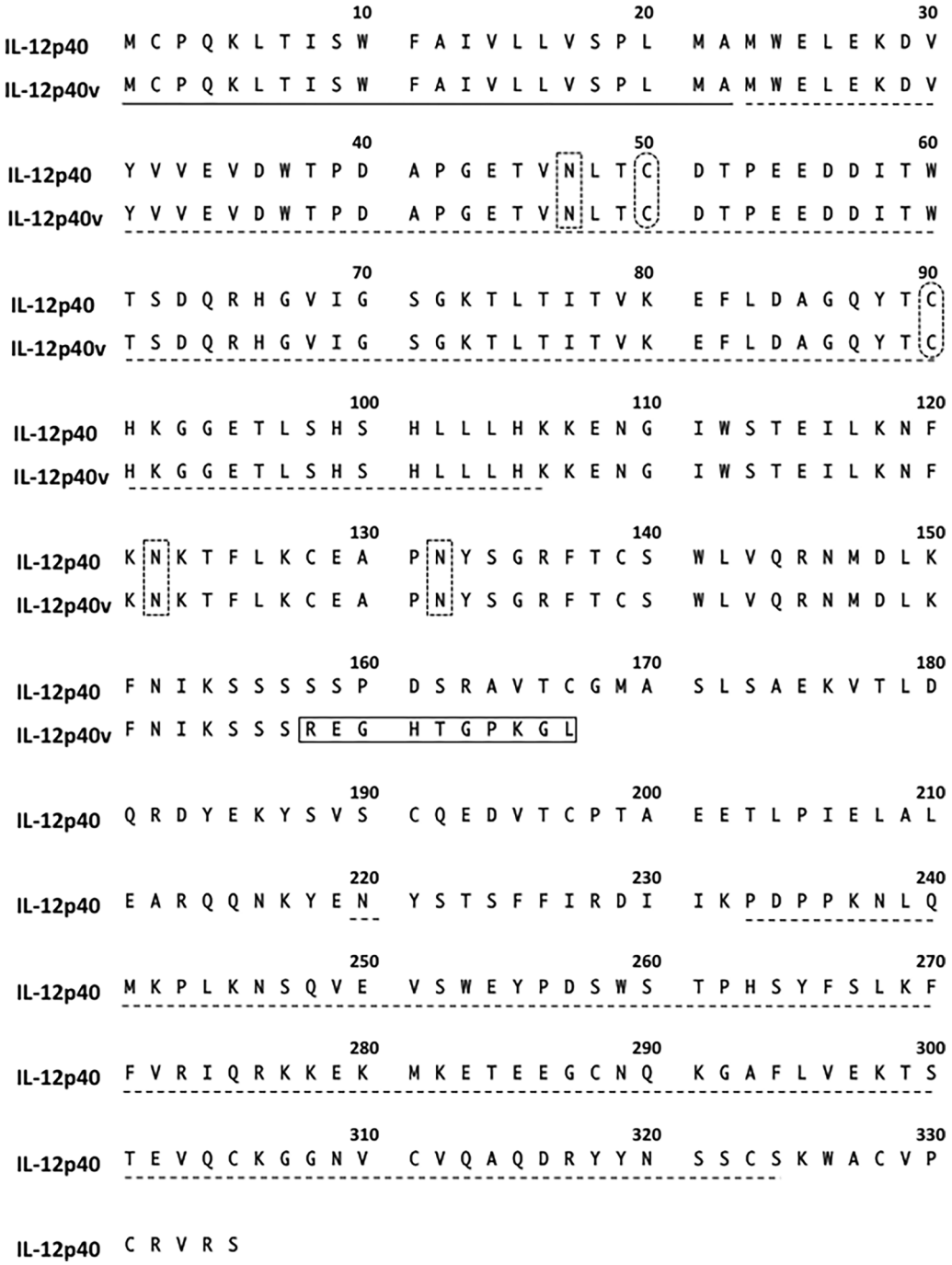

Fig. 2. Comparison of the mouse IL-12p40 and IL-12p40 variant (IL-12p40v) amino acid sequences. The signal peptide is underlined (solid line), the immunoglobulin-like C2 type region is underlined (dashed line at the N-terminal region), and the fibronectin type III region is underlined (dashed line at the C-terminal region). Asparagines (N) involved in N-linked carbohydrate binding and cysteines (C) involved in an intramolecular disulfide bond of the immunoglobulin-like C2 type region are surrounded by dashed boxes and dashed ovals, respectively. The IL-12p40 variant-specific amino acid sequence is surrounded by a box.

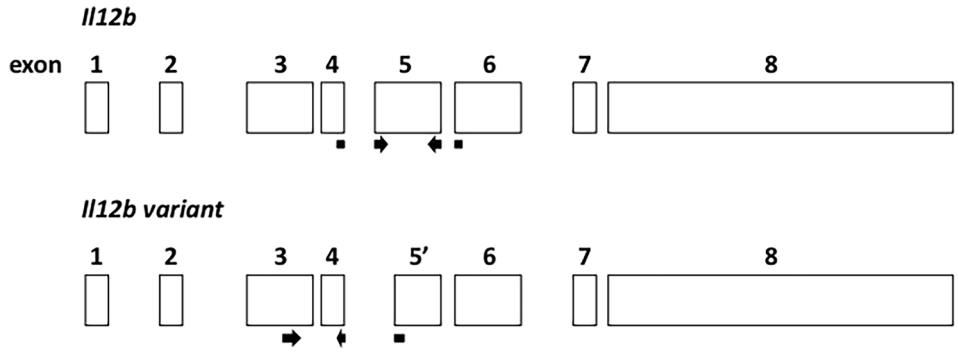

Fig. 3. Structure of the *Il12b* and *Il12b variant* exons. Structures of the mouse *Il12b* and *Il12b variant* exons are shown. The exon 5 specifically used in the *Il12b variant* was defined as exon 5′. The positions of each specific PCR primer set are shown as arrows.

Signaling Technology, D2E4, #4134), horseradish peroxidase-labeled anti-rabbit IgG Ab (Cell Signaling Technology, #7074), and horseradish peroxidase-labeled anti-rat IgG Ab (Cell Signaling Technology, #7077). Other reagents used in this study were lipopolysaccharide (LPS) (Sigma-Aldrich, #L4516) and Pam3CSK4 (InvivoGen, #tlrl-pms).

2.4. Quantitative real-time RT-PCR (qRT-PCR) and RT-PCR analysis

Total RNA was isolated with TRIzol reagent (Life Technologies, #15596018), and the cDNA was synthesized with ReverTra Ace-α (Toyobo Life Science, #FSQ-301) according to the manufacturer's instructions. Thermo-cycling was performed using a LightCycler (Roche Molecular Systems) and Thunderbird SYBR qPCR mix (Toyobo Life Science, #QPS-201). The *Il12a*, *Il12b,* and *Il12b variant* transcript levels were shown relative to the *Actb* mRNA level. RT-PCR analysis was performed using a thermal cycler (Takara Dice) and Ex Taq polymerase (Takara, #RR001). The mouse primer sets used in these PCR reactions were as follows: *Il12a* sense: 5′-CCACCCTTGCCCTCCTAAAC-3′ and antisense: 5′-GTTTTTCTCTGGCCGTCTTCA-3′; *Il12b* sense: 5′-GGGACA TCATCAAACCAGACCC-3′ and antisense: 5′-GCCTTTGCATTGGACTT CGG-3′; *Il12b variant* sense: 5′-GCTCTGGAAAGACCCTGACC-3′ and antisense: 5′-CAGTGTGACCTTCTCTGCTAC-3′; *Actb* sense: 5′-TGACAG GATGCAGAAGGAGA-3′ and antisense: 5′-GCTGGAAGGTGGACAGT GAG-3′.

2.5. Preparation and transfection of expression vector plasmids.

Mouse IL-12p35 and IL-12p40 cDNAs were amplified by PCR from Pam3CSK4-stimulated BMDC cDNA with KOD plus polymerase (Toyobo Life Science, #KOD-211) using the primer pairs, 5′-ATCTCGAGTCTCA CCGTGCACATCCAAG-3′ and 5′-AACTCGAGGCCAAGACCACCTGA CTC-3′, and 5′-ATCTCGAGTCCAGGCACATCAGACCAG-3′ and 5′-AACT CGAGACGCAGCCCTGATTGAAGAG-3′, respectively. Mouse IL-12p40 variant cDNA was also amplified with the same primer set used for IL-12p40. The PCR products were digested with Xho-I and then ligated into the pKU2-Hyg expression vector [11]. The expression plasmid vectors for mouse IL-12p35 (pKUmIL12a) and mouse IL-12p40 (pKU-mIL12b) were verified by sequencing. HEK-293T cells were transfected with plasmid vectors by a calcium phosphate method as described previously [12]. Thirty-six hours after transfection, the cells and supernatants were collected.

2.6. Cell extraction and immunoblot analysis

The cell extraction and immunoblot analysis were performed as described previously [13]. Splenocytes were suspended in whole cell extraction buffer [10 mM phosphate buffer (pH 7.4), 1 mM EDTA, 400 mM KCl, 10% (v/v) glycerol, 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich, #P8340), 1% (v/v) phosphatase inhibitor cocktail II (Sigma-Aldrich, #P0044) and III (Sigma-Aldrich, #P5726), 5 mM NaF,

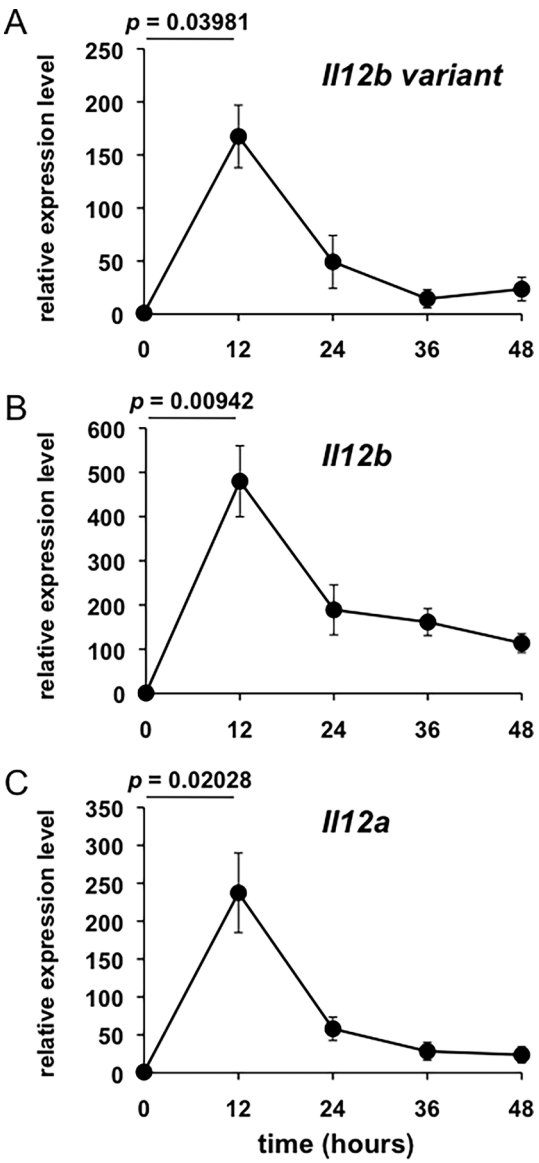

Fig. 4. Kinetics of the *Il12b*, *Il12b variant*, and *Il12a* mRNA expressions. Mouse BMDCs were stimulated with LPS for the indicated periods, and the amount of *IL12b variant* (A), *IL12b* (B) and *IL12a* (C) mRNA was measured by quantitative RT-PCR as described in the Materials and Methods. The obtained data from each sample were normalized to the amount of *β-actin* (*Actb*) as an internal control and showed each comparison with before LPS stimulation. Data represent the means ± SD from 3 independent experiments. *p* values were calculated by unpaired Student's *t*-test (before stimulation vs. 12 hours after LPS stimulation) and are shown in the figure.

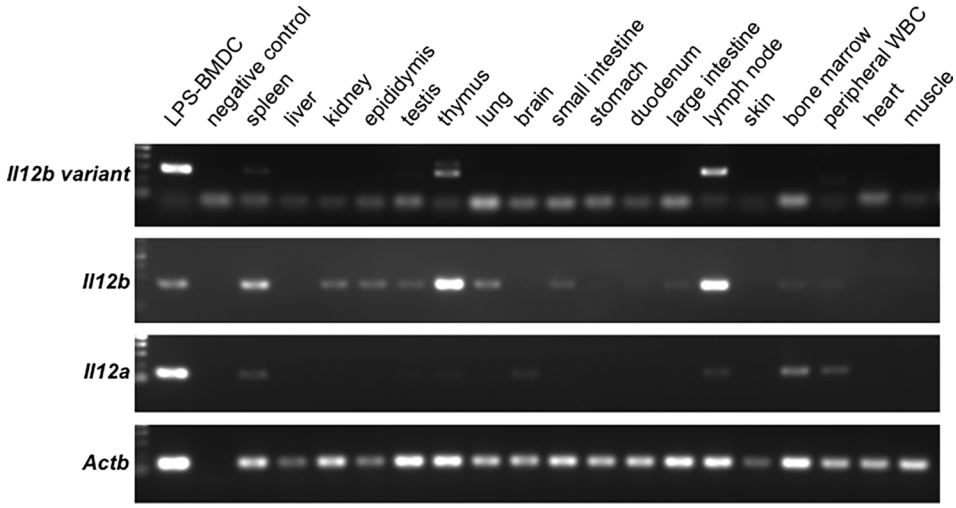

Fig. 5. *Il12b variant* expression in mouse tissues. The expression of *IL12b variant*, *IL12b*, *IL12a* and *Actb* mRNA of mouse various tissues was analyzed by RT-PCR as described in the Materials and Methods. cDNAs prepared from LPS-stimulated BMDC and H_2O are used as positive and negative controls, respectively.

1 mM DTT, and 1 mM PMSF] and subjected to three freeze-thaw cycles. The cell suspensions were centrifuged to remove the insoluble fraction, and the supernatant proteins were separated by SDS-PAGE under reducing conditions. HEK-293T cells were suspended in non-reducing NP-40 cell lysis buffer [1% (w/v) NP-40, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1% (v/v) protease inhibitor cocktail, 1% (v/v) phosphatase inhibitor cocktail II and III, 5 mM NaF, and 1 mM PMSF]. The cell suspensions were centrifuged to remove the insoluble fraction. The supernatant proteins of the HEK-293T cell lysates and the HEK-293T cell culture supernatants were separated by SDS-PAGE under non-reducing conditions, and the separated proteins were transferred to Immobilon-P membranes (Millipore, #IPVH000 10). The membranes were blocked with 5% nonfat milk in TBS supplemented with 0.05% (w/v) Tween 20, and then incubated with the first Abs, followed by incubation with HRP-conjugated anti-rabbit or anti-rat IgG secondary Abs. The signals were visualized using the ECL Prime detection system (GE Healthcare, #RPN2232). To reprobe the membranes with different antibodies, the membranes were soaked in stripping buffer [62.5 mM Tris-HCl (pH 6.7), 2% SDS, 100 mM 2-Mercaptoethanol] and incubated for 30 min at 55 °C.

2.7. Statistical analysis

The statistical analysis of pair-wise comparisons between two samples was performed by Student's *t*-test using the R software (version 3.2.2). P-values < 0.05 were considered to be statistically significant.

3. Results and discussion

3.1. Identification of a novel IL-12p40 splice variant form

When we amplified mouse IL-12p40 cDNA by the RT-PCR method, we unexpectedly found a 50-base pair-shorter form of the cDNA (Fig. 1). We named this novel splice variant form *Il12b variant*. We found that the amino acid sequence up to position 157 was identical to that of conventional IL-12p40, while the 10 amino acids from position 158 to 167 were variant-specific, and the protein was terminated at position 168 (Fig. 2). Predicted structures of the *Il12b* and *Il12b variant* genes are shown in Fig. 3. In the splicing of the *Il12b variant* mRNA, the exon 4 donor site is joined to the acceptor site 50 bases downstream of the original exon 5 acceptor site. The human *IL12B* gene structure is quite similar to that of the mouse, and human *IL12B* exon 5 has the same acceptor site for the variant form, suggesting that the IL-12p40 variant form may be evolutionarily conserved in humans and mice.

3.2. Expression analysis of the Il12b variant

To examine the expression of the *Il12b variant* transcript, we prepared a variant specific primer set as described in the Material and Methods and shown in Fig. 3. Quantitative RT-PCR with LPS-stimulated BMDCs showed that the *Il12b variant* mRNA was increased 12 hours after stimulation and then quickly decreased, along with the *Il12b* and *Il12a* mRNAs (Fig. 4). Next, we analyzed *Il12b variant* mRNA expression

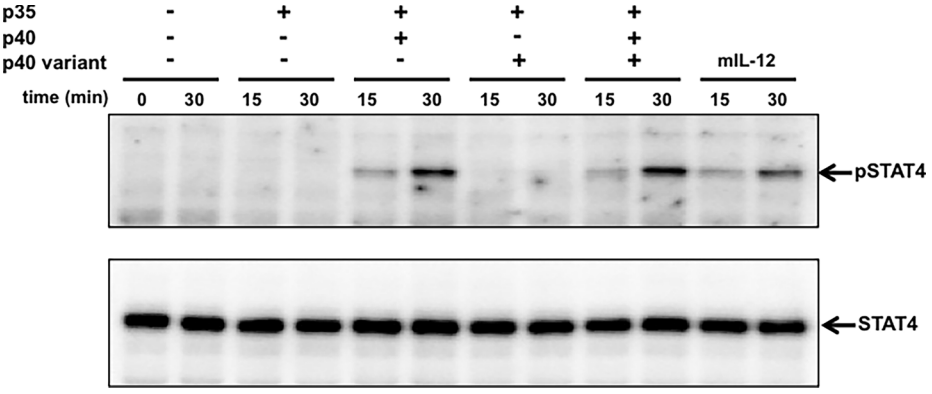

Fig. 6. Effect of the IL-12p40 variant on STAT4 phosphorylation. Mouse splenocytes were stimulated with the culture supernatant of HEK-293T cells transfected with each expression plasmid as indicated. Proteins extracted from the splenocytes were analyzed by immunoblot with an anti-phospho-STAT4 antibody (upper panel). The antibody was then removed from the membrane, and the membrane was reprobed with an anti-STAT4 antibody (lower panel). Splenocytes were stimulated with 10 ng/mL recombinant mouse IL-12 (rmIL-12) as a positive control. A representative result from at least 4 independent experiments is shown.

in various mouse tissues and compared it with *Il12b* expression (Fig. 5). *Il12b variant* is expressed only in lymphoid tissues such as spleen, thymus and lymph node. On the other hand, *Il12b* relatively highly expressed in spleen, thymus and lymph node similar to the *Il12b variant*, and it expressed in bone marrow and peripheral white blood cells and also in non-lymphoid tissues such as kidney, epididymis, testis, lung, small and large intestines. These results suggest that *Il12b variant* shows limited expression pattern than that of *Il12b*. *IL12a* is also expressed in many lymphoid tissues and brain.

3.3. Functional analysis of the IL-12p40 variant

IL-12p70 is a heterodimer consisting of IL-12p35 and IL-12p40. To analyze the function of the IL-12p40 variant, we introduced the combination of IL-12p35, IL-12p40, and IL-12p40 variant expression vectors into HEK-293T cells, and collected the culture supernatants. STAT4 is a main signaling pathway downstream of IL-12p70-IL-12 receptor system. So mouse splenocytes were treated with these supernatants, and the STAT4 phosphorylation was examined (Fig. 6). The supernatant from the combination of IL-12p35 and IL-12p40 expression vectors clearly induced STAT4 phosphorylation, while the supernatant from the combination of IL-12p35 and IL-12p40 variant did not show any STAT4 activation. Furthermore, IL-12p40 variant expression had no effect on the IL-12p35 and IL-12p40 expression vector-transfected supernatant. These results suggested that the IL-12p40 variant has no effect on IL-12p70 formation or on IL-12p70 function.

Next, we analyzed the expression of the IL-12p40 variant in the expression vector-introduced HEK-293T cells and their culture supernatants. IL-12p40 variant was detected in the transfected cell lysates (Fig. 7B) but not in the supernatants (Fig. 7A). The IL-12p40 variant had no effect on the IL-12p70 or IL-12p40 monomer expression or secretion, whereas it decreased the expression of IL-12p80, which is a homodimer of IL-12p40 that migrates as an approximately 120-kDa molecule in non-reducing SDS-PAGE [5]. With the decrease in IL-12p80 intracellular expression, IL-12p80 secretion also decreased. We are not sure why the IL-12p40 variant reduces the IL-12p80 homodimer expression. Because the IL-12p40 variant did not reduce the IL-12p40 monomer production in the cells, the IL-12p40 variant may inhibit the homodimerization process. Interestingly, IL-12p80 was detected only in the absence of IL-12p35 expression (Fig. 7). IL-12p40 may preferentially associate with IL-12p35 than the homodimerization. Because IL-12p40 and p35 expressions are differentially regulated, IL-12p40 variant may have function only in the absence of IL-12p35. IL-12p80 was originally reported to inhibit the IL-12p70 function. However, it was later found to function as an agonistic cytokine for macrophages and DCs, and as a pathogenic inflammatory cytokine [7]. The IL-12p40

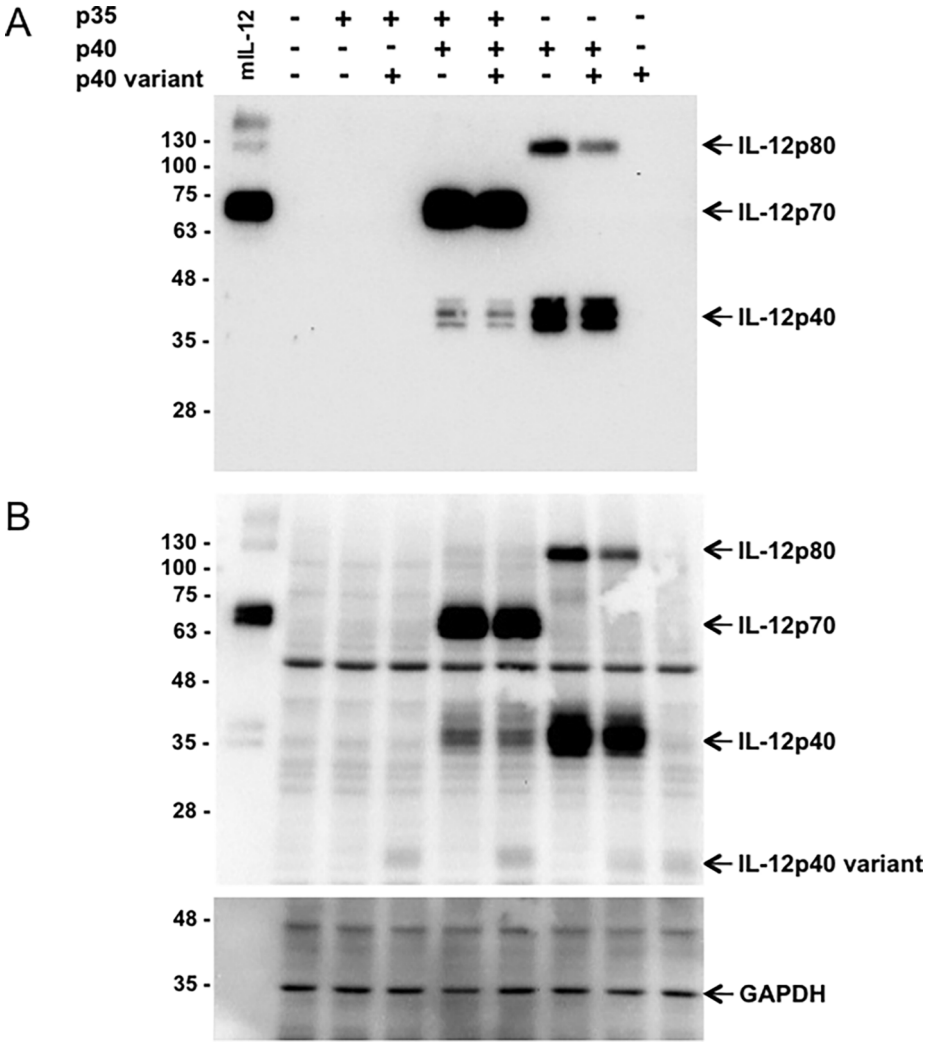

Fig. 7. Effect of the IL-12p40 variant on IL-12p70, IL-12p40, and IL-12p80. HEK-293T cells were transfected with the indicated expression plasmids. The culture supernatants (A) and cell lysates (B, upper panel) were separated by non-reducing SDS-PAGE and analyzed by immunoblot with an anti-IL-12p40 antibody. The antibody was removed from the membrane and the membrane was reprobed with an anti-GAPDH antibody (B, lower panel). The positions of each molecule are indicated by arrows. As a positive control of mIL-12p70, 1 or 0.5 ng of rmIL-12 was used. A representative result from at least 3 independent experiments is shown.

variant may have a role in fine-tuning the IL-12p80 functions. Further study is required to clarify the details of the IL-12p40 variant's roles, which will lead to a better understanding of the whole IL-12 system.

CRediT authorship contribution statement

Yumi Oshikiri: Investigation, Writing - original draft. **Hidetoshi Nara:** . **Yuji Takeda:** . **Akemi Araki:** . **Nobuhito Nemoto:** . **Md. Yeashin Gazi:** . **Shoko Saito:** Investigation. **Shinichi Saitoh:** Investigation. **Osamu Nakajima:** . **Hironobu Asao:** Conceptualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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