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ORIGINAL ARTICLE

Hypothermia Augments NF-kappaB Activity and the Production of IL-12 and IFN-gamma

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ABSTRACT

Background: The differentiation of Th1 and Th2 is strictly regulated by humoral and cellular factors. The imbalance between Th1 and Th2 is considered to be the pathogenesis of allergic and autoimmune disorders. It is important to elucidate the effect of environmental factors, such as temperature, on the expression of cytokines of Th1 and Th2.
Methods: We investigated the expression of IFN-gamma, IL-4, IL-5, IL-10 and IL-12 from LPS- or PHA-stimulated PBMCs at 30℃ or 37℃ using ELISA and Real-time PCR. We measured the change of NF-kappaB activity at 30℃ or 37℃ with LPS stimulation using the reporter gene assay.
Results: IFN-gamma production from LPS-stimulated PBMCs at 30℃ was up-regulated compared with 37℃. IL-5 and IL-10 production from PHA-stimulated PBMCs at 30℃ were down-regulated compared with 37℃. This augmented IFN-gamma production was caused by the up-regulation of IL-12 production from $CD14^+$ blood monocytes. Both IL-12 mRNA and IL12 protein at 30℃ were up-regulated compared with 37℃. NF-kappaB, the key molecule for the expression of IL-12, was also augmented at 30℃ compared with 37℃.
Conclusions: Hypothermia up-regulated the expression of IL-12 and IFN-gamma due to the augmented NF-kappaB activity. It is suggested that hypothermia modifies the pattern of cytokine gene expression.

KEY WORDS

hypothermia, IFN-gamma, IL-12, lipopolysaccharide, NF-kappaB

ABBREVIATIONS

Th, helper T cell; PBMCs, peripheral blood mononuclear cells; IL, interleukin; LPS, Lipopolysaccharide; IFN, interferon; PHA, phytohemagglutinin; NF-kappaB, nuclear factor-kappaB; TLR, Toll-like receptor; SD, standard deviation; FCS, Fetal calf serum.

INTRODUCTION

The $CD4^+$ helper-inducer T lymphocyte subset is in itself heterogeneous.[1-3] Type 1 helper (Th1) clones synthesize IL-2, IFN-gamma and lymphotoxin, but these lymphokines are not detectably expressed in type 2 T helper (Th2) clones. Conversely, only Th2 clones synthesize detectable amounts of IL-4 and IL-5. The differentiation of Th1 and Th2 is strictly regulated by humoral and cellular factors. An Imbalance between Th1 and Th2 subsets has been suggested to be responsible for the pathogenesis that leads to allergic and auto-immune diseases.[4,5]

IgE plays an important role in immediate hypersensitivity.[6] T lymphocyte- or monocyte-derived cytokines play an important role in the regulation of immunoglobulin isotype switching. IL-4 promotes class switching to IgE in B lymphocytes and Th2 lymphocyte differentiation.[7,8] The production of IgE in B lymphocytes is down-regulated by IFN-gamma.[9] Activated macrophages/monocytes produce IL-12, which plays a central role in promoting Th1 type immune responses and thus cell-mediated immunity.[10-12] Lipopolysaccharide (LPS) stimulates NF-kappaB activation through Toll-like receptor 4 (TLR4), which activates MyD88-dependent and -independent path-

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ways.[13-15] IL-12 also induces IFN-gamma production by T lymphocytes and natural killer cells.[16] Therefore, the IFN-gamma-IL-12 circuit plays crucial roles in the balance of Th1 and Th2 subtypes.

Several lines of evidence show that some environmental stressors have variant modulatory effects on the cells of the immune system.[17-24] Temperature is one of the environmental stressors that influences the immune system. The direct influence of mild hypothermia on cytokine expression has been reported.[17,18] Mild hypothermia significantly impaired IL-2 gene expression. In adult monocytes cultured at 32℃, early IL-6 and IL-1β secretion decreased compared with levels at 37℃. It has been reported that hypothermia augments the generation of inflammatory cytokines in a THP-1 human promyelocytic leukemic cell line.[22,23] Hangalapura *et al.*[24] reported that cold stress equally enhanced in vivo proinflammatory cytokine gene expression in chicken lines with both low and high primary antibody responses.

This study was designed to analyze the effect of temperature on the expression of cytokines by investigating the production of IFN-gamma and IL-12 and determining NF-kappaB activity using freshly isolated PBMCs exposed to moderate hypothermia (30℃).

METHODS

ASSAYS FOR CYTOKINES

Cell Preparation

Peripheral blood mononuclear cells (PBMCs) were separated from the blood of 18 healthy volunteers by gradient centrifugation in Ficoll-Paque™ PLUS (GE Healthcare Bio-Sciences, Uppsala, Sweden). $CD14^+$ blood monocytes were separated from the PBMCs using the MACS system (Miltenyi Biotec, Bergisch-Gladbach, Germany) with CD14-specific immunomagnetic beads (Miltenyi Biotec). PBMCs and $CD14^+$ blood monocytes were suspended at a density of 1×10^6 /ml in an RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 20 mmol/L HEPES, 2 mmol/L L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

Cell Culture

PBMCs and $CD14^+$ blood monocytes (1×10^6 /ml) were cultured in the presence or absence of 1 μg/ml of LPS (SIGMA, St. Louis, MO) or 10 μg/ml of PHA (Gibco BRL, Grand Island, NY) for 48 hours in a final volume of 1 ml in a round-bottom tube (Falcon 2059, Becton Deckinson Labware, Franklin Lakes, NJ) at 30℃ or 37℃ in a humidified atmosphere containing 5% CO_2.

ELISA

IFN-gamma concentrations in cell culture supernatants were measured with a human IFN-gamma ELISA kit (JIMRO, Tokyo, Japan). The minimum detection limit was 15.6 pg/ml. IL-4 concentrations in the supernatants of the cell culture were measured with a human IL-4 US ELISA kit (Biosource International, Camarillo, CA). The minimum detection limit was 0.39 pg/ml. IL-5 concentrations in the supernatants of the cell culture were measured with a human IL-5 ELISA kit (Biosource International). The minimum detection limit was 11.7 pg/ml. IL-10 concentrations in the supernatants of the cell culture were measured with a human IL-10 US ELISA kit (Biosource International). The minimum detection limit was 0.78 pg/ml. IL-12 concentrations in the supernatants of the cell culture were measured with a human IL-12 + p40 ELISA kit (Biosource International). The minimum detection limit was 7.81 pg/ml.

PROLIFERATION ASSAY

PBMCs (1×10^6 /ml) were grown in triplicate at 0.2 ml per well in round-bottom microtest plates (A/S Nunc, Roskilde, Denmark) with or without LPS at 30 ℃ or 37℃ for 48 hours. The amount of DNA synthesized was measured by adding 0.5 μCi of ^{3}H-thymidine per well in the microtest plates for 4 hours before harvesting using glass fibre filters. The cells were then harvested, the amount of ^{3}H-thymidine incorporated was measured by liquid scintillation counting, and the results were expressed as the mean of triplicate cultures.[25]

PBMC Culture with Anti-Human IL-12 Neutralizing Antibody

PBMCs were incubated with an anti-human IL-12 neutralizing antibody (0, 1 or 10 μg/ml) (clone number 24910) (R&D Systems, Minneapolis, MN) by LPS stimulation at 30℃ or 37℃ for 48 hours. This antibody was selected for its ability to neutralize the biological activity of recombinant human IL-12. The IFN-gamma concentrations in the supernatants of the cell culture were measured.

Real-Time RT-PCR

Total RNA was isolated from PBMCs or $CD14^+$ monocytes with ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's recommendations. Reverse transcription to cDNA was performed with a 1st Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Real-time RT-PCR was performed with a LightCycler-Primer Set "IL-12 p40 mRNA" (Search-LC GmbH, Heidelberg, Germany), "IL-12 p 35 mRNA" (Search-LC GmbH) and "GAPDH mRNA" (Search-LC GmbH) using the LightCycler instrument (Roche Diagnostics). Then, the IL-12 p40 and p35 amounts were divided by an endogenous reference (GAPDH) amount to obtain normalized target values.

NF-KAPPA B REPORTER GENE LUCIFERASE ASSAY

Cell and Vector Preparation

Human embryonic kidney 293-hTLR4A-HA cells were purchased from InvivoGen (San Diego, CA). These cells were cultured in Dulbecco's modified Eagle medium (high glucose containing D-MEM) (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were incubated at 37℃ in a humidified atmosphere of 5% CO_2. The NF-kappaB luciferase reporter vector, which was a pGL3-Basic Vector (Promega, Madison, AL) containing four kappaB binding sites, was a gift from Sewon Ki and Tetsuro Kokubo (Yokohama City University, Japan). The internal control luciferase vector (pGL4-hRluc-TK) was purchased from Promega.

Transient Transfection and NF-kappaB Reporter Gene Luciferase Assay

One day before transfection, HEK293-hTLR4A-HA cells were seeded at a density of 2.0×10^5 /mL per six-well plate SUMILON CELLTIGHT C-1 (SUMITOMO BAKELITE, Tokyo, Japan). Then 0.5 μg of NF-kappaB luciferase reporter vector and 0.5 μg of pGL4-hRluc-TK were transfected using Lipofectamine 2000 (Invitrogen). All plasmid vectors were purified using an EndoFree Plasmid Maxi Kit (QIAGEN Sciences, Germantown, MD). After 12 hours, the culture medium was replaced with a fresh medium in the presence or absence of LPS (1.0 μg/mL), and incubated at 30℃ or 37℃ in a humidified atmosphere containing 5% CO_2 for 24 hours. Then the cells were washed with PBS and harvested with passive lysis buffer (Promega). NF-kappaB reporter gene activity assays were performed using a Dual-Luciferase Reporter Assay System (Promega). These luciferase activities were measured using Lumat LB 9507 (Berthold Technologies, Bad Wildbad, Germany). The NF-kappaB-dependent Firefly luciferase activity was normalized to the activity of the internal control (Renilla luciferase). All reagents and samples in these experiments were checked for endotoxin contamination with Endospecy ES-24S (Seikagaku Corporation, Tokyo, Japan).

Statistical Analysis

Significant differences between the two groups were analyzed using Student's t test. A statistical significance was assumed for p-values of less than 0.05.

RESULTS

To investigate the effect of temperature on cytokine production, we measured the concentration of the Th1 cytokine, IFN-gamma, by ELISA in the supernatants of cell cultures exposed to 30℃ or 37℃. IFN-gamma production from PBMCs stimulated with LPS at 30℃ was significantly up-regulated compared to that at 37℃ (Fig. 1A; $p < 0.05$). We also investigated the Th2 cytokines (IL-4 and IL-5) and IL-10 production at 30℃ and 37℃. As shown in Figure 1B, no change in the production of IL-4 stimulated with PHA at 30℃ and 37℃ was observed. IL-5 production stimulated with PHA at 30℃ was significantly down-regulated compared to 37℃ exposure (Fig. 1C; $p < 0.01$). IL-10 production from PBMCs stimulated with LPS at 30℃ tended to be down-regulated compared to 37℃ exposure (Fig. 1D; $p = 0.058$).

To investigate whether IL-12 production, which is a strong inducer for IFN-gamma production in PBMCs, played a role in the augumentation of IFN-gamma at 30℃, we measured the IL-12 p40 concentrations in PBMCs supernatants stimulated with LPS using ELISA. IL-12 p40 production from PBMCs incubated at 30℃ was up-regulated compared to 37℃ exposure (Fig. 2A; $p < 0.01$). Furthermore, $CD14^+$ blood monocytes which were separated from the PBMCs using the microbead system also showed evidence of greater production of IL-12 p40 at 30℃ than at 37℃ (Fig. 2B; $p < 0.05$).

In order to control the possible effects on cell growth due to LPS at different temperatures, we analyzed the proliferation of LPS-stimulated PBMCs incubated at 30℃ and 37℃ using ^{3}H-thymidine. As shown in Figure 3, ^{3}H-thymidine up-take in PBMCs did not increase by LPS stimulation compared with no-stimulation. When PBMCs were cultured in the absence of LPS, ^{3}H-thymidine up-take in PBMCs at 30℃ was inhibited compared with 37℃ ($p < 0.05$). Using trypan-blue, cell viability was almost equal (above 95%) at both 30℃ and 37℃ in PBMCs in the presence or absence of LPS.

In order to determine whether IL-12 was responsible for the increased expression of IFN-gamma, we incubated LPS-stimulated PBMCs with 0, 1 or 10 μg/ml of an anti-human IL-12-neutralizing antibody at 30℃ or 37℃ for 48 hours. The anti-IL-12-neutralizing antibody down-regulated the production of IFN-gamma at 30℃ with LPS (Fig. 4). These results suggest that the augmentation of IFN-gamma production at 30℃ was mainly caused by the up-regulation of IL-12 production from $CD14^+$ blood monocytes.

To analyze the time course of expression of IL-12 p40 mRNA and protein, we cultured PBMCs for up to 48 hours at 30℃ or 37℃. IL-12 p40 mRNA was measured by real-time PCR and IL-12 p40 protein by ELISA. An increase in IL-12 p40 mRNA was detected 4 hours after incubation at 37℃ and only after 8 hours with 30℃ incubation, but the extent of the response was markedly greater at 30℃ (Fig. 5A). This was also the case for IL-12. Protein appeared 8 hours after incubation at 37℃ and plateaued after 12 hours, whereas IL-12 was only detected after 12 hours at 30℃ but it increased markedly in parallel with that for IL-12 mRNA (Fig. 5B). Since biologically active IL-12 consists of p35 and p40, we examined the expres-

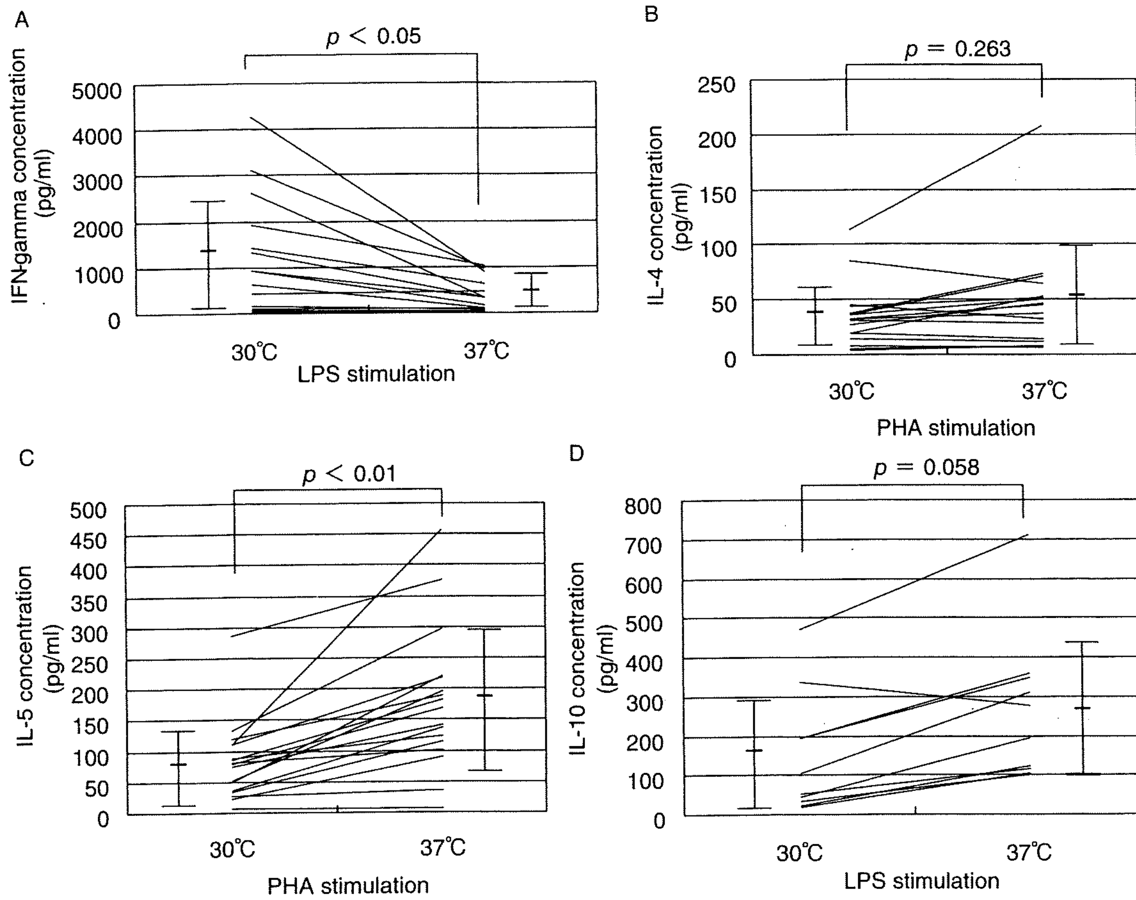

Fig. 1 PBMCs were cultured in the presence of LPS (1 μg/mL) (IFN-gamma and IL-10) or PHA (10 μg/ml) (IL-4 and IL-5) for 48 hours at 30℃ or 37℃. (**A**) IFN-gamma, (**B**) IL-4, (**C**) IL-5 and (**D**) IL-10 concentrations in the supernatants of the cell cultures were measured by ELISA. Results are mean ± SD.

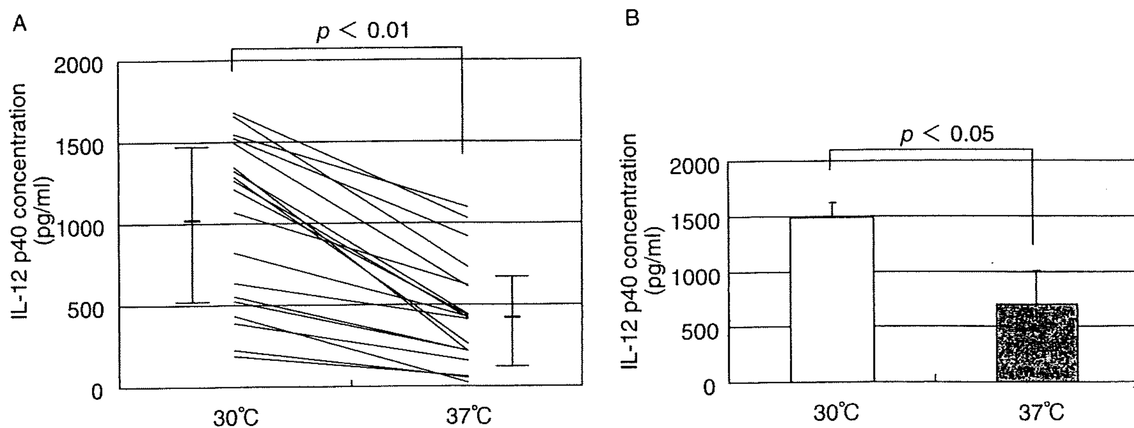

Fig. 2 PBMCs were cultured in the presence of LPS (1 μg/mL) for 48 hours at 30℃ or 37℃. (**A**) IL-12 p40 concentrations in the supernatants of the cell cultures were measured by ELISA. (**B**) $CD14^+$ blood monocytes were separated from the PBMCs, and cultured in the presence of LPS (1 μg/mL) for 48 hours at 30℃ or 37℃. IL-12 p40 concentrations in the supernatants of the cell cultures were measured by ELISA. Results are mean ± SD.

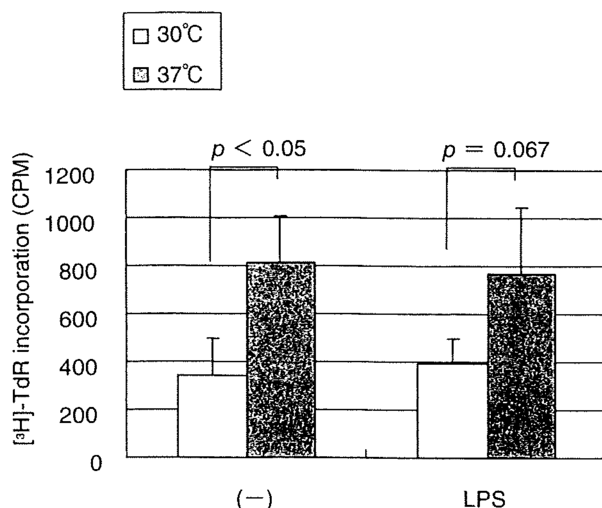

Fig. 3 PBMCs were grown in triplicate at 0.2 ml per well in round-bottom microtest plates with or without LPS at 30℃ or 37℃ for 48 hours. The amount of DNA synthesized was measured by adding 0.5 μCi of ^{3}H-thymidine per well in the microtest plates for 4 hours before harvesting using glass fibre filters. The cells were then harvested, the amount of ^{3}H-thymidine incorporated was measured by liquid scintillation counting, and the results were expressed as the mean ± SD of triplicate cultures

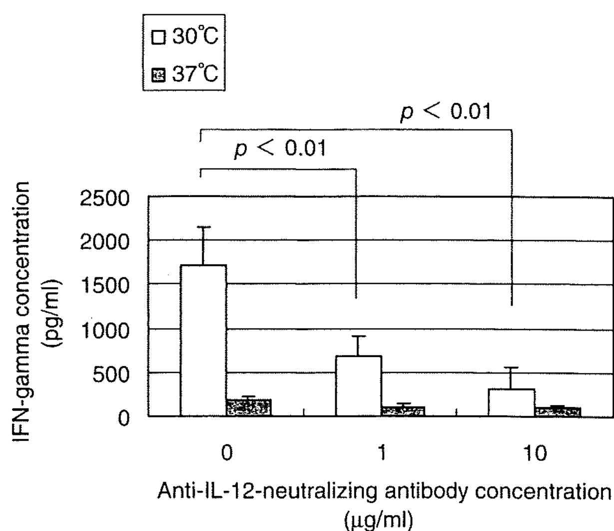

Fig. 4 PBMCs were cultured in the presence of LPS (1 μg/mL) and 0, 1 or 10 μg/ml of an anti-IL-12-neutralizing antibody for 48 hours at 30℃ or 37℃. IFN-gamma concentrations in the supernatants of the cell cultures were measured by ELISA. Results are mean ± SD.

sion of IL-12 p35 in CD14$^+$ monocytes to show the hypothermia up-regulated IL-12 production. As shown in Figure 5C, IL-12 p35 expression was up-regulated at 30℃ compared with 37℃.

LPS is an inducer of the inflammatory response through a well described signalling pathway involving Toll-like receptors 4, NF-kappaB and a number of downstream genes including IL-12 and IFN-gamma.[13] We determined whether the responses observed here might be mediated through NF-kappaB. We measured the change in NF-kappaB activity at 30℃ and 37℃ after LPS stimulation using the Luciferase Assay. NF-kappaB activity at 30℃ was higher than at 37℃ after 24 hours incubation (Fig. 6).

DISCUSSION

In this study, we showed that IFN-gamma production after LPS stimulation of PBMCs at 30℃ was enhanced compared to that after incubation at 37℃. The augmented IFN-gamma production was caused by the up-regulation of IL-12 in CD14$^+$ blood monocytes, and hypothermia up-regulated expression of IL-12 was due to an increase in NF-kappaB activity. As shown in Figure 4, when LPS-induced IL-12 was blocked by an anti-IL-12 antibody, production of IFN-gamma was inhibited. The functional IL-12 receptor was expressed mainly in T cells. It is considered that the T cells were the major producers of IFN-gamma upon LPS stimulation in the PBMC culture.

Consistent with our results, Fairchild *et al.*[22,23] reported that hypothermia enhanced phosphorylation of I-kappaB kinase and prolonged nuclear localization of NF-kappaB in LPS-activated macrophages and augmented the generation of inflammatory cytokines. Matsui *et al.*[21] reported that mild hypothermia raised the levels of IL-1beta, IL-6, IL-12 p70 and TNF-alpha produced by monocytes stimulated with LPS. On the other hand, Irazuzta *et al.*[26] reported that hypothermia produced a transitory attenuation of nuclear factor-kappaB activation in a rat model of bacterial meningitis, but they exposed rats to hypothermia for only a 6-hour period. It was reported that human cerebral endothelial cells exposed to moderate hypothermia showed attenuated NF-kappaB activation at 4 hours after stimulation with IL-1beta, but other time points were not studied.[27] In this study we demonstrated that NF-kappaB activity was up-regulated at 30℃ compared to 37℃ at 24 hours, using the reporter gene Luciferase Assay system.

Several reports have appeared on the mechanism of hypothermia-induced augumention of NF-kappaB activity. Luhm *et al.*[28] concluded that hypothermia augments LPS-induced cytokine generation by increasing LPS bioactivity rather than by modifying the cellular response to stimulation. In contrast it was suggested that hypothermia augments cytokine generation by modifying monocytes cellular responses to diverse stimuli.[22]

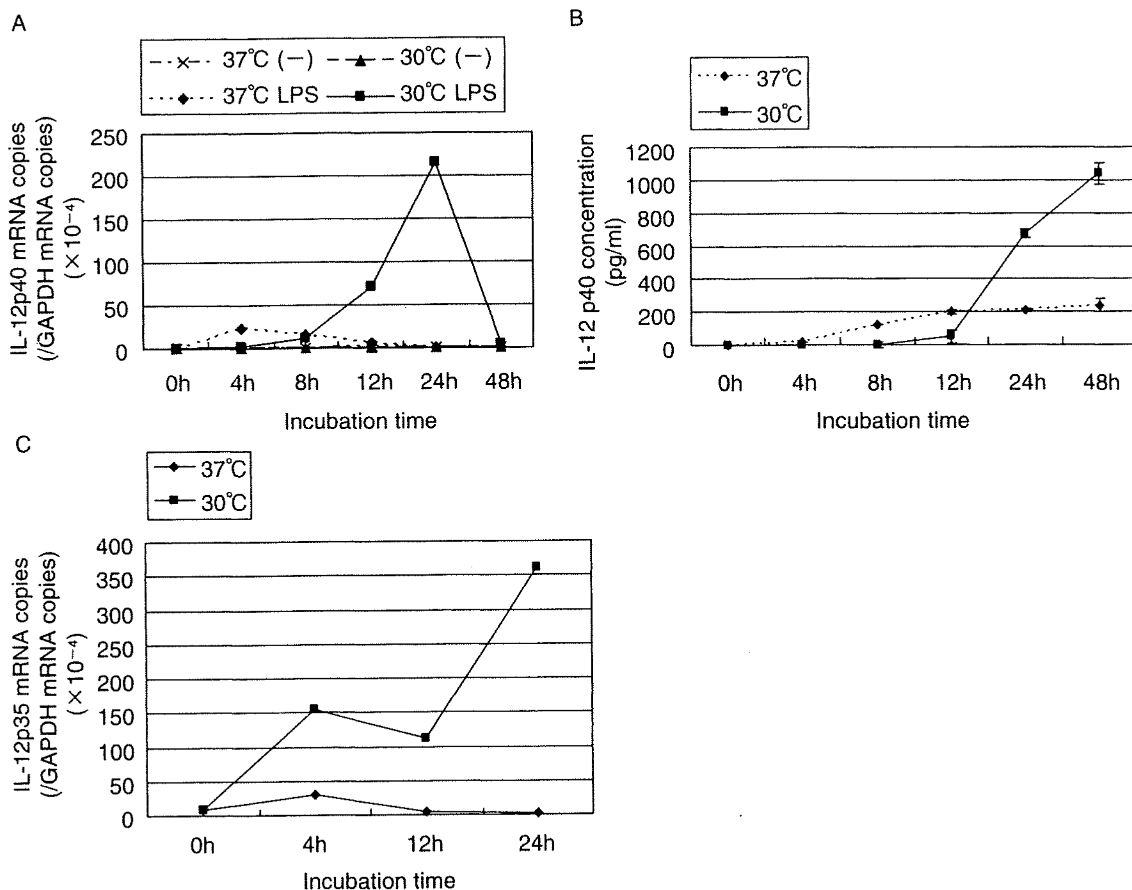

Fig. 5 PBMCs were cultured in the presence of LPS (1 μg/mL) at 30℃ or 37℃ for up to 48 hours. At the indicated time points, (**A**) the IL-12 p40 mRNA amounts in the cultured cells were measured by real-time RT-PCR, and (**B**) IL-12 p40 concentrations in the supernatants of the cell cultures were measured by ELISA. Three independent experiments showed similar results. (**C**) $CD14^{+}$ monocytes were cultured in the presence of LPS (1 μg/mL) at 30℃ or 37℃ for up to 24 hours. At the indicated time points, IL-12 p35 mRNA amounts in the cultured cells were measured by real-time RT-PCR.

We previously reported that reduced IFN-gamma production in PBMCs is associated with an elevated serum IgE level in atopic patients.[29] Furthermore, we showed that the serum IgE levels were negatively correlated with IL-12 production[30] and that atopic patients with high levels of serum IgE had some abnormality in this IL-12-IFN-gamma loop.[31] Our results suggest that hypothermia up-regulates IFN-gamma and IL-12 production and that hypothermia might modify the balance of the differentiation of Th cells leading to the onset of allergic diseases. We have not found an epidemiological association between hypothermia and the onset or development of allergic disease. However, it is known that inhalation of cold air is one of the causative factors for bronchial asthma.[32] It might be that a modified balance caused by hypothermia in the respiratory tract influences the pathogenesis of bronchial asthma.

IL-10 is considered to be a cytokine with a regulatory function.[33] Matsui *et al.*[19] reported that mild hypothermia inhibits IL-10 production in PBMCs. In our study IL-10 production from PBMCs stimulated with LPS was also inhibited at 30℃ compared with 37℃. Reduction of IL-10 production might play a role in the onset of allergic disease.[34] It remains to be elucidated whether *in vivo* mild hypothermia has an effect on the balance of the differentiation of Th cells or not. Experiments along these lines are now under way.

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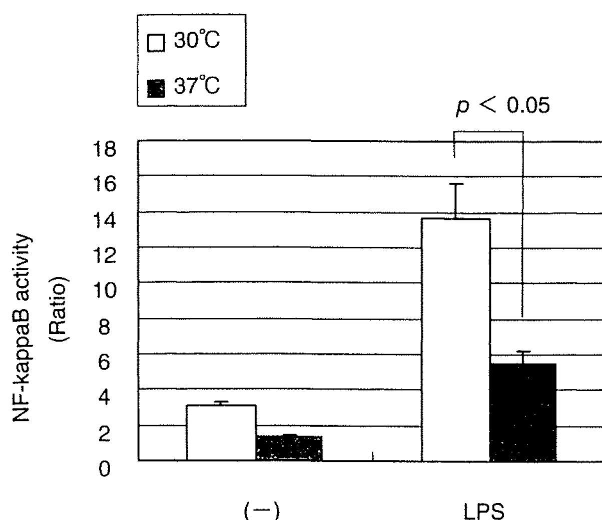

Fig. 6 HEK293-hTLR4A-HA cells were cultured in the presence or absence of LPS (1 μg/mL) at 30℃ or 37℃ for 24 hours. After 24 hours incubation, the cells were lysed and analyzed for activated NF-KappaB using a Luciferase Assay. The results show representative data of three independent transfection experiments. Results are mean ± SD.

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ORIGINAL ARTICLE

Expression, Purification and Structural Analysis of Human IL-18 Binding Protein: A Potent Therapeutic Molecule for Allergy

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ABSTRACT

Background: While interleukin-18 (IL-18) plays an important role in the innate and adaptive immune responses, it can also cause severe allergic inflammatory reactions. Thus it is a molecule currently being targeted for therapy. The natural intrinsic inhibitor of IL-18 receptor activation, IL-18 binding protein (IL-18BP), shows a great potential for the treatment of allergy.
Methods: Expression and purification of recombinant human IL-18BP (rhIL-18BP) were performed using the baculovirus system to develop a therapeutic molecule for the treatment of IL-18-related diseases and to investigate the structural basis of its inhibitory mechanism.
Results: Purified rhIL-18BP potently inhibited the production of interferon-gamma by peripheral blood mononuclear cells in the presence of lipopolysaccharide and by human myelomonocytic KG-1 cells in the presence of IL-18 (IC50 = 0.4 nM). Surface plasmon resonance showed a high affinity (Kd = 0.46 nM) for rhIL-18BP in binding hIL-18. Structural analysis indicated that the stoichiometry between IL-18 and IL-18BP is 1 : 1 in solution and the model structure of the complex suggests that the key residues on IL-18 (L5, K53, S55) and the estimated key residues on IL-18BP (F93,Y97, F104) could have interactions. The structural mechanism of IL-18BP inhibition might be a competition for Site 2 on rIL-18 so that IL-18BP can prevent IL-18 receptor alpha from binding to Site 2 and inhibit IL-18 receptor activation.
Conclusions: IL-18BP has unique features with respect to its structure, binding mode and inhibitory mechanism. It is a molecule that has a great potential for the therapy of allergy.

KEY WORDS
baculovirus, binding mode, IL-18 binding protein, purification, structure

INTRODUCTION

Interleukin-18 (IL-18) is a cytokine originally found to induce the production of interferon-gamma (IFN-γ) in T lymphocytes, and plays an important role in innate and adaptive immune systems.[1,2] To initiate the IL-18 pathway, the IL-18 receptor needs to be activated, which requires IL-18 receptor alpha (IL-18Rα, formerly known as IL-1Rrp) and IL-18 receptor beta (IL-18Rβ, formerly known as IL-1RAcPL) to heterodimerize.[2]

The structure of human IL-18 has been determined and it has shown that hIL-18 contains the β-trefoil fold that is similar to those found in interleukin-1 (IL-1) family members, making a new structural family in the interleukins.[3,4] Also, using mutant recombinant human IL-18 (rhIL-18) proteins in receptor-binding and cellular response assays, three important binding sites have been identified.[3] Two of these sites are important for binding to IL-18Rα, and the third is in-

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volved in cellular responses but not in IL-18Rα binding.[3]

By comparing the structure and the receptor binding sites of IL-18 with those of the IL-1 family members, a two-step ternary complex formation that involves IL-18, IL-18Rα, and IL-18Rβ has been revealed.[4] The formation of the ternary complex can then trigger a signal cascade that activates IL-1 receptor activating kinase (IRAK), tumor necrosis factor (TNF)-receptor associated factor 6 (TRAF6) and nuclear factor-kappa B (NF-κB).[5,6]

In medicine, the aberrant expression of IL-18 has been suggested to be responsible for several inflammatory conditions, such as allergies, autoimmune diseases, and neurological disorders.[2,7] Serum concentrations of IL-18 and IgE have been correlated with disease severity in atopic dermatitis (AD) patients.[7-9] In these patients, the serum concentration of IL-18 is significantly higher than that found in healthy individuals.[9] Furthermore, in response to lipopolysaccharide (LPS), leukocytes prepared from the peripheral blood of AD patients produced more IL-18 than the cells of healthy individuals.[7,8] In patients with bronchial asthma, the level of serum IL-18 is also elevated, and seems to correlate with disease severity.[7,10-14] As well, polymorphic genes for IL-18 and IL-18Rα have been found in association with allergy.[15-17] Collectively, these studies strongly suggest that IL-18 is responsible for causing and/or maintaining inflammatory conditions. Thus, IL-18 may be an ideal therapeutic target for the treatment of allergy.[7]

In clinical settings, humanized antibodies are widely used as therapeutic agents for the treatment of many diseases, including allergy.[18] Although anti-IL-18 antibody has been proven to be effective for preventing liver damage in mice, to date, there are no specific inhibitors against IL-18 to treat patients.[1,7,18] To this end, Hamasaki *et al.* recently reported a human anti-hIL-18 antibody (h18–108) that is capable of inhibiting IFN-γ production in a cell line.[19]

For therapeutic purposes, IL-18 binding protein (IL-18BP), a natural intrinsic inhibitor of hIL-18, may be an ideal candidate for use as a therapeutic agent.[20] One reason is that it is a natural inhibitor of IL-18, and its functional homologs have also been found in poxviruses, which utilize proteins to evade the immune system.[21-26] Also, the severity of autoimmune diseases appears to be influenced by the relative levels of IL-18 and IL-18BP.[27] Furthermore, IL-18BP has been shown to prevent disease development in various mouse models, which strongly supports IL-18BP's potential as a therapeutic agent.[28-30]

In this study, we performed high expression and purification of recombinant human IL-18BP (rhIL-18BP) using the baculovirus system to develop a therapeutic molecule for the treatment of IL-18-related diseases. A structural analysis of rhIL-18BP was performed to investigate the structural basis of its inhibitory mechanism.

METHODS

CONSTRUCTION OF EXPRESSION VECTOR FOR hIL-18BP

The IL-18BP isoform-a was selected from six isoforms of IL-18BP, because isoform-a most strongly inhibits IL-18 activity on human peripheral blood mononuclear cells (PBMCs) or human cell lines and has been widely used as the representative of the isoforms.[31,32] Total mRNA was extracted from the blood of a healthy volunteer, and single-stranded cDNAs were synthesized at 72℃ for 60 minutes using reverse transcriptase and oligo-dT primers. Only the coding region of the mature hIL-18BP isoform a (NM173042, residues 27–193 aa,) was amplified by PCR. The amplified fragment was cloned into T-vector (Invitrogen, USA), because the amino acid residues 1 to 26 of the hIL-18BP are recognized as a signal peptide in mammalian cells (Fig. 1A). One of the primers used in the PCR contained an *Eco*RI site and a signal peptide sequence for Sf9 insect cells, and by design, these were placed immediately upstream of the first codon of the mature hIL-18BP. The second primer contained the His-6 tag sequence, a stop codon, and a *Not*I site. The purified PCR product was subcloned into the pFastBac1 vector (Invitrogen, USA). The DNA sequence of the clone was confirmed by bi-directional sequencing. The construct was called pFastBac1-hIL-18BP-His6 (Fig. 1B).

PRODUCTION OF RECOMBINANT BACULOVIRUS

Generation of recombinant baculovirus expressing hIL-18BP in Sf9 cells was carried out with a Bac-to-Bac baculovirus expression kit (Invitrogen, USA). In accord with the manufacturer's protocol, pFastBac1-hIL-18BP-His6 was introduced into *E.coli* DH10Bac (Invitrogen, USA) for the transposition of IL-18BP into baculovirus genomic DNA (bacmid). Colonies containing the recombinant bacmid were isolated using a miniprep plasmid isolation kit (Promega, USA). The recombinant bacmid DNA was then used to transfect Sf9 cells, which were plated at a density of 1×10^6 cells per 35-mm well and then transfected with the bacmid DNA using Cellfectin reagent (Invitrogen, USA). The recombinant virus was harvested 72 hours post-transfection. Plaque assays were performed with the supernatants to determine the titer of recovered virion particles. Plaque assay and propagation of viruses were carried out according to the manual provided with the kit.

INSECT CELL CULTURE

Sf9 insect cells were cultured and routinely subcultured at 27℃. They were maintained either as a monolayer or as a suspension culture in Sf-900 II SFM (Invitrogen, USA) without antibiotics. Insect

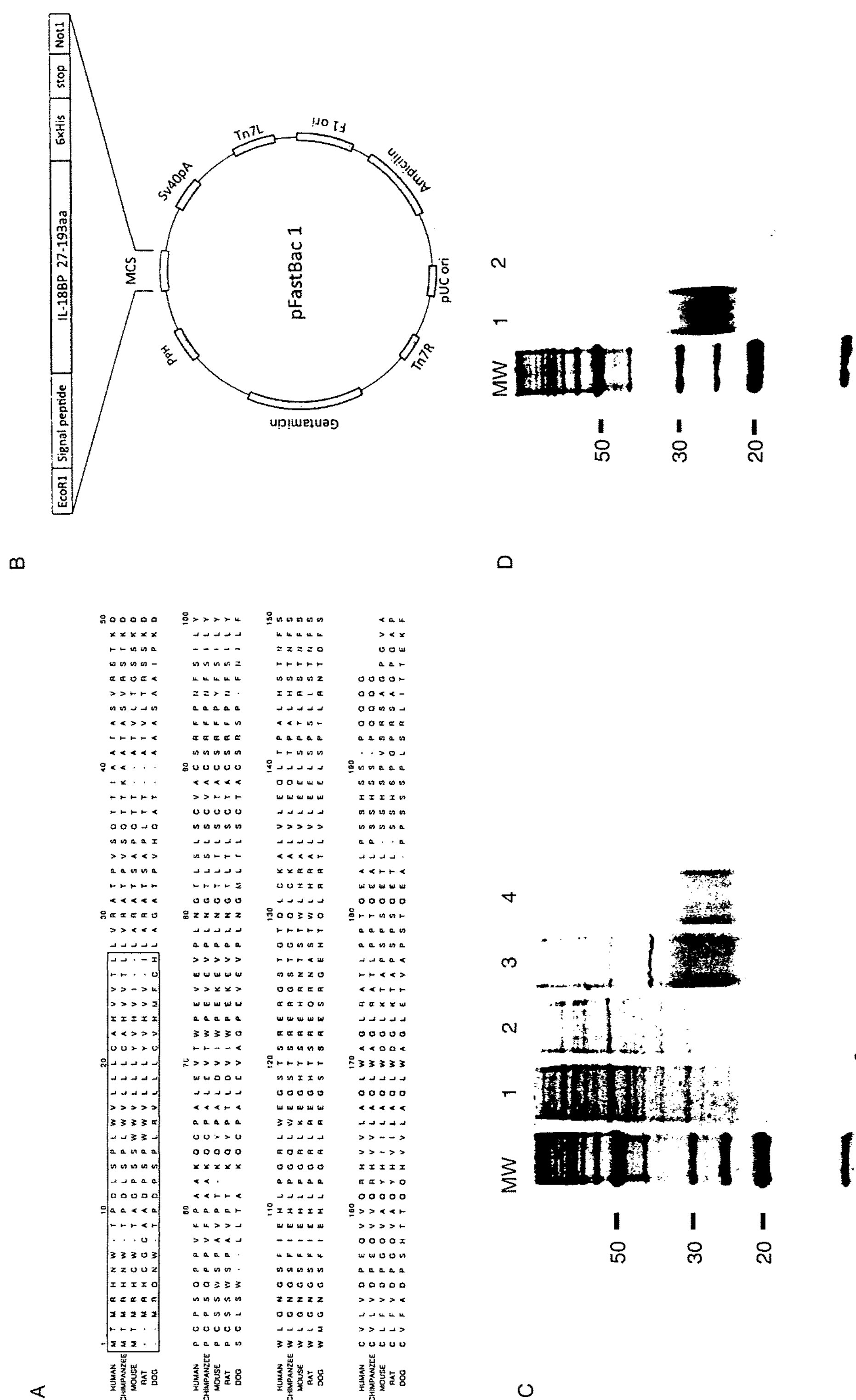

Fig. 1 Expression and purification of rhIL-18BP. **A**, Alignment for IL-18BPs. The signal peptide residues of the hIL-18BP in mammalian cells are boxed. Estimated key residues on hIL-18BP for hIL-18 binding are highlighted in yellow.[26] **B**, A map of the construct used for expression of hIL-18BP in the baculovirus system. **C**, Purification of rhIL-18BP. Lane 1: medium, lane 2: DEAE, lane 3: NiNTA, lane 4: Gel filtration. **D**, Deglycosylation by trifluoromethane sulfonic acid. Lane 1: before deglycosylation, lane 2: after deglycosylation.

cells in a monolayer culture were plated at a density of 1.0 × 10^6 cells/well in six-well plates. These cells were used to transfect recombinant bacmid DNA or for the determination of the optimal multiplicity of infection (MOI). The cells in the suspension culture were grown in a 25 ml volume of Sf-900 II SFM using 125 ml disposable Erlenmeyer flasks (Nalgene, USA). The flasks were maintained in a Bioshaker BR40LF (TAITEC, Japan) and were subjected to rotary shaking at a speed of 95 rpm (amplitude 35 mm). Stock

Sf9 cells that were grown in suspension were seeded at 0.5 × 10^6 cells/ml and culture passage was performed every 3 days during the log phase (4–6 × 10^6 cells/ml).

PURIFICATION OF hIL-18BP FROM BACULOVIRUS-INFECTED SF9 CELL CULTURE MEDIUM

For the purification of rhIL-18BP, Sf9 cells were subcultured in 1 L Fernbach flasks. Each flask received 400 ml of the cell culture at a concentration of 0.5 × 10^6 cells/ml. Shaking speed was set to 71 rpm (Amplitude 50 mm). When the subcultured cells reached a concentration of 2.0 × 10^6 cells/ml, Sf9 cells were infected with the recombinant baculovirus at an MOI of 0.1 plaque-forming units (pfu) per ml and were incubated for up to 72 hours.

Secreted rhIL-18BP was processed by centrifugation and filtration. Then, the medium was applied to a DEAE-Sepharose (GE health care, Sweden) open column (Bio-Rad, USA) equilibrated with buffer A (50 mM sodium-phosphate, pH 6.0 containing 50 mM NaCl). The flow through was concentrated 10-fold using a tangential flow filtration system; Masterflex L/S (Barnant Company, USA) and viva flow 50 (Vivasciece AG, Germany). This was then dialyzed against buffer B (50 mM Sodium Phosphate, 0.5 M NaCl, 10 mM Imidazol, pH 7.4) and insoluble material removed by centrifugation at 2 × 10^4 g; himac CR 20B2 (Hitachi, Japan). Dialyzed sample was loaded onto a Ni-agarose (GE Healthcare, Sweden) column equilibrated with buffer B. The column was then washed with buffer B and bound proteins were eluted by an elution buffer B with varying imidazol concentrations (50, 100, 200, 300 and 500 mM). The eluted fraction of rhIL-18BP was concentrated and further purified using a superdex75 16/60 (GE Healthcare, Sweden) size exclusion column equilibrated with 50 mM potassium phosphate buffer pH 7.0, containing 150 mM KCl and 0.1 mM EDTA. The purity of the preparation was assessed by SDS-PAGE.

DEGLYCOSYLATION OF rhIL-18BP BY TRIFLUOROMETHANE SULFONIC ACID

Deglycosylation was performed in a draft chamber (DF-19RST, DALTON, Japan). Freeze dried IL-18BP (30 μg) in glass vial and trifluoromethane sulfonic acid (TFMS) (Sigma Aldrich, Japan) was chilled on ice prior to mixing. Fifty micro liters of TFMS was added in a glass vial and gently mixed. The vial was then incubated for 50 hours on ice and neutralized with a solution of 500 μl of 1M-Tris. Neutralized rhIL-18BP was concentrated with a MicroconYM-10 (Millipore, USA) and dialyzed against 20 mM sodium phosphate buffer of pH 7.0 containing 150 mM NaCl before SDS-PAGE.

N-TERMINAL SEQUENCING BY EDMAN DEGRADATION

Purified proteins were electro-blotted onto a polyvinylidene difluoride membrane (Amersham Biosciences, UK) after SDS-PAGE for 1 hour at 200 mA in solution that contained 25 mM Tris, 192 mM glycine, and 10% methanol. The membrane was briefly stained with CBB R250 (Wako, Japan) and de-stained extensively in 45% and 90% methanol containing 7% acetic acid. The amino acid sequence analysis of the recombinant protein was carried out by an Edman degradation technique using a pulse liquid automatic sequencer (Model 491HT, Applied Biosystems, USA).

FUNCTIONAL ANALYSIS OF rhIL-18BP

Biological activity of rhIL-18BP was assayed by measuring its ability to inhibit the production of IFN-γ. This was performed as previously described with minor modifications.[17] Briefly, PBMCs were isolated from three volunteers and suspended at 1 × 10^6 /ml in the culture medium. PBMCs were cultured in the presence or absence of 1 ng/ml of the LPS with or without rhIL-18BP (400 ng/ml) for 24 hours at 37℃ in a humidified atmosphere containing 5% CO2. The cell culture supernatants were collected in test tubes, and the samples were spun to get rid of cells and then stored at −80℃ until assay. The concentration of IFN-γ was measured by enzyme-linked immunosorbent assay (ELISA), as previously described.[32]

MEASUREMENT OF rhIL-18BP-MEDIATED INHIBITION OF IFN-γ PRODUCTION IN KG-1 CELLS

In hIL-18 inhibition assay, the level of inhibition is determined by the amount of IFN-γ produced by the target cells. A detailed description has been reported.[32] Briefly, human myelomonocytic KG-1 cells (ATCC CCL246) were grown in RPMI1640 (Invitrogen, USA) supplemented with 10% heat-inactivated fetal calf serum (Sigma Aldrich, USA), L-glutamine (2 mmol/L) (Wako, Japan), penicillin (100 U/ml) (Meiji, Japan), and streptomycin (100 μg/ml) (Meiji, Japan). The rhIL-18 and rhIL-18BP prepared in our laboratory were mixed in the RPMI1640 medium noted above. The concentration of rhIL-18 was 4 ng/ml and rhIL-18BP was from 0 to 250 ng/ml. The mixed samples were incubated at 37℃ for 1 hour. Then, 100 μl of the mixture was added to the wells of a 96-well plate (Nunc, Denmark), which contained 100 μl of KG-1 cells (3 × 10^6 cells/ml) (The final concentration of rhIL-18 was 2 ng/ml and rhIL-18BP was from 0 to 125 ng/ml). The plate was further incubated at 37℃ for 24 hours in 5% CO2. The culture supernatants were collected, and the amount of IFN-γ produced by KG-1 cells was determined by ELISA.[32] IC50, the concentration of antagonists required to inhibit 50% of IFN-γ production by KG-1 cells stimulated with hIL-18, was then calculated.

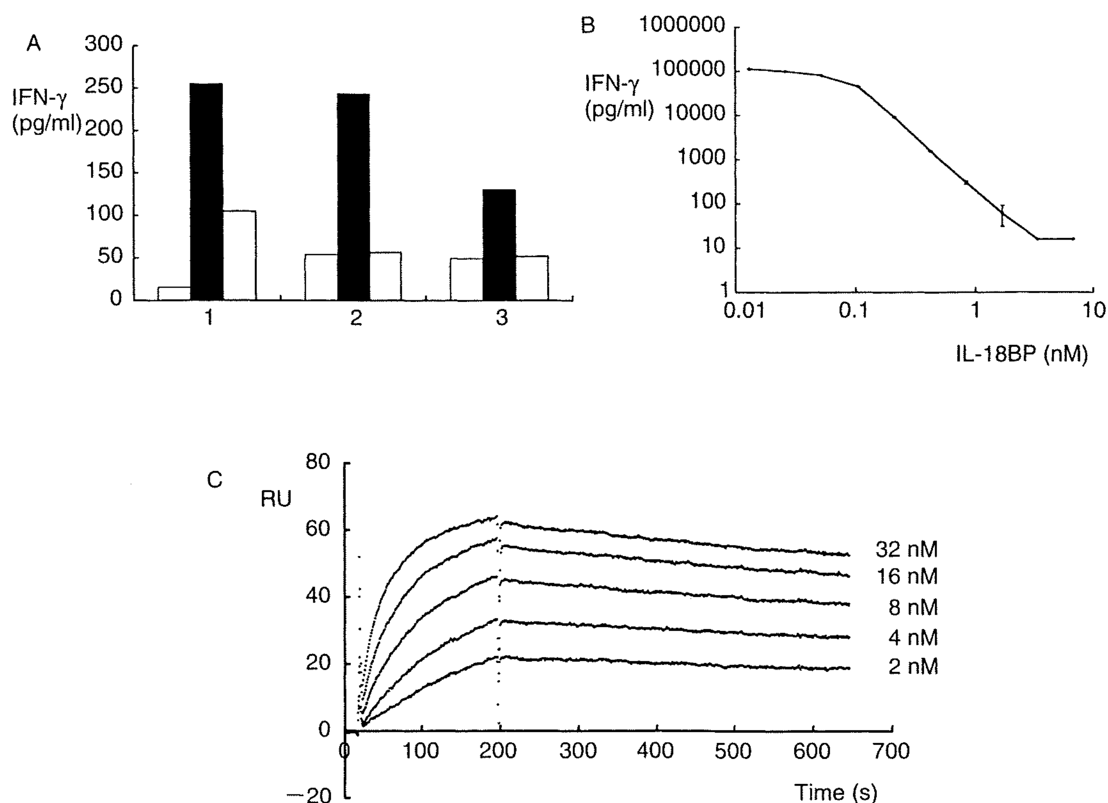

Fig. 2 Functional activity of purified rhIL-18BP. **A**, Inhibition of IFN-γ production by rhIL-18BP in LPS-stimulated human PBMCs. The numbers on the horizontal axis show the subject numbers. Open box, control; black box, LPS stimulation, grey box, LPS stimulation with rhIL-18BP. **B**, Inhibition of IFN-γ production by a human cell line, KG-1. **C**, Dose-response of the rIL-18BP/rhIL-18 interaction by surface plasmon resonance (Kd = 0.46 nM).

MEASUREMENT OF rhIL-18BP'S BINDING ACTIVITY BY SURFACE PLASMON RESONANCE EXPERIMENT

In vitro affinity of the hIL-18 for rhIL-18BP was measured at 25℃ by surface plasmon resonance (SPR) using a BIAcore3000 (BIAcore, Sweden). A specific binding surface was prepared by coupling the anti-penta-His antibody (QIAGEN K.K, Japan) to a CM5 sensor chip by the standard amine coupling method, as recommended by BIAcore. Then, 6-his tagged rhIL-18BP was injected over the sensor chip and immobilized. The coupling density was limited to 120 resonance units (RU) . Samples of hIL-18 were diluted to varying concentrations ranging from 2 to 32 nM in HBS-EP buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.0 mM EDTA, 0.005% (v/v) surfactant P-20). For dissociation constant analysis, diluted hIL-18 samples were injected over the sensor chip at a flow rate of 20 μl/min. The sensor surface was regenerated by two 60-second pulses of 0.2 M glycine-HCl, pH 2.2. The sensorgrams obtained from various concentrations of rhIL-18 were fitted with BIAEVALUATION software (BIAcore, Sweden) in a 1-to-1 binding model.

ANALYTICAL GEL FILTRATION OF rhIL-18BP

The molecular mass of purified rhIL-18BP and the complex of rhIL-18 and rhIL-18BP were determined by size exclusion chromatography. A superdex200 10/30 GL column (GE Healthcare, Sweden) attached to a FPLC system (GE Healthcare, Sweden) was utilized and the study conducted at 4℃. The column was equilibrated with 50 mM potassium phosphate buffer of pH 7.0, containing 150 mM KCl and 0.1 mM EDTA. The column was calibrated with the following gel filtration standards (Bio-Rad Laboratories, USA): thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa), and vitamin B_{12} (1.35 kDa) at a flow rate of 0.25 ml/min. Samples with only hIL-18BP, only rhIL-18 or a mixture of rhIL-18BP and rhIL-18 at a 1 : 2 ratio were used for the analysis. Protein elution was monitored and detected by UV absorption at 280 nm. We plotted the logarithm of the molecular weight versus the elution volume, and calculated the correlation line. The elution volume of rhIL-18BP and the complex of rhIL-18/rhIL-18BP were plotted and the deduced molecular weight determined.

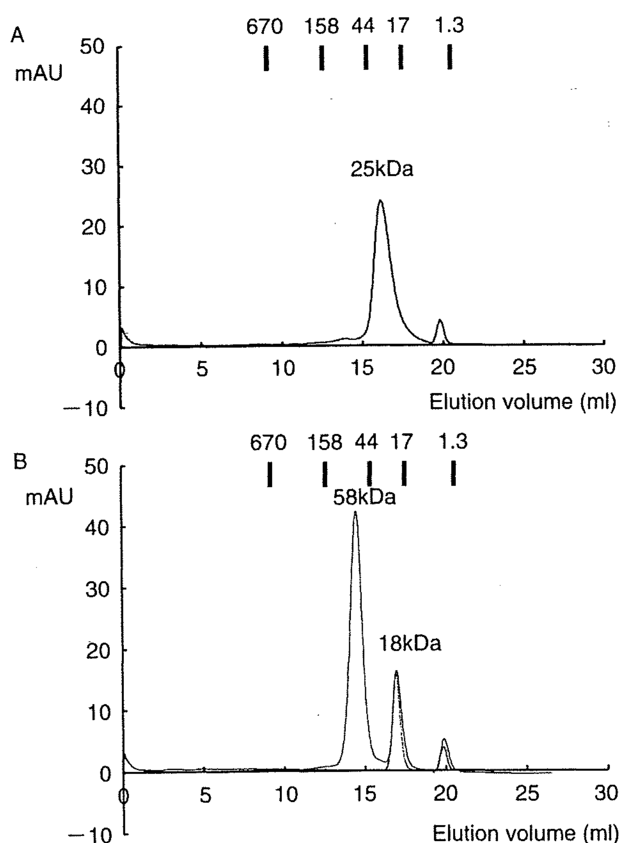

Fig. 3 Stoichiometric analysis of the rhIL-18/rhIL-18BP complex. **A**, Gel filtration of rhIL-18BP. **B**, Gel filtration of the rhIL-18BP/rhIL-18 complex. A solid line indicates the sample that contained a mixture of rhIL-18BP and rhIL-18 at 1 : 2 ratio, a dashed line, indicates the sample with only rhIL-18.

HOMOLOGY MODELING OF THE STRUCTURES

The sequence alignment of IL-18BP from different species was performed using ClustalW (http://www2.ebi.ac.uk/clustalw/) with a BLOSUM matrix. Homology modeling was performed using MOE software (CCG, Inc., Canada) with a combination of segment-matching and modeling of indels.[33,34] The templates used for the modeling were as follows: the NMR structure of hIL-18 (PDB: 1J0S) and the crystal structure of the domain 3 of hIL-1Rα (PDB: 1ITB) for the hIL-18: hIL-18BP complex; the NMR structure of hIL-18 (PDB: 1J0S) and the crystal structure of the hIL-10/anti-IL-10 Fab complex (PDB: 1LK3) for the hIL-18/human anti-hIL-18 Fab interaction. The modeled structures were refined by further energy minimization.

RESULTS

CHARACTERIZATION OF THE PURIFIED rhIL-18BP

High expressions of rhIL-18BP were obtained using the baculovirus expression system. We were able to obtain a yield of 2.5 mg/L of rhIL-18BP after Ni-NTA column purification (Fig. 1C). A further purification by gel filtration showed excellent purity and yielded 1.8 mg/L (Fig. 1C). To check the purity of the protein, electrophoresis was performed. Although the band appeared smeary, when we deglycosylated it with TFMS, a single sharp band of 18 kDa in size was detected (Fig. 1D). Edman degradation analysis of rhIL-18BP revealed the expected five N-terminal amino acid residues: (LVRAT). The sequence verified the identity of the expressed protein and indicated that the signal peptide from the vector was correctly cleaved at the N-terminal residue of mature rhIL-18 BP.

FUNCTIONAL ACTIVITY OF THE rhIL-18BP

The biological activity of rhIL-18BP was tested by measuring its ability to inhibit the production of IFN-γ by LPS-stimulated PBMCs from volunteers (Fig. 2A). The rhIL-18BP showed distinct inhibitory actions against IFN-γ production by PBMCs in response to LPS. rhIL-18BP only partly inhibited IFN-γ production by PBMCs from volunteer #1, while it almost completely inhibited those from volunteers #2 and #3 (Fig. 2A) . This diversity might be partly explained by the different amounts of IL-18 released from PBMCs of individual volunteers and/or in part by the different productions of other IFN-γ-related cytokines, such as IL-12 and IL-15.[8,17,35,36] As well, rhIL-18BP inhibited the production of IFN-γ by human KG-1 cells in the presence of IL-18 (IC50 = 0.4 nM, Fig. 2B).

His-tagged rhIL-18BP was immobilized on a BIAcore sensor chip coated with anti-His-tag antibodies and its binding activity monitored in real time with a BIAcore 3000 sensor. A sensorgram of rhIL-18 showed a rapid increase in signal during the association phase and a slow decline in the dissociation phase (Fig. 2C), demonstrating a fast on-rate and a slow off-rate. The estimated Kd of 0.46 nM was similar to results previously reported, indicating that the produced rhIL-18BP had proper structural folding.[25,26,31]

STRUCTURAL ANALYSIS OF rhIL-18BP

Gel filtration analysis revealed that rhIL-18BP is a monomer in solution (Fig. 3A) and that a complex of rhIL-18 and rhIL-18BP could be made in the same molar ratio (Fig. 3B). Together with the molecular weight of the complex, it is suggested that the complex could be made by one hIL-18 and one hIL-18BP (Fig. 3B).

Based on the results of analytical gel filtration, a model structure of the rhIL-18/rhIL-18BP complex was constructed using the NMR structure of hIL-18 and the crystal structure of the domain 3 of IL-1Rα as templates[3] (Fig. 4). The analysis of the amino acid sequence alignment for IL-18BP revealed that the residues that are involved in the binding of hIL-18 are

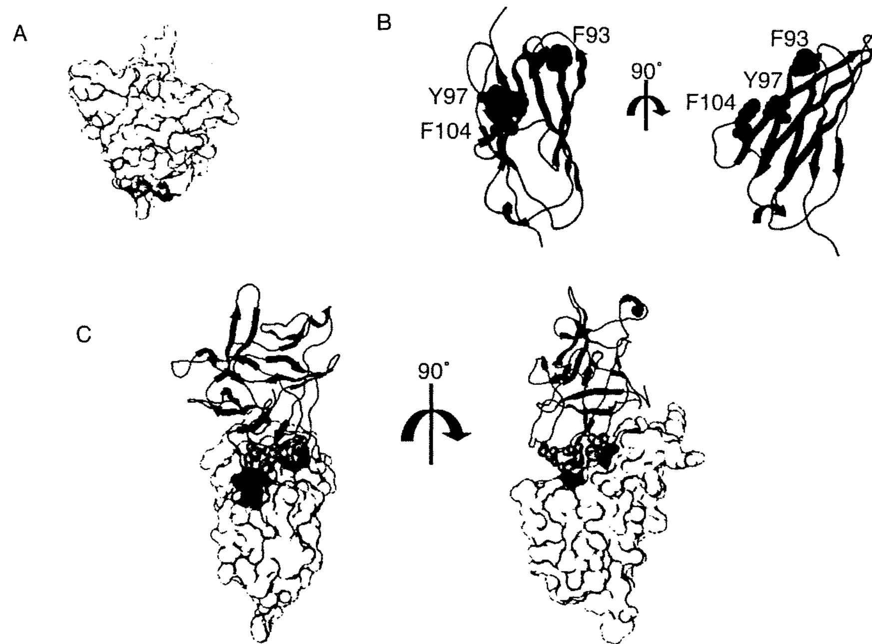

Fig. 4 A structural model of the hIL-18/hIL-18BP complex. **A**, Surface of hIL-18. Functional binding residues on hIL-18 for hIL-18BP are colored in orange. **B**, A structural model of the rhIL-18BP monomer. Estimated functional key residues of hIL-18BP for hIL-18 are shown as atoms in red. **C**, A model of the hIL-18/hIL-18BP complex (hIL-18 as a ribbon model, hIL-18BP as a surface representation, colored as in A or B).

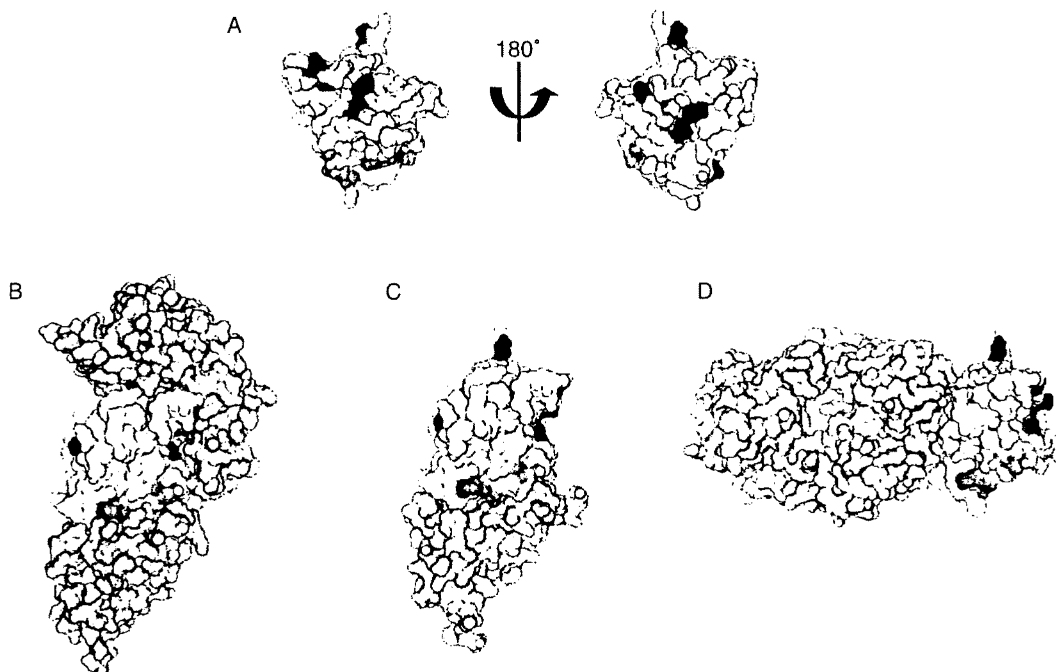

Fig. 5 Human IL-18 interacts differently with different proteins through its three binding sites. **A**, Receptor binding sites on hIL-18.[3] Sites 1, 2 and 3 are indicated in red, orange, and blue, respectively. **B, C, D**, Models of complexes between hIL-18 and hIL-18Rα (in grey), hIL-18BP (in yellow) and human Anti-hIL-18 Fab (in yellow).

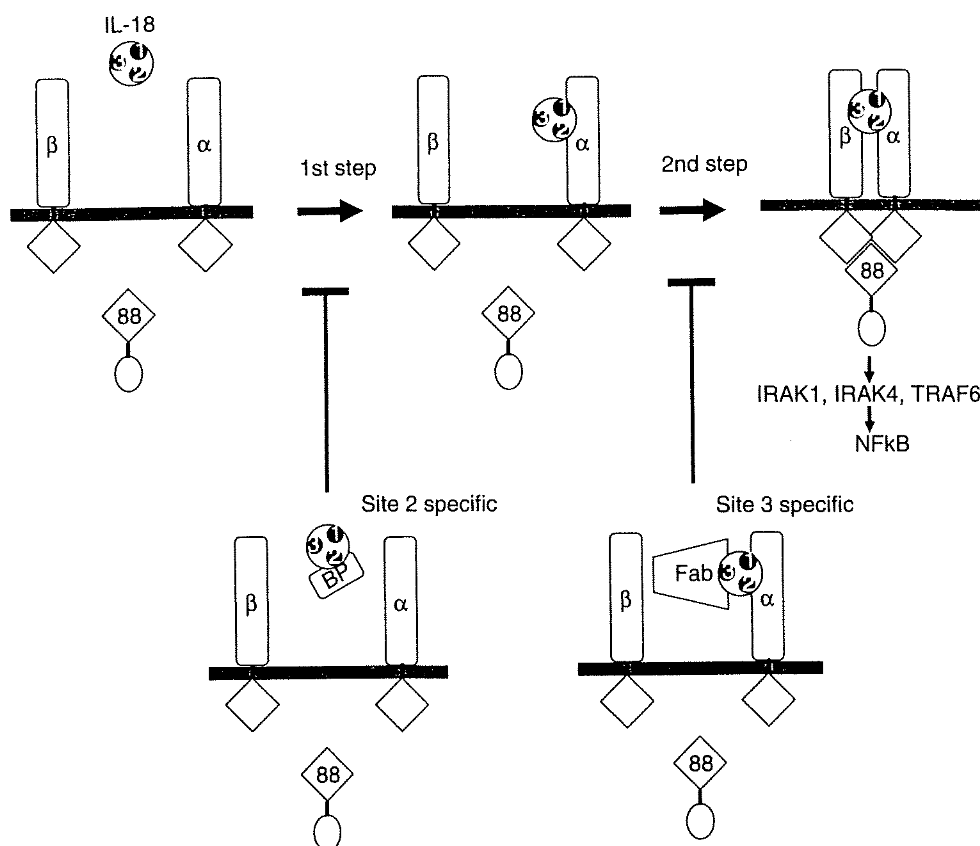

Fig. 6 A schematic model of IL-18 receptor activation and inhibition. A two-step complex formation model of IL-18 signaling is shown at the top. The inhibitory mode of IL-18BP or Fab fragment of h18-108 is shown at the bottom. Sites 1, 2 and 3 of hIL-18 are indicated in red, orange, and blue, respectively.

highly conserved between species[25,26] (Fig. 1A). Our model showed that the three key residues (F93, Y97, and F104) that are involved in binding IL-18 make hydrophobic patches that align with the binding interfaces of hIL-18 (Fig. 4C). The three key residues of hIL-18 for binding (L5, K53, and S55) have been shown to be involved in the interaction with hIL-18BP.[26]

DISCUSSION

Our previous structural analysis of the IL-18/IL-18 receptor interaction showed that there are 3 distinct and important binding sites on the surface of IL-18 (Fig. 5A).[3] Two of the sites (Sites 1 and 2) are responsible for binding to IL-18Rα while the third site is involved in binding to IL-18Rβ (Fig. 5A, B). Site 2 residue is also important for IL-18BP binding[3] (Fig. 5A, C). A recent study showed that a single-chain Fv (scFv) of an anti-human IL-18 antibody binds to Site 3 of IL-18 (Fig. 5A, D).[19] scFv (h18-108) showed a moderate binding affinity (Kd = 50 nM), whereas the Fab fragment of h18-108 showed a higher affinity (Kd = 3.7nM), suggesting that the stability of the binding surface structure could improve the affinity.[19] Further, hIL-18BP, which has a smaller molecular mass than scFv, showed a greater than 100-fold higher affinity (Kd = 0.46 nM)[25,26,31] than scFV. This suggested that the evolutionally improved structure of IL-18BP provided an optimal biding surface complementarity. Based on this, perhaps one could optimize the binding affinity of h18-108 by modifying the surface structure through *in vitro* mutagenesis or through a computational design, as it has been demonstrated for the anti-epidermal growth factor receptor drug, Cetuximab.[37]

IL-18 activates IL-18 receptor through a two-step binding mechanism as shown[3] in Figure 6. According to this model, the inhibitory mechanisms of IL-18BP and the Fab fragment of h18-108 antibody are different (Fig. 6). IL-18BP inhibits the receptor activation by competing for Site 2 on IL-18, which is also a binding site for IL-18Rα. On the other hand, the Fab fragment of h18-108 antibody binds to Site 3 on IL-18 to block IL-18 from binding to IL-18Rβ[3] (Fig. 6). The IC50 of IL-18BP has been shown to fall in the range of 0.04-0.46 nM, while it is 100 nM for the Fab frag-

ment.[19] This huge difference in IC50 may reflect the type of inhibitory mechanism that is employed (Fig. 6). Interestingly, an intact form of h18–108 antibody, an IgG molecule, has a much higher affinity with a Kd of 0.64 nM and an IC50 of 5 nM.[19] This improvement is most likely the result of its overall stability and the bivalency of the intact antibody molecule. Regardless of the affinity, these different molecules will be useful for determining the precise mechanism by which IL-18 receptor is activated or inhibited. Information gained from such studies will undoubtedly help us design potent therapeutic reagents.

A number of drugs targeting and neutralizing the deleterious effects of cytokines have been developed. Etanercept is a soluble fusion protein that is composed of two tumor necrosis factor alpha (TNF-α) receptors (p75) and a Fc fragment of a human IgG1 molecule; it has been shown to be effective and safe in patients with rheumatoid arthritis.[38] Recently, it was reported that the blockade of TNF-α by etanercept had efficacy in patients with asthma.[39,40] In addition to the anti-TNF drugs, the role of IL-18 in allergy suggests that drugs that neutralize the effect of IL-18, such as monoclonal antibodies or IL-18BP, may also be useful for the management of allergy.[7] The chemical characteristics of IL-18BP (a single polypeptide, a monomer, and with a low molecular weight of 25 kDa) support its use in clinical settings. Because of these characteristics, it is possible that its dosage could be lower than that of molecules, such as monoclonal antibodies and etanercept (multiple polypeptides, oligomer, high molecular weight of > 300 kDa) that are much bigger and more complex. Establishing a system that enables researchers to highly express the desired protein to study its structural mechanism could open avenues of research to develop new reagents and strategies for the treatment of various diseases, including allergy.

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The response of bovine beta-lactoglobulin-specific T-cell clones to single amino acid substitution of T-cell core epitope

Kondo M, Kaneko H, Fukao T, Suzuki K, Sakaguchi H, Shinoda S, Kato Z, Matsui E, Teramoto T, Nakano T, Kondo N. The response of bovine beta-lactoglobulin-specific T-cell clones to single amino acid substitution of T-cell core epitope.
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Cow's milk is one of the most common food allergens in the first year of life, with approximately 2.5% of infants experiencing an allergic reaction to it. Beta-lactoglobulin (BLG) is one of the major allergens in cow's milk. Previously, we reported that four of six T-cell clones (TCC) which were established from cow's milk allergy patients recognized BLGp97-117 as the core sequence and also recognized BLG in association with the human leucocyte antigen (HLA)-DRB1*0405 allele. Using two of these four TCCs, we evaluated the T-cell response to BLG peptides with single amino acid substitution or deletion and identified BLGp102-112 as the minimum essential region in BLGp97-117. In the alanine-scan assay, the proliferative responses of TCCs to pE108A disappeared, and the proliferative responses of TCCs to pC106A decreased. In the analog peptide proliferation assay, pY102S had retained some T-cell response to the two TCCs. Collecting these results, we propose a motif for the interaction between the HLA-DRB1*0405 allele and antigen peptide, and suggest that BLGp105-108 are important residues to retain the TCR/BLG-peptide/HLA complex. pY102A and pY102S are partial agonists for the T-cell receptor. These peptides might be considered as candidate peptides for the modification of the T-cell response to BLG in cow's milk allergy.

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Cow's milk allergy is one of the most common food allergies in young children, with approximately 2% to 2.5% of all infants experiencing allergic reactions to it. The majority of children out-grow their allergy to cow's milk before the age of 3 yr, but 15% of these infants with IgE-mediated cow's milk allergy retain their sensitivity into the second decade of life (1, 2).

Beta-lactoglobulin (BLG) is the major whey protein of the milk of the ruminants. It is relatively resistant to acidic pH and to proteolytic enzymes, leaving its structure relatively unchanged during digestion and possibly allowing the passage of intact protein into the circulation (3). Moreover, it is not present in human milk and is considered to be a major allergen of cow's milk (3). IgE-binding regions of BLG have been identified. Selo et al. showed that the major IgE-epitopes of BLG appeared to be fragments p41–60, p102–124, and p149–162 (4).

The isotype switching of antigen-specific B cells to IgE is controlled by T cells that play a key role in the initiation of allergic symptoms, as well as in tolerance induction. Antigenic peptide-made

Abbreviations: BLG, beta-lactoglobulin; IFN-gamma, interferon gamma; PBMCs, peripheral blood mononuclear cells.

Table 1. Patients' profile and characterization of T-cell clones

	Sex	Age (yr)	IgE (IU/ml)	CAP-RAST		Antigen presenting HLA†	Recognition site of BLG peptide†
				Milk	BLG		
YA	Female	1	1136.8	94	55.4	DRBI*0405	p97-117 (TDYKKYLLFCMENSAEPEQSL)
HA	Female	2	249	2.3	1.5	DRBI*0405	p97-117 (TDYKKYLLFCMENSAEPEQSL)

BLG, beta-lactoglobulin; RAST, radioallergosorbent test.
†Previously reported data (6).

complexes with HLA class II molecules are displayed on the surface of antigen-presenting cells for recognition by CD4$^+$ T cells.

We have previously reported that the BLG peptide (BLGp97-117) was presented by HLA-DRB1*0405 using BLG-specific T-cell lines and clones (TCCs) obtained from patients with cow's milk allergy (5, 6). Four of six TCCs recognized the BLG peptide (BLGp97-117) presented by HLA-DRB1*0405, and suggested that HLA-DRB1*0405 is an immunoregulatory gene product for T-cell responses to BLG (6). We hypothesize that single amino acid substitutions on an allergen peptide carrying the T-cell epitope might modify the T-cell response to allergen. In this study, we identified the minimal core epitope of TCCs specific to BLG using BLG-derived peptides, and the analog peptides which modified the T-cell responses. We proposed a motif for the interaction between the HLA-DRB1*0405 allele and antigen peptide.

Material and methods

Subjects

As shown in Table 1, we obtained BLG-specific TCCs from two patients (YA and HA) with cow's milk allergy. Allergic symptoms such as eczema, urticaria, and wheezing occurred in less than 1 h after cow's milk ingestion in these patients.

Both of the TCC recognized BLGp97-117 as the core sequence and recognized BLG in association with the HLA-DRB1*0405 allele (6).

TCCs specific to BLG Culture

The TCCs were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 2 mM L-glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 10% pooled AB, heat-inactivated normal human male plasma in 24-well flat-bottomed cultured plates (Falcon, Becton Dickinson, Lincoln Park, NJ, USA). The plates were incubated at 37°C in 5% CO_2 humidified air. After 7–10 days, irradiated (30 Gy) autologous peripheral blood mononuclear cells (PBMCs) (1.5×10^5/well) pulsed with a BLG-derived peptide mixture (1 μM each for 5h), human rIL-2 (50 U/ml) (Genzyme, Cambridge, MA, USA), and human rIL-4 (10 U/ml) (Biosource International, Camario, CA, USA) were added to the culture wells and the TCCs were maintained for another 7 days.

Intracellular IFN-gamma and IL-4 staining of BLG-specific TCCs

The TCCs specific to BLG were cultured at a density of 2×10^6 cells/ml in an RPMI 1640 medium for 4 h at 37°C. During the 4-h incubation, the cells were stimulated with a combination of 25 ng/ml of phorbol 12-myristate 13-acetate (SIGMA, St Louis, MO, USA) and 2 μg/ml of ionomycin (SIGMA) in the presence of 10 ng/ml of Brefeldin-A (SIGMA). Then the cells were directly stained with a PC-5-conjugated anti-CD4 monoclonal antibody (Coulter-Immunotech, Marseille, France) for 15 min at room temperature, and fixed with a Fluorescence-activated cell sorting (FACS) Lysing Solution (Becton Dickinson, Mountain View, CA) for 10 min. After washing, they were pre-incubated with a FACS Permeabilizing Solution (Becton Dickinson) for 10 min and after washing again, they were incubated with FASTIMMUNE interferon (IFN)-gamma FITC/IL-4 PE (Becton Dickinson) for 30 min at room temperature. Flow cytometric analysis was performed using a FACS Calibur.

Synthesis of BLG-derived peptides

BLG-truncated peptides for searching the core sequences of BLG p97-117 (TDYKKYLLFCMENSAEPEQSL), BLG-derived peptides for analyzing alanine-scan mutant peptides, and BLG-derived analog peptides of 11 amino acid residues were synthesized using the multiple method based on the Fmoc strategy (5). The substitution of a single amino acid residue was done for each peptide. All peptides were purified by C18 reverse-phase HPLC (Gilson, Middleton, WI, USA).