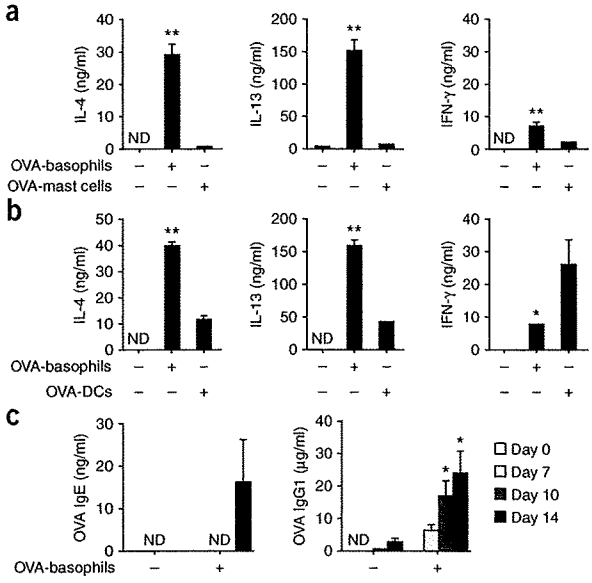

Figure 4 Intravenous administration of OVA-pulsed basophils induces a T_H2 response. (**a**,**b**) Enzyme-linked immunosorbent assay (ELISA) of IL-4, IL-13 and IFN-γ in supernatants of cells prepared as follows: purified bone marrow–derived basophils, mast cells (**a**) or splenic DCs (**b**) stimulated with DNP-OVA plus IgE anti-DNP as described in **Figure 3d** were adoptively transferred into BALB/c mice through the tail vein (2.5×10^5 cells of each per mouse); 4 d later, mice were intravenously challenged with OVA protein (100 μg per mouse) and, 2 d after this challenge, splenic $CD4^+$ T cells from each mouse were restimulated for 5 d in 96-well plates with OVA protein (100 μg/ml) in the presence of irradiated ΔT-spleen cells (1×10^5 cells per 0.2 ml per well for all cells). OVA- (left margin), processed OVA. (**c**) ELISA of OVA-specific IgE and IgG1 in serum from mice treated as follows: after priming of basophils as described in **a**, basophils (5×10^5 cells per mouse) were adoptively transferred into BALB/c mice through the tail vein; 1 week later (day 0), all mice were intravenously challenged with OVA protein (100 μg per mouse) and serum was then collected on days 0–14 (key). *, $P < 0.05$ and **, $P < 0.0001$, OVA-pulsed basophils versus cells from mice given mast cells and injected with OVA protein (**a**), given DCs and injected with OVA protein (**b**) or injected with OVA protein alone (**c**; Student's *t* test). Data are representative of two independent experiments (mean and s.e.m. of five mice).

neutral conditions. Furthermore, basophils had this T_H2-inducing capacity even when the ratio of basophils to $CD4^+$ T cells was decreased to 1:8. As expected, in T_H2 conditions, both types of APCs showed similar APC function. Furthermore, we were able to decrease the ratio of APC to $CD4^+$ T cells to 1:16 without substantially diminishing T_H2 cell development (**Fig. 3e**). In contrast, DCs 'preferentially' induced IFN-γ-producing cells in neutral conditions. Thus, basophils incubated with DNP-OVA and IgE anti-DNP showed very potent OVA-specific T_H2 cell–inducing activity *in vitro*.

OVA-pulsed basophils induce T_H2 cells *in vivo*

We compared the activity of OVA-pulsed basophils and mast cells to induce T_H2 cells *in vivo*. We pulsed basophils and mast cells with OVA by culturing cells with complexes of DNP-OVA and IgE anti-DNP, then transferred these basophils or mast cells into normal mice through the tail vein. Then, 4 d later, we challenged mice intravenously with intact OVA protein in PBS, and 2 d after this challenge, we prepared splenic $CD4^+$ T cells and stimulated them with OVA–pulsed APCs (ΔT-spleen cells) and measured IL-4, IL-13 and IFN-γ in the culture supernatants. We found that only OVA-pulsed basophils promptly and strongly induced T_H2 cells and modestly induced T_H1 cells in the spleen (**Fig. 4a**). We also examined the effect of OVA-pulsed DCs on T_H1 and T_H2 response *in vivo*. Intravenous administration of OVA-pulsed DCs into naive mice dominantly induced T_H1 cells, whereas intravenous administration of OVA-pulsed basophils again induced T_H2 cells strongly and T_H1 cells moderately (**Fig. 4b**), which suggests the importance of basophils in inducing T_H2 responses *in vivo*.

We next examined the ability of mice immunized with OVA-pulsed basophils to produce OVA-specific IgE and IgG1 after OVA challenge. Intravenous administration of OVA solution in naive mice did not induce IgE response but induced a very modest IgG1 response (**Fig. 4c**). In contrast, mice primed with OVA-pulsed basophils produced both IgE and IgG1 in response to OVA (**Fig. 4c**). Indeed, these mice developed $CD4^+CD62L^{lo}IL\text{-}33R\alpha^+$ T_H2 cells in their spleens (**Supplementary Fig. 4a,b** online) that help OVA-activated B cells produce IgE and IgG1 *in vivo*.

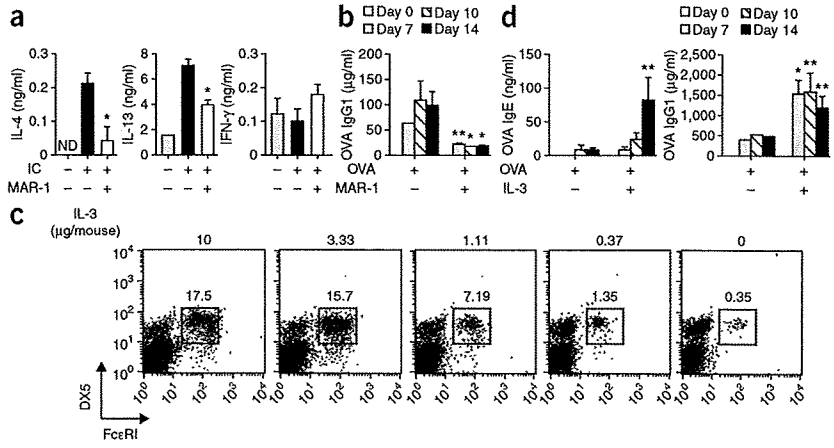

Figure 5 Intravenous administration of antigen-IgE complex induces T_H2 responses. (**a**,**b**) Analysis of cytokines (**a**) and antibodies (**b**) in BALB/c mice pretreated with PBS or anti-FcεRIa (MAR-1) and then injected intravenously with a mixture of DNP-OVA (10 μg) and IgE anti-DNP (20 μg; immune complex (IC)). (**a**) ELISA of IL-4, IL-13 and IFN-γ in supernatants of splenic $CD4^+$ T cells obtained 4 d after injection of the immune complex and restimulated as described in **Figure 4a**. (**b**) OVA-specific IgE and IgG1 antibodies in serum of mice intravenously challenged with OVA protein 4 d after injection of the immune complex, analyzed as described in **Figure 4c**. *, $P < 0.05$ and **, $P < 0.005$, compared with mice given immune complex without MAR-1 pretreatment (Student's *t*-test). (**c**) Frequency of basophils ($FcεRI^+DX5^+$ cells) in BALB/c mice injected with IL-3 (0–10 μg per mouse (above plots) for 2 weeks) via osmotic pump. Numbers above outlined areas indicate percent $FcεRI^+DX5^+$ cells gated on splenic non–B, non–T cells. (**d**) OVA-specific IgE and IgG1 in serum of BALB/c mice pretreated with PBS or IL-3 and then injected intravenously with immune complexes as described in **a**,**b**, then intravenously challenged 4 d later with OVA protein, assessed as described in **Figure 4c**. *, $P < 0.01$ and **, $P < 0.05$, compared with mice without IL-3 pretreatment (Student's *t*-test). Data are representative of two independent experiments (mean and s.e.m. of five mice; **a**,**b**,**d**) or two experiments with five mice (**c**).

Antigen-IgE induces T_H2 cells in a basophil-dependent way

Finally, to demonstrate the contribution of basophils to the development and upregulation of T_H2-IgE response *in vivo*, we intravenously injected complexes of DNP-OVA and IgE anti-DNP into naive mice or mice depleted of basophils by treatment with anti-FcεRIα (MAR-1). As reported by others[38], daily injection of MAR-1 for 3 d almost completely depleted the spleen and liver of basophils (**Supplementary Fig. 5a,b** online). At day 4 after intravenous injection of DNP-OVA (10 μg) and IgE anti-DNP (20 μg), we prepared splenic $CD4^+$ T cells, stimulated them with OVA-pulsed APCs and measured IL-4, IL-13 and IFN-γ in the culture supernatants. We found that IgE immune complexes induced T_H2 cells in the spleens of naive mice (**Fig. 5a**). Depletion of basophils (**Supplementary Fig. 5c,d**) resulted in significantly diminished T_H2 cell development and T_H2-dependent IgG1 responses (**Fig. 5a,b**). These results suggest that basophils efficiently take up DNP-OVA–IgE anti-DNP immune complexes and induce OVA-specific T_H2 cells, which in turn stimulate OVA-stimulated B cells to produce IgG1.

To confirm the contribution of basophils to the initiation and amplification of T_H2-IgE responses, we injected IL-3 into naive mice using an osmotic pump (10 μg IL-3 per 100 μl PBS) to increase the number of basophils and then examined their responsiveness to the treatment with antigen-IgE complex. IL-3-treated mice markedly increased the number of basophils in their spleens (**Fig. 5c**) and other organs, somewhat resembling atopic people, who also increase the number of basophils in inflammation sites[8–11]. We found IL-3-treated mice died of systemic anaphylactic shock when challenged with a high dose of IgE complex (for example, 100 μg DNP-OVA and 200 μg IgE anti-DNP). Therefore, we intravenously injected low doses of IgE complex (5 μg DNP-OVA and 10 μg IgE anti-DNP). In contrast to mice that received no pretreatment with IL-3, IL-3-treated mice significantly increased their production of OVA-specific IgG1 and IgE in response to OVA challenge (**Fig. 5d**), which suggests that the number of basophils might determine the responsiveness to IgE complex that 'preferentially' induces T_H2 cells. These results collectively indicated that basophils are responsible for inducing OVA-specific T_H2 cells by taking up DNP-OVA–IgE anti-DNP complexes, presenting OVA peptide with MHC class II and producing abundant IL-4.

DISCUSSION

Basophils can induce T_H2 cells *in vitro* and *in vivo* by producing early IL-4 (refs. 18,20,29,39). Other studies have shown that basophils that transmigrate to draining lymph nodes after papain stimulation are stimulated to produce IL-4 and/or thymic stromal lymphopoietin, which promote T_H2 differentiation *in vivo*[29]. However, it has remained uncertain whether basophil-derived IL-4 is indeed involved in the development of T_H2 cells in response to stimuli other than protease allergens.

Here we have demonstrated that protein antigen without enzymatic activity induced antigen-specific T_H2 cells *in vitro* and *in vivo* in a basophil- and IL-4-dependent way, which suggests involvement of basophil-derived IL-4 in the development of T_H2 cells. Another important issue that needs to be addressed is the mechanism by which basophils induce T_H2 cells. We could propose at least two mechanisms for basophil-mediated promotion of T_H2 responses. One is that basophils produce IL-4 and/or thymic stromal lymphopoietin and simply transfer foreign protein to DCs, which do the actual antigen presentation. The other is that basophils are APCs that also produce IL-4. We have demonstrated that basophils induce T_H2 cells in the absence of 'professional' APCs *in vitro*. Furthermore, we have shown that basophils are potent APCs that directly and 'preferentially' induce T_H2 cells *in vivo*.

We first demonstrated that basophils derived from mice inoculated with *S. venezuelensis* produced substantial amounts of IL-4, IL-6 and IL-13 in IL-3-containing medium. Then we demonstrated that they expressed MHC class II and strongly induced the development of naive $CD4^+$ T cells into T_H2 cells in neutral conditions. In contrast, basophils from naive mice produced relatively small amounts of IL-4, IL-6 and IL-13 in IL-3-containing medium.

We initially regarded only basophils from infected mice as potent APCs. Then we recognized that basophils from naive mice and infected mice expressed almost identical amounts of MHC class II, which suggested they had a potent APC function. Indeed, splenic basophils in naive mice were also immunologically competent APCs. Both types of basophils produced substantial amounts of IL-10, which suggests the possibility that this IL-10 might enhance the ability of IL-4 from basophils to induce the development of T_H2 cells *in vitro*. Bone marrow basophils also recapitulated well the APC function of splenic basophils. In particular, they were able to efficiently take up a low dose of antigen-IgE complex, present antigen–MHC class II and produce IL-4, which suggests that they are also very potent T_H2 cell–inducing APCs.

We further demonstrated that intravenous administration of OVA-pulsed basophils, which we prepared by culturing basophils with complexes of DNP-OVA and IgE anti-DNP, strongly induced OVA-specific T_H2 cells in the spleens of naive mice. In contrast, OVA-pulsed mast cells failed to do so. These results indicated their difference in inducing antigen-specific T_H2 cells *in vivo*. Basophils have been shown to have a very short half-life after adoptive transfer[40]. Because we cultured basophils with immune complexes of DNP-OVA and IgE anti-DNP, we suspect such crosslinking might induce signals that sustain their survival *in vivo*.

Finally, we demonstrated a single intravenous administration of a low dose of DNP-OVA–IgE anti-DNP complex into naive mice rapidly and 'preferentially' induced OVA-specific T_H2 cells in an endogenous basophil–dependent way. Such sensitized mice then promptly produced antigen-specific IgG1 in response to intravenous administration of antigen solution. As expected, IL-3 treatment prepared mice highly susceptible to the T_H2 cell–inducing action of IgE complex by increasing the number of basophils.

Although basophil MHC class II expression was less than that on conventional APCs, basophils showed more potent T_H2 cell–inducing activity than did conventional APCs in both neutral and T_H2 conditions. We found that basophils had a greater APC activity when pulsed with DNP-OVA in the presence of IgE anti-DNP. We also found that basophils still had the notable T_H2 cell–inducing ability in neutral conditions even when the ratio of APCs to $CD4^+$ T cells was low (1:8). In contrast, OVA-pulsed DCs failed to induce T_H2 cells at any APC/$CD4^+$ T cell ratio in neutral conditions, although they induced IFN-γ-producing cells. Thus, basophils are very potent T_H2 cell–inducing cells *in vitro*.

Our study has indicated that endogenous basophils are important for promotion of the T_H2-IgE response *in vivo*. We demonstrated that intravenous administration of immune complexes of DNP-OVA and IgE anti-DNP 'preferentially' induced OVA-specific T_H2 cells in an endogenous basophil–dependent way. Studies have shown that basophils can also capture antigen by binding to surface antigen-specific IgE-FcεRI (refs. 38,41). Activated basophils then produce IL-4 and IL-6 and possibly express $CD40L$[42], the ligand for the costimulatory molecule CD40, which in combination induce B cells to proliferate and to produce IgE. Thus, basophils promote both T_H2 and IgE responses *in vivo*.

Atopic people are characterized by having more basophils in sites of allergic inflammation[8–11]. Once atopic people start to produce antigen-specific IgE, they can steadily increase the amount of complexes of

antigen and antigen-specific IgE, which allows basophils to augment their uptake of IgE complex. Although mature human basophils lack HLA-DR, we have shown here that some can re-express HLA-DR when stimulated with IL-3. Thus, it is plausible that mature basophils in the allergic inflammation site, which might be characterized by abundant production of IL-3 and other factors, do express HLA-DR. Basophils, then, could become potent APCs and induce progressive allergic inflammation in these people.

Here we have demonstrated that basophils are important in the amplification of the T_H2-IgE response. Indeed, depletion of basophils by a specific antibody inhibited IgE complex–induced T_H2-IgE responses. Published work has suggested that anti-IgE therapy is effective for T_H2-IgE–mediated diseases[43–45]. The rationale for such therapy is that it is believed to interfere with IgE-mediated activation of mast cells and basophils. On the basis of our results here, we can add another rationale: inhibition of the generation of antigen-pulsed basophils. Thus, basophils might represent an important therapeutic target cell.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Accession codes. UCSD-Nature Signaling Gateway (http://www.signaling-gateway.org): A000543 and A001262.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

K.N. and T.Y. envisaged the possible APC function of basophils; T.Y. and K.N. designed the experiments; T.Y. did the main part of this study and analyzed the data; K.Y., M.N. and Y.I. helped with some experimental procedures; H.T. and Y.F. analyzed human cells; and T.Y. prepared the draft of manuscript and K.N. completed it.

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ONLINE METHODS

Mice. BALB/c mice were from Jackson Laboratory. Mice transgenic for αβ TCR recognizing OVA(323–339) (DO11.10) and BALB/c G4-homozygous (IL-4-deficient) mice[34] were bred in specific pathogen–free conditions at the animal facilities of Hyogo College of Medicine. All animal experiments were done in accordance with guidelines of the Institutional Animal Care Committee of Hyogo College of Medicine.

Antibodies and reagents. Anti–mouse IL-4 (11B11)[25] was purified in our laboratory in the Department of Immunology and Medical Zoology, Hyogo College of Medicine. Phycoerythrin (PE)–anti–mouse CD4 (GK1.5), fluorescein isothiocyanate (FITC)–anti–mouse CD62L (MEL-14), FITC–anti–mouse I-A^d (AMS-32.1), FITC–anti–mouse CD40 (HM40-3), FITC–anti–mouse CD80 (16-10A1), FITC–anti–mouse CD86 (GL1), FITC–anti–mouse CD11c (HL3), PE–anti–mouse c-Kit (2BB), FITC–anti–mouse c-Kit (2BB), FITC–anti–mouse CD49b (DX5), FITC–anti–human HLA-DR (TÜ36) and biotin–human CD203c (FR3–16A11) were from BD Biosciences. FITC–anti–mouse ST2 (DJ8), biotin–anti–mouse FcεRIα (MAR-1), streptavidin-PE and streptavidin-allophycocyanin were from eBioscience. The following PE-labeled monoclonal antibodies to human cell surface markers were from BD Biosciences: anti-CD3 (HIT3a), anti-CD7 (M-T701), anti-CD14 (M5E2), anti-CD15 (HI98), anti-CD16 (3G8), anti-CD19 (HIB19), anti-CD36 (CB38), anti-CD45RA (HI100) and anti-CD235a (GAR-2). Recombinant mouse IL-2, IL-3, IL-4 and human IL-3 were from R&D Systems. IL-18 was from MBL. Recombinant human IL-33 was purified in our laboratory in the Department of Immunology and Medical Zoology, Hyogo College of Medicine[28]. Monoclonal IgE anti-DNP (SPE-7), OVA (grade V), lipopolysaccharide from *Salmonella minnesota* Re-595 or *Escherichia coli* 055:B5, and peptidoglycan from *Staphylococcus aureus* were from Sigma. DNP-OVA was prepared according to a published method[46].

Flow cytometry and cell purification. For the preparation of bone marrow–derived basophils, bone marrow cells were cultured for 14 d with IL-3 (10 U/ml) in RPMI-1640 medium supplemented with 10% (vol/vol) FBS, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin (complete RPMI) and were washed twice. Cells were first treated for 30 min at 4 °C with anti–FcγRII/III (10 μg/ml), followed by treatment for 1 h at 4 °C with biotin–anti–mouse FcεRIα (5 μg/ml) in staining buffer (1% (vol/vol) FCS in PBS). After being washed twice, cells were stained for 30 min with streptavidin-allophycocyanin and PE–anti–mouse c-Kit. Samples were analyzed on a FACSCalibur (BD Biosciences) and were separated into $FcεRI^+$c-Kit^- cells (basophils) or $FcεRI^+$c-Kit^+ cells (mast cells) with a FACSAria (BD Biosciences). The purity of each population was over 96%. The resultant populations were further stained with FITC-labeled antibodies for analysis of surface makers. For preparation of splenic basophils, spleen cell samples from BALB/c mice were first depleted of Thy-1.2^+ T cells and $B220^+$ cells with a MACS system (MiltenyiBiotec), then the residual cells were further stained and separated into $FcεRI^+$c-Kit^- or $FcεRI^+$c-Kit^+ cells with a FACSAria. The purity of each population was over 96%. For the preparation of splenic $CD4^+CD62L^+$ resting T cells and for intracellular cytokine staining, published methods were followed[47].

Human peripheral blood from normal volunteers and umbilical cord blood obtained from normal full-term deliveries were obtained and processed after informed consent was given. The Institutional Review Board approved the experimental plan. Mononuclear cells were isolated from peripheral blood and cord blood by Ficoll density-gradient centrifugation. Peripheral blood mononuclear cell samples were further depleted of T cells, monocytes, eosinophils, natural killer cells, B cells, platelets, DCs and erythroid cells magnetically with a 'cocktail' of PE-labeled monoclonal antibodies to human CD3, CD7, CD14, CD15, CD16, CD19, CD36, CD45RA and CD235a and anti-PE MicroBeads (MiltenyiBiotec). Umbilical cord blood mononuclear cells were further enriched to $CD34^+$ cells with MicroBeads. These $CD34^+$ progenitor cells were plated at a density of 5×10^5 cells per ml in 12-well plates and were cultured for 7 d in StemPro-34 SFM (GIBCO) supplemented with 10% (vol/vol) FBS, 50 μM 2-mercaptoethanol, 0.5 mM L-glutamine, 50 U/ml of penicillin, 50 μg/ml of streptomycin and 10 ng/ml of human IL-3.

***In vitro* culture.** Naive splenic $CD4^+CD62L^+$ T cells (1×10^5 cells per ml) from DO11.10 mice were stimulated in 48-well plates with IL-2 (100 pM), IL-3 (20 U/ml) and OVA(323–339) (1 μM) or DNP-OVA (6.25–100 μg/ml) in the presence of conventional APCs (irradiated T cell–depleted BALB/c splenocyte samples), irradiated splenic $CD11c^+$DCs prepared as described[37] or irradiated purified basophils (5×10^5 cells per ml each). For the induction of T_H2 cells, IL-4 (1000 U/ml) was also added to the culture. On the third or fourth day of culture, cells were diluted 1:2 or 1:3 in complete RPMI medium with IL-2 (100 pM) and their populations were expanded into 48-well plates. Then, 7 d after the initial stimulation, cells were collected and washed, then were recultured for 4 h with PMA (phorbol 12-myristate 13-acetate; 50 ng/ml) plus ionomycin (500 ng/ml) and were analyzed by flow cytometry for cytosolic IL-4 and IFN-γ. In some experiments, after initial priming, $CD4^+$ T cells (1×10^5 cells per 0.2 ml per well) were restimulated for 48 h in 96-well plates with IL-2 (100 pM) and OVA(323–339) (1 μM) in the presence of 1×10^5 irradiated conventional APCs. Supernatants were collected and cytokine production was assessed with ELISA kits (R&D Systems) or the Bio-Plex system (BioRad) as described before[28].

***In vivo* treatment of mice.** Bone marrow–derived and flow cytometry–sorted basophils, mast cells and splenic $CD11c^+$ DCs (5×10^5 cells per ml each) were cultured for 16 h in 48-well plates with IL-3 (20 U/ml), DNP-OVA (100 μg/ml) and IgE anti-DNP (10 μg/ml). After priming, basophils, mast cells and splenic DCs (2.5×10^5 cells per mouse) were transferred through the tail vein into BALB/c mice. At 4 d or 1 week after reconstitution, mice were intravenously challenged with OVA protein (100 μg) in PBS. In some experiments, BALB/c mice were injected intravenously with a mixture of DNP-OVA (5–100 μg per mouse) and IgE anti-DNP (10–200 μg per mouse). For *in vivo* depletion of basophils, a published method of was followed[38]. Mice were injected intraperitoneally twice daily for 3 d with 5 μg anti–mouse FcεRIα (MAR-1) or PBS. Mice were allowed to 'rest' for 2 d and then were injected with a mixture of DNP-OVA plus IgE anti-DNP, then these mice were injected twice daily for additional 3 d with MAR-1 or PBS. IL-3 was infused subcutaneously into mice via osmotic pumps (Durect) filled with IL-3 (100 μg) in 100 μl PBS in mice as described[48].

ELISA. OVA-specific serum IgE was measured with a Mouse OVA-IgE ELISA kit (Dainippon Sumitomo Pharma). OVA-specific serum IgG1 was measured with a Mouse OVA-IgG1 ELISA kit (AKRIE-04; Shibayagi).

Parasites. BALB/c mice were subcutaneously inoculated with 5,000 *S. venezuelensis* third-stage larvae to initiate complete infection as described[27,30].

Electron microscopy. Sorted human $CD203c^+HLA\text{-}DR^+$ cells were fixed with 2% (wt/vol) paraformaldehyde and 1.25% (wt/vol) glutaraldehyde, were post-fixed with 1% (wt/vol) OsO_4 and were embedded in Epon. Ultrathin sections were double-stained with uranyl acetate and lead citrate and were examined with a JEM 1220 transmission electron microscopy (Jeol).

Proliferation assay. Naive splenic $CD4^+CD62L^+$ T cells from DO11.10 mice (5×10^4 cells per 0.2 ml per well) were stimulated for 4 d in 96-well plates with IL-2 (100 pM), IL-3 (20 U/ml) and OVA(323–339) (1 μM) or DNP-OVA (6.25–100 μg/ml) with or without monoclonal anti-DNP IgE (10 μg/ml) in the presence of conventional APCs or purified basophils (2.5×10^5 cells per well each). DNA synthesis was assessed by measurement of the incorporation of 0.2 μCi [^{3}H]thymidine during the final 16 h.

Analysis of expression of TLR mRNA. Total RNA was extracted from sorted basophils and mast cells with TRIzol reagent, was treated with DNase I and was reverse-transcribed with SuperScript II Reverse Transcriptase and oligo$(dT)_{12–18}$ primer (Invitrogen). As a positive control for each TLR, RNA extracted from total spleen cells was used. For analysis of expression of TLR mRNA, mRNA was amplified by a modified standard RT-PCR amplification procedure. The specific TLR primer sequences and their annealing

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temperatures were according to a published report[49]. PCR conditions were as follows: cDNA was amplified by 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, followed by further extension at 72 °C for 10 min, then samples were stored at 4 °C until analysis. After amplification, PCR products were separated by electrophoresis through 1.7% agarose gels and were visualized by illumination with ultraviolet light.

Statistics. Statistical comparisons between two experimental groups were made with a paired Student's *t*-test using GraphPad Instat Software. *P* values of less than 0.05 were considered significant.

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A Functional Polymorphism in *IL-18* Is Associated with Severity of Bronchial Asthma

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Rationale: IL-18 is a unique cytokine that enhances innate immunity and both Th1- and Th2-driven immune responses. Recent murine and human genetic studies have shown its role in the pathogenesis of asthma.

Objectives: We conducted an association study in a Japanese population to discover variants of *IL-18* that might have an effect on asthma susceptibility and/or progression and conducted functional analyses of the related variants.

Methods: The *IL-18* gene locus was resequenced in 48 human chromosomes. Asthma severity was determined according to the 2002 Global Initiative for Asthma Guidelines. Association and haplotype analyses were performed using 1,172 subjects.

Measurements and Main Results: Although no polymorphisms differed significantly in frequency between the control and adult asthma groups, rs5744247 C>G was significantly associated with the severity of adult asthma (steps 1, 2 vs. steps 3, 4; $P = 0.0034$). We also found a positive association with a haplotype ($P = 0.0026$). By *in vitro* functional analyses, the rs5744247 variant was found to increase enhancer-reporter activity of the *IL-18* gene in bronchial epithelial cells. Expression levels of *IL-18* in response to LPS stimulation in monocytes were significantly greater in subjects homozygous for the susceptibility G allele at rs5744247 C>G. Furthermore, we found a significant correlation between the serum IL-18 level and the genotype of rs5744247 ($P = 0.031$).

Conclusions: Although the association results need to be replicated by other studies, *IL-18* variants are significantly associated with asthma severity, and the rs5744247 variant reflects higher transcriptional activity and higher expression of *IL-18* in LPS-stimulated monocytes and a higher serum IL-18 level.

Keywords: asthma severity; IL-18; LPS; monocytes; genetic polymorphisms

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

IL-18 plays multiple roles in chronic inflammation and in a number of infections and enhances both Th1- and Th2-mediated immune responses. The influence of genetic changes in this crucial cytokine on the etiology of asthma is unclear.

What This Study Adds to the Field

Our results suggest that a functionally relevant IL-18 polymorphism contributes to the disease severity of asthma. The variant affects the level of mRNA expression induced by LPS in human monocytes and correlates with the serum IL-18 level in individuals with asthma. The LPS-induced IL-18 expression was not suppressed by dexamethasone and salmeterol.

Bronchial asthma is a complex disorder caused by a combination of genetic and environmental factors (1, 2). Cytokines recruit and activate immune cells and play an important role in the coordination and persistence of the airway inflammation of asthma (3, 4). IL-18 is produced by both immune and non-immune cells, such as peripheral blood mononuclear cells and bronchial epithelial cells, and plays multiple roles in chronic inflammation and in a number of infections and enhances both Th1- and Th2-mediated immune responses (5). Although originally discovered as a factor that induced IFN-γ production from Th1 cells (5), IL-18 also has the potential to induce IL-4 and IL-13 production in T cells, natural killer (NK) cells, NK T cells, mast cells, and basophils (6–9).

Th2-type airway inflammation is a characteristic feature of bronchial asthma; however, important roles of IFN-γ in allergic inflammation have been shown in recent reports (10–16). Intranasal administration of an antigen and IL-18 stimulates Th1 cells to induce severe airway inflammation through IFN-γ and IL-13 in a murine model (11, 12). In humans, IFN-γ production by peripheral blood T cells is associated with the alteration of lung function in individuals with chronic stable asthma (13). Overproduction of IFN-γ has been observed in asthma, and the number of IFN-γ–producing $CD8^+$ T cells is related to asthma severity (14). Furthermore, both Th-1 and Th-2 chemokines and cytokines are involved in antigen-induced airway inflammation by segmental allergen bronchoprovocation related to disease severity (15, 16). These findings imply that Th-1 responses together with Th-2 responses cause severe allergic inflammation, and a polymorphism of the *IL-18* gene might be a genetic marker of asthma and disease severity.

Several association studies using polymorphic markers of the *IL-18* gene have been performed to discover genetic compo-

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nents in the pathogenesis of bronchial asthma (17–21). In this study, we focused on the *IL-18* gene, resequenced the gene regions including introns, performed linkage disequilibrium (LD) mapping, and conducted an association study and functional analyses of the related variants. Increased *IL-18* expression and serum levels in human allergic diseases have been reported (22–25). Here we measured serum IL-18 levels of patients with adult asthma and examined the correlation between the IL-18 level and related genotype.

Some of the results of these studies have been previously reported in the form of an abstract (26).

METHODS

Additional details on methods are provided in the online supplement.

Study Subjects

All subjects with asthma were diagnosed according to the American Thoracic Society criteria as described (27–29). We recruited 453 adults with asthma and recorded the age, sex, serum total IgE level, eosinophil count, lung functions, and clinical severity (Table 1). The clinical severity of adult asthma was classified according to the criteria of the National Institutes of Health/Global Initiative for Asthma 2002 by physicians who were experts in allergic diseases, and was defined by controller medication use at the time of entry into the study (30). Genomic DNA was prepared in accordance with standard protocols.

Screening for Polymorphisms and Genotyping

We resequenced the *IL-18* gene regions in 48 human chromosomes from 24 control subjects (*see* Table E1 in the online supplement). Pairwise LD was calculated as D′ and r^2 by using the Haploview 4.1 program (http://www.broad.mit.edu/mpg/haploview/). The polymorphisms were genotyped by use of the TaqMan system (Applied Biosystems, Foster City, CA).

Cells, Reagents, and Stimulation

Normal human bronchial epithelial cells (NHBE) (n = 4, aged 17 to 58 yr, white male), normal human lung fibroblasts (NHLF) (n = 1, aged 10 yr, white male) and bronchial smooth muscle cells (BSMC) (n = 1, aged 63 yr, white male) were purchased and maintained using Clonetics medium kits (Lonza Walkersville, Inc, Walkersville, MD). Monocytes were isolated from peripheral blood mononuclear cells (PBMCs) of healthy Japanese volunteers (aged 32 to 46 yr) by magnetic activated cell sorting according to the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were stimulated with the indicated concentrations of poly(I:C) (InvivoGen, San Diego, CA), LPS (InvivoGen), and macrophage-activating lipopeptide 2 (Alexis, Lausen, Switzerland). PBMCs, $CD4^+$ T cells, and $CD8^+$ T cells were stimulated by plate-bound anti-CD3 monoclonal antibodies (incubated at 1 μg/ml, clone number UCHT1) with soluble anti-CD28 monoclonal antibodies (1 μg/ml, clone number CD28.2). NHBE and monocytes were also cultured with dexamethasone (DEX) (ICN Biomedicals, Costa Mesa, CA) and/or salmeterol (SAL) (TOCRIS Inc., Ellisville, MO).

Quantitative Real-Time Reverse Transcriptase–Polymerase Chain Reaction, ELISA, and Luciferase Assay

The expression of *IL-18* and *IL-6* was determined by real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) using SYBR Premix Ex Taq (Takara, Shiga, Japan). Concentrations of IL-18 were measured in duplicate with a human-specific IL-18 ELISA kit (MBL, Nagoya, Japan) (31). Luciferase assays were conducted using pGL3-enhancer vector (Promega, Madison, WI).

Statistical Analysis

We calculated allele frequencies and tested agreement with Hardy-Weinberg equilibrium using a χ^2 goodness-of-fit test at each locus. To test the association, we compared differences in the allele frequency and genotype distribution of each polymorphism by using a contingency χ^2 test or Fisher exact test. We applied Bonferroni corrections, the multiplication of P values by three, the number of Tag single nucleotide polymorphisms (SNP)s. In the association study, corrected P values of less than 0.05 were judged to be significant. All tests were two-sided and odds ratios (ORs) with 95% confidence intervals (CIs) were also calculated.

Haplotype frequencies for multiple loci were estimated, and haplotype association tests were performed using Haploview 4.1. Serum total IgE levels, eosinophil counts, FVC (% predicted) and FEV_1 (% predicted) were analyzed as quantitative levels by the Kruskal-Wallis test, Friedman test, or Mann-Whitney U test. The Jonckheere-Terpstra trend test was used for *IL-18* genotype–phenotype correlation analyses. Comparison in expression analysis was performed with Student t test. A P value of less than 0.05 was considered statistically significant.

TABLE 1. BASELINE CHARACTERISTICS AMONG STUDY PARTICIPANTS WITH ASTHMA

Characteristics	Values
No. of Subjects	453
Age, yr, (range), mean ± SD	20–75 (49.7 ± 14.6)
Male (%)	43
Asthma characteristics, mean ± SD	
Serum total IgE, IU/ml	658 ± 1,550
Eosinophils, no./μl	411 ± 387
Asthma severity, n (%)	
Step 1, mild intermittent	11 (2.4)
Step 2, mild persistent	237 (52.3)
Step 3, moderate persistent	122 (26.9)
Step 4, severe persistent	83 (18.3)
Pulmonary function, mean ± SD	
FVC, % predicted	85 ± 20
FEV_1, % predicted	71 ± 21
FEV_1/FVC % ratio	70 ± 12

RESULTS

Fine Mapping of *IL-18* and Identification of *IL-18* Polymorphisms Associated with Asthma Severity

After extensive examination of *IL-18* by direct sequencing, we identified 18 polymorphisms (8 SNPs in the promoter region and 4 SNPs within the transcript) (Table 2, Figure 1). To examine the LD between identified SNPs, pairwise LD coefficients D′ and r^2 were calculated using the Haploview 4.1 program. Because five of the SNPs were quite rare, pairwise LD was measured by D′ and r^2 among the 13 SNPs with minor allele frequencies of greater than 5% (Table 2 and Table E2, Figure E1). One SNP, -9731T>G (rs1946519), was in complete LD (D′ = 1.00 and r^2 = 1.00) with -9682A>C (rs1946518) and in strong LD (D′ = 1.00 and r^2 = 0.84) with -12310C>T (rs2904613). Another SNP, -8963T>G (rs360718), was in complete LD (D′ = 1.00 and r^2 = 1.00) with -11877C>T (rs11214105), -11778G>T (rs1290349), -11608T>C (rs1293344), -9212G>C (rs187238), and -8949C>T (rs360717) and in strong LD (D′ = 1.00 and r^2 = 0.87) with -140C>G (rs360721) and 4861A>C (rs549908). The -380C>G (rs5744247) variant was in strong LD (D′ = 1.00 and r^2 = 0.84) with 1867->insertion C (rs5744252). We selected three tag SNPs, -9731T>G (rs1946519), -8963T>G (rs360718), and -380C>G (rs5744247), for association studies using tagger in the Haploview 4.1 program, and these three SNPs captured 13 of the 13 alleles with a mean r^2 of 0.955 ($r^2 > 0.84$) (Table E2, Figure E1). We also genotyped all 18 polymorphisms. The 18 SNPs were successfully genotyped in more than 96% of the people studied, and were in Hardy-Weinberg equilibrium (Table E3).

TABLE 2. FREQUENCIES OF POLYMORPHISMS OF THE *IL-18* GENE IN A JAPANESE POPULATION

SNP*	NCBI†	Position in the gene structure	mRNA	MAF‡
-12561C>T		5′ flanking region		0.02
-12310C>T	rs2904613	5′ flanking region		0.40
-11877C>T	rs11214105	5′ flanking region		0.19
-11778G>T	rs1290349	5′ flanking region		0.19
-11608T>C	rs1293344	5′ flanking region		0.19
-9731T>G§	rs1946519	5′ flanking region		0.44
-9682A>C	rs1946518	5′ flanking region		0.44
-9212G>C	rs187238	5′ flanking region		0.19
-8963T>G§	rs360718	exon 1	5′UTR	0.19
-8949C>T	rs360717	exon 1	5′UTR	0.19
-380C>G §	rs5744247	intron 1		0.37
-140C>G	rs360721	intron 1		0.17
-139A>G	rs4988359	intron 1		0.04
-109A>G	rs12721559	intron 1		0.02
1867-> ins. C	rs5744252	intron 3		0.37
1950G>C	rs1834481	intron 3		0.04
4861A>C	rs549908	exon 4	Ser35Ser	0.17
11641A>G	rs5744292	exon 6	3′UTR	0.04

Definition of abbreviations: dbSNP = single nucleotide polymorphism database; NCBI = National Center for Biotechnology Information; MAF = minor allele frequencies; SNP = single nucleotide polymorphism; UTR = untranslated region.

* Numbering according to the genomic sequence of *IL-18* (NT_033899.7). Position 1 is the A of the initiation codon.

† NCBI, number from the dbSNP of NCBI (http://www.ncbi.nlm.nih.gov/SNP/)

‡ MAF in the screening population (n = 24).

§ SNPs were genotyped in this study.

The results for genotype frequencies in the group with asthma and the control group are shown in Table E3. None of the three SNPs tested in this study showed a significant association with adult asthma. We next surveyed associations between the SNPs and disease severity. The distribution of subjects was as follows: step 1, mild intermittent 2.4% (11 individuals); step 2, mild persistent 52.3% (237 individuals); step 3, moderate persistent 26.9% (122 individuals); and step 4, severe persistent 18.3% (83 individuals). Because the step 1 subgroup comprised 11 patients (2.4%) and step 4 subgroup comprised 83 patients (18.3%), we divided the subjects with asthma into two groups, steps 1 and 2 versus steps 3 and 4 by sample size (54.7 vs. 45.3%). The results for genotype frequencies are shown in Table 3 and Table E4. We found a significant association between the -380C>G (rs5744247) genotype and asthma severity (allelic model, P = 0.0034, corrected P = 0.010; OR, 1.49; 95% CI, 1.14–1.94; recessive model, P = 0.012, corrected P = 0.036; OR, 1.79; 95% CI, 1.14–2.74). The -9731T>G (rs1946519) polymorphism also showed a significant association with asthma severity (allelic model, P = 0.0078, corrected P = 0.023; OR, 0.69; 95% CI, 0.53–0.91; dominant model, P = 0.0077, corrected P = 0.023; OR, 0.59; 95% CI, 0.40–0.87). However, the -8963T>G SNP (rs360718) was not associated with asthma severity. Rs 2904613 and rs1946518, in strong LD with rs1946519 (r^2 = 0.84 and 1.0, respectively), and rs5744252, in strong LD with 5744247 (r^2 = 0.84), also showed similar results (Table E4).

In addition, we surveyed associations between the three SNPs and patients with asthma who had high eosinophil counts and high serum IgE levels as quantitative phenotypes. However, we could not find any association between the three SNPs and eosinophil counts or total serum IgE levels.

Associations of *IL-18* Haplotypes with Asthma Severity

We next constructed the haplotypes of the three SNPs and estimated the frequency of each haplotype in the step 1, 2 and step 3, 4 adult asthma groups (Table E5). We identified three common haplotypes covering more than 99% of the population in both groups, and found a positive association with a haplotype of *IL-18* in adult asthma severity (χ^2 = 9.07, P = 0.0026) (haplotype T-T-G [-9731T, -8963T, and -380G] versus others) using the Haploview 4.1 program.

IL-18 mRNA Is Highly Expressed in Bronchial Epithelial Cells and rs5744247 Increases Transcriptional Activity

We next investigated whether *IL-18* mRNA was up-regulated by Toll-like receptor (TLR) ligands in cultured NHBE, NHLF, and BSMC. Although *IL-18* was expressed in NHBE, it was barely expressed in NHLF and BSMC (Figure 2A). Furthermore, *IL-18* mRNA expression in NHBE was not induced by stimulation with LPS, poly(I:C), and macrophage-activating lipopeptide-2 for 4 hours (Figure 2A) or 24 hours (data not shown).

To clarify whether the SNPs in the intronic region associated with asthma severity affected the expression of *IL-18*, we constructed plasmid clones containing genomic DNA fragments corresponding to these SNPs. PCR products were subcloned into the upstream or downstream regions of the luciferase gene in the pGL3-enhancer vector (Figure 2B). We compared enhancer-like effects of sequences containing the intron 1 -380C>G (rs5744247) or intron 3 1867->insertion C (rs5744252) SNPs in NHBE (Figure 2B). The clone containing the susceptible -380G (rs5744247) showed significantly greater transcriptional activity than the other allele, -380C, when the genomic DNA fragments were inserted downstream of the luciferase gene in the vector (Figure 2B). In contrast, the reporter activities of clones of the intron 3 SNP (rs5744252) had no effect on transcriptional activity (Figure 2B).

Inhaled corticosteroids (ICS) are widely used as first-line therapeutic agents in patients with inflammatory lung diseases such as asthma (32), and patients with severe asthma need higher-dose ICS and oral steroids to control their symptoms. In addition, recent studies have shown that the combination of an

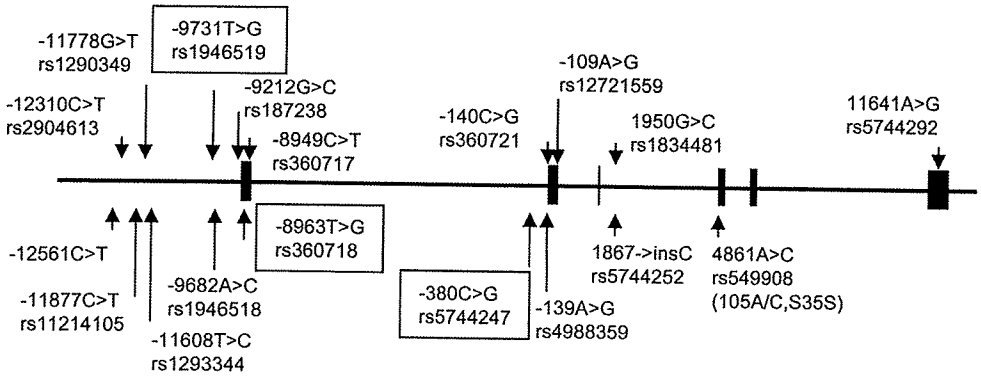

Figure 1. A graphical overview of polymorphisms identified in relation to the exon/intron structure of the human *IL-18* gene. Six exons are shown by black boxes, and positions for polymorphisms are relative to the translation start site (+1). Polymorphisms enclosed within boxes were genotyped in the whole samples.

TABLE 3. GENOTYPE FREQUENCIES FOR *IL-18* SINGLE NUCLEOTIDE POLYMORPHISMS AND ASTHMA SEVERITY

Genotype	Steps 1,2 n = 248 (%)	Steps 3,4 n = 205 (%)	Allele	Steps 1,2 n = 248 (%)	Steps 3,4 n = 205 (%)	P Value and ORs (95% CI)			
						Genotype	Dominant	Recessive	Allelic
rs1946519									
TT	77 (31)	89 (44)	T	268 (55)	259 (63)	0.026	0.0077	0.153	0.0078
TG	114 (47)	81 (40)	G	222 (45)	149 (37)		0.59		0.69
GG	54 (22)	34 (17)					0.40–0.87		0.53–0.91
rs360718									
TT	182 (73)	159 (78)	T	427 (86)	359 (88)	0.266	0.305	0.323	0.515
TG	63 (25)	41 (20)	G	69 (14)	51 (12)				
GG	3 (1)	5 (2)							
rs5744247									
CC	87 (35)	51 (25)	C	294 (59)	200 (50)	0.014	0.024	0.012	0.0034
CG	120 (48)	98 (49)	G	202 (41)	204 (50)		1.60	1.79	1.49
GG	41 (17)	53 (26)					1.06–2.41	1.14–2.74	1.14–1.94

Definition of abbreviations: CI = confidence interval; OR = odds ratio.

inhaled corticosteroid and long-acting β_2-agonist is more efficacious in asthma than either alone (33, 34). Another study has shown synergistic suppression of virus-induced chemokines in airway epithelial cells by combination therapy (35). We next investigated the effects of DEX and SAL on the expression of *IL-18*. We examined effects of DEX and SAL on *IL-18* mRNA expression levels in NHBE. The expression of *IL-18* was not affected by DEX or SAL (Figure 2C).

IL-18 mRNA Is Highly Expressed in NK Cells, Dendritic Cells, and Monocytes and Is Highly Induced by LPS in Human Monocytes

We further investigated whether *IL-18* mRNA was up-regulated by TLR ligands in immune blood cells, using quantitative real-time PCR. *IL-18* mRNA was highly expressed in nonstimulated NK cells, dendritic cells, and monocytes as compared with $CD4^+$ or $CD8^+$ T cells and B cells (Figure 3A). After stimulation, levels of *IL-18* mRNA in monocytes were increased threefold in response to LPS (Figure 3A).

Polymorphism rs5744247 Is Associated with *IL-18* mRNA Expression Level in LPS-Stimulated Monocytes

To investigate whether the -380C>G (rs5744247) polymorphism affected the mRNA levels of *IL-18*, we measured relative mRNA expression and compared it in subjects with different genotypes. We isolated monocytes from PBMCs of a total of nine healthy volunteers, five of whose genotypes were homozygous for -380C and four of whose were homozygous for -380G, and stimulated them with 5 μg/ml LPS. After LPS stimulation, we found significant increases in both *IL-18* (-380C group, $P = 0.0044$; -380G group, $P = 0.011$) and *IL-6* (-380C group, $P = 0.0036$; -380G group, $P = 0.014$) mRNAs within each group by the Friedman test. Monocytes homozygous for the -380G allele exhibited significantly higher expression of *IL-18* mRNA in response to LPS for 1.5, 3, and 6 hours ($P = 0.014$, 0.014, and 0.033, respectively, by the Mann-Whitney *U* test); however, this genotype effect was not observed for *IL-6* mRNA induction (Figure 3B). A recent study has shown antiinflammatory effects of SAL after inhalation of LPS in humans (36). However, a combination effect of glucocorticoid and SAL on inflammatory cytokines in response to LPS has not been reported. We next investigated the effects of DEX and SAL on the induction of *IL-18* mRNA by LPS in monocytes. Although the addition of DEX with/without SAL to the medium with LPS significantly reduced induction of *IL-6* mRNA, DEX and SAL did not affect the level of induction of *IL-18* mRNA (Figure 3C).

Polymorphism rs5744247 Is Associated with Serum IL-18 Level

To evaluate whether the serum IL-18 protein level correlated with the *IL-18* genotype, we conducted ELISA assays of sera of 88 patients with asthma. Baseline characteristics among serum study participants are shown in Table E6. We compared the distribution of severity between subjects with serological studies and that with genetic studies by the Mann-Whitney *U* test. In the subgroup examined for serum IL-18, asthma tended to be more severe than in the genotyped group ($P = 0.023$; Table E7). The Jonckheere-Terpstra trend test is a nonparametric trend statistic to test for ordered differences among groups assumed to be arranged ordinally. The test is superior to the Kruskal-Wallis procedure when the conjectured ordering of the genotype effects is, indeed, appropriate (37). Serum levels of the IL-18 protein positively correlated with the *IL-18* genotype in the Jonckheere-Terpstra trend test ($P = 0.031$; Figure 4).

DISCUSSION

In this study we identified 18 polymorphisms, conducted LD mapping of the gene region, and found significant associations between polymorphisms and asthma severity. Several genetic studies have already surveyed the genes involved in the IL-18 signaling pathway as candidate genes for asthma. For the *IL-18* receptor and related molecules, a recent study has shown significant replicated associations between polymorphisms in the *IL18R1* gene and asthma, atopic asthma, and bronchial hyperreactivity (38). Another study, in which adult asthma probands aged 18.1–64.7 years were examined, has reported associations of *IL1RL1*, *IL18R1*, and *IL18RAP* gene cluster polymorphisms with asthma and atopy in a Dutch population (21). Several association studies of the *IL-18* gene have also been conducted. Five polymorphisms, rs1946518, rs187238, rs360718, rs360717, and rs360721, located in the promoter and exon 1 region, were screened in a cohort of 228 children with asthma, but no polymorphism showed a significant difference in frequency between the case and control groups (17). The association study did not contain adult subjects with asthma and did not examine associations between the SNPs and asthma severity. Among the five SNPs, we found a significant association between rs1946518 and asthma severity. The recent SAPALDIA Cohort study using a Swiss population (mean ± SD, 41.2 ± 11.4) has shown associations between the *IL-18* promoter variant -137G>C and atopic asthma (19). SNP rs187238 (-137G>C) was not associated with either adult asthma susceptibility or severity in our study. The proportion

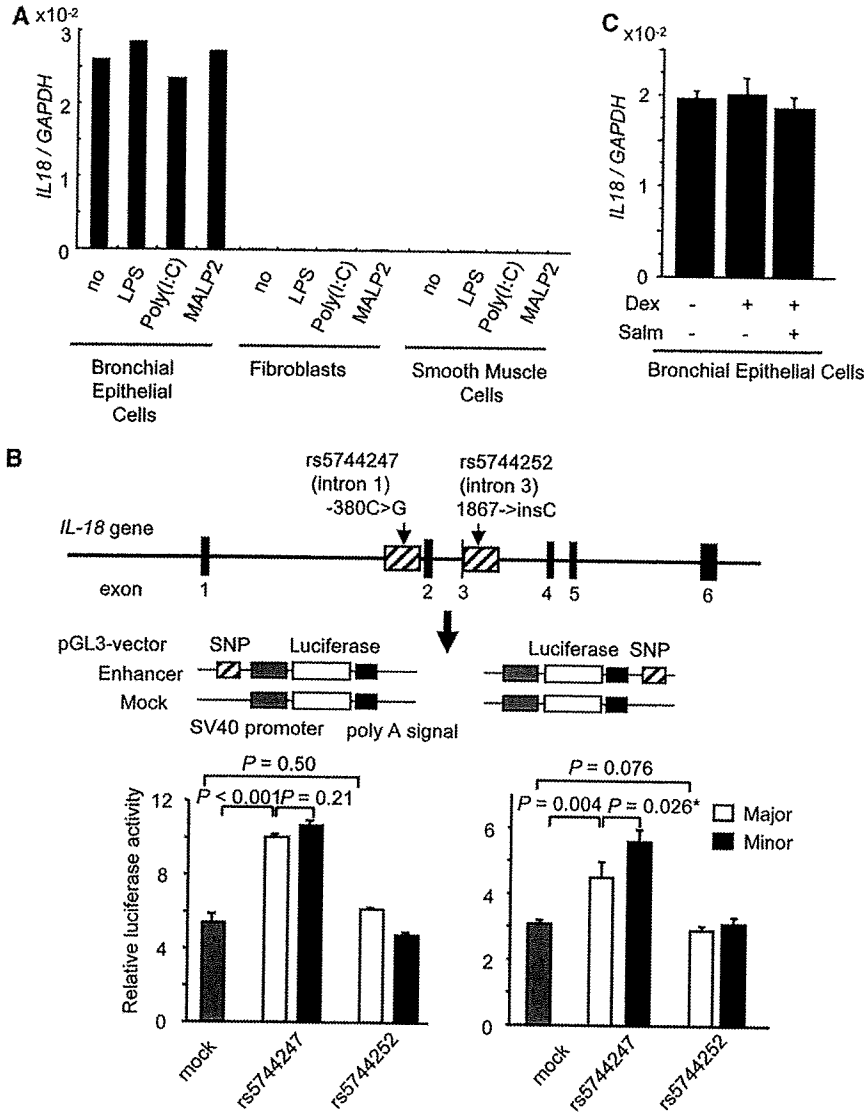

Figure 2. (*A*) *IL-18* mRNA expression analyses of normal human bronchial epithelial cells (NHBE), normal human lung fibroblasts (NHLF), and bronchial smooth muscle cells (BSMC) stimulated with 100 ng/ml LPS, 10 μg/ml poly(I:C) or 1 μg/ml macrophage-activating lipopeptide-2 for 4 hours. NHBE, NHLF, and BSMC were each derived from one individual, respectively. Data represent means of duplicate samples. Two independent experiments were performed with similar results. (*B*) Enhancer activity of *IL-18* introns 1 and 3 in NHBE, containing rs5744247 and rs5744252 variants. Schematic representation of the reporter constructs and relative luciferase activities of the two SNPs. Values represent the means ± SD of three independent experiments. The asterisk (*) indicates a significant difference between minor and major alleles ($P = 0.026$) by Student *t* test. (*C*) Effects of dexamethasone (Dex) and salmeterol (Salm) on *IL-18* mRNA expression levels in NHBE. Cells were treated with dexamethasone and salmeterol for 0.5 hours before addition of 100 ng/ml LPS. Values represent the means ± SD using NHBE derived from four individuals. GAPDH = glyceraldehyde 3-phosphate dehydrogenase; MALP = macrophage-activating lipopeptide-2; SNP = single nucleotide polymorphism.

of subjects with severe asthma in the prospective cohort study appeared to be lower than that in our study using clinic-based samples, and the difference might have affected the results. In a Japanese population, an association study of the *IL-18* gene was conducted using three polymorphisms, rs1946518, rs187238, and rs549908 (Ser35Ser), with 221 children and 276 adults with asthma and 85 adult control subjects (20). The study screened only polymorphisms in the coding and promoter regions of the *IL-18* gene, and found a significant association between rs549908 (Ser35Ser) and asthma susceptibility. Among the three SNPs, we could not find any association between the *IL-18* variants and adult asthma susceptibility; however, we found a significant association between rs1946518 and asthma severity. Association between the SNPs and asthma severity was not examined in the earlier study (20), and sample size might account for the contradictory results. Furthermore, according to the data of Hapmap (www.hapmap.org), the allele frequencies of SNP rs5744247 in Japanese, Han Chinese, Yoruba people, and the Centre d'Etude du Polymorphisme Humain population are 34%, 53%, 1%, and 9%, respectively. Thus the functional effect of the rs5744247 variant on asthma severity might be specific to the Japanese population.

Recent studies have shown important roles of bronchial epithelial cells as both mediators and regulators of innate immune responses and adaptive immune responses (39), and constitutive expression of IL-18 protein was observed within airway epithelial cells (40, 41). In this study, human *IL-18* was highly expressed by airway epithelial cells, and we could confirm the enhancer-like effects of the rs5744247 variant and significantly greater transcriptional activity of the susceptible G allele in NHBE. Although the majority of individuals with asthma are well controlled with current therapies, the existent therapeutic strategies are inadequate for those with severe asthma (42). Approximately 5% to 10% of the asthmatic population is in the severe end of the disease spectrum and it is difficult to control the asthma with maximal inhaler therapy (43, 44). Combination therapy with long-acting β_2-agonists and inhaled corticosteroids reduces exacerbation frequency in asthma, and it is also efficacious as intervention therapy for exacerbation of the disease (33, 34, 45). The suppressive effect

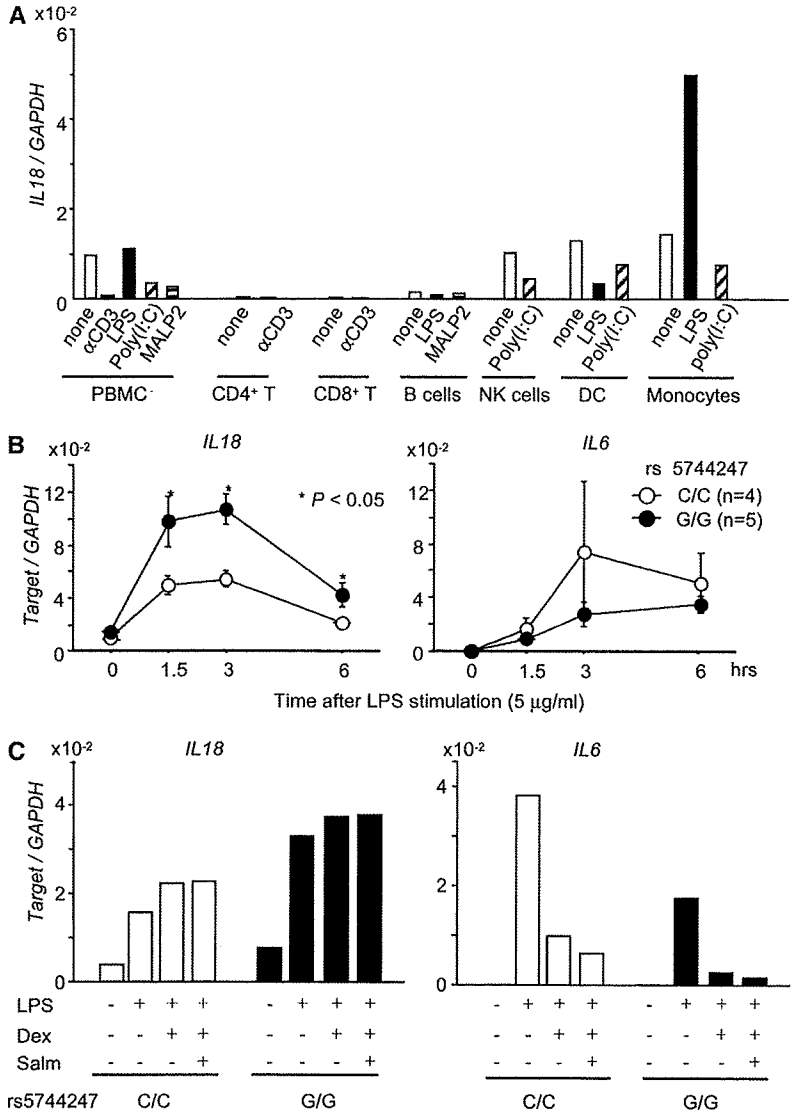

Figure 3. (*A*) Quantitative reverse transcriptase–polymerase chain reaction assays of *IL-18* in immune cells. The expression levels were normalized with glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) expression. Freshly isolated immune cells were stimulated using 1 μg/ml plate-bound anti-CD3 monoclonal antibody (mAb) with 1 μg/ml soluble anti-CD28 mAb, 5 μg/ml LPS, 25 μg/ml poly(I:C), or 1 μg/ml macrophage-activating lipopeptide (MALP)-2 for 5 hours. Data were averaged among duplicate samples and are representative of two independent experiments. Similar results were obtained using immune cells from two individuals. (*B*) Relationship of rs5744247 genotype to *IL-18* mRNA expression level. The *IL-18* and *IL-6* mRNA expression levels were measured in monocytes stimulated with 5 μg/ml LPS for the indicated time. The asterisks (*) represent $P = 0.014$, 0.014, and 0.033, respectively, by the Mann-Whitney *U* test. (C) Effects of dexamethasone (Dex) and salmeterol (Salm) on *IL-18* and *IL-6* mRNA expression levels in monocytes stimulated with 5 μg/ml LPS. Data are means in duplicate from an individual. Two independent experiments each using human monocytes, homozygous for C ($n = 4$) and homozygous for G ($n = 3$), were performed with similar results.

of steroids mediated by glucocorticoid response elements within the *IL-6* promoter has been reported (46). In the present study, we could confirm the effects of DEX and SAL on *IL-6* expression in LPS-stimulated monocytes; however, DEX and SAL were not able to suppress *IL-18* mRNA expression in either monocytes or NHBE cells. A recent study has shown that corticosteroid-resistant (CR) asthma is associated with classic antimicrobial activation of airway macrophages, and higher endotoxin levels are detected in bronchoalveolar lavage fluid from subjects with CR asthma (47). The study implies that prolonged exposure to LPS might contribute to CR asthma (47). In human monocytes, we here demonstrated that the mRNA expression of *IL-18* was highly induced by LPS and identified a -380C>G (rs5744247) SNP that had an allele-specific effect on mRNA expression. Primary monocytes from subjects homozygous for the susceptible -380G allele exhibited significantly high expression of *IL-18* mRNA in response to LPS. In addition, the induction of *IL-18* mRNA in monocytes was not suppressed by DEX and SAL. IL-18 is involved in severe asthma through functional polymorphism and might contribute to enhanced innate immunity and both Th1- and Th2-driven immune responses. It is likely that targeting IL-18 itself might be therapeutically efficacious as a new treatment for severe asthma.

Recent studies have reported elevated circulating levels of IL-18 in patients with allergic diseases (22–25). Serum IL-18 levels are higher in patients with acute asthma than in control subjects and the IL-18 level has a tendency to inversely correlate with peak expiratory flow (22, 23). In patients with atopic dermatitis, serum IL-18 levels are elevated and the levels are correlated with disease severity and with the number of eosinophils in peripheral blood (25). In another study, IL-18 secretion from mononuclear cells of patients with bronchial asthma and atopic dermatitis was significantly higher than that in nonallergic controls (48). In this study, we found a positive correlation between the serum IL-18 level and *IL-18* rs5744247 genotype. Although we randomly recruited 88 subjects with asthma who provided serum samples, in the subgroup examined for serum IL-18, asthma tended to be more severe than in the genotyped group. There might be a subpopulation selection bias with regard to disease severity, and the bias might influence the positive correlation between serum levels of the IL-18 protein

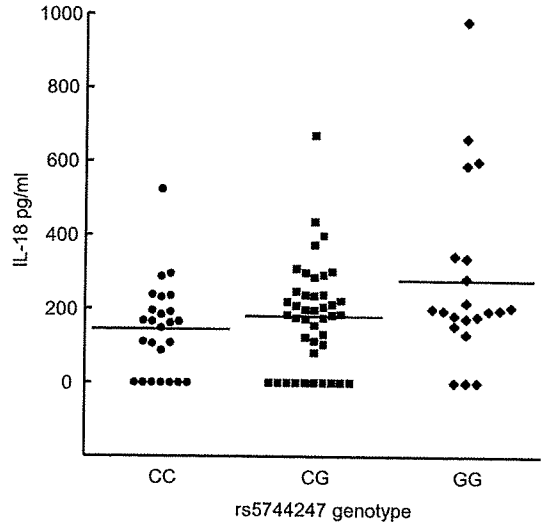

Figure 4. Relationship between rs5744247 genotype and serum IL-18 level. Serum levels of the IL-18 protein positively correlated with rs5744247 genotype by the Jonckheere-Terpstra trend test (P = 0.031).

and the *IL-18* genotype. Thus, validation studies of the connection between the serum IL-18 protein level and genotype are needed in a large number of samples.

We concluded that a genetic variant in the *IL-18* gene appears to influence the serum level of IL-18 and the asthma severity, putatively by altered enhancer activity in NHBE and increased *IL-18* mRNA expression in monocytes in response to LPS. Further investigations of IL-18 function would be helpful to understand the pathophysiology of inflammatory diseases whose development and progression are affected by microbial infections.

Conflict of Interest Statement: M.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.O. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. T.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. T.Y. received up to $1,000 from Boehringer Ingelheim in lecture fees and $5,001–$10,000 from Novartis Foundation Japan in grants. Y.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.D. received up to $1,000 from Ono Pharmaceutical Co., Ltd., up to $1,000 from Sanofi Aventis, and up to $1,000 from Kyorin Pharmaceutical Co., Ltd. in lecture fees. A.M. received up to $1,000 from GlaxoSmithKline and up to $1,000 from Banyu Pharmaceutical Co., Ltd. in lecture fees. K.F. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. T.E. received $1,001–$5,000 from Sanofi Aventis, $5,001–$10,000 from Kyowa Hakko Kirin Pharma, $1,001–$5,000 from Boehringer Ingelheim, $1,001–$5,000 from Dainippon Sumitomo Pharma, and up to $1,000 from Ono Pharmaceutical Co., Ltd. in lecture fees. M.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. N.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. Y.F. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.N. received $1,001–$5,000 in consultancy fees from Abbott, $1,001–$5,000 from Nippon Boehinger Ingelheim, $1,001–$5,000 from Nippon Boehinger Ingelheim, $1,001–$5,000 from Astellas Pharm, $1,001–$5,000 from Kyowa Kirin, and $1,001–$5,000 from Dainippon Sumitomo Seiyaku in lecture fees. Y.N. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.T. received up to $1,000 from Kyorin Pharmaceutical Co., Ltd. and up to $1,000 from GlaxoSmithKline in lecture fees.

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ESTABLISHMENT OF A CONTINUOUS CULTURE SYSTEM FOR *ENTAMOEBA MURIS* AND ANALYSIS OF THE SMALL SUBUNIT rRNA GENE

KOBAYASHI S.*, SUZUKI J.** & TAKEUCHI T.*

Summary:

We established a culture system for *Entamoeba muris* (MG-EM-01 strain isolated from a Mongolian gerbil) using a modified Balamuth's egg yolk infusion medium supplemented with 4 % adult bovine serum and *Bacteroides fragilis* cocultured with *Escherichia coli*. Further, encystation was observed in the culture medium. The morphological characteristics of *E. muris* are similar to those of *Entamoeba coli* (*E. coli*); moreover, the malic isoenzyme electrophoretic band, which shows species-specific electrophoretic mobility, of *E. muris* had almost the same mobility as that observed with the malic isoenzyme electrophorectic band of *E. coli* (UZG-EC-01 strain isolated from a gorilla). We determined the small subunit rRNA (SSU-rRNA) gene sequence of the MG-EM-01 strain, and this sequence was observed to show 82.7 % homology with that of the UZG-EC-01 strain. Further, the resultant phylogenetic tree for molecular taxonomy based on the SSU-rRNA genes of the 21 strains of the intestinal parasitic amoeba species indicated that the MG-EM-01 strain was most closely related to *E. coli*.

KEY WORDS : *Entamoeba muris, Entamoeba coli,* dixenic culture, Balamuth's egg yolk infusion medium, SSU-rRNA gene, phylogenetic analysis.

Résumé : ÉTABLISSEMENT D'UN SYSTEME DE CULTURE SUCCESSIVE POUR *ENTAMOEBA MURIS* ET ANALYSE DU GÈNE CODANT POUR LA PETITE SOUS-UNITÉ DE L'ARNr

*Un système de culture d'*Entamoeba muris *(souche MG-EM-01, isolée da la gerbille de Mongolie) a été établi en utilisant un milieu d'infusion de vitellus de Balamuth modifié, supplémenté avec 4 % de sérum bovin adulte et de* Bacteroides fragilis *en coculture avec* Eschericia coli. *L'enkystement s'est également présenté dans le milieu de culture. Les aspects morphologiques d'*E. muris *sont semblables à ceux d'*Entamoeba coli *et la bande isoenzymatique malique présentant une mobilité électrophorétique spécifique à l'espèce avait à peu près la mobilité d'*Entamoeba coli *(souche UZG-EC-01, isolée d'un gorille). La séquence du gène codant pour la petite sous-unité de l'ARNr (SSU-rRNA) de la souche MG-EM-01 a été déterminée et l'homologie de la séquence était également identique à 82,7 % de celle de la souche UZG-EC-01. L'arbre phylogénétique qui en résulte pour la taxonomie moléculaire basée sur les gènes SSU-rRNA de 21 souches d'espèces de parasites intestinaux amibiens indiquait également que la souche MG-EM-01 était étroitement liée à* E. coli.

MOTS CLÉS : Entamoeba muris, Entamoeba coli, *culture dixenic, milieu d'infusion de vitellus de Balamuth, petite sous-unité de l'ARNr, analyse phylogénétique.*

Entamoeba muris is a highly contagious intestinal protozoan parasite of laboratory mice, rats and other rodents; this species is morphologically similar to *Entamoeba coli* (*E. coli*) (Neal, 1950) and primarily proliferates and encysts in the caecum of mice (Lin, 1971).

In vitro culture of *E. muris* has been attempted (Neal, 1950; Simitch & Petrovitch, 1951; Smith *et al.*, 1985); however, trials to achieve successive culture have not been successful.

In the present study, we established a system for the stable and successive culture of an *E. muris* strain. Further, we attempted phylogenetic analysis of this strain to help in investigating its molecular taxonomy.

MATERIALS AND METHODS

E. muris (strain MG-EM-01) isolated from a Mongolian gerbil spontaneously infected in our laboratory was used to establish the culture system. An *E. coli* strain (UZG-EC-01) isolated from a gorilla in a zoo in Tokyo, Japan, was used as a reference. *Escherichia coli* and *Bacteroides fragilis* strains were isolated from the stool of a primate [DeBrazza's guenon (*Cercopithecus neglectus*)] and fresh human stool samples from a patient with intestinal amoebic colitis, respectively. The *E. coli* strains were maintained in a chemically defined medium (R medium) (Robinson, 1968), and the *B. fragilis* strains were maintained on trypticase, yeast extract and iron (TYI) broth (Diamond *et al.*, 1978); these strains were used as supplements for the culture system of *E. muris*.

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The egg yolk infusion medium of Balamuth's medium (Balamuth, 1946) was replaced with infusion of a liver concentrate with 4 % preheat-treated adult bovine serum (56° C for three hours). At least one day prior to *E. muris* culture, 0.1 ml of *E. coli* maintained in the R suspension was added from the stock culture preserved at 4° C (used within one month); however, 0.2 ml of a 1- to 3-day culture of *B. fragilis* maintained in TYI broth was added to the abovementioned culture at the time of *E. muris* primary culture. The primary culture of *E. muris* was performed by inoculating cysts obtained from the stool sample of a Mongolian gerbil; this inoculation was performed after killing the concomitant enteric bacteria with 0.1 N HCl for 60 min at 35.5° C, and subsequent inoculation with a newly designed excystation medium (0.25 % trypsin, 0.24 % gall powder, and lipase (840 units/ml) from *Chromobacterium viscosum* (Sigma-Aldrich Corp., St. Louis, MO, USA) in Hanks' balanced salt solution) for 60 min at 35.5° C.

For 120 hours, the number of trophozoites in 5 µl of the abovementioned culture was counted microscopically at 24-hours intervals, as described previously (Kobayashi *et al.*, 2005). The resistance of the cysts to osmotic pressure was confirmed using 0.05 % sarcosyl (Eichinger, 1997) over a 24-hours period. The viability of the cysts was assessed by double-fluorescence staining using acridine orange and ethidium bromide (Parks *et al.*, 1979). The number of cysts in 5 µl of the homologous amoeba suspension in 0.05 % sarcosyl, which was adjusted to the same volume as that of the culture medium, was counted microscopically in a manner similar to that described previously (Kobayashi *et al.*, 2005).

In order to characterize the trophozoites, we performed isoenzyme analyses (zymodeme) (Sargeaunt, 1988) of *E. muris* and *E. coli.*

The primers for amplification of the SSU-rRNA gene sequences of the *E. muris* (MG-EM-01) and *E. coli* (UZG-EC-01) isolates were designed on the basis of the two SSU-rRNA sequences of *E. coli* [IH:96/135 (AF149914) and HU-1:CDC (AF149915)] acquired from GenBank. Table I lists the three primer sets designed, namely, Ecoli1F/Ecoli1R, Ecoli2F/Ecoli2R and Ecoli3F/Ecoli3R. The polymerase chain reaction (PCR) amplification and the sequencing analysis of the PCR products of the SSU-rRNA genes derived from MG-EM-01 and UZG-EC-01 were performed as described previously (Suzuki *et al.*, 2008).

Analysis and multiple alignments of the acquired sequences of the SSU-rRNA genes of *E. muris* (MG-EM-01) and *E. coli* (UZG-EC-01) were performed by following the Yebis system for DNA Alignment, which uses a tree-based round-robin iterative algorithm (Hirosawa *et al.*, 1995). The phylogenetic tree was constructed using PhyML software package version 2.4.5 (Guindon and Gascuel, 2003) using maximum likelihood (ML) analysis and a general time-reversible (GTR) model to calculate genetic distances. The reliability of the branches of the tree of the GTR model was tested with bootstrap values obtained from 1,000 replications. The ML tree data file from PhyML was read, and the tree was constructed using MEGA software (Tamura *et al.*, 2007).

RESULTS

We have been successfully culturing *E. muris* for greater than 16 months now. Fig. 1 shows the growth and encystation kinetics of *E. muris* maintained in modified Balamuth's medium. Encystation of *E. coli* isolates was not observed in this medium. The mean sizes of trophozoites (33.3 ± 8.9 × 21.6 ± 5.6 µm) and cysts (21.6 ± 3.0 × 20.8 ± 2.3 µm) of *E. muris* in the culture medium were almost the same as those of *E. coli* trophozoites (32.8 ± 12.6 × 28.4 ± 7.6 µm) and cysts (21.6 ± 6.1 × 19.8 ± 3.8 µm). However, similar to the result reported by Neal (1950), a greater number of trophozoites and cyst nuclei with extremely eccentric karyosomes and thin peripheral chromatin layers were observed in *E. muris* compared to *E. coli.* Additionally, a large chromatin mass in the polar position of the karyosome in the nucleus of the *E. muris* cyst was frequently observed in the cysts reproduced in the *in vitro* culture (Fig. 2).

Figure 3 shows a representation of the zymodeme analyses to estimate malic enzyme (ME) and hexokinase (HK) levels. The electrophoretic mobility of the single band of *E. muris* ME was almost the same as that observed with *E. coli* ME. However, the electrophoretic

Primer name	Primer sequence (5' to 3')	Nucleotide position*
Ecoli1F (forward)	GTT GAT CCT GCC AGT ATT ATA TG	7-28
Ecoli1R (reverse)	ATA CCA TGC TTC ATC ATT C	841-859
Ecoli2F (forward)	GTA ATT CCA GCT CCA ATA GTC	617-637
Ecoli2R (reverse)	AAG TTC AAG TCT CGT TCG TTA TCG GA	1467-1492
Ecoli3F (forward)	TGA CTC AAC ACG GGA AAA CTT	1339-1359
Ecoli3R (reverse)	ATC CTT CCG CAG GTT CAC CTA C	2083-2104

* Nucleotide position was based on sequence of *Entamoeba coli* UZG-EC-01 Srain (AB444953).

Table I. – Oligonucleotide primers used for PCR assays in present study.

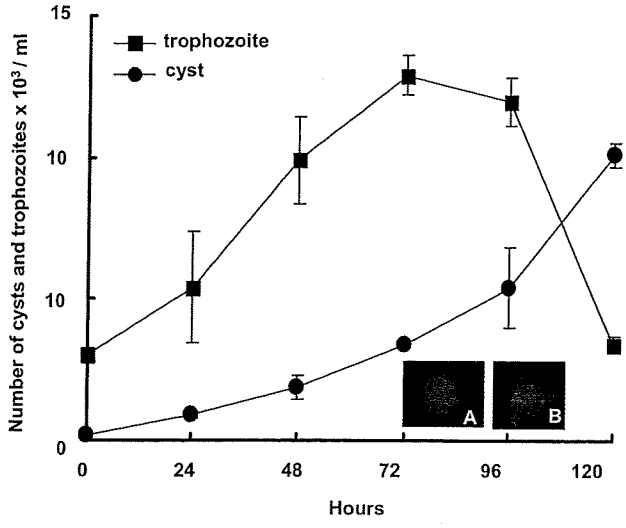

Fig. 1. – Growth kinetics of *Entamoeba muris* strain MG-EM-01 with reproduction of cysts in newly modified Balamuth's medium. The mean number of trophozoites and cysts in duplicate cultures are plotted. A and B, micrographs of cysts that were double-fluorochrome stained with acridine orange (AO) and ethidium bromide (EB). Live cysts (A) are stained with only AO (green colour); however, dead cysts (B) are stained with both AO and EB (red colour).

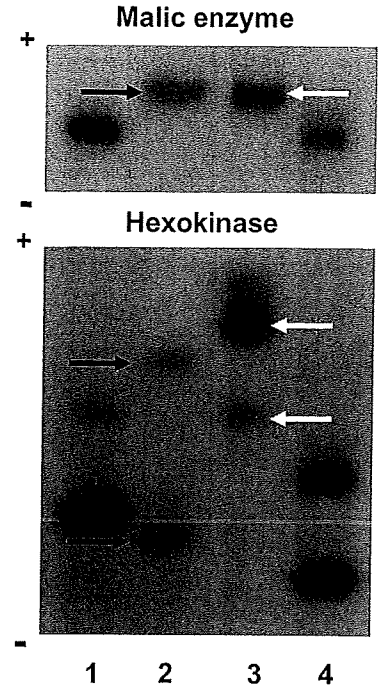

Fig. 3. – Isoenzyme pattern of malic enzyme and hexokinase of four strains of four different *Entamoeba* species. Lane 1, HM-1:IMSS clone 6 (*Entamoeba histolytica*); Lane 2, MG-EM-01 (*Entamoeba muris*); Lane 3, UZG-EC-01 (*Entamoeba coli*); Lane 4, AS 16 IR (*Entamoeba dispar*).

mobility of the double bands of *E. muris* HK showed a pattern that was different from that observed with *E. coli* HK, HM-1:IMSS clone 6 (ATCC 50527) (*E. histolytica*) HK, and AS 16 IR (*E. dispar*) HK (Kobayashi *et al.*, 2005) (Fig. 3). Under these electrophoretic conditions, distinguishable bands of phosphoglucomutase (PGM) and glucose phosphate isomerase (GPI) were observed neither in *E. muris* nor in *E. coli*.

On the basis of phylogenetic analysis, these four strains of *E. coli* and *E. muris* were included in the genus *Entamoeba* and the eight nuclei per cyst group were sister taxa in 100 % of the bootstrap resamplings (Fig. 4).

DISCUSSION

Escherichia coli, a facultative anaerobic bacterium, can produce anaerobic conditions that facilitate the culture of certain obligate anaerobic bacteria such as *B. fragilis*; this principle has been used in a coculture system with *Escherichia coli* in Robin-

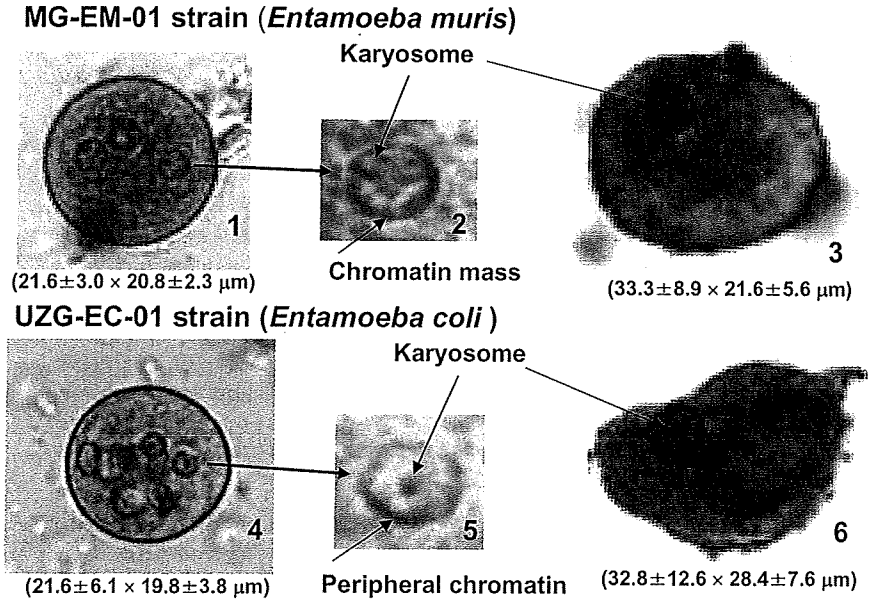

Fig. 2. – Micrographs of cysts and trophozoites of *Entamoeba muris* strain MG-EM-01 and *Entamoeba coli* strain UZG-EC-01. 1 and 4, cysts of MG-EM-01 (1) and UZG-EC-01 (4) strains stained with Lugol's iodine solution (Lugol). 2 and 5, nuclei of MG-EM-01 (2) and UZG-EC-01 (5) strains stained with Lugol. 3 and 6, trophozoites of MG-EM-01 (3) and UZG-EC-01 (6) strains stained with Kohn's chlorazol black E (Gleason *et al.*, 1965).

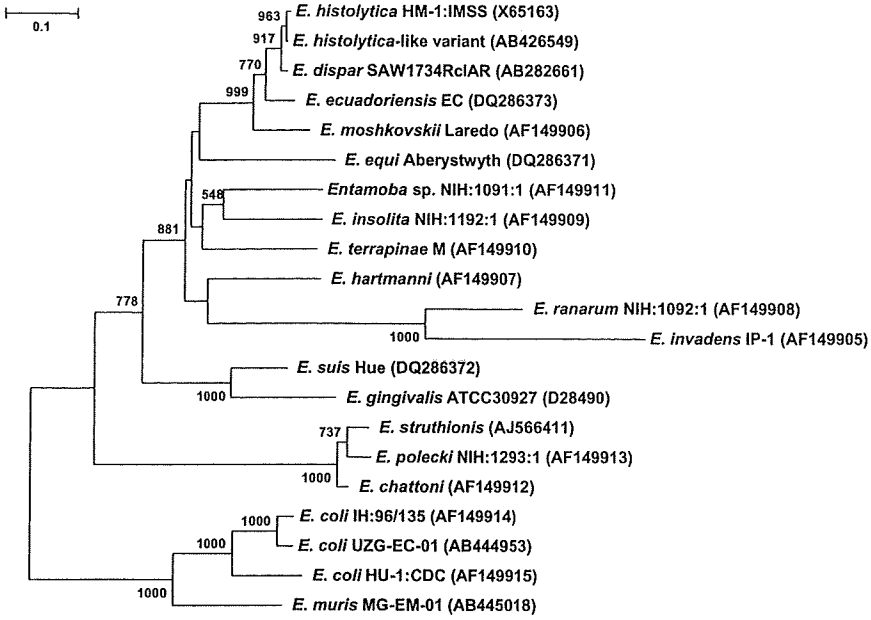

Fig. 4. – An unrooted phylogenetic reconstruction based on the SSU-rRNA gene sequences that explores the relationships among *Entamoeba* species is shown. A maximum likelihood (ML) tree derived using a general time reversible (GTR) model employing estimates of the proportion of invariable sites and the gamma distribution parameter of 0.423 and 0.169, respectively. Significant bootstrap support (> 500) from 1,000 replicates is indicated on the left of the supported node. The scale bar represents the evolutionary distance for the number of changes per site. The numbers within parentheses represent the corresponding GenBank accession numbers.

son's medium (Robinson, 1968). Some obligate anaerobic bacteria such as *Fusobacterium symbiosum* (*Clostridium symbiosum* ATCC 14940) (Diamond, 1983) can promote the growth of human parasitic *Entamoeba* isolates. Therefore, an isolate of *B. fragilis* that demonstrated a growth-promoting effect on the wild isolates of *E. histolytica* and *E. coli* in Balamuth's egg yolk infusion medium (data not shown) was used as a supplement for the culture of *E. muris;* thereafter, a successful culture system for *E. muris* was established for the first time. Phylogenetic analysis of the SSU-rRNA gene sequence of the *E. muris* (MG-EM-01) isolates, although derived from only one strain, suggested that *E. muris* is phylogenetically similar to *E.coli* that produced cysts with eight nuclei. The correlation between phylogenetic propinquity and the number of nuclei observed in the *Entamoeba* species possessing four nuclei per cyst has been previously reported (Silberman *et al.*, 1999; Clark *et al.*, 2006).
The established culture system continues to be dixenic. However, it enabled the analysis of the biological and molecular characteristics of an *E. muris* strain.

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Isolation and characterization of a potentially virulent species *Entamoeba nuttalli* from captive Japanese macaques

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SUMMARY

We have recently proposed revival of the name *Entamoeba nuttalli* Castellani, 1908 for a virulent amoeba (P19-061405 strain) isolated from a rhesus monkey (*Macaca mulatta*) and located phylogenetically between *E. histolytica* and *E. dispar*. In this study, *E. nuttalli* was isolated from feces of captive Japanese macaques (*M. fuscata*) in an open-air corral in Japan. The sequence of the 18S rRNA gene in the isolates differed from the P19-061405 strain in 2 nucleotide positions, but was identical to the EHMfas1 strain isolated previously from a cynomolgus monkey (*M. fascicularis*). One of the *E. nuttalli* isolates from Japanese macaques, named the NASA6 strain, was axenized and cloned. In isoenzyme analysis, the mobilities of hexokinase and phosphate glucose isomerase in the NASA6 strain were identical to those in the P19-061405 and EHMfas1 strains, but the mobility of phosphoglucomutase was different. These results were supported by gene analyses of these enzymes. Inoculation of NASA6 strain trophozoites into the liver of hamsters led to formation of an amoebic liver abscess. The liver lesions were characterized by extensive necrosis associated with inflammatory reactions. These results demonstrate that the NASA6 strain is potentially virulent and that *E. nuttalli* should be recognized as a common parasite in macaques.

Key words: *Entamoeba nuttalli*, Japanese macaque, 18S rRNA gene, virulency, zymodeme.

INTRODUCTION

The protozoan parasite *Entamoeba histolytica* is responsible for millions of cases of amoebic colitis and liver abscess in humans annually, resulting in up to 100 000 deaths (World Health Organization, 1997). Another intestinal-dwelling amoeba, *E. dispar*, is morphologically indistinguishable from *E. histolytica* but is non-pathogenic (Diamond and Clark, 1993). *E. histolytica/E. dispar* is also commonly found in the feces of non-human primates (Smith and Meerovitch, 1985; Muriuki *et al.* 1998; Tachibana *et al.* 2000; Verweij *et al.* 2003). *E. histolytica* infection in these primates is a serious problem for animal health and also has zoonotic potential, which makes it important to discriminate *E. histolytica* from other non-pathogenic amoebae.

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In older literature, several *E. histolytica*-like amoebae in monkeys have been described. The name *E. nuttalli* Castellani, 1908 was proposed for an amoeba found in a liver abscess in a *Macacus pileatus* monkey in Colombo, Sri Lanka (Castellani, 1908). Thereafter, *E.* (*Löschia*) *duboscqi* Mathis, 1913; *E. chattoni* Swellengrebel, 1914; *E. cercopitheci* Macfie, 1918; and *E. ateles* Suldey, 1924 have been described as *E. histolytica*-like species from non-human primates (Wenyon, 1965). However, except for *E. chattoni*, which has uninucleated cysts, these species are thought to be synonymous with *E. histolytica* (Hegner and Schumaker, 1928; Neal, 1966).

We have recently isolated an *E. histolytica*-like amoeba (P19-061405 strain) from a rhesus monkey (*Macaca mulatta*) in Nepal (Tachibana *et al.* 2007). However, the rRNA gene sequence of the isolate is located phylogenetically between *E. histolytica* and *E. dispar*. Furthermore, inoculation of trophozoites of the P19-061405 strain into livers of hamsters causes amoebic liver abscesses, indicating that the strain is potentially virulent. Therefore, we have proposed the revival of the name *E. nuttalli* for the

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amoeba isolated from rhesus monkey. Two other *E. histolytica*-like amoebae, the EHMfas1 and JSK2004 strains, which are also located phylogenetically between *E. histolytica* and *E. dispar*, have been isolated from a cynomolgus monkey (*M. fascicularis*) and a De Brazza's guenon (*Cercopithecus neglectus*), respectively, although there are 1–3 nucleotide differences among the 18S-rRNA genes in these 3 isolates (Suzuki *et al.* 2007; Takano *et al.* 2007).

Japanese macaque (*M. fuscata*) is a monkey that is distributed widely in Japan. A high prevalence of *E. dispar* infection but not of *E. histolytica* has been found in this primate (Rivera and Kanbara, 1999; Tachibana *et al.* 2001), which makes it of interest to examine whether *E. nuttalli* infections are found in Japanese macaques. In this study, we report a high prevalence of *E. nuttalli* infection in captive Japanese macaques, and we describe the characteristics of the amoeba after isolation in an axenic culture.

MATERIALS AND METHODS

Subjects and stool examination

Thirty stool samples from captive Japanese macaques were collected in an open-air corral located in a park in Nagasaki City, Japan, between December 2006 and March 2008. The samples were examined microscopically with iodine staining. Aliquots of each sample were suspended in 2% potassium dichromate solution and stored at room temperature.

Culture conditions

Fresh stool samples (within 6 h after collection) were suspended in water for 24 h and then cultured in modified Tanabe-Chiba medium at 37 °C (Tachibana *et al.* 2007). Grown trophozoites were transferred to Robinson's medium (Robinson, 1968). After several passages, the trophozoites were treated with a cocktail of antibiotics and then cultured monoxenically with living *Crithidia fasciculata* in TYI-S-33 medium (Diamond *et al.* 1978) supplemented with 15% adult bovine serum at 37 °C. Finally, the trophozoites were cultured axenically in TYI-S-33 medium and then cloned by limiting dilution, followed by microscopic observation. Trophozoites of the *E. nuttalli* P19-061405 strain and the *E. histolytica* SAW1453 and HM-1:IMSS strains were also cultured axenically in TYI-S-33 medium. Trophozoites of *E. dispar* SAW1734RclAR were cultured monoxenically with autoclaved *C. fasciculata* in YIGADHA-S medium supplemented with 15% adult bovine serum at 37 °C (Kobayashi *et al.* 2005).

Sandwich ELISA

Fresh stool samples (within 6 h after collection) were processed using an *E. histolytica* II kit (TechLab) for detection of *E. histolytica* antigen. Approximately 10^4 trophozoites cultured axenically were also analysed using the same kit.

Determination of the trophozoite diameter

Diameters of trophozoites were measured using a method reported in the literature (López-Revilla and Gómez-Domínguez, 1988). Briefly, cultured trophozoites in log growth phase were chilled on ice and then fixed with 2·5% glutaraldehyde in 0·1 M cacodylate buffer (pH 7·4). After washing with phosphate-buffered saline (PBS), the diameters of 100 round trophozoites were determined with an ocular micrometer. Statistical analysis was performed by Student's *t*-test.

Isolation of genomic DNA

Small aliquots of fecal samples in potassium dichromate solution were mixed with ether and centrifuged. Precipitates were washed 3 times with PBS and genomic DNA was then isolated using a DNeasy tissue kit (Qiagen). Genomic DNA was also isolated from cultured trophozoites using the same kit.

PCR amplification

For detection of various *Entamoeba* species, genomic DNA was subjected to 35 cycles of PCR amplification using Takara *ExTaq* DNA polymerase. Partial 18S rRNA genes of *E. histolytica*, *E. dispar* and *E. nuttalli* were amplified using primer sets described previously (Tachibana *et al.* 2007). Amplification of the partial 18S rRNA gene of *E. coli* was performed using the primers 5′-GAA TGT CAA AGC TAA TAC TTG ACG-3′ and 5′-GAT TTC TAC AAT TCT CTT GGC ATA-3′, which were designed based on sequences in the GenBank database (Accession numbers: AF149914 and AF149915). Detection of the *E. chattoni* partial 18S rRNA gene was performed using primers Echattoni1 and Echattoni2 (Verweij *et al.* 2001). PCR conditions were as follows: denaturation at 94 °C for 15 s (195 s in cycle 1), annealing at 55 °C (for *E. chattoni*) or 60 °C (for others) for 30 s, and extension at 72 °C for 30 s (450 s in cycle 35). An approximately 2·4-kb region containing the 18S and 5·8S rRNA genes was amplified from genomic DNA of cultured trophozoites over 30 cycles using Takara *ExTaq* DNA polymerase (Tachibana *et al.* 2007), using the following PCR conditions: denaturation at 94 °C for 15 s (195 s in cycle 1), annealing at 60 °C for 30 s, and extension at 72 °C for 180 s (600 s in cycle 30). Amplification of a serine-rich protein gene was performed essentially as reported by Ghosh *et al.* (2000). The genes encoding hexokinase (HXK) and glucose phosphate isomerase (GPI) were amplified in 30 cycles using PrimeSTAR HS DNA polymerase