1. Introduction

Toll-like receptor (TLR) signaling that is independently controlled by myeloid differentiation factor 88 (MyD88) and TIR domain-containing adapter inducing IFN-β (TRIF) [1], is important for the liver in health (host defense and liver regeneration) and disease (inflammation) [2]. Liver possesses robust innate immune cells expressing TLRs, such as Kupffer cells and NK cells [2–4]. After partial hepatectomy, Kupffer cells in the remnant liver promptly produce TNF-α in a MyD88-dependent manner, which in turn induces IL-6 production, eventually leading to homeostatic liver regeneration [5–7]. Following microbial infection, Kupffer cells produce IL-12 MyD88-dependently, which initiates IFN-γ production for microbial eradication in the early infection phase [3,4,8,9]. Thus, the TLR/MyD88-mediated signalings are beneficial in the liver homeostasis. Simultaneously, TLR-mediated signals induce severe liver inflammation. As previously reported wild-type (WT) mice primed with heat-killed *Propionibacterium acnes* develop dense hepatic granulomas. These *P. acnes*-primed mice undergo acute inflammatory liver injury whenever being subsequently challenged with a sublethal dose of LPS (<50 μg/kg [3,10]. However, as shown previously, *Il12p40*$^{-/-}$ mice and *Ifnγ*$^{-/-}$ mice lack the dense hepatic granuloma formation after *P. acnes* treatment and never exhibit liver injury after LPS challenge [11,12], suggesting importance of IL-12/IFN-γ for the sensitization to LPS induced by *P. acnes* treatment. Furthermore, *P. acnes*-primed *Myd88*$^{-/-}$ mice have poor formation of hepatic granulomas and are resistant to liver injury following LPS challenge [13], suggesting importance of MyD88-dependently produced IL-12/IFN-γ for *P. acnes*-induced sensitization to LPS. In contrast, IL-18 accounts for this liver injury by induction of hepatocytotoxic Fas ligand and TNF-α after LPS challenge [3,10,14–16]. Indeed, IL-18 blockade at LPS challenge can prevent *P. acnes*-primed mice that possess dense hepatic granulomas from this liver injury [10,17]. Furthermore, *Il18*$^{-/-}$ mice normally develop hepatic granulomas after *P. acnes* treatment, but can evade liver injury after LPS challenge [11]. Thus, it is very important to reveal the mechanism how IL-18 is released in response to LPS. We and others previously reported that IL-18, like IL-1β, is produced as biologically inactive precursor (pro) and becomes active after cleavage by caspase-1 [18–21]. Caspase-1 is also produced as inactive zymogen and needs appropriate stimuli to become active. However, it is unclear how TLR4 signaling activates caspase-1 for IL-18 release and which cell type is mainly involved in IL-18 release *in vivo*.

Accumulated lines of evidence demonstrated that Nalp3 inflammasome is important for activation of caspase-1 [22]. Here we first showed that *Trif*$^{-/-}$ mice, but not *Myd88*$^{-/-}$ mice, develop hepatic granulomas after *P. acnes* treatment but that both genotypes of mice evade the liver injury following LPS challenge. *Myd88*$^{-/-}$ Kupffer cells as well as WT Kupffer cells could secrete IL-18 in response to TLR4 agonist [8], while *Trif*$^{-/-}$cells could not, suggesting that the TLR4/TRIF-signaling activates caspase-1. Furthermore, *Nalp3*$^{-/-}$ mice, like *Trif*$^{-/-}$mice, could not increase serum IL-18 or develop liver injury after *P. acnes* pretreatment and subsequent LPS challenge. Thus, the TLR4/TRIF signaling induces liver injury by Nalp3-dependent activation of caspase-1.

As named inflammasome, this component plays a critical role in induction of inflammation through caspase-1 activation [22,23]. Production of IL-18 and IL-1β is principally dependent on caspase-1 activation, and levels of these cytokines are tightly associated with severity of inflammation [22,23]. TLR delivers signals via two distinct pathways. MyD88-dependent signal is for induction of host defense and liver regeneration. In contrast, TRIF-dependent signal induces inflammation via activating caspase-1. Therefore, it is conceivable that IL-18 released by Kupffer cells/hepatic macrophages is profoundly involved in the development of liver injury initiated by the TLR4 engagement.

2. Materials and methods

2.1. Mice

Trif$^{-/-}$ mice [24] on a C57BL/6 (B6) 129 background were back-crossed with B6 mice ten times and more. *Myd88*$^{-/-}$mice [25] and *Nalp3*$^{-/-}$ mice [26] on a B6 background were described elsewhere. *P2x7r*$^{-/-}$ B6 129 mice from Jackson Laboratory were back-crossed with B6 mice, and F3 littermates were used. B6 mice were purchased from Clea Japan (Osaka, Japan). Female mice (8–12 week-old) were used. All mice were maintained under specific pathogen-free conditions, and received humane care as outlined in the Guide for the Care and Use of Experimental Animals in Hyogo College of Medicine.

2.2. Reagents

LPS from *Escherichia coli* (O55: B5) was from Sigma (St. Luis, MO). Synthetic lipid A (Compound 506) was kindly provided by Dr. Fujimoto at Osaka University (Osaka, Japan) [8]. Heat-killed *P. acnes* was prepared [15]. Liposome-encapsulated dichloromethylene bisphosphonate (clodronate liposome) and PBS liposome were described elsewhere [27]. Caspase inhibitor z-VAD-fmk was purchased from Peptide Institute (Osaka, Japan) [15]. Preparation of monosodium urate crystal was described elsewhere [28].

2.3. Preparation of Kupffer cells

Kupffer cells (Supplementary method) (4×10^6 cells/well) were incubated in 1 ml of opti-MEM (Invitrogen, Calsbad, CA) supplemented with 0.001% fetal calf serum and 10 μg/ml of lipid A in a 6-well plate for 4.5 h. Supernatants and cells were separately collected for Western blotting analysis or ELISA [8]. In some experiments Kupffer cells in RPMI1640-based medium [8] (2×10^6 cells/well in a 24 well-plate) were incubated with monosodium urate crystal for 16 h and supernatants were harvested for measurement of cytokines by ELISA.

2.4. Western blotting analysis

Supernatants were concentrated using trichloroacetate [26]. The cell lysate sample was prepared [8]. Molecular sizes of IL-1β and caspase-1 in each preparation were determined by Western blotting [5]. The primary antibodies (Abs) used were: anti-mouse IL-1β Abs (Santa Cruise) and anti-caspase-1 p10 Abs (Santa Cruise). Densitometric analysis for active caspase-1 of independent experiments was performed [5], and calculated as follows:

Relative expression of active caspase-1 = densitometric data of active caspase-1 released from stimulated Kupffer cells/those from unstimulated cells.

2.5. Sequential treatment with P. acnes and LPS

Propionibacterium acnes-primed mice were challenged with 50 μg/kg of LPS at day 7 [8,17,29]. In some experiments, mice were administered intravenously with 200 μl of clodronate liposome at −2 and +5 days after *P. acnes* treatment. In some experiments, *P. acnes*-primed mice were additionally administered *iv* with 200 μg of z-VAD-fmk at 1 h before LPS challenge [15,30]. Sera were sampled for measurement of cytokine levels by ELISA [8] and of ALT levels (SRL, Tokyo Japan).

2.6. Immuno-histological analysis

Liver slices were incubated with a battery of anti-F4/80 mAb (BMA, Augst, Switzerland) and rhodamine-labeled anti-rat IgG [5], followed by counter-staining with hematoxylin.

2.7. Confocal microscopic analysis

Frozen sections of liver specimens were incubated with F4/80 mAb, biotinylated anti-rat IgG, and then Alexa Fluor 488-conjugated streptavidin (Molecular Probes). Nuclei were stained by DAPI (KPL, Gaitherburg, MD). The immunostaining of each section was evaluated using a laser scanning confocal microscopy [31].

2.8. Statistics

All data are shown as the mean ± SD of triplicate samples. Five to ten mice were used for each experimental group *in vivo*. Significance was examined by the unpaired Student *t* test. *p* values less than 0.05 were considered significant. Two to three experiments were performed, and representative data were shown in the figures.

3. Results

3.1. Importance of macrophages for the development of liver injury with elevated serum IL-18

Since many cell types can produce IL-18 [16], we analyzed whether macrophage is a major cell source of IL-18 in *P. acnes*-primed and LPS-challenged mice. It is well documented that intravenous administration of clodronate liposome can eliminate macrophages including Kupffer cells [27]. Consistent with our previous report [11,17], *P. acnes* treatment induced dense hepatic granulomas consisting of $F4/80^+$ macrophages (Fig. 1A, B). A single injection of clodronate liposome could not abolish hepatic granulomas (Supplementary Fig. 1). Twice injections could eliminate the hepatic granuloma formation (Fig. 1C), whereas control PBS liposome did not reduce it (Fig. 1B, described below). Notably, clodronate liposome administration inhibited increase in serum IL-18 (Fig. 1D) and protected against *P. acnes*/LPS-induced liver injury (Fig. 1F and G), while control mice exhibited both of them (Fig. 1D, E and G). These results demonstrated that macrophages predominantly contribute to the elevated serum IL-18 and to the development of liver injury.

3.2. Dispensability of TRIF for hepatic granuloma formation

Propionibacterium acnes-induced hepatic granuloma is necessary for the liver injury after LPS challenge (Fig. 1B, C, E, F and G). In fact, naïve mice did not develop liver injury or release IL-18 after LPS challenge (Supplementary Fig. 2). Thus, *P.* acnes treatment induces sensitization to LPS in WT mice possibly by induction of hepatic granuloma formation. Recent study showed involvement of MyD88 in the formation of hepatic granuloma [13]. Consistent with this, microscopic analysis revealed loss of granuloma formation in $Myd88^{-/-}$ mice (Fig. 2A and C). In contrast, $Trif^{-/-}$ mice possessed comparable hepatic granulomas (Fig. 2A and B), indicating dispensability of TRIF.

3.3. Requirement of both TRIF and MyD88 signals for the development of liver injury

Although it is dispensable for the hepatic granuloma formation (Fig. 2B), TRIF is necessary for the development of *P. acnes*/LPS-induced liver injury (Fig. 2D, E and G). Consistently $Myd88^{-/-}$ mice evaded this liver injury (Fig. 2F and G). Taken together, these results clearly demonstrated that both TRIF and MyD88 are necessary for the liver injury [11,17].

3.4. Both TRIF and MyD88 are required for in vivo release of IL-18

Next, we investigated whether both genotypes of mice lack IL-18 release relevant to the liver injury. C3H/HeJ mice genetically deficient in TLR4 signaling failed to increase serum IL-18 as compared to the congenic WT C3H/HeN mice (Supplementary Fig. 3), confirming LPS used as an accurate ligand for TLR4. Expectedly, *P. acnes*-primed $Myd88^{-/-}$ mice completely lacked the serum elevation of IL-18 after LPS challenge (Fig. 2H). $Trif^{-/-}$ mice, despite normal hepatic granuloma formation, lacked serum increase of IL-18 (Fig. 2H), suggesting that TRIF signal contribute to the liver injury by induction of release of IL-18.

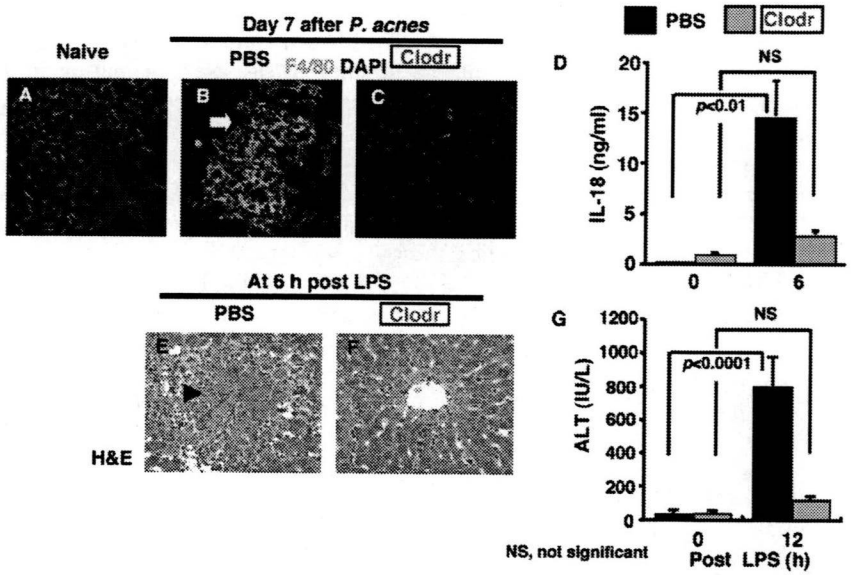

Fig. 1. Importance of macrophages for both elevated serum IL-18 and liver injury induced by sequential treatment with *P. acnes* and LPS. WT mice administered without (A) or with heat-killed *P. acnes* (B–G) were challenged without (A–C) or with LPS (D–G) at day 7. At day −2 and +5, mice were additionally administered with PBS liposome (PBS, black bars, B, D, E, and G) or clodronate liposome (Clodr, gray bars, C, D, F, and G). Liver specimens and sera were sampled for analysis of distribution of F4/80$^+$ cells (green) (A–C) and histological analysis (E and F), and measurement of IL-18 (D) and ALT (G), respectively. Original magnification; 200× (A–C), 100× (E and F). Arrow indicates hepatic granuloma, and arrowhead shows necrotic locus.

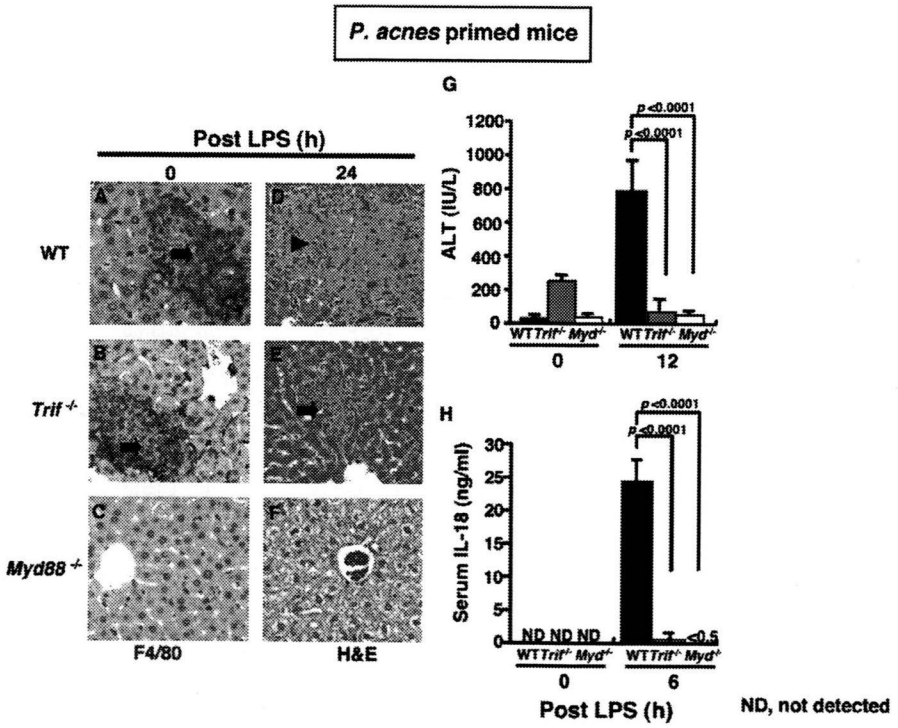

Fig. 2. Both MyD88 and TRIF are necessary for the liver injury. Liver specimens and sera were sampled from *P. acnes*-primed WT (A, D, G, and H), *Trif*$^{-/-}$ (B,E, G, and H), or *Myd88*$^{-/-}$ mice (C, F, G, and H) at the indicated time points after LPS challenge for detection of macrophage localization by staining liver sections with anti-F4/80 mAb (F4/80) (A–C) and histological study (H&E) (D, E, and F), and for measurement of ALT levels (G) and IL-18 concentrations (H), respectively. Arrows indicate hepatic granulomas, and an arrowhead indicates necrotic lesion. Original magnification: 100×.

3.5. Requirement of caspase-1 for in vivo release of IL-18

Consistent with our previous report showing loss of serum IL-18 elevation in *Caspase-1*$^{-/-}$ mice after sequential treatment with *P. acnes* and LPS [20], additional administration of caspase inhibitor z-VAD at LPS challenge hampered serum increase of caspase-1-dependent IL-18 and IL-1β, but completely not caspase-1–independent IL-6, in *P. acnes*-primed WT mice (Fig. 3). These results clearly indicated that caspase-1 is activated during the effector phase after LPS challenge.

3.6. Importance of TRIF, but not MyD88, for the TLR4-mediated caspase-1 activation

We wanted to know whether TRIF is involved in the caspase-1 activation after LPS challenge. To verify this we incubated Kupffer cells with lipid A, an active component of LPS and analyzed active caspase-1 in their supernatant and cell lysate by Western blotting analyses. Lack of caspase-1 activation in lipid A-stimulated *Tlr4*$^{-/-}$ cells demonstrated this lipid A as an appropriate TLR4 agonist (Supplementary Fig. 4). Consistent with our previous report [8], *Myd88*$^{-/-}$ Kupffer cells exhibited comparable activation of caspase-1 (Fig. 4B and D). In contrast, *Trif*$^{-/-}$ Kupffer cells could not process pro-caspase-1 (Fig. 4A and C), indicating importance of TRIF for the TLR4-mediated caspase-1 activation. To prove the enzymatic capacity of active caspase-1 we analyzed IL-1β processing. Expectedly, WT Kupffer cells, but not *Tlr4*$^{-/-}$ cells, processed IL-1β after stimulation with lipid A (Supplementary Fig. 4). As they were severely impaired in pro-IL-1β production, *Myd88*$^{-/-}$ Kupffer cells could not release mature IL-1β despite their normal activation of caspase-1 (Fig. 4B, D and F). Inversely, *Trif*$^{-/-}$ Kupffer cells normally produced pro-IL-1β but failed in release of mature IL-1 β Fig. 4A, C and E), clearly indicating that their lack of IL-1β release is attributable to their impaired caspase-1 activation (Fig. 4A and C). These results clearly demonstrated importance of TRIF, but not MyD88, for the TLR4-mediated caspase-1 activation and that the absence of caspase-1 activation in *Trif*$^{-/-}$ mice results in loss of the elevated serum IL-18 and in their escape from the liver injury.

3.7. Dispensability of endogenous ATP for the development of liver injury

ATP signaling has been reported to be important for caspase-1 activation in mouse peritoneal exudate macrophages [22,23]. To exclude the possible involvement of endogenous ATP in the release of IL-18 *in vivo*, we investigated whether mice deficient in cell surface ATP receptor, $P2x_7R$, can evade the IL-18-involved liver injury [32]. *P2x7r*$^{-/-}$ mice exhibited comparable elevation of serum IL-18 as in WT littermates after sequential treatment with *P. acnes* and LPS (Fig. 5A). Furthermore, *P2x7r*$^{-/-}$ mice could form the dense hepatic granulomas (Fig. 5C) and suffered from the liver injury (Fig. 5E and F) comparably as did *P2x7r*$^{+/-}$ mice (Fig. 5B, D and F). *In vitro* study also revealed that *P2x7*$^{-/-}$ Kupffer cells exhibit normal caspase-1 activation and release comparable IL-18 and IL-1β (Supplementary Fig. 5), verifying dispensability of endogenous ATP signaling. Thus, ATP signaling is not involved in the IL-18 production or the liver injury.

3.8. Importance of Nalp3 inflammasome

Nalp3 inflammasome is composed of cytoplasmic sensor, Nalp3 protein, and caspase-1 activation adaptor, Apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC). Nalp3 inflammasome controls caspase-1 activation in various situations [22]. Furthermore, as previously reported, Asc$^{-/-}$ mice lacked the elevation of serum IL-18 and evaded the liver injury [29]. Therefore, we wanted to know a role for Nalp3 protein. The elevated serum IL-18 was absent in Nalp3$^{-/-}$ mice (Fig. 6A) despite normal hepatic granuloma formation after *P. acnes* treatment (Fig. 6B and

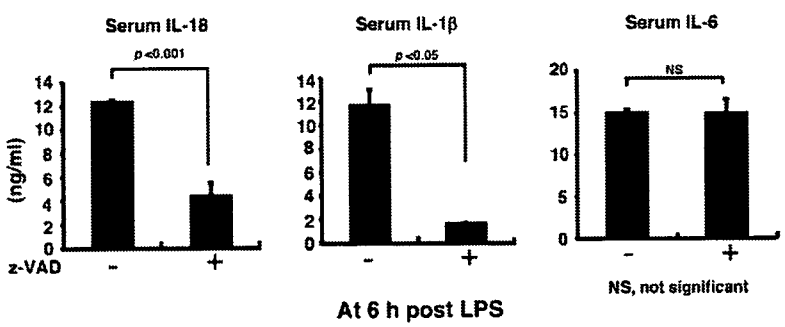

Fig. 3. Impaired serum increase of IL-1β and IL-18 but not IL-6 by treatment with caspase inhibitor. *P. acnes*-primed WT mice were administered with z-VAD 1 h before LPS challenge. At 6 h after LPS challenge sera were sampled for measurement of IL-18, IL-1β, and IL-6.

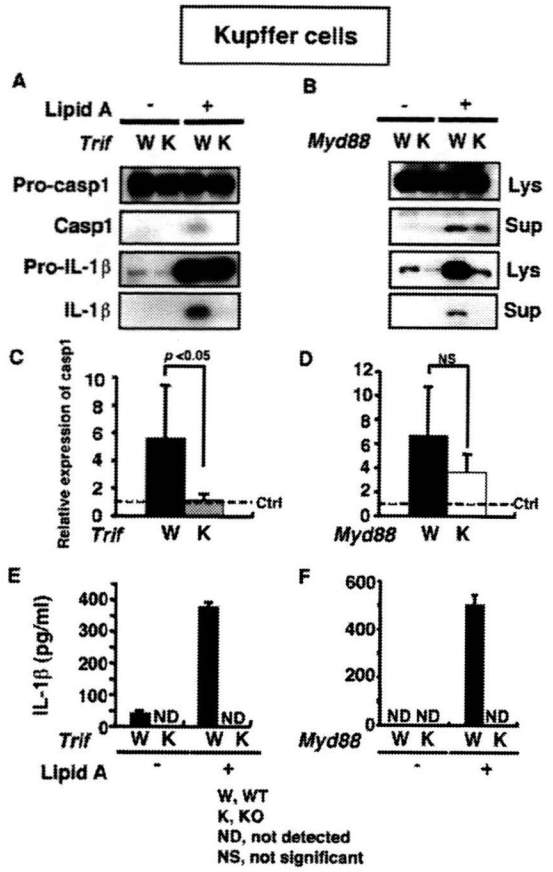

Fig. 4. TRIF but not MyD88 is essential for the lipid A-induced caspase-1 activation in Kupffer cells. Kupffer cells from WT mice (W) (A–F), *Trif*$^{-/-}$ mice (K) (A, C, and E), or *Myd88*$^{-/-}$ mice (K) (B, D and F) were incubated with lipid A for 4.5 h. Resulting supernatant (Sup) and cells (Lys) were separately collected. Western blotting for caspase-1 (casp1) or IL-1β (A, B, C and D) and ELISA for IL-1β (E and F) were performed. Representative data were shown (A, B, E and F). After densitometric analysis, relative expression of active caspase-1 was shown of three independent experiments (C and D). Dotted lines indicate control expression (Ctrl).

C). Furthermore, Nalp3$^{-/-}$ mice, like Asc$^{-/-}$ mice [29], were resistant to *P. acnes*/LPS-induced liver injury (Fig. 6D and E). Taken together these results clearly demonstrated that Nalp3 inflammasome is essential for *in vivo* release of IL-18 and the resulting liver injury.

4. Discussion

This study clearly demonstrated that MyD88 and TRIF play distinct roles in the development of liver injury after sequential treatment with *P. acnes* and LPS. MyD88 but not TRIF, presumably in hematopoietic cells [33], is essential for formation of hepatic granulomas after *P. acnes* treatment (Figs. 2 and 8). Inversely, TRIF, but not MyD88, is important for caspase-1 activation perhaps in macrophages (Figs. 4 and 8). The MyD88-dependent hepatic granuloma and the TRIF-dependent caspase-1 activation for release of IL-18 are both necessary for the serum elevated IL-18 and the development of liver injury (Fig. 2). Thus, MyD88 is essential for the priming phase, while TRIF is important for the effector phase (Fig. 8). Nalp3 inflammasome was activated during the effector phase, which eventually leading to the liver injury (Fig. 6).

Several possibilities can explain loss of the serum increase of IL-18 in *Myd88*$^{-/-}$ mice although their Kupffer cells have capacity to achieve caspase-1 activation (Fig. 4B and D). First, dense hepatic accumulation of macrophages occurs in the WT liver but not in *Myd88*$^{-/-}$ liver after *P. acnes* treatment. This is consistent with the previous report by Szabo et al. [13]. Indeed, naïve WT mice do not increase serum IL-18 or undergo liver injury after challenge with the same sublethal dose of LPS (Supplementary Fig. 2). MyD88-dependently induced chemokines and/or their receptor might be involved in accumulation of F4/80^{+} macrophages and F4/80^{-} dendritic cells [34]. Second, Kupffer cells/macrophages might proliferate after *P. acnes* treatment MyD88-dependently. Third, *Myd88*$^{-/-}$ macrophages might remain functionally premature. *Myd88*$^{-/-}$ mice poorly produce IFN-γ in response to exogenous pathogens and perhaps endogenous microbes through poor production of IFN-γ-inducing factors such as IL-12 [1]. As IFN-γ is important for priming of macrophages, *Myd88*$^{-/-}$ macrophages might not be fully activated by *P. acnes* or not become to be susceptible to following LPS. Fourth, the dose of LPS is different between*in vivo* and *in vitro* studies. We used a sublethal dose of LPS (<50 μg/kg) for *in vivo* study. Indeed, when injected with a lethal dose of LPS (>50 mg/kg), *Myd88*$^{-/-}$ mice showed elevation of serum IL-18 (Supplementary Fig. 6). Fifth, the lack of responsiveness to IL-18 in *Myd88*$^{-/-}$ mice might reduce the augmentation of IL-18 release by Fas/Fas ligand system. IL-18 induces Fas ligand, which activates macrophages to release IL-18 via activating their Fas receptor [15]. Thus, MyD88 signaling renders mice highly susceptible to LPS by numerical increase of activated macrophages. Even though they are susceptible to LPS, *P. acnes*-primed mice evade the liver injury in the absence of TRIF signalings.

Our results clearly demonstrated a critical role of the TLR4-mediated TRIF pathway for activation of Nalp3 inflammasome for caspase-1 activation (Fig. 8). Indeed, *Asc*$^{-/-}$ Kupffer cells could not complete caspase-1 activation, resulting in the absence of release both IL-18 and IL-1β (Supplementary Fig. 7). Thus, it is plausible that TRIF signaling might activate Nalp3 inflammasome. However, in spite of our intensive efforts we could not detect the association between Nalp3 protein and TRIF or between ASC and TRIF in LPS-stimulated human

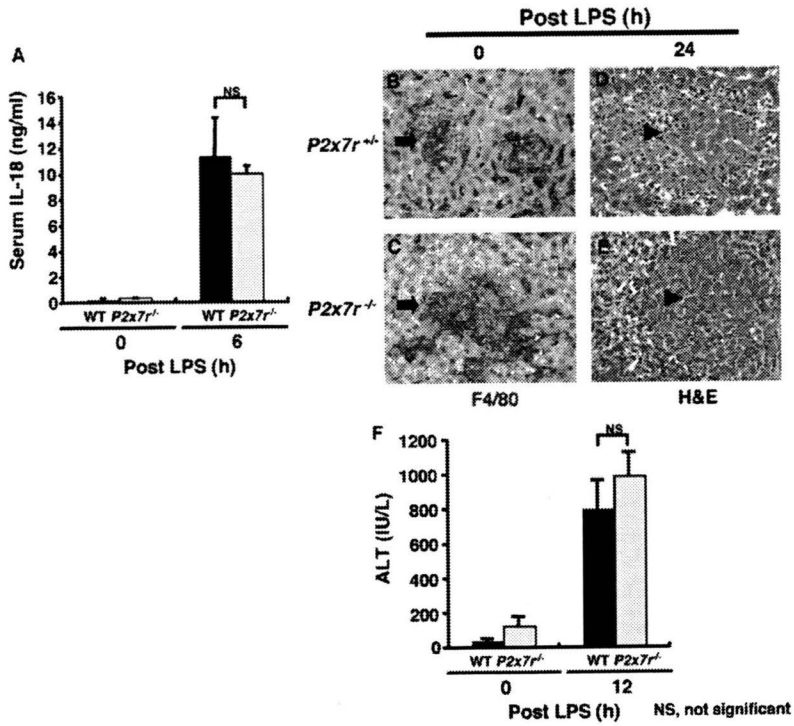

Fig. 5. Dispensability of endogenous ATP signaling for the liver injury. *P. acnes*-primed WT littermates (A and F), *P2x7r*$^{+/-}$ littermates (B and D) or *P2x7r*$^{-/-}$ mice (A, C, E and F) were challenged with LPS, and at the indicated time points sera (A and F) and liver specimens (B–E) were sampled for measurement of IL-18 concentrations (A) and ALT levels (F). F4/80^{+} cells were determined by immunohistochemistry (B and C). Liver sections were stained by H&E (D and E). Arrows indicate hepatic granulomas, and arrowheads indicate necrotic lesion. Original magnification: 100×.

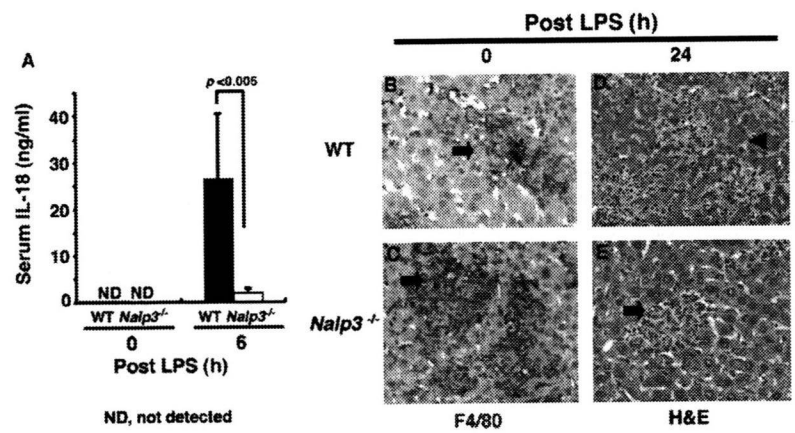

Fig. 6. Essential role for Nalp3 protein in the development of liver injury. *P. acnes*-primed WT (A, B, and D) or *Nalp3*$^{-/-}$ mice (A, C and E) were challenged with LPS, and at the indicated time points sera and liver specimens were sampled. Serum IL-18 concentrations (A) were measured. F4/80^{+} cells were determined by immunohistochemistry (B and C). Liver sections were stained by H&E (D and E). Arrows indicate hepatic granulomas, and an arrowhead indicates necrotic lesion. Original magnification: 100×.

monocytic THP-1 cells or THP-1 cells that over-express TRIF, ASC and Nalp3 proteins. Therefore, although TRIF cannot interact with Nalp3 inflammasome, TRIF-mediated signal may activate Nalp3 inflammasome by induction of some mediators (cytoplasmic molecules or metabolites).

Recent reports revealed that Nalp3 inflammasome is involved in inflammatory tissue damages of several diseases. Self-derived monosodium urate crystal relevant to gouty arthritis activates Nalp3 inflammasome to release IL-1β, which eventually initiates joint inflammation [26]. Previously, we also found that murine macrophages can

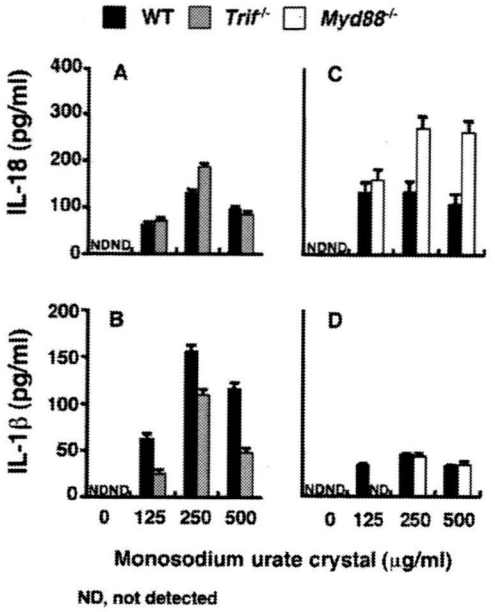

Fig. 7. Dispensability of TRIF and MyD88 for monosodium urate crystal-induced IL-18/IL-1 β secretion. WT (closed bars), *Trif*$^{-/-}$ (hatched bars), and *Myd88*$^{-/-}$ Kupffer cells (white bars) were incubated with various doses of monosodium urate crystal for 16 h. Concentrations of IL-18 (A and C) and IL-1β (B and D) were measured.

secrete both IL-18 and IL-1β in a caspase-1-dependent manner in response to monosodium urate crystal [28]. In addition to the aforementioned TRIF-mediated Nalp3 inflammasome activation, we formally succeeded in demonstrating the TRIF-independent Nalp3-dependent caspase-1 activation in Kupffer cells. *Myd88*$^{-/-}$ cells and *Trif*$^{-/-}$ Kupffer cells were able to secrete both IL-18 and IL-1β in response to monosodium urate crystal (Fig. 7). Therefore, at least two distinct signalings can activate Nalp3 inflammasome; one is TRIF-dependent pathway activated by TLR4 agonist and the other is TRIF-independent pathway initiated by monosodium urate crystal (Fig. 8). We need further study to reveal which pathway is activated by the individual inflammatory stimuli.

In summary, MyD88 and TRIF signalings play a different biological role. MyD88 is beneficial for health, such as homeostatic liver regeneration [5] and host defense [1]. As shown here, TRIF is necessary for activation of Nalp3 inflammasome for caspase-1 activation. Either single signaling could not induce liver injury. However, simultaneous or sequential activation of both signalings cause harmful outcomes (Fig. 8). Chronic inflammatory diseases with poorly identified mechanisms might be caused by the persistent activation of both MyD88 pathway and TRIF-mediated Nalp3 activation pathway.

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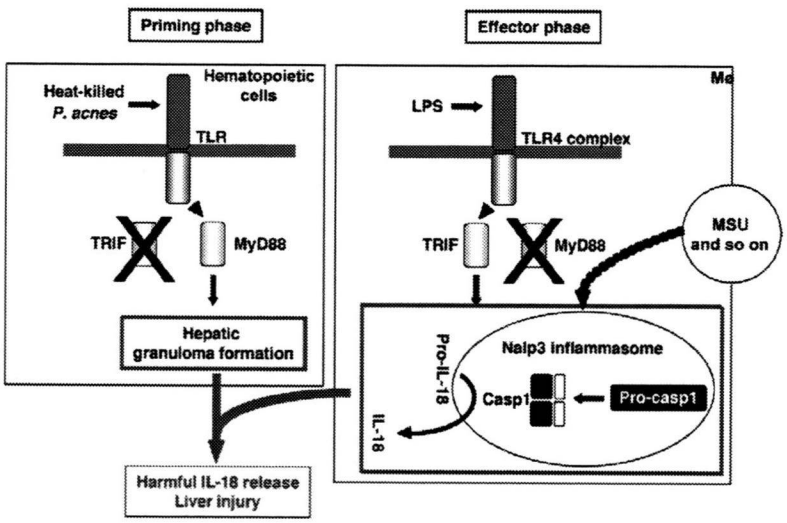

Fig. 8. A proposal model for IL-18-dependent *P. acnes*/LPS-induced liver injury. After treatment of mice with heat-killed *P. acnes*, hematopoietic cells are activated for the formation of dense hepatic granuloma consisting of many F4/80^{+} cells in a MyD88-dependent but TRIF-independent manner (priming phase). LPS stimulation induces caspase-1 activation and resultant release of mature IL-18 via activating Nalp3 inflammasome in macrophages in a TRIF-dependent but MyD88-independent manner, which eventually leading to the development of liver injury (effector phase). Nalp3 inflammasome is also activated by various inflammatory stimuli, such as monosodium urate crystal (MSU), in which neither TRIF nor MyD88 are involved. Therefore, Nalp3 inflammasome is activated by at least two pathways; TLR-mediated TRIF-dependent pathway and TRIF- and MyD88-independent one.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2009.03.027.

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nature immunology

Basophils contribute to T_H2-IgE responses *in vivo* via IL-4 production and presentation of peptide–MHC class II complexes to $CD4^+$ T cells

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Basophils express major histocompatibility complex class II, CD80 and CD86 and produce interleukin 4 (IL-4) in various conditions. Here we show that when incubated with IL-3 and antigen or complexes of antigen and immunoglobulin E (IgE), basophils internalized, processed and presented antigen as complexes of peptide and major histocompatibility complex class II and produced IL-4. Intravenous administration of ovalbumin-pulsed basophils into naive mice 'preferentially' induced the development of naive ovalbumin-specific $CD4^+$ T cells into T helper type 2 (T_H2) cells. Mice immunized in this way, when challenged by intravenous administration of ovalbumin, promptly produced ovalbumin-specific IgG1 and IgE. Finally, intravenous administration of IgE complexes rapidly induced T_H2 cells only in the presence of endogenous basophils, which suggests that basophils are potent antigen-presenting cells that 'preferentially' augment T_H2-IgE responses by capturing IgE complex.

Atopic people, after repeated exposure to a particular antigen, develop strong T helper type 2 (T_H2) responses and produce immunoglobulin E (IgE). IgE then sensitizes mast cells and basophils by binding to their FcεRI receptor (A000543)[1–3]. Subsequent exposure to the same antigen activates the mast cells and basophils to secrete the chemical mediators, cytokines and chemokines that result in the pathological reactions of immediate hypersensitivity. IgE is a unique antibody that upregulates expression of FcεRI on mast cells and basophils, thereby providing a mechanism for the amplification of IgE-mediated reactions[4,5]. Indeed, a strong positive correlation exists between FcεRI expression on basophils and IgE titers in human peripheral blood[6]. Furthermore, as with the inhalation of ragweed pollen, low antigen dose without adjuvant can induce IgE production, which suggests that there is an amplification loop for IgE production *in vivo*. Thus, once atopic people begin to produce IgE, they develop progressive allergic inflammation by increasing production of IgE and expression of FcεRI on effector cells.

Basophils and mast cells are important effector cells in IgE-mediated allergic inflammation[1–3]. Progenitors of mast cells in the bone marrow migrate to the peripheral tissues as immature cells and undergo differentiation *in situ*[1,7]. Thus, normally, mature mast cells are not found in the circulation. In contrast, basophils are rare circulating granulocytes that originate from progenitors in the bone marrow. Basophils constitute less than 1% of blood leukocytes and are normally not present in tissues. However, they may be recruited to some inflammatory sites where antigen is present and contribute to immediate hypersensitivity reactions[8–11]. Studies also suggest that basophils induce IgE-mediated chronic allergic inflammation and IgG1-mediated systemic anaphylactic shock[12–14]. Thus, basophils are primary effector cells in allergic disorders.

However, some lines of evidence have shown that these cells are important regulators of T_H2 responses *in vivo*, particularly in helminth-infected mice[15–20]. In general, the entry of an invading pathogen triggers recognition by dendritic cells (DCs) through Toll-like receptors (TLRs) and their subsequent maturation to express costimulatory molecules and produce interleukin 12 (IL-12) and IL-18, which favor T_H1 responses[21–24]. In contrast, infection with helminths strongly induces T_H2 cells and the proliferation of basophils in the spleens and livers of host mice[18], which suggests a contribution of basophils to the induction and/or augmentation of T_H2 responses. The development of naive $CD4^+$ T cells into T_H2 cells is dependent on IL-4 (A001262) in the milieu[25]. However, the nature of cells that produce 'early' IL-4, required for the development of naive $CD4^+$ T cells into T_H2 cells, remains unknown[26]. IL-18 with IL-3 or IL-33 with IL-3 strongly induces basophils but not mast cells to produce both IL-4 and IL-13 *in vitro*[27,28], which suggests basophils are involved in the induction of T_H2 cells by functioning as early IL-4-producing cells. Other published studies have also indicated that basophils are critically involved in T_H2 responses by their unique ability to produce early IL-4 and thymic stromal lymphopoietin in response to papain or

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bromelain[29]. Thus, here we studied the mechanism that accounts for the induction and progression of allergic response by positive feedback loops between IgE and basophils *in vivo*. We demonstrate the contribution of basophils to the T_H2-IgE response *in vitro* and *in vivo* through the production of IL-4 and presentation of complexes of peptide and major histocompatibility complex (MHC) class II to naive $CD4^+$ T cells, in contrast to the T_H1 cell–inducing action of DCs.

RESULTS

Basophils induce the development of T_H2 cells *in vitro*

We first examined the ability of splenic basophils from naive mice and mice infected with *Strongyloides venezuelensis*[30] to produce T_H2 cytokines and to induce the development of naive $CD4^+$ cells into T_H2 cells *in vitro*. We prepared non–T cell, non–B cell fractions from spleens of naive mice and infected mice and determined the proportion of $Fc\varepsilon RI^+c\text{-}Kit^-$ cells (basophils) in those fractions. Non–T cell, non–B cell fractions from spleens of naive mice contained 0.20% $Fc\varepsilon RI^+c\text{-}Kit^-$ cells, whereas those from *S. venezuelensis*–infected mice had a much greater proportion of these cells (5.84%; **Fig. 1a**), as reported for mice infected with *Nippostrrongylus brasiliensis*[18]. Furthermore, *S. venezuelensis*–infected mice had a greater proportion of $Fc\varepsilon RI^+c\text{-}Kit^+$ cells (mast cells) in those fractions (0.02% in naive mice compared with 0.39% in infected mice). We purified basophils from the spleens of naive mice and infected mice (**Fig. 1a**) and examined their production of cytokines and expression of MHC class II molecules. Splenic basophils from infected mice cultured for 24 h with IL-3 produced large amounts of IL-4, IL-6 and IL-13, whereas those from naive mice produced small amounts of these T_H2 cytokines, although both types of basophils produced similar amounts of IL-10 (**Fig. 1b**). However, the production of IL-17A, interferon-γ (IFN-γ) and tumor necrosis factor was low (**Fig. 1b**). As reported before[27], basophils from infected mice were able to produce IL-4 without IL-3 stimulation, whereas basophils from naive mice did not produce IL-4 in the absence of IL-3 *in vitro* (**Supplementary Table 1** online), which suggests that basophils in infected mice gain the ability to produce substantial IL-4 even in the absence of IL-3. Flow cytometry of basophils from naive mice and infected mice showed that they had abundant and comparable expression of MHC class II (**Fig. 1c**). Peripheral blood basophils from naive mice also expressed MHC class II molecules (**Fig. 1c**).

As splenic basophils from infected mice expressed MHC class II and had the potential to produce substantial IL-4, IL-6 and IL-13 in cultures containing IL-3, we next examined their ability to induce ovalbumin (OVA)-specific naive $CD4^+$ T cells to develop into T_H2 cells *in vitro* in the presence of OVA peptide (amino acids 323–339 (OVA(323–339)), IL-2 and IL-3 without IL-4 ('neutral' culture conditions). We simultaneously cultured naive $CD4^+$ T cells with conventional antigen-presenting cells (APCs; T cell–depleted splenic cell samples (ΔT-spleen cells)) in the presence of OVA(323–339) in neutral conditions (**Fig. 1d**). Splenic basophils from *S. venezuelensis*–infected mice showed a notable ability to induce naive $CD4^+$ T cells to develop into T_H2 cells (**Fig. 1d**). In contrast, as reported elsewhere[25], conventional APCs failed to induce T_H2 cells in these neutral conditions, although both types of APC strongly induced the development of T_H2 cells in T_H2 conditions (**Fig. 1d**). We found that like typical T_H2 cells that developed in T_H2 conditions

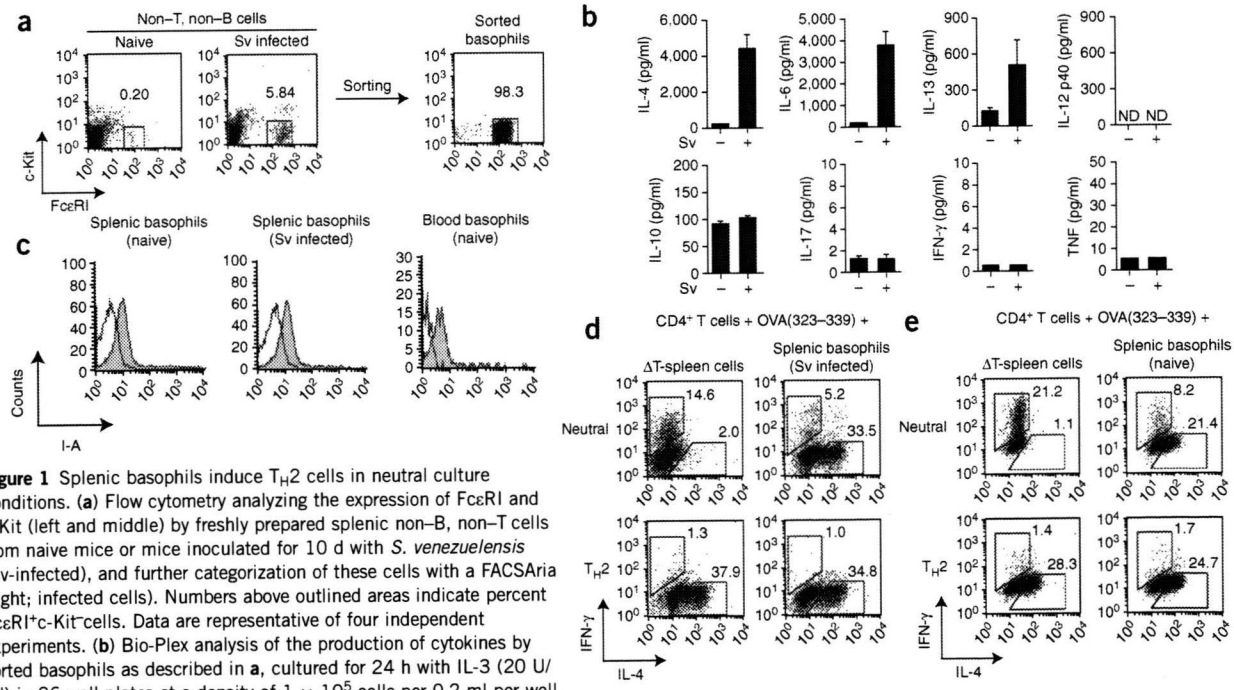

Figure 1 Splenic basophils induce T_H2 cells in neutral culture conditions. (**a**) Flow cytometry analyzing the expression of FcεRI and c-Kit (left and middle) by freshly prepared splenic non–B, non–T cells from naive mice or mice inoculated for 10 d with *S. venezuelensis* (Sv-infected), and further categorization of these cells with a FACSAria (right; infected cells). Numbers above outlined areas indicate percent $Fc\varepsilon RI^+c\text{-}Kit^-$ cells. Data are representative of four independent experiments. (**b**) Bio-Plex analysis of the production of cytokines by sorted basophils as described in **a**, cultured for 24 h with IL-3 (20 U/ml) in 96-well plates at a density of 1×10^5 cells per 0.2 ml per well. ND, not detected. Data are representative of two independent experiments (mean and s.e.m. of three mice). (**c**) Flow cytometry of sorted splenic basophils from naive mice (left) or *S. venezuelensis*–infected mice (middle), stained for MHC class II molecules (I-A), and of peripheral blood mononuclear cells from naive mice, stained for MHC class II molecules and gated on $Fc\varepsilon RI^+DX5^+B220^-CD3^-$ cells (right). Filled histograms, markers; lines, unstained cells. Data are representative of two independent experiments with five mice. (**d**,**e**) Flow cytometry analyzing cytosolic IL-4 and IFN-γ in naive splenic DO11.10 $CD4^+CD62L^+$ T cells (1×10^5 cells per ml) stimulated for 7 d in 48-well plates with IL-2 (100 pM), IL-3 (20 U/ml) and OVA(323–339) (1 μM) in the presence of irradiated BALB/c ΔT-spleen cells or irradiated splenic basophils (5×10^5 cells per ml each) from *S. venezuelensis*–infected mice (**d**) or naive mice (**e**), with (T_H2) or without (Neutral) IL-4 (1,000 U/ml), then washed and recultured for 4 h with the phorbol ester PMA (50 ng/ml) plus ionomycin (0.5 μg/ml). Numbers adjacent to outlined areas indicate percent $IL\text{-}4^+$ or $IFN\text{-}\gamma^+$ cells gated on $CD4^+$ T cells. Data are representative of three independent experiments.

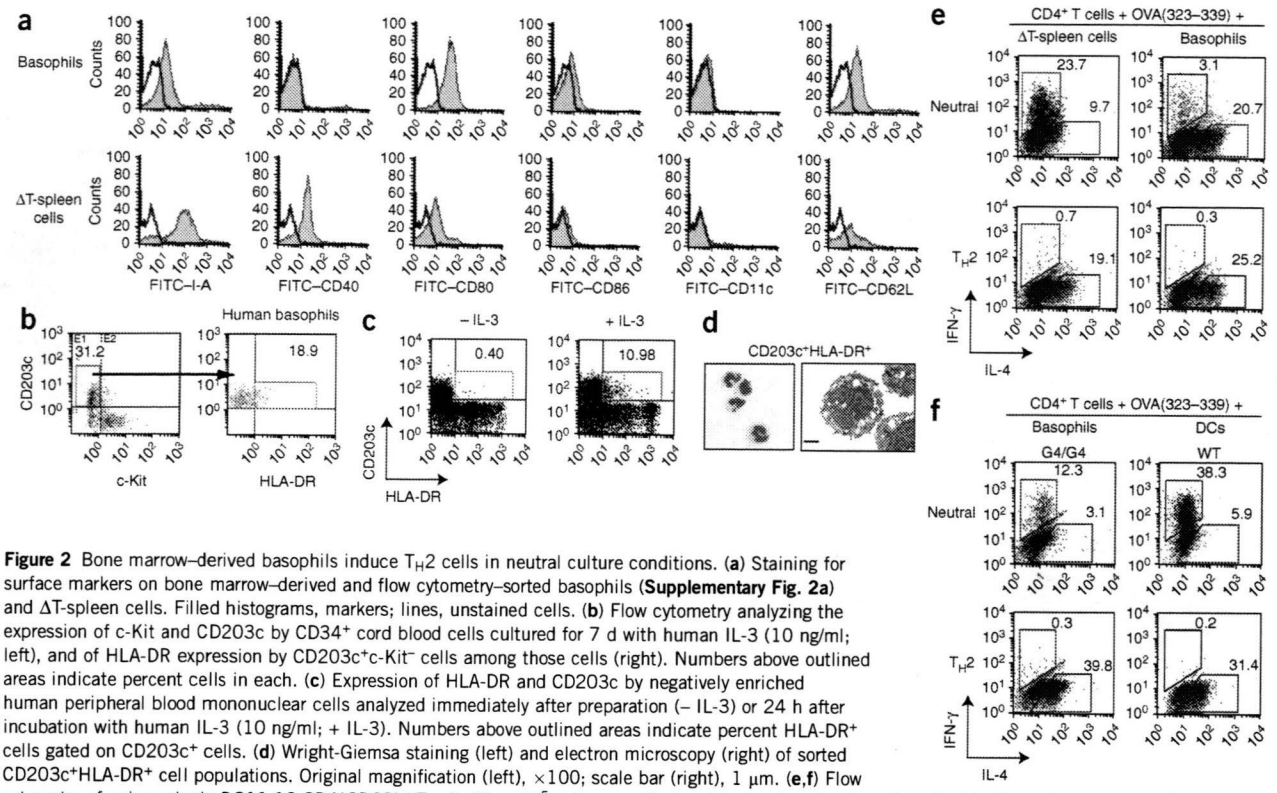

Figure 2 Bone marrow–derived basophils induce T_H2 cells in neutral culture conditions. (**a**) Staining for surface markers on bone marrow–derived and flow cytometry–sorted basophils (**Supplementary Fig. 2a**) and ΔT-spleen cells. Filled histograms, markers; lines, unstained cells. (**b**) Flow cytometry analyzing the expression of c-Kit and CD203c by $CD34^+$ cord blood cells cultured for 7 d with human IL-3 (10 ng/ml; left), and of HLA-DR expression by $CD203c^+c\text{-}Kit^-$ cells among those cells (right). Numbers above outlined areas indicate percent cells in each. (**c**) Expression of HLA-DR and CD203c by negatively enriched human peripheral blood mononuclear cells analyzed immediately after preparation (– IL-3) or 24 h after incubation with human IL-3 (10 ng/ml; + IL-3). Numbers above outlined areas indicate percent $HLA\text{-}DR^+$ cells gated on $CD203c^+$ cells. (**d**) Wright-Giemsa staining (left) and electron microscopy (right) of sorted $CD203c^+HLA\text{-}DR^+$ cell populations. Original magnification (left), ×100; scale bar (right), 1 μm. (**e**,**f**) Flow cytometry of naive splenic DO11.10 $CD4^+CD62L^+$ T cells (1×10^5 cells per ml) stimulated and analyzed as described in **Figure 1d**, with irradiated ΔT-spleen cells, purified bone marrow–derived wild-type (WT) or IL-4-deficient (G4/G4) basophils or wild-type splenic DCs as APCs. Data are representative of three or four independent experiments.

(**Fig. 1d**), T_H2 cells that developed after culture of naive $CD4^+$ T cells together with basophils in neutral culture conditions (**Fig. 1d**) produced IL-4, IL-5, IL-6, IL-10 and IL-13 (**Supplementary Fig. 1a** online), which suggested that these were true T_H2-polarized cells.

Next we assessed whether basophils from naive mice were also able to induce the development of T_H2 cells *in vitro*. We found that those splenic basophils also had a potent T_H2 cell–inducing function (**Fig. 1e**). As expected, some of the T_H2 cells induced in neutral conditions (**Fig. 1d**) expressed T_H2 cell marker IL-33Rα[31] (**Supplementary Fig. 1b**) and increased their production of T_H2 cytokines other than IL-4 when challenged with antigen plus IL-33 *in vitro* (**Supplementary Fig. 1c**).

Bone marrow basophils induce T_H2 cells *in vitro*

We next examined the ability of highly purified bone marrow basophils (**Supplementary Fig. 2a** online), devoid of other potential APCs, to induce T_H2 cell development *in vitro*. We first examined their expression of MHC class II molecules and the costimulatory molecules CD80 and CD86 (**Fig. 2a**). We simultaneously examined the expression of these molecules by conventional APCs. Bone marrow–derived basophils and conventional APCs expressed MHC class II, CD80 and CD86 but not CD11c. Basophils also expressed the lymph node–homing molecule CD62L, which suggested their potential to enter into lymphoid tissues[32]. As reported before[33], a fraction of human immature basophils ($CD203c^+c\text{-}Kit^-$) derived from cord blood expressed HLA-DR (18.9%; **Fig. 2b**). Although immature basophils decrease their expression of HLA-DR after maturation[33], we found mature peripheral blood basophils re-expressed HLA-DR after being cultured for 24 h with IL-3 (**Fig. 2c,d** and **Supplementary Fig. 2b**). In contrast, mouse peripheral basophils that expressed MHC class II failed to increase this expression in IL-3-containing medium (**Supplementary Fig. 2c**).

We compared the ability of conventional APCs and basophils to induce T_H2 cell development *in vitro* in neutral and T_H2 conditions (**Fig. 2e**). In the absence of any other APC, bone marrow–derived basophils were able to induce naive $CD4^+$ T cells to develop into T_H2 cells in neutral culture conditions as described above (20.7%), whereas conventional APCs induced T_H2 cells only in T_H2 conditions (**Fig. 2e**). Additional IL-4 stimulation (T_H2 conditions) resulted in an only modestly enhanced capacity of basophils to induce T_H2 cell development (25.2%; **Fig. 2e**), which suggests that bone marrow basophils produce sufficient IL-4 for maximum development of T_H2 cells. Indeed, basophils from IL-4-deficient G4/G4 mice[34] could not induce the development of T_H2 cells (3.1%) in neutral conditions (**Fig. 2f**). However, such IL-4-deficeint basophils did induce T_H2 cells in T_H2 conditions (39.8%), which allowed us to conclude that endogenous IL-4 from basophils was essential for the development of naive $CD4^+$ T cells into T_H2 cells. As reported elsewhere[25], splenic DCs induced T_H2 cells only in T_H2 cell–inducing conditions (**Fig. 2f**).

Basophils pulsed with antigen-IgE complexes are potent APCs

It was important to demonstrate the ability of basophils to take up and process OVA protein into OVA(323–339). We used 2,4-dinitrophenyl (DNP)-conjugated OVA (DNP-OVA) instead of OVA protein in this experiment and subsequent experiments, as DNP-OVA can also yield OVA(323–339) after processing. We were able to induce OVA-specific

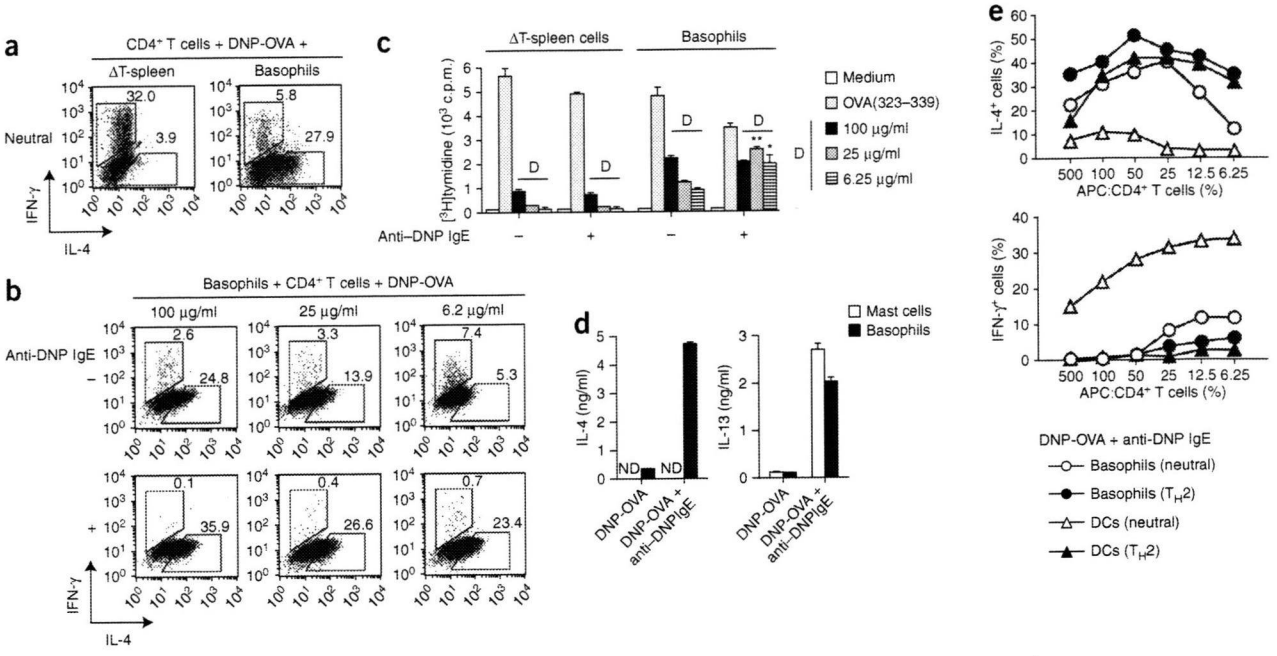

Figure 3 IgE complex enhances uptake of OVA by basophils. (**a**,**b**) Flow cytometry (as described in **Fig. 1d**) of naive splenic DO11.10 CD4+CD62L+ T cells (1×10^5 cells per ml) stimulated for 7 d with IL-2, IL-3 and DNP-OVA (100 μg/ml; **a**), or DNP-OVA (6.25–100 μg/ml) with or without IgE anti-DNP (10 μg/ml; **b**) in the presence of irradiated ΔT-spleen cells or bone marrow–derived basophils (5×10^5 cells per ml each). (**c**) Proliferative responses of naive splenic DO11.10 CD4+CD62L+ T cells (5×10^4 cells per 0.2 ml per well) cultured for 4 d in 96-well plates with OVA(323–339) (1 μM) or DNP-OVA (D; 6.25–100 μg/ml; in key) with or without IgE anti-DNP (10 μg/ml). *, $P < 0.05$ and **, $P < 0.005$, versus cells without anti–DNP IgE (Student's *t*-test). (**d**) Cytokine production by bone marrow–derived basophils or mast cells (5×10^5 cells per ml each) stimulated for 16 h in 48-well plates with IL-3 and DNP-OVA (100 μg/ml) with or without IgE anti-DNP (10 μg/ml). (**e**) Flow cytometry (as described in **Fig. 1d**) of naive splenic DO11.10 CD4+CD62L+ T cells (1×10^5 cells per ml) stimulated for 7 d with IL-2, IL-3, DNP-OVA (100 μg /ml) and IgE anti-DNP (10 μg /ml) in the presence of various numbers (6.25×10^3 to 5×10^5 cells per ml) of irradiated splenic DCs or bone marrow basophils with IL-4 (T_H2) or without IL-4 (Neutral). Data are representative of four (**a–d**) or two (**e**) independent experiments (mean and s.e.m. in **c**,**d**).

T_H2 cells by culturing naive CD4+ T cells with basophils in the presence of IL-2, IL-3 and DNP-OVA (100 μg/ml) without IL-4 (**Fig. 3a**). Again, conventional APCs failed to induce the development of T_H2 cell in these neutral culture conditions (**Fig. 3a**). Thus, basophils are able to process DNP-OVA into OVA(323–339) and to display peptide fragment in association with MHC class II and to produce IL-4.

It was also important to demonstrate the unique potential of basophils to increase their capacity to act as APCs when pulsed with antigen in the presence of antigen-specific IgE. Thus, we pulsed basophils with various doses of DNP-OVA in the presence or absence of monoclonal antibody to DNP (IgE anti-DNP; **Fig. 3b**). Basophils pulsed with a low dose (6.2 μg/ml) of DNP-OVA modestly induced T_H2 cells (5.3%), whereas pulsation with a higher dose (100 μg/ml) of DNP-OVA resulted in a higher proportion of 24.8%. The addition of IgE anti-DNP resulted in much higher proportions, particularly at lower concentrations of DNP-OVA (no IgE anti-DNP, 5.3%, 13.9% and 24.8%, versus with IgE anti-DNP, 23.4% 26.6% and 35.9%, for 6.2 μg/ml, 25 μg/ml and 100 μg/ml of DNP-OVA, respectively; **Fig. 3b**). Thus, the enhancing effect of IgE anti-DNP on basophil-induced T_H2 cell development was most apparent when basophils were pulsed with low concentrations of DNP-OVA.

We next examined whether IgE anti-DNP could enhance the capacity of conventional APCs to function as APCs. Thus, we cultured OVA(323–339)-specific T cells with conventional APCs or basophils in the presence of OVA(323–339) or DNP-OVA with or without IgE anti-DNP. Basophils pulsed with DNP-OVA in the presence of IgE anti-DNP had a significantly greater capacity to induce the proliferation of OVA–specific T cells (**Fig. 3c**). In contrast, conventional APCs pulsed with DNP-OVA in the presence of IgE anti-DNP did not have a greater capacity to induce T cell proliferation (**Fig. 3c**). These results suggest that basophils, taking advantage of their expression of FcεRI, might efficiently take up low doses of antigen in an IgE-dependent way.

As reported before[35], basophils had a much greater capacity to produce IL-4 and IL-13 after being pulsed with DNP-OVA in the presence of IgE anti-DNP (**Fig. 3d**). In contrast, mast cells pulsed with DNP-OVA–IgE anti-DNP immune complexes produced only IL-13, not IL-4 (**Fig. 3d**). We prepared basophils and mast cells from bone marrow cells cultured for 14 d with IL-3 (**Supplementary Fig. 2a**). We stimulated those cells with mixture of DNP-OVA and IgE anti-DNP. Mast cells prepared from bone marrow cells cultured for 4–6 weeks in medium conditioned by mouse leukemic WEHI-3 cells (containing IL-3) are reported to produce IL-4 after sequential IgE anti-DNP sensitization and subsequent DNP-protein challenge[36]. In addition to that unique function, we found that only basophils, when stimulated with IL-3 plus IL-18, IL-33, peptidoglycan or lipopolysaccharide, produced both IL-4 and IL-13 (**Supplementary Fig. 3a,b** online). Thus, basophils became producers of large amounts of IL-4 in *S. venezuelensis*–infected mice (**Fig. 1b**) or *in vitro* when stimulated with DNP-OVA–anti-DNP IgE in the presence of IL-3. Next we compared the APC activity of basophils and sorted CD11c+ splenic DCs[37] by changing the ratio of APCs to naive CD4+ T cells. Again, only basophils 'preferentially' induced the development of T_H2 cells in

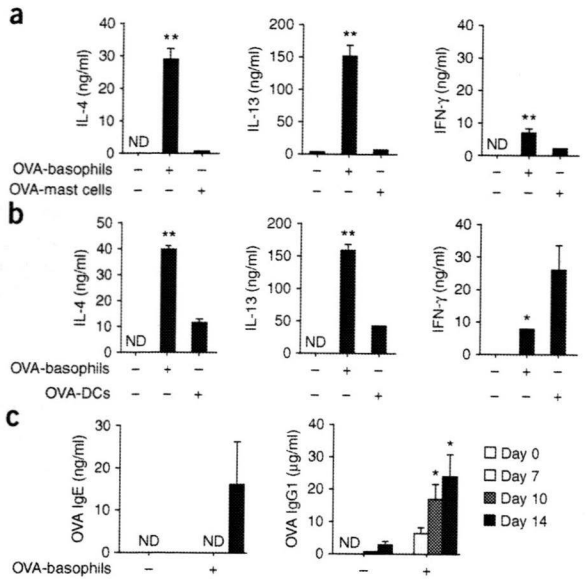

Figure 4 Intravenous administration of OVA-pulsed basophils induces a T_H2 response. (**a**,**b**) Enzyme-linked immunosorbent assay (ELISA) of IL-4, IL-13 and IFN-γ in supernatants of cells prepared as follows: purified bone marrow–derived basophils, mast cells (**a**) or splenic DCs (**b**) stimulated 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*.

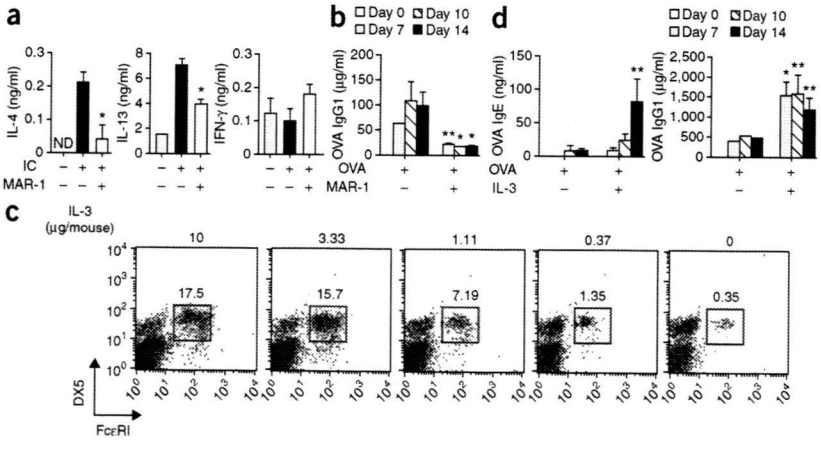

Figure 5 Intravenous administration of antigen-IgE complex induces T_H2 responses. (**a**,**b**) Analysis of cytokines (**a**) and antibodies (**b**) in BALB/c mice pretreated with PBS or anti-FcεRIa (MAR-1) and then injected intravenously with a mixture of DNP-OVA (10 μg) and IgE anti-DNP (20 μg; immune 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 ($Fc\varepsilon RI^+DX5^+$ 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 $Fc\varepsilon RI^+DX5^+$ 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

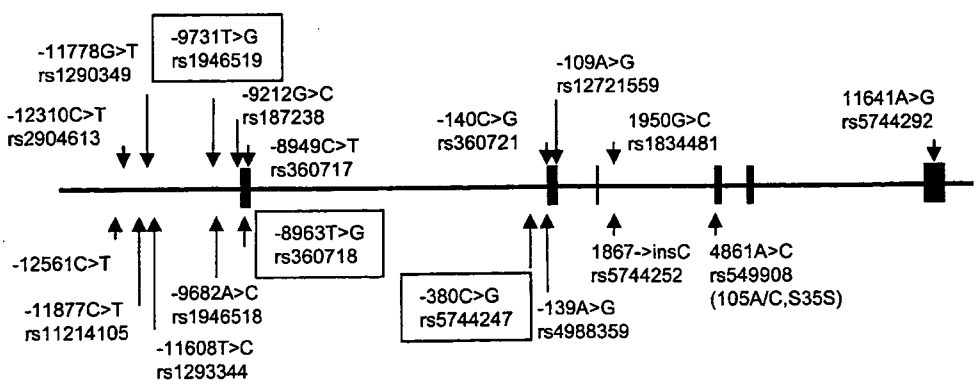

Figure 1. A graphical overview of polymorphisms identified in relation to the exon/intron structure of the human *IL-18* gene. Six exons are shown by black boxes, and positions for polymorphisms are relative to the translation start site (+1). Polymorphisms enclosed within boxes were genotyped in the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