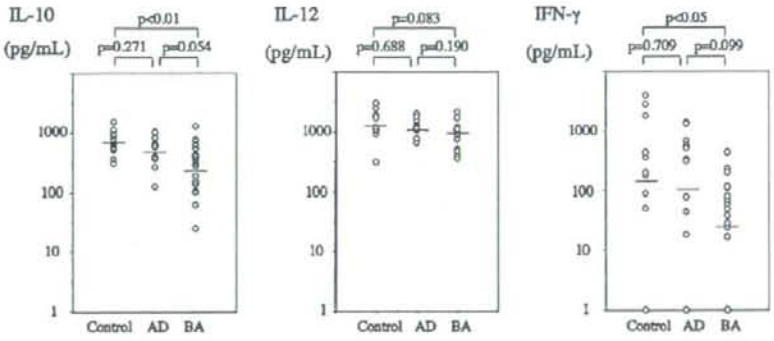


Figure 1. IL-10, IL-12 or IFN-γ production by LPS-stimulated PBMCs in healthy control subjects (n=12), AD (n=12) and BA patients (n=19).

Table II. The prevalence of haplotypes in the *IL-10* gene.

| Haplotype | GCC | ACC | ATA |
|---|---|---|---|
| Control (n=10) | 0 (0%) | 4 (20.0%) | 16 (80.0%) |
| AD (n=10) | 0 (0%) | 5 (25.0%) | 15 (75.0%) |
| BA (n=13) | 2 (7.7%) | 7 (26.9%) | 17 (65.4%) |

AD, atopic dermatitis; BA, bronchial asthma.

## Results

*IL-10 production by PBMCs in atopic patients and healthy control subjects.* We studied IL-10 production by PBMCs in atopic patients and healthy control subjects. Twelve healthy control subjects, 12 patients with AD and 19 patients with BA were studied. IL-10, IL-12 and IFN-γ production by LPS-stimulated PBMCs was measured with ELISA kits. The features of the patients are summarized in Table I.

IL-10 production by PBMCs stimulated with LPS is shown in Fig. 1. IL-10 production was lower in atopic patients than in healthy control subjects. In particular, IL-10 production was lower in patients with BA (average, 275.4 pg/ml; 1 SD range, 102.3-741.3 pg/ml) than in healthy control subjects (average, 691.8 pg/ml; 1 SD range, 446.7-1071.5 pg/ml) ($p<0.01$) (Fig. 1). IFN-γ production was also lower in patients with BA than in healthy control subjects. IL-10, IL-12 and IFN-γ production was lower in patients with AD than that in control subjects, but there was no statistically significant difference between the two groups when analyzed using the Mann-Whitney U test.

*IL-10 gene polymorphisms and atopic diseases.* The *IL-10* gene from allergic patients and healthy control subjects was sequenced. We detected three polymorphisms in the *IL-10* gene promoter region, -1082 (G/A), -819 (C/T) and -592 (C/A) as previously reported (12,13). These polymorphisms produced three different haplotypes, GCC, ACC and ATA.

We determined the prevalence of these haplotypes in the *IL-10* gene in both allergic patients and healthy control subjects by direct sequencing (Table II). None of the healthy control subjects or AD patients had the GCC haplotype. Two of the BA patients had the GCC haplotype. Most of the healthy control subjects and atopic patients had the ATA haplotype.

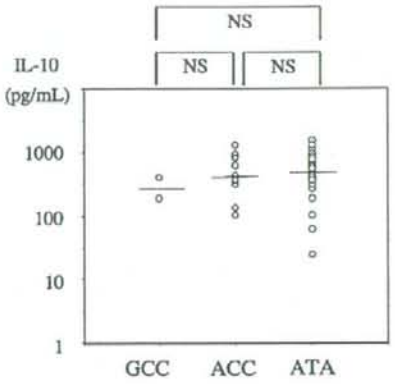


Figure 2. Relationship between the haplotype of the IL-10 gene promoter region and IL-10 production by LPS-stimulated PBMCs.

Next, we investigated whether these polymorphisms were associated with the production of IL-10 by LPS-stimulated PBMCs. As shown in Fig. 2, these polymorphisms did not have an effect on IL-10 production by PBMCs in this study.

*Effect of IL-10 on Th1 and Th2 cytokine production by PBMCs.* The effect of IL-10 on Th1 and Th2 cytokine production in atopic patients was examined. IFN-γ production by PBMCs stimulated with PHA was significantly inhibited by IL-10 in a dose-dependent manner (Fig. 3A). Similarly, IL-12 production by PBMCs stimulated with PHA was inhibited by IL-10 in a dose-dependent manner (Fig. 3B). IL-12 production by PBMCs stimulated with LPS was inhibited by IL-10 (data not shown). These data suggest that the production of Th1 cytokines such as IL-12 and IFN-γ by PBMCs are directly inhibited by IL-10.

Furthermore, we investigated the effect of IL-10 on the production of the Th2 cytokine IL-4. IL-4 production by

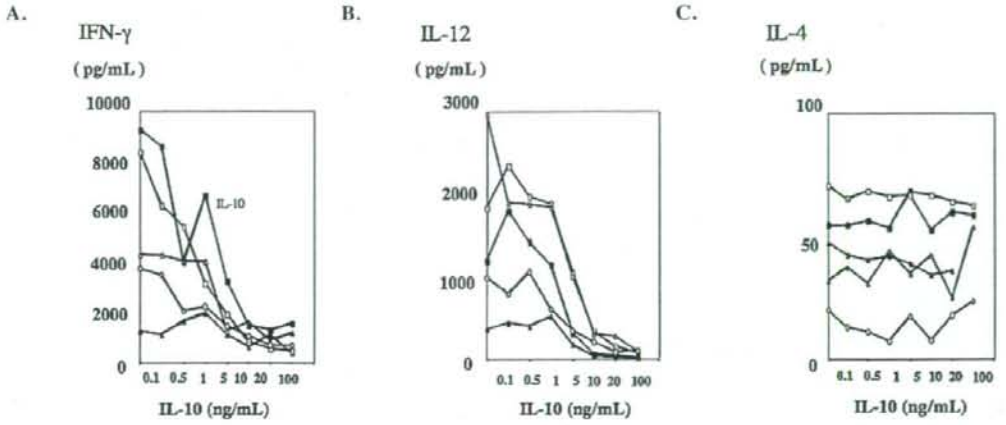


Figure 3. Effect of IL-10 on IL-12, IFN-γ or IL-4 production by PHA-stimulated PBMCs in one randomly selected healthy control subject, one AD patient and three BA patients (control subject -■-, AD patient -▲-, BA patient 1 -□-, BA patient 2 -△-, BA patient 3 -◇-). (A) IFN-γ production by PHA-stimulated PBMCs was inhibited by IL-10 dose-dependently in one healthy control subject and three BA patients. In one AD patient, IFN-γ production by PHA-stimulated PBMCs was low and was not evaluated. (B) IL-12 production by PHA-stimulated PBMCs was inhibited by IL-10 dose-dependently in all patients. (C) IL-4 production by PHA-stimulated PBMCs was not affected by IL-10.

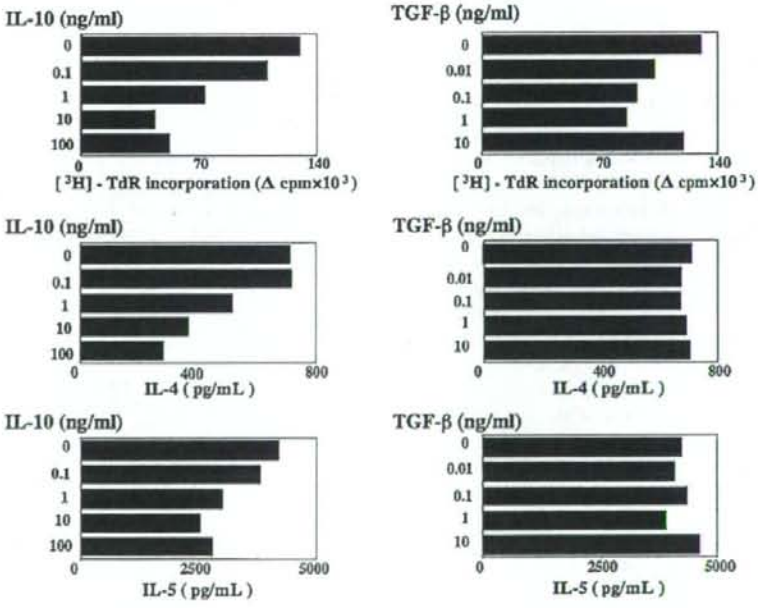


Figure 4. Antigen-induced proliferative responses and cytokine production of ß-lactoglobulin (BLG)-specific T cell clones. IL-10 inhibited the BLG-specific antigen-induced proliferation of T cell clones. IL-10 also inhibited the production of IL-4 and IL-5 by T cell clones.

PBMCs stimulated with PHA did not change with the addition of IL-10 (Fig. 3C).

*Effect of IL-10 on specific antigen-induced proliferation of T cell clones.* The effect of IL-10 on specific antigen-induced proliferation of T cell clones and Th2 cytokine production by T cell clones was examined. The BLG-specific antigen-induced proliferation of T cell clones was significantly inhibited by IL-10 in a dose-dependent manner (Fig. 4). In contrast, TGF-ß did not inhibit the proliferation of T cell clones.

Furthermore, IL-10 inhibited the production of the Th2 cytokines IL-4 and IL-5 by T cell clones in a dose-dependent manner (Fig. 4).

## Discussion

Immune responses generated by cytokines are essential to the development of allergic disease (14). IL-4, IL-5 and IL-13 produced by Th2 cells induce, prolong and amplify allergic responses by enhancing the production of IgE and the recruitment, growth and differentiation of eosinophils and mast cells. They also directly cause airway hyperreactivity in and of themselves. It has been suggested that Th2-mediated allergic diseases result from inadequate Th1 cytokine production. Our previous studies demonstrated that IL-12 and IFN-γ play important roles in the regulation of IgE synthesis by B cells (15,16).

IL-10 is an anti-inflammatory cytokine. Human IL-10, in contrast to murine IL-10 (which is primarily a Th2 product), is produced by both the Th1 and Th2 cells (17) as well as by mononuclear phagocytes, which may be its most important source (18). A role for IL-10 in the regulation of immune responses to allergens was first suggested by studies revealing that IL-10 inhibited cytokine production by eosinophils stimulated with LPS (19). Later, it was demonstrated that IL-10 might also inhibit the production of cytokines, such as TNF and IL-6, by stimulated mast cells (20,21). IL-10 inhibits monocyte major histocompatibility complex-class (MHC-class II), B7.1 (CD80), B7.2 (CD88), intercellular adhesion molecule-1, and CD23 expression and accessory cell function (22). Monocytes pre-treated with IL-10 fail to induce specific antigen T cell proliferation.

In this study, IL-10 production by PBMCs in atopic patients, particularly BA patients, was significantly lower than in healthy control subjects. This might be explained by reduced IL-10 production, which was noted in the lungs of asthmatic patients as a result of a decreased gene expression level (23,24).

Next, we investigated whether the difference in IL-10 production between healthy control subjects and atopic patients was due to a difference in the distribution of the haplotype in the *IL-10* gene promoter region. In a previous study, the GCC/GCC genotype was associated with higher production and the ATA haplotype with lower production of IL-10 by PBMCs compared with other genotypes. However, none of our subjects had the GCC/GCC genotype. Most of the healthy control subjects and atopic patients had the ATA haplotype. Two of the BA patients had the GCC haplotype. IL-10 production by PBMCs was not affected by these haplotypes of the *IL-10* gene promoter region. Lim *et al* reported that the IL-10 haplotype has a role in determining disease severity, but does not seem to be important to disease susceptibility (12). More studies are required to clarify these points.

Lastly, we investigated the effect of IL-10 on the Th1 or Th2 cytokines. In this study, IFN-γ and IL-12 production by PBMCs stimulated with PHA were inhibited by IL-10. IL-12 production by PBMCs stimulated with LPS was also inhibited by IL-10. These data suggest that IL-10 is an inhibitor of Th1 cytokines. Moreover, IL-10 inhibited the antigen-induced proliferation of T cell clones and Th2 cytokine production by T cell clones. Previous studies have demonstrated that IL-10 inhibits cytokine production and the proliferation of $CD4^+$ T cells and T cell clones via down-regulatory effects on APC function (2,25). In addition, IL-10 directly affects the function of T cells and inhibits IL-4 and IL-5 production depending on activation conditions (26). It is reported that IL-10 production by regulatory T cells ($CD4^+$/$CD25^+$ T cells) plays an important role in the regulation of allergies by inhibiting Th0, Th1 and Th2 cells (27,28). Recently, it was reported that IL-10 and IL-13Rα2 coordinatedly suppressed Th2-mediated inflammation and pathology, respectively (29).

IL-10 could play a critical role in the pathogenesis of atopic diseases and is a modulator of Th1 and Th2 cytokines. However, further research into the function of IL-10 is required.

## References


1. Fiorentino DF, Bond MW and Mosmann TR: Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J Exp Med 170: 2081-2095, 1989.
2. Fiorentino DF, Zlotnik A, Vieira P, Mosmann TR, Howard M, Moore KW and O'Garra A: IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. J Immunol 146: 3444-3451, 1991.
3. D'Andrea A, Aste-Amezaga M, Valiante NM, Ma X, Kubin M and Trinchieri G: Interleukin 10 (IL-10) inhibits human lymphocyte interferon-γ production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. J Exp Med 178: 1041-1048, 1993.
4. Firentino DF, Zlotnik A, Mosmann TR, Howard M and O'Garra A: IL-10 inhibits cytokine production by activated macrophages. J Immunol 147: 3815-3822, 1991.
5. Gruber MF, Williams CC and Gerrard TL: Macrophage-colony-stimulating factor expression by anti-CD45 stimulated human monocytes is transcriptionally up-regulated by IL-1 beta and inhibited by IL-4 and IL-10. J Immunol 152: 1354-1361, 1994.
6. Marfaing-Koka A, Maravic M, Humbert M, Galanaud P and Emilie D: Contrasting effects of IL-4, IL-10 and corticosteroids on RANTES production by human monocytes. Int Immunol 8: 1587-1594, 1996.
7. Kopydlowski KM, Salkowski CA, Cody MJ, van Rooijen N, Major J, Hamilton TA and Vogel SN: Regulation of macrophage chemokine expression by lipopolysaccharide in vitro and in vivo. J Immunol 163: 1537-1544, 1999.
8. Gasche C, Bakos S, Dejaco C, Tillinger W, Zakeri S and Reinisch W: IL-10 secretion and sensitivity in normal human intestine and inflammatory bowel disease. J Clin Immunol 20: 362-370, 2000.
9. Grondal G, Gunnarsson I, Ronnelid J, Rogberg S, Klareskog L and Lundberg I: Cytokine production, serum levels and disease activity in systemic lupus erythematosus. Clin Exp Rheumatol 18: 565-570, 2000.
10. Kondo N, Fukutomi O, Agata H, Motoyoshi F, Shinoda S, Kobayashi Y, Kuwabara N, Kameyama T and Orii T: The role of T lymphocytes in patients with food-sensitive atopic dermatitis. J Allergy Clin Immunol 91: 658-668, 1993.
11. Sakaguchi H, Inoue R, Kaneko H, Watanabe M, Suzuki K, Kato Z, Matsushita S and Kondo N: Interaction among human leucocyte antigen-peptide-T cell receptor complexes in cow's milk allergy: the significance of human leucocyte antigen and T cell receptor-complementarity determining region 3 loops. Clin Exp Allergy 32: 762-770, 2002.
12. Lim S, Crawley E, Woo P and Barnes PJ: Haplotype associated with low interleukin-10 production in patients with severe asthma. Lancet 352: 113, 1998.
13. Turner DM, Williams DM, Sankaran D, Lazarus M, Sinnott PJ and Hutchinson IV: An investigation of polymorphism in the interleukin-10 gene promoter. Eur J Immunogenet 24: 1-8, 1997.
14. Umetsu DT and DeKruyff RH: TH1 and TH2 $CD4^+$ cells in human allergic diseases. J Allergy Clin Immunol 100: 1-6, 1997.
15. Teramoto T, Fukao T, Tashita H, Inoue R, Kaneko H, Takemura M and Kondo N: Serum IgE level is negatively correlated with the ability of peripheral mononuclear cells to produce interferon gamma (IFN-γ): evidence of reduced expression of IFN-γ mRNA in atopic patients. Clin Exp Allergy 28: 74-82, 1998.
16. Matsui E, Kaneko H, Teramoto T, Fukao T, Inoue R, Kasahara K, Takemura M, Seishima M and Kondo N: Reduced IFN-gamma production in response to IL-12 stimulation and/or reduced IL-12 production in atopic patients. Clin Exp Allergy 30: 1250-1256, 2000.

17. Del Prete G, De Carli M, Almerigogna F, Giudizi MG, Biagiotti R and Romagnani S: Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. J Immunol 150: 353-360, 1993.
18. Wanidworanun C and Strober W: Predominant role of tumor necrosis factor-alpha in human monocyte IL-10 synthesis. J Immunol 151: 6853-6861, 1993.
19. Takanaski S, Nonaka R, Xing Z, O'Byrne P, Dolovich J and Jordana M: Interleukin 10 inhibits lipopolysaccharide-induced survival and cytokine production by human peripheral blood eosinophils. J Exp Med 180: 711-715, 1994.
20. Arock M, Zuany-Amorim C, Singer M, Benhamou M and Pretolani M: Interleukin 10 inhibits cytokine generation from mast cells. Eur J Immunol 26: 166-170, 1997.
21. Marshall JS, Leal-Berumen I, Nielsen L, Glibetic M and Jordana M: Interleukin (IL)-10 inhibits long-term IL-6 production but not preformed mediator release from rat peritoneal mast cells. J Clin Invest 97: 1122-1128, 1996.
22. Ding L, Linsley PS, Huang LY, Germain RN and Shevach EM: IL-10 inhibits macrophage costimulatory activity by selectively inhibiting the up-regulation of B7 expression. J Immunol 151: 1224-1234, 1993.
23. John M, Lim S, Seybold J, Jose P, Robichaud A, O'Connor B, Barnes PJ and Chung KF: Inhaled corticoastroids increase interleukin-10 but reduce macrophage inflammatory protein-1, granulocyte-macrophage colony-stimulating factor, and interferon-gammma release from alveolar macrophages in asthma. Am J Respir Crit Care Med 157: 256-262, 1998.
24. Borish L, Aarons A, Rumbyrt J, Cvietusa P, Negri J and Wenzel S: Interleukin-10 regulation in normal subjects and patients with asthma. J Allergy Clin Immunol 97: 1288-1296, 1996.
25. De Waal Malefyt R, Haanen J, Spits H, Roncarolo MG, Te Velde A, Figdor C, Johnson K, Kastelein R, Yssel H and De Vries JE: Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. J Exp Med 174: 915-924, 1991.
26. Schandene L, Alonso-Vega C, Willems F, Gerard C, Delvaux A, Velu T, Devos R, De Boer M and Goldman M: B7/CD28-dependent IL-5 production by human resting T cells is inhibited by IL-10. J Immunol 152: 4368-4374, 1994.
27. Sakaguchi S: Regulatory T cells: mediating compromises between host and parasite. Nat Immunol 4: 10-11, 2003.
28. Bellinghausen I, Klostermann B, Knop J and Saloga J: Human $CD4^+CD25^+$ T cells derived from the majority of atopic donors are able to suppress TH1 and TH2 cytokine production. J Allergy Clin Immunol 111: 862-868, 2003.
29. Wilson MS, Elnekave E, Mentink-Kane MM, Hodges MG, Pesce JT, Ramalingam TR, Thompson RW, Kamanaka M, Flavell RA, Keane-Myers A, Cheever AW and Wynn TA: IL-13 Ralpha2 and IL-10 coordinately suppress airway inflammation, airway-hyperreactivity, and fibrosis in mice. J Clin Invest 117: 2941-2951, 2007.

*Allergology International*. 2008;57:331-338
DOI: 10.2332/allergolint.O-08-532

ORIGINAL ARTICLE

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

Takahiro Arai[1], Hideo Kaneko[1], Hidenori Ohnishi[1], Eiko Matsui[1], Toshiyuki Fukao[1], Norio Kawamoto[1], Kimiko Kasahara[1] and Naomi Kondo[1]

## 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-

[1]Department of Pediatrics, Graduate School of Medicine, Gifu University, Gifu, Japan.
Correspondence: Takahiro Arai, MD, Department of Pediatrics, Graduate School of Medicine, Gifu University, 1-1 Yanagido, Gifu, Gifu 501-1194, Japan.
Email: takahiroarai0425ver2@yahoo.co.jp
Received 7 January 2008. Accepted for publication 10 April 2008.

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 pro-inflammatory 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 $1\times10^6$ /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 ($1 \times 10^6$ /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 ($1 \times 10^6$ /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 $2.0 \times 10^5$ /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-

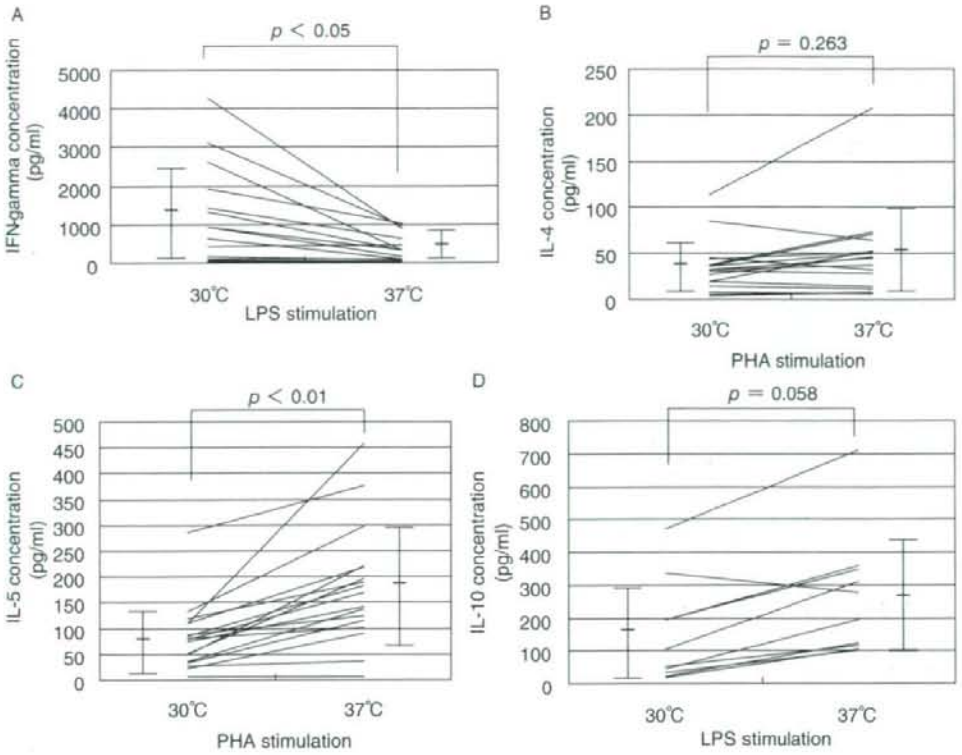


**Fig. 1** PBMCs were cultured in the presence of LPS (1 μg/mL) (IFN-gamma and IL-10) or PHA (10 μg/ml) (IL-4 and IL-5) for 48 hours at 30℃ or 37℃. (**A**) IFN-gamma, (**B**) IL-4, (**C**) IL-5 and (**D**) IL-10 concentrations in the supernatants of the cell cultures were measured by ELISA. Results are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

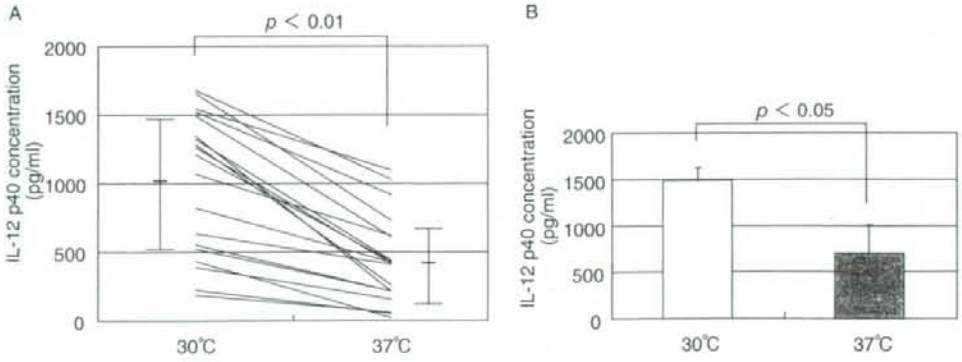


**Fig. 2** PBMCs were cultured in the presence of LPS (1 μg/mL) for 48 hours at 30℃ or 37℃. (**A**) IL-12 p40 concentrations in the supernatants of the cell cultures were measured by ELISA. (**B**) $CD14^+$ blood monocytes were separated from the PBMCs, and cultured in the presence of LPS (1 μg/mL) for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

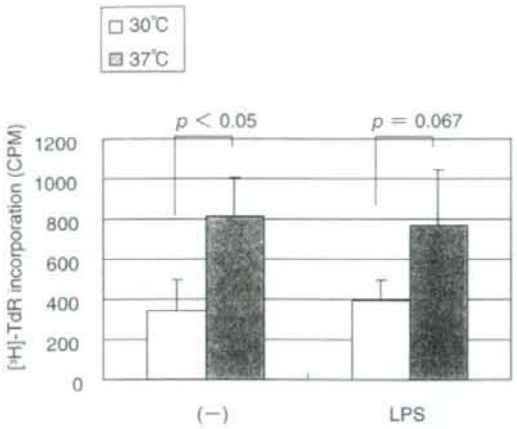


**Fig. 3** PBMCs were grown in triplicate at 0.2 ml per well in round-bottom microtest plates with or without LPS at 30℃ or 37℃ for 48 hours. The amount of DNA synthesized was measured by adding 0.5 μCi of $^{3}$H-thymidine per well in the microtest plates for 4 hours before harvesting using glass fibre 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

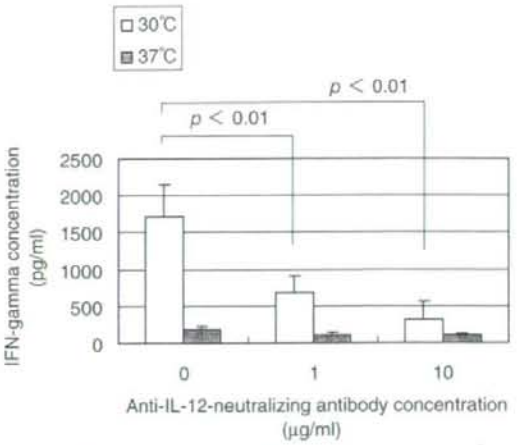


**Fig. 4** PBMCs were cultured in the presence of LPS (1 μg/mL) and 0, 1 or 10 μg/ml of an anti-IL-12-neutralizing antibody for 48 hours at 30℃ or 37℃. IFN-gamma concentrations in the supernatants of the cell cultures were measured by ELISA. Results are mean ± SD.

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 auguments 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]

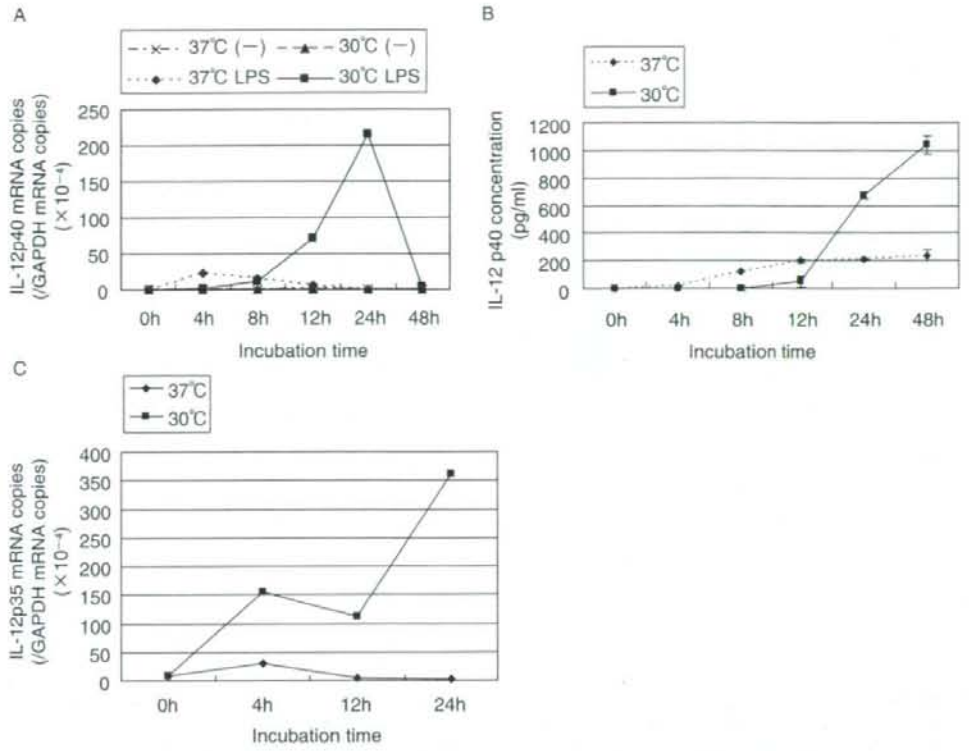


**Fig. 5** PBMCs were cultured in the presence of LPS (1 μg/mL) at 30℃ or 37℃ for up to 48 hours. At the indicated time points, (**A**) the IL-12 p40 mRNA amounts in the cultured cells were measured by real-time RT-PCR, and (**B**) IL-12 p40 concentrations in the supernatants of the cell cultures were 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.

## ACKNOWLEDGEMENTS

We thank Sewon Ki and Tetsuro Kokubo (Yokohama City University, Japan) for providing the NF-kappaB luciferase reporter vector and Martin F Lavin (Queensland Institute of Medical Research and University of Queensland) for helpful advice on the preparation of the manuscript. This study was in part funded by the Research and Development Program for New Bio-industry Initiatives (2005–2009) of the Bio-oriented Technology Research Advancement Institution (BRAIN), Japan.

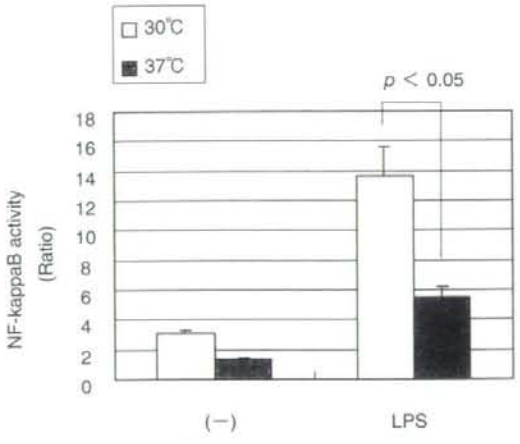


**Fig. 6** HEK293-hTLR4A-HA cells were cultured in the presence or absence of LPS (1 μg/mL) at 30℃ or 37℃ for 24 hours. After 24 hours incubation, the cells were lysed and analyzed for activated NF-KappaB using a Luciferase Assay. The results show representative data of three independent transfection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

## REFERENCES


1. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 1986;**136**:2348-57.
2. Del Prete GF, De Carli M, Mastromauro C *et al.* Purified protein derivative of Mycobacterium tuberculosis and excretory-secretory antigen (s) of Toxocara canis expand in vitro human T cells with stable and opposite (type 1 T helper or type 2 T helper) profile of cytokine production. *J Clin Invest* 1991;**88**:346-50.
3. Romagnani S. Human TH1 and TH2 subsets: doubt no more. *Immunol Today* 1991;**12**:256-7.
4. Kondo N, Fukutomi O, Agata H *et al.* The role of T lymphocytes in patients with food-sensitive atopic dermatitis. *J Allergy Clin Immunol* 1993;**91**:658-68.
5. Lagier B, Pons N, Rivier A *et al.* Seasonal variations of interleukin-4 and IFN-γ release by peripheral blood mononuclear cells from atopic subjects stimulated by polyclonal activators. *J Allergy Clin Immunol* 1995;**96**:932-40.
6. Maezawa Y, Nakajima H, Seto Y *et al.* IgE-dependent enhancement of Th2 cell-mediated allergic inflammation in the airways. *Clin Exp Immunol* 2004;**135**:12-8.
7. Gauchat JF, Lebman DA, Coffman RL, Gascan H, de Vries JE. Structure and expression of germline epsilon transcripts in human B cells induced by interleukin-4 to switch to IgE production. *J Exp Med* 1990;**172**:463-73.
8. Finkelman FD, Katona IM, Mossmann TR, Coffman RL. Interferon-γ regulates the isotypes of Ig secreted during in vivo humoral immune responses. *J Immunol* 1998;**140**: 1022-7.
9. Pene J, Rousset F, Briere F *et al.* IgE production by normal human lymphocytes is induced by interleukin 4 and suppressed by interferons gamma and alpha and prostaglandine E2. *Proc Natl Acad Sci USA* 1998;**85**:6880-4.
10. Manetti R, Parronchi P, Giudizi MG *et al.* Natural killer cells stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1(Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. *J Exp Med* 1993;**177**:1199-204.
11. D'Andrea A, Rengaraju M, Valiante NM *et al.* Production of natural killer cell stimulatory factor (interleukin 12) by peripheral blood mononuclear cells. *J Exp Med* 1992;**176**: 1387-98.
12. Macatonia SE, Hosken NA, Litton M *et al.* Dendritic cells produce IL-12 and direct the development of Th1 cells from naïve CD4+ T cells. *J Immunol* 1995;**154**:5071-9.
13. Kaisho T, Akira S. Toll-like receptor function and signaling. *J Allergy Clin Immunol* 2006;**117**:979-87.
14. Andreakos E, Sacre SM, Smith C *et al.* Distrinct pathways of LPS-induced NF-kB activation and cytokine production in human myeloid and nonmyeloid cells defined by selective utilization of MyD88 and Mal/TIRAP. *Blood* 2004; **103**:2229-37.
15. Roberts JR, Rowe PA, Demaine AG. Activation of NF-kB and MAP kinase cascades by hypothermic stress in endothelial cells. *Cryobiology* 2002;**44**:161-9.
16. Chan SH, Kobayashi M, Santoli D, Perussia B, Trinchieri G. Mechanisms of IFN-gamma induction by natural killer cell stimulatory factor(NKSF/IL-12). Role of transcription and mRNA stability in the synergistic interaction between NKSF and IL-2. *J Immunol* 1992;**148**:92-8.
17. Russwurm S, Stonans I, Schwerter K, Stonane E, Meissner W, Reinhart K. Direct influence of mild hypothermia on cytokine expression and release in cultures of human peripheral blood mononuclear cells. *J Interferon Cytokine Res* 2002;**22**:215-21.
18. Fairchild KD, Viscardi RM, Hester L, Singh IS, Hasday JD. Effects of hypothermia and hyperthermia on cytokine production by cultured human mononuclear phagocytes from adults and newborns. *J Interferon Cytokine Res* 2000; **20**:1049-55.
19. Matsui T, Ishikawa T, Takeuchi H, Tsukahara M, Maekawa T. Mild hypothermia inhibits IL-10 production in peripheral blood mononuclear cells. *Acta Anaesthesiol Scand* 2004;**48**:205-10.
20. Li CL, Wang XY, Shao J *et al.* Heat shock inhibits IL-12 p40 expression through NF-kB signaling pathway in murine macrophages. *Cytokine* 2001;**16**:153-9.
21. Matsui T, Ishikawa T, Takeuchi H, Tsukahara M, Maekawa T. Mild hypothermia promotes pro-inflammatory cytokine production in monocytes. *J Neurosurg Anesthesiol* 2006;**18**:189-93.
22. Fairchild KD, Singh IS, Patel S *et al.* Hypothermia prolongs activation of NF-kB and augments generation of inflammatory cytokines. *Am J Physiol Cell Physiol* 2004;**287**: 422-31.
23. Fairchild KD, Singh IS, Carter HC, Hester L, Hasday JD. Hypothermia enhances phosphorylation of IkB kinase and prolongs nuclear localization of NF-kB in lipopolysaccharide-activated macrophages. *Am J Physiol Cell Physiol* 2005;**289**:1114-21.
24. Hangalapura BN, Kaiser MG, Poel JJ, Parmentier HK, Lamont SJ. Cold stress equally enhances in vivo pro-inflammatory cytokine gene expression in chicken lines divergently selected for antibody responses. *Dev Comp Immunol* 2006;**30**:503-11.
25. Sakaguchi H, Inoue R, Kaneko H *et al.* Interaction among human leukocyte antigen-peptide-T cell receptor complexes in cow's milk allergy: the significance of human

leukocyte antigen and T cell receptor-complementarity determining region 3 loops. *Clin Exp Allergy* 2002;**32**:762-70.

**26**. Irazuzta JE, Pretzlaff RK, Zingarelli B, Xue V, Zemlan F. Modulation of nuclear factor-kappaB activation and decreased markers of neurological injury associated with hypothermic therapy in experimental bacterial meningitis. *Crit Care Med* 2002;**30**:2553-9.

**27**. Sutcliffe IT, Smith HA, Stanimirovic D, Hutchinson J. Effects of moderate hypothermia on IL-1β-induced leukocyte rolling and adhesion in pial microcirculation of mice and on proinflammatory gene expression in human cerebral endothelial cells. *J Cereb Blood Flow Metab* 2001;**21**:1310-9.

**28**. Luhm J, Schromm AB, Seydel U *et al.* Hypothermia enhances the biological activity of lipopolysaccharide by altering its fluidity state. *Eur J Biochem* 1988;**256**:325-33.

**29**. Teramoto T, Fukao T, Tashita H *et al.* Serum IgE level is negatively correlated with the ability of peripheral mononuclear cells to produce interferon gamma(IFNgamma): evidence of reduced expression of IFNgamma RNA in atopic patients. *Clin Exp Allergy* 1998;**28**:74-82.

**30**. Matsui E, Kaneko H, Teramoto T *et al.* Reduced IFNgamma production in response to IL-12 stimulation and/or reduced IL-12 production in atopic patients. *Clin Exp Allergy* 2000;**30**:1250-6.

**31**. Kondo N, Matsui E, Kaneko H *et al.* Reduced interferon-gamma production and mutations of the interleukin-12 receptor beta (2) chain gene in atopic subjects. *Int Arch Allergy Immunol* 2001;**124**:117-20.

**32**. Lumme A, Haahtela T, Ounap J *et al.* Airway information, bronchial hyperresponsiveness and asthma in elite ice hockey players. *Eur Respir J* 2003;**22**:113-7.

**33**. Groux H, Bigler M, de Vries JE, Roncarolo MG. Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells. *J Exp Med* 1996;**184**:19-29.

**34**. Gentile DA, Schreiber R, Howe-Adams J *et al.* Diminished dendritic cell interleukin 10 production in atopic children. *Ann Allergy Asthma Immunol* 2004;**92**:538-44.

Allergology International. 2008;57:367-376
DOI: 10.2332/allergolint.O-08-546

ORIGINAL ARTICLE

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

Takeshi Kimura[1,2], Zenichiro Kato[1,3,4], Hidenori Ohnishi[1], Hidehito Tochio[2,5], Masahiro Shirakawa[2,5] and Naomi Kondo[1,3,4]

## 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-

[1]Department of Pediatrics, Graduate School of Medicine, [3]Center for Emerging Infectious Diseases (CEID), [4]Center for Advanced Drug Research (CADR), Gifu University, Gifu, [2]Department of Molecular Engineering, Graduate School of Engineering, Kyoto University, Kyoto and [5]CREST, Japan Science and Technology Agency, Saitama, Japan.
Correspondence: Zenichiro Kato, MD, PhD, Department of Pediatrics, Graduate School of Medicine, Gifu University, 1-1 Yanagido, Gifu 501-1194, Japan.
Email: zen-k@gifu-u.ac.jp
Received 22 February 2008. Accepted for publication 9 May 2008.

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 $1 \times 10^6$ 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

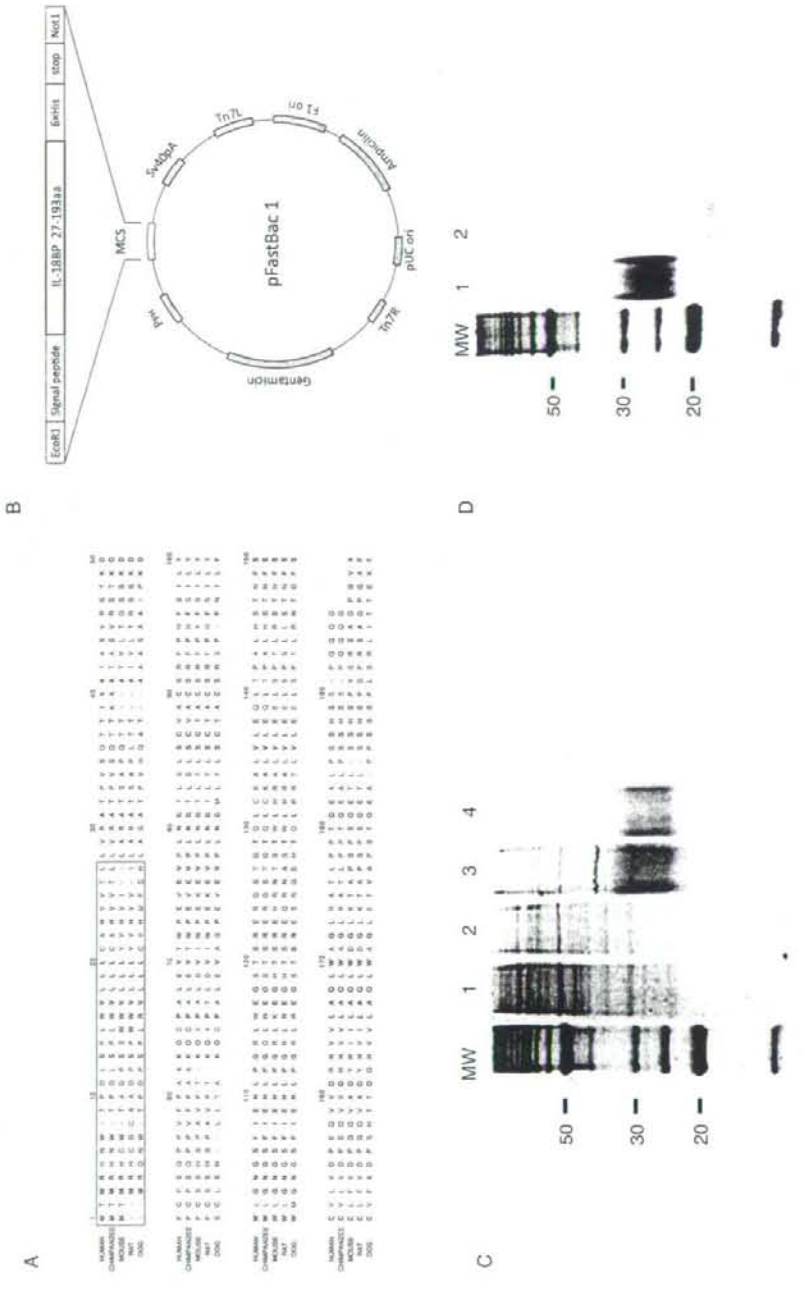


**Fig. 1** Expression and purification of rhIL-18BP. **A**, Alignment for IL-18BPs. The signal peptide residues of the hIL-18BP in mammalian cells are boxed. Estimated key residues on hIL-18BP for hIL-18 binding are highlighted in yellow.[26] **B**, A map of the construct used for expression of hIL-18BP 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 $1.0 \times 10^6$ 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.

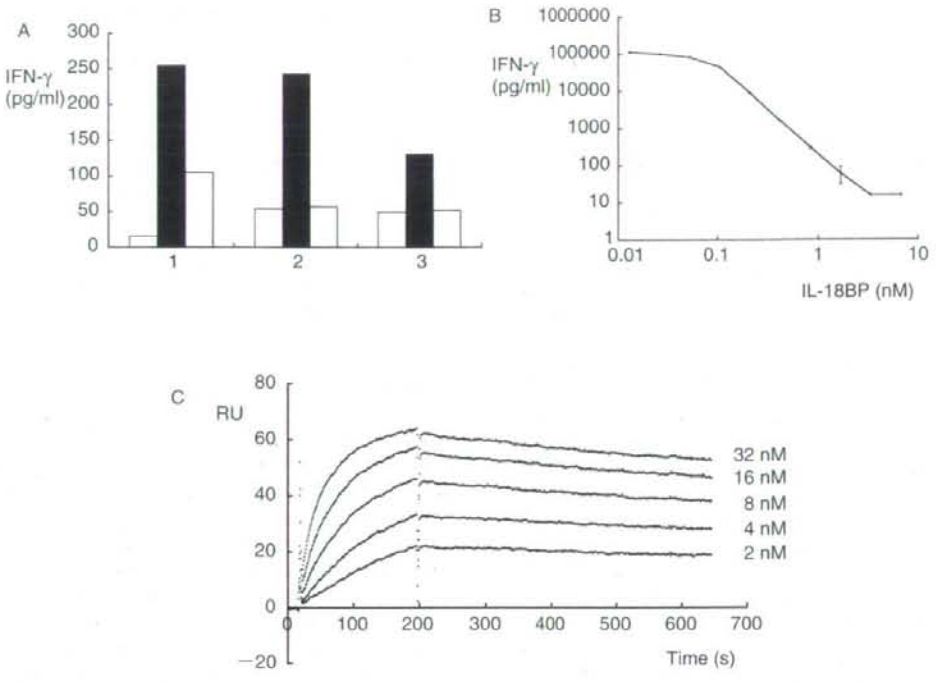


**Fig. 2** Functional activity of purified rhIL-18BP. **A**, Inhibition of IFN-γ production by rhIL-18BP in LPS-stimulated human PBMCs. The numbers on the horizontal axis show the subject numbers. Open box, control; black box, LPS stimulation, grey box, LPS stimulation with rhIL-18BP. **B**, Inhibition 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.

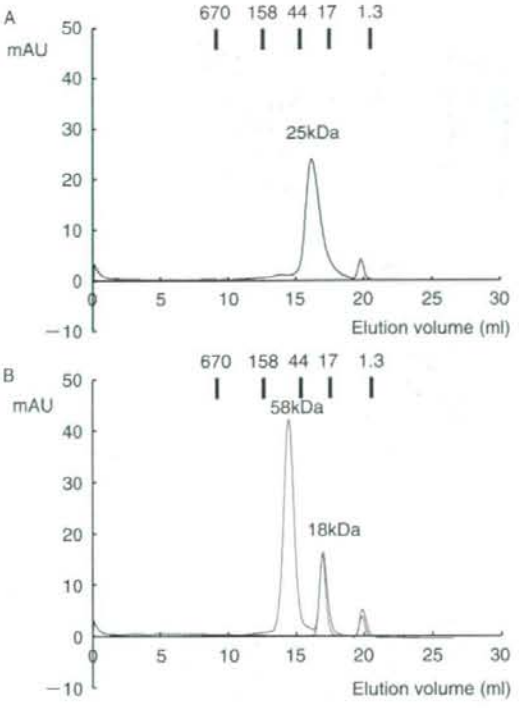


**Fig. 3** Stoichiometric analysis of the rhIL-18/rhIL-18BP complex. **A**, Gel filtration of rhIL-18BP. **B**, Gel filtration of the rhIL-18BP/rhIL-18 complex. A solid line indicates the sample that contained a mixture of rhIL-18BP and rhIL-18 at 1 : 2 ratio, a dashed line, indicates the sample with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

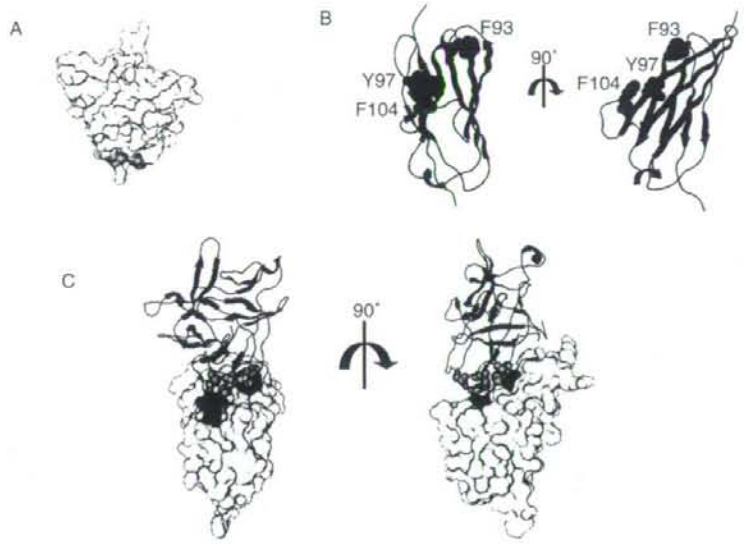


**Fig. 4** A structural model of the hIL-18/hIL-18BP complex. **A**, Surface of hIL-18. Functional binding residues on hIL-18 for hIL-18BP are colored in orange. **B**, A structural model of the rhIL-18BP monomer. Estimated functional key residues of hIL-18BP for hIL-18 are shown as atoms in red. **C**,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

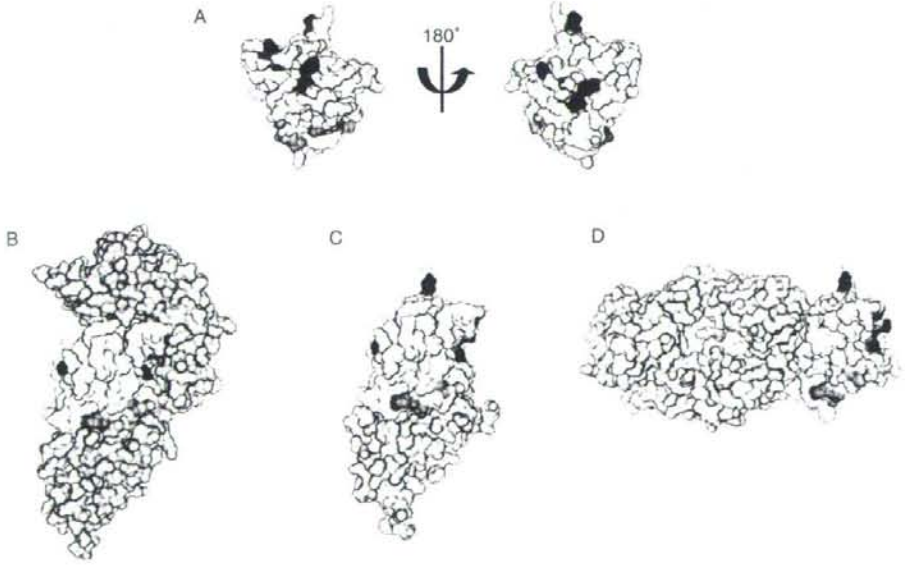


**Fig. 5** Human IL-18 interacts differently with different proteins through its three binding sites. **A**, Receptor binding sites on hIL-18.[3] Sites 1, 2 and 3 are indicated in red, orange, and blue, respectively. **B, C, D,** Models of complexes between hIL-18 and hIL-18Rα (in grey), hIL-18BP (in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

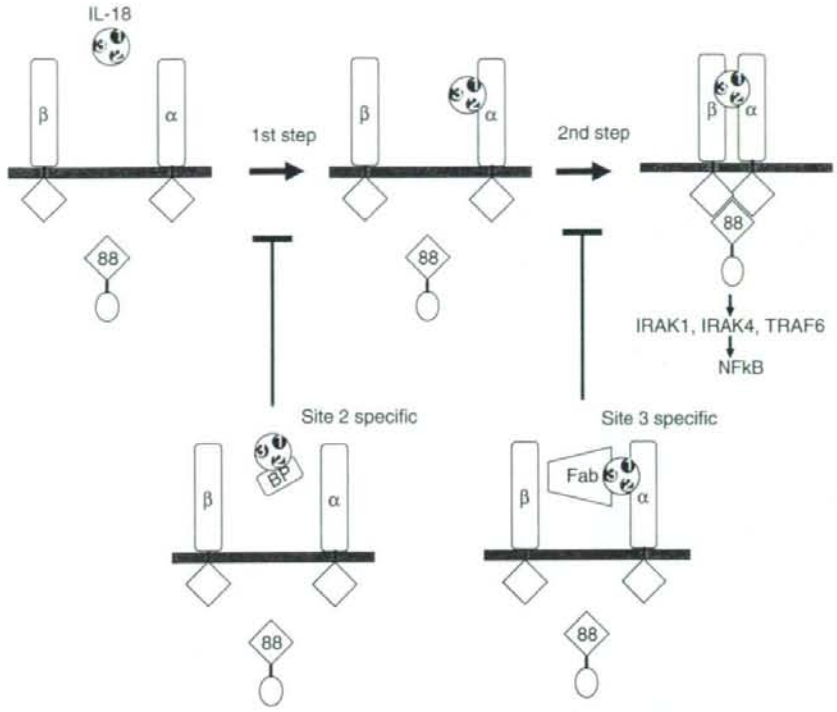


**Fig. 6** A schematic model of IL-18 receptor activation and inhibition. A two-step complex formation model of IL-18 signaling is shown at the top. The inhibitory mode of IL-18BP or Fab fragment of h18－108 is shown at the bottom. Sites 1, 2 and 3 of hIL-18 are indicated in red, orange, and blue, respectively.

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-