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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.
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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 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 1×10^6 /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-

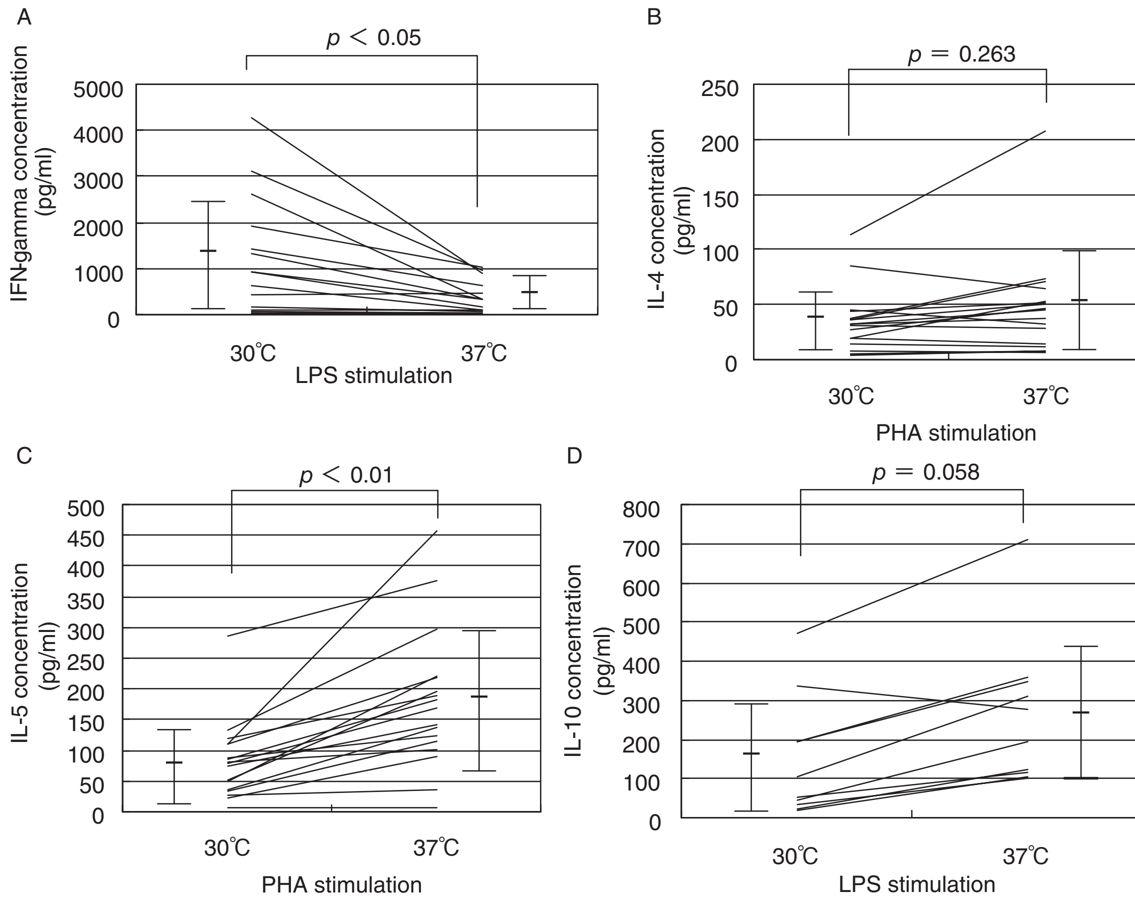

Fig. 1 PBMCs were cultured in the presence of LPS (1 μg/mL) (IFN-gamma and IL-10) or PHA (10 μg/ml) (IL-4 and IL-5) for 48 hours at 30℃ or 37℃. (**A**) IFN-gamma, (**B**) IL-4, (**C**) IL-5 and (**D**) IL-10 concentrations in the supernatants of the cell cultures were measured by ELISA. Results are mean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

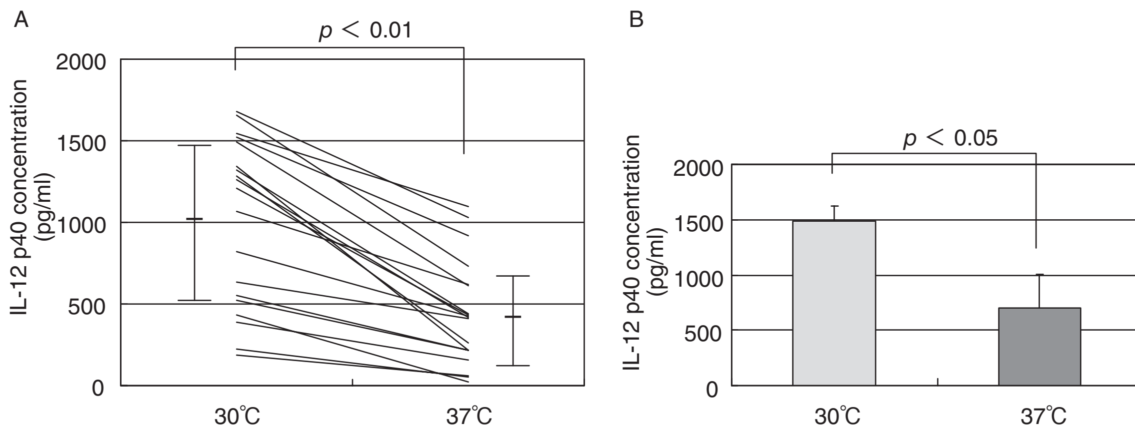

Fig. 2 PBMCs were cultured in the presence of LPS (1 μg/mL) for 48 hours at 30℃ or 37℃. (**A**) IL-12 p40 concentrations in the supernatants of the cell cultures were measured by ELISA. (**B**) $CD14^+$ blood monocytes were separated from the PBMCs, and cultured in the presence of LPS (1 μg/mL) for 48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

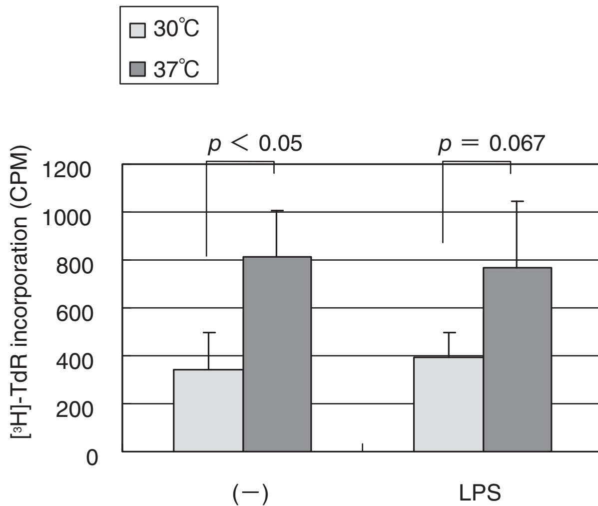

Fig. 3 PBMCs were grown in triplicate at 0.2 ml per well in round-bottom microtest plates with or without LPS at 30℃ or 37℃ for 48 hours. The amount of DNA synthesized was measured by adding 0.5 μCi of ^{3}H-thymidine per well in the microtest plates for 4 hours before harvesting using glass fibre filters.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

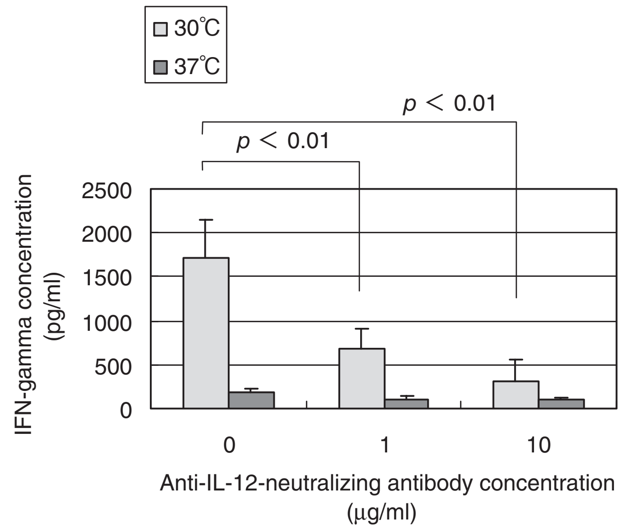

Fig. 4 PBMCs were cultured in the presence of LPS (1 μg/mL) and 0, 1 or 10 μg/ml of an anti-IL-12-neutralizing antibody for 48 hours at 30℃ or 37℃. IFN-gamma concentrations in the supernatants of the cell cultures were measured by ELISA. Results are mean ± SD.

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]

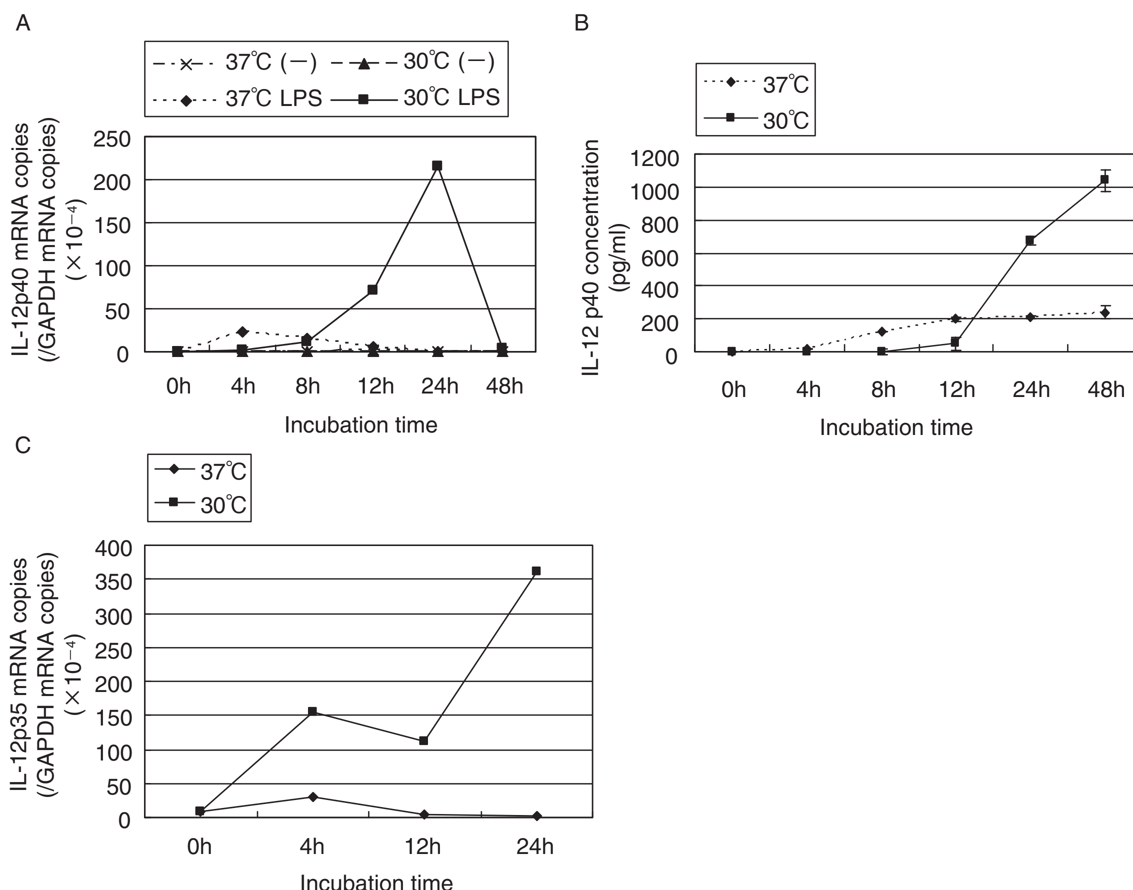

Fig. 5 PBMCs were cultured in the presence of LPS (1 μg/mL) at 30℃ or 37℃ for up to 48 hours. At the indicated time points, (**A**) the IL-12 p40 mRNA amounts in the cultured cells were measured by real-time RT-PCR, and (**B**) IL-12 p40 concentrations in the supernatants of the cell cultures were measured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

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.

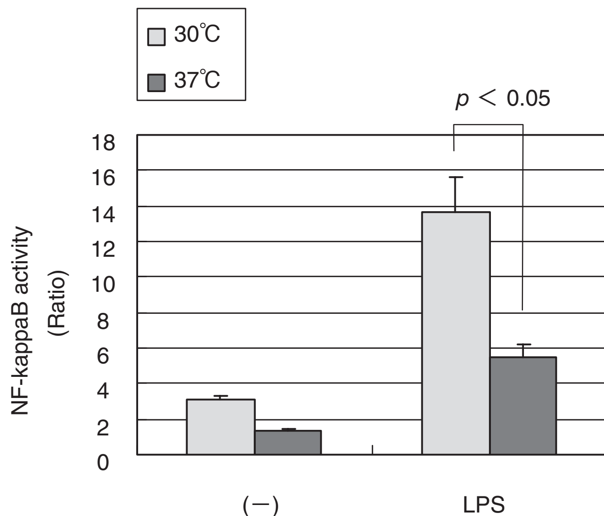

Fig. 6 HEK293-hTLR4A-HA cells were cultured in the presence or absence of LPS (1 μg/mL) at 30℃ or 37℃ for 24 hours. After 24 hours incubation, the cells were lysed and analyzed for activated NF-KappaB using a Luciferase Assay. The results show representative data of three independent transfection experiments.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

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