of peritonitis-induced intestinal adhesion (11). Thus, it is important to determine whether and how IFN-γ triggers and/or modulates the action of TNF-α to induce hyper-coagulation and other symptoms (12, 13) and high mortality as well (14).

As previously reported, priming with heat-killed *Propionibacterium acnes* (*P. acnes*) renders mice highly susceptible to the lethal effects of LPS (15–17). *Propionibacterium acnes*-primed mice, when challenged with a sub-lethal dose of LPS, develop endotoxin shock syndrome accompanied by high elevation of serum pro-inflammatory cytokines (15, 17, 18). In contrast, *P. acnes*-primed *Il12p40*$^{-/-}$ mice completely escape from such LPS hyper-responsiveness (19). This strongly suggests the importance of IL-12 and/or IL-23 for *P. acnes* priming, as IL-12p40 is their common and essential subunit (20). Since IL-12 induces development of T_h1 cells and IL-23 activates of T_h17 cells (21), it is important to determine whether IL-12p40 contributes to the LPS sensitization, via induction of IFN-γ and/or IL-17.

Here, we showed that IFN-γ induces LPS sensitization and positively regulates the development of major symptoms induced by LPS challenge. Upon challenge with a sub-lethal dose of LPS, *P. acnes*-primed wild-type (WT) mice, but not naive mice, developed severe hypothermia, systemic inflammation and hyper-coagulation with elevation of plasma levels of TAT and PAI-1 and eventually died of shock. In contrast, *P. acnes*-primed *Ifnγ*$^{-/-}$ mice as well as *Il12p40*$^{-/-}$ mice evaded all these symptoms, indicating requirement of IL-12–IFN-γ axis for the LPS sensitization. Besides, *P. acnes*-primed *Ifnγ*$^{-/-}$ mice, contrasting to WT mice, evaded all the symptoms upon challenge with TNF-α, indicating that IFN-γ plays a central role in determining the sensitization to TNF-α as well. Finally we showed that administration of neutralizing anti-IFN-γ mAb at the time of LPS challenge could prevent *P. acnes*-primed WT mice from all the symptoms. These results clearly demonstrated that IFN-γ is a master regulator of endotoxin shock syndrome and suggested that IFN-γ might be a potential therapeutic target for the treatment of serious septic shock syndrome.

Methods

Mice

Ifnγ$^{-/-}$ mice on a BALB/c background (22) and *Il17A*$^{-/-}$ mice on a C57BL/6 (B6) background (23) were described elsewhere. *Il12p40*$^{-/-}$ B6 129 mice (24) were backcrossed with BALB/c mice, and F10 mice were used. BALB/c WT, BALB/c *nu/nu* and B6 WT mice were purchased from Clea Japan (Osaka, Japan). Female mice (8–12 weeks old) were used. All mice were maintained under specific pathogen-free conditions and received human care as outlined in the Guide for the Care and Use of Experimental Animals in Hyogo College of Medicine.

Reagents

LPS from *Escherichia coli* (O55: B5), which selectively activates Toll-like receptor 4 both *in vivo* and *in vitro* (25), were purchased from Sigma (St Louis, MO, USA). Heat-killed *P. acnes* was prepared as described elsewhere (26). Recombinant murine TNF-α was purchased from PeproTech (Rocky Hill, NJ, USA). Hybridoma producing neutralizing anti-IFN-γ mAb (R6A2) was purchased from American Type Culture Collection (ATCC, Livermore, CA). Neutralizing anti-IFN-γ mAb for *in vivo* treatment was prepared as shown previously (26). The culture medium was RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 50 μM 2-ME, 2 mM L-glutamine, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin.

Sequential administration of P. acnes and LPS or TNF-α

Mice were administered intra-peritoneally with heat-killed *P. acnes* (1 mg in 200 μl PBS). At day 5, mice were challenged with various doses of LPS or rTNF-α via a tail vein (26). In some experiments, *P. acnes*-primed mice were administered intra-peritoneally with various doses of anti-IFN-γ mAb 30 min before LPS challenge (27). At the indicated time points, plasma was sampled for measurement of concentrations of various pro-inflammatory cytokines, TAT and PAI-1 according to the method described by Sommeijer *et al.* (28). Briefly, anesthetized mice were administered with 180 μl of 3.2% (w/v) sodium citrate via the vena cava, and 10 s later, plasma was sampled through the same syringe. The rectal temperature was periodically monitored. Survival was monitored until 72 and 24 h after challenge with LPS and TNF-α, respectively. We killed all mice that appeared inactive and lost reactions to a supine position and counted them as dead ones.

Assay for TAT, PAI-1 and cytokines

An ELISA kit for PAI-1 was purchased from Innovative Research Inc. (Novi, MI, USA). ELISA kits for IFN-γ, TNF-α, IL-6 and IL-12p40 were from R&D (San Diego, CA, USA). HMGB-1 ELISA kits were from SinoTest (Sagamihara-shi, Japan). We measured TAT concentrations by a commercially available kit from Enzyme Research Laboratories (South Bend, IN, USA) according to the manufacturer's instruction.

Core body temperature

Rectal temperature readings were performed using a rectal probe digital thermometer (BAT-10; Physitemp, Clifton, NJ, USA). Difference in rectal temperature post and prior to the challenge was calculated and shown as ΔRectal temperature.

Responsiveness of splenocytes to LPS and TNF-α

Splenocytes (2×10^6 ml^{-1}) from variously treated mice with various genotypes were incubated with LPS or rTNF-α *in vitro*. Supernatants were collected for measurement of pro-inflammatory cytokines.

T-cell reconstitution

In total, 2×10^7 splenic T cells from naive WT BALB/c mice, enriched by a nylon wool column method (>90% $CD3^+$) (29), were transferred into naive BALB/c *nu/nu* mice through a tail vein for the T-cell reconstitution, and after 24 h, these T cell-reconstituted *nu/nu* mice were sequentially administered with *P. acnes* and challenged with LPS.

Statistics

All data are shown as the mean ± SD of samples in each experimental group. Five to 10 mice were used for each group. Significance between the experimental and control

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groups was examined by the unpaired Student's *t*-test. *P*-values <0.05 were considered significant. Two to three experiments were separately performed, and representative data were shown in each data.

Results

In vivo sensitization to LPS by priming with heat-killed P. acnes

We administered heat-killed *P. acnes* into WT BALB/c mice and challenged them with LPS (2 or 20 μg per head) at day 5 after this pretreatment. Half of the *P. acnes*-primed mice died within 12 h after challenge with 2 μg LPS, while all died after challenge with 20 μg LPS (Fig. 1A), indicating that LPS kills the animals in a dose-dependent manner. In sharp contrast, none of naive mice succumbed to these LPS challenges (Fig. 1A). Thus, *P. acnes*-primed mice are highly susceptible to LPS.

Since hypothermia is an important clinical indicator of sepsis in human (2, 30, 31), we measured rectal temperature of *P. acnes*-primed mice after LPS challenge. Mice challenged with 2 μg LPS showed 9°C diminution at 6 h and gradually recovered thereafter (Fig. 1B). Mice challenged with 20 μg LPS exhibited 12°C reduction without any recovery. PBS treatment instead of LPS challenge did not affect body temperature or mortality rate of *P. acnes*-primed mice (Fig. 1A and B).

Elevation of TAT and PAI-1 after LPS challenge

Endotoxin occasionally induces DIC in septic patients (1), and DIC exacerbates septic shock (6–8). Thus, we measured plasma levels of TAT and PAI-1 in *P. acnes*-primed mice after challenge with 2 μg LPS. Plasma TAT levels were strikingly elevated in *P. acnes*-primed mice with a peak at 4 h after LPS challenge (Fig. 1C). In contrast, naive mice showed only limited elevation of plasma TAT levels (Fig. 1C). Plasma PAI-1 levels were also dramatically elevated in *P. acnes*-primed mice after LPS challenge. Compared with the kinetics of TAT induction, PAI-1 level remained at basal levels until 4 h and sharply increased at 8 h after LPS challenge (Fig. 1C). In contrast, plasma PAI-1 levels remained low in naive mice after LPS challenge (Fig. 1C). *P. acnes* priming alone only modestly increased plasma TAT and PAI-1 levels (Fig. 1C). PAI-1 and tPA mRNA expression

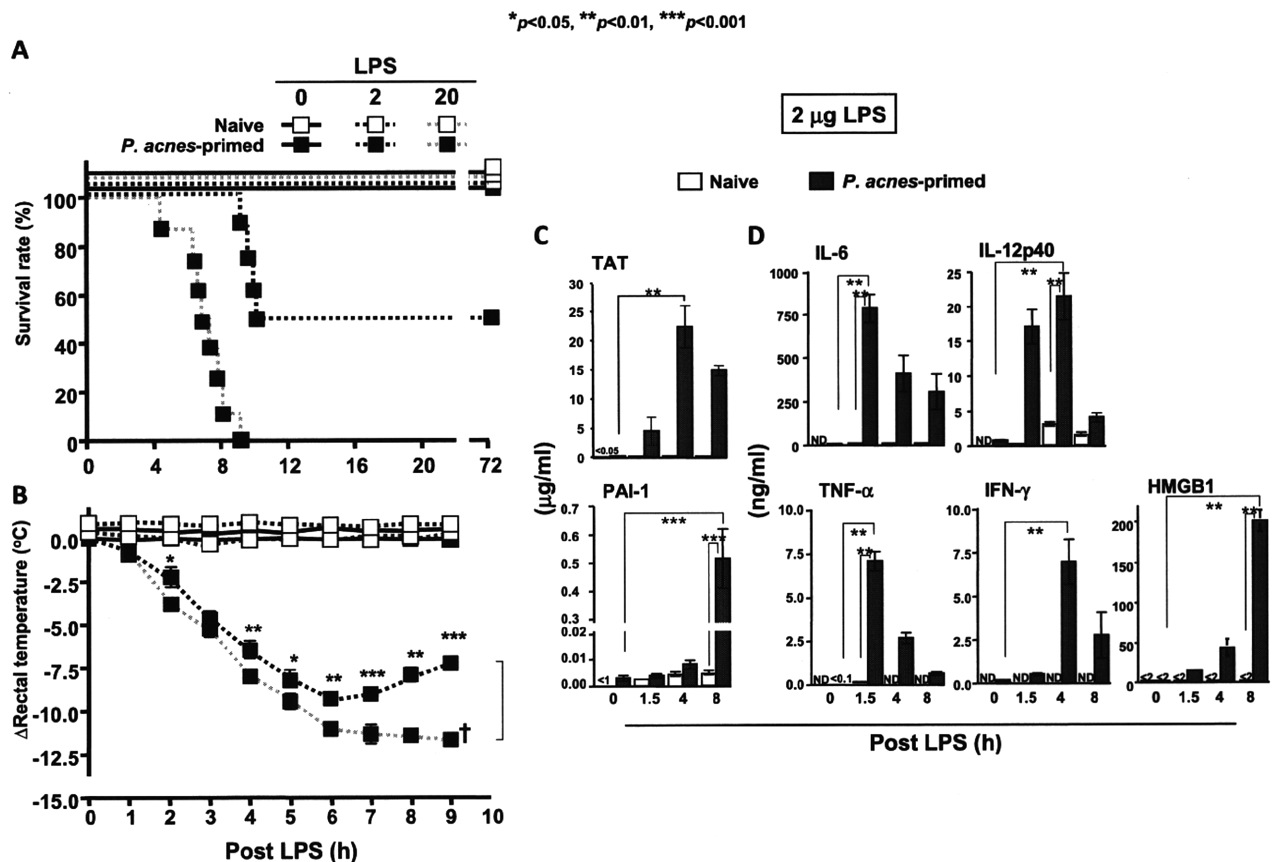

Fig. 1. Increase of the *in vivo* susceptibility to LPS by priming with heat-killed *Propionibacterium acnes*. WT BALB/c mice were administered with heat-killed *P. acnes*. *Propionibacterium acnes*-primed mice were challenged with 20 or 2 μg of LPS. Survival was monitored until 72 h (A). Rectal temperatures were measured, and difference of rectal temperatures at each time point to that before LPS challenge was shown (B). At various time points after LPS challenge, plasma was sampled for measurement of TAT and PAI-1 concentrations (C) and of IL-6, IL-12p40, TNF-α, IFN-γ and HMGB1 levels (D) by ELISA. A dagger indicates the time point at which all the mice die of shock.

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levels increased strikingly (50-fold) and modestly (5.5-fold) in the liver of *P. acnes*-primed mice after LPS challenge, respectively (Supplementary Figure 1, available at *International Immunology* Online), suggesting that LPS induces hyper-coagulation status in *P. acnes*-primed mice by much higher induction of PAI-1 than that of tPA.

Induction of production of pro-inflammatory cytokines by LPS challenge

Propionibacterium acnes-primed mice, but not naive mice, promptly increased plasma levels of IL-6 and TNF-α after LPS challenge (Fig. 1D). They started to increase plasma level of IL-12p40 at 1.5 h, increased it further until 4 h and decreased it rapidly thereafter. We also noticed that they start to increase IFN-γ level at 4 h (Fig. 1D), prior to the increase of PAI-1 level (Fig. 1C). Since high-mobility group box protein 1 (HMGB1) is a potent cytokine that mediates severe sepsis at late stage (32–34), we measured plasma HMGB1 level. Like other pro-inflammatory cytokines, plasma HMGB1 level was dramatically elevated after LPS challenge in *P. acnes*-primed mice but not in naive mice (Fig. 1D).

Requirement of IL-12p40 for the LPS sensitization

Previously, we demonstrated that *P. acnes*-primed *Il12p40*$^{-/-}$ mice showed 100% survival after LPS challenge (19). Thus, we examined whether *P. acnes*-primed *Il12p40*$^{-/-}$ mice also evade other symptoms. None of *P. acnes*-primed *Il12p40*$^{-/-}$ mice died of endotoxin shock (Fig. 2A) and developed hypothermia (Fig. 2B) after challenge with 2 μg of LPS, which is 50% lethal dose for *P. acnes*-primed WT mice (Fig. 2A). Furthermore, the elevation of their plasma TAT and PAI-1 levels was very modest (Fig. 2C). This was also the case for TNF-α, IFN-γ or HMGB1 level in the plasma of *P. acnes*-primed *Il12p40*$^{-/-}$ mice at 8 h after LPS challenge (Fig. 2D). Taken together, these results indicated that *P. acnes*-primed *Il12p40*$^{-/-}$ mice are unresponsive to LPS and strongly suggested that IL-12p40 is necessary for the sensitization to LPS. To verify this possibility, we incubated splenocytes from naive or *P. acnes*-primed WT and *Il12p40*$^{-/-}$ mice with LPS and measured pro-inflammatory cytokine levels in their culture supernatants. Compared with those from naive WT mice, splenocytes from *P. acnes*-primed WT mice produced much larger amounts of TNF-α, IL-6 and IFN-γ upon LPS challenge

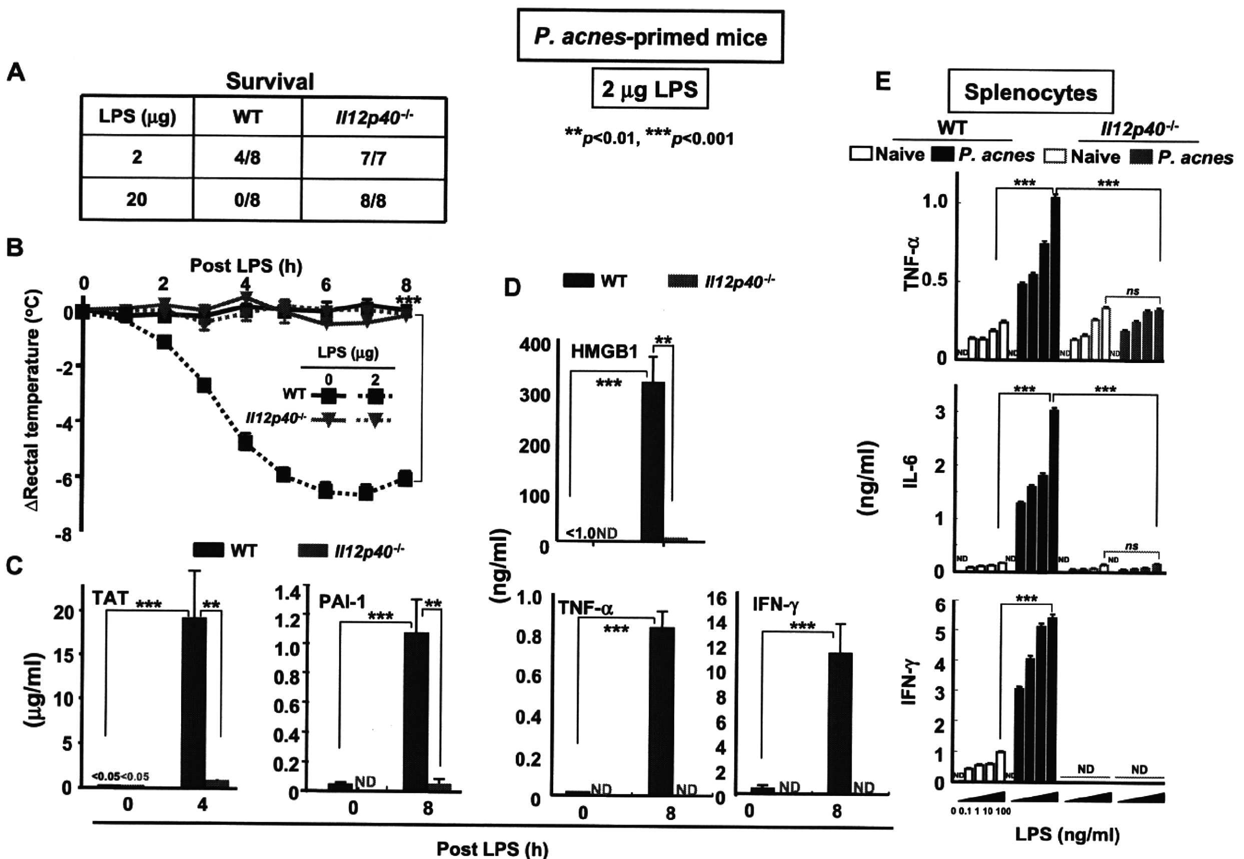

Fig. 2. Requirement of IL-12p40 for *Propionibacterium acnes*-induced LPS sensitization. *Propionibacterium acnes*-primed WT BALB/c mice or Il12p40$^{-/-}$ mice were challenged with 2 μg LPS. Survival rate (A) and rectal temperatures (B) were monitored until 72 and 8 h, respectively. At the indicated time point, plasma was sampled for measurement of TAT and PAI-1 (C) and pro-inflammatory cytokines (D). Splenocytes from naive or *P. acnes*-primed WT or *Il12p40*$^{-/-}$ mice were incubated with LPS *in vitro*, and TNF-α, IL-6 and IFN-γ concentrations in each supernatant were measured by ELISA. The data were shown as mean ± SD of those of splenocytes from four to five mice in each experimental group (E).

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in vitro. In contrast, splenocytes from *P. acnes*-primed *Il12p40*$^{-/-}$ mice produced small amounts of these cytokines, like as those from naive WT or *Il12p40*$^{-/-}$ mice (Fig. 2E). These results taken together clearly indicated that IL-12p40 is essentially required for *P. acnes*-induced LPS sensitization.

Importance of T_h1 cells but not T_h17 cells for the LPS sensitization

Since *Il12p40*$^{-/-}$ mice failed to develop T_h1 cell (Supplementary Figure 2, available at *International Immunology* Online) and to become sensitized to LPS after treatment with *P. acnes* (Fig. 2A–E), we next investigated whether T cells are required for the sensitization to LPS. As *nu/nu* mice lack thymic T cells, we examined whether *P. acnes* priming is able to induce *nu/nu* mice to be susceptible to LPS. Expectedly, all *P. acnes*-primed *nu/nu* mice could survive after challenge with 20 μg of LPS that could 100% kill *P. acnes*-primed WT mice, suggesting that thymus-derived T cells are required for the *P. acnes*-induced sensitization to LPS (Fig. 3A). To verify this possibility, we transferred WT splenic T cells into *nu/nu* mice and sequentially treated them with *P. acnes* and 20 μg of LPS. All the *nu/nu* mice reconstituted with thymic T cells, like WT mice, became to succumb to the sequential treatment with *P. acnes* and 20 μg of LPS (Fig. 3B). Collectively, these results strongly suggested the importance of T cells for the *P. acnes*-induced sensitization to LPS.

IL-12p40 is a common and essential subunit of IL-12 and IL-23. As IL-23 can activate T_h17 cells and as *P. acnes*-primed mice reportedly possess T_h17 cells and T_h1 cells both specific for *P. acnes* (35), we examined possible contribution of IL-17 to the LPS sensitization. Upon LPS challenge, *P. acnes*-primed *Il17A*$^{-/-}$ mice showed survival rate and hypothermia comparable to those of WT mice (Fig. 3C and D). Thus, IL-17 is not profoundly involved in the sensitization to LPS.

IL-12 contributes to the sensitization to LPS through induction of IFN-γ

We next investigated whether T_h1 cytokine IFN-γ is essential for the LPS sensitization. Like *Il12p40*$^{-/-}$ mice (Fig. 2), *P. acnes*-primed *Ifnγ*$^{-/-}$ mice evaded the lethality, hypothermia, hyper-coagulation and systemic inflammation after LPS challenge (Fig. 4A and B). They failed to increase production of TAT, PAI-1, TNF-α and HMGB1 after sequential treatment with *P. acnes* and LPS (Fig. 4C and D). Furthermore, *P. acnes* treatment did not increase the responsiveness to LPS of *Ifnγ*$^{-/-}$ splenocytes, as illustrated by the failure of splenocytes from *P. acnes*-primed *Ifnγ*$^{-/-}$ mice to produce large amounts of TNF-α and IL-6 upon challenge with LPS *in vitro* (Fig. 4E). These results indicated requirement of IFN-γ for the LPS sensitization.

As IFN-γ was reported to be capable of sensitizing macrophages to LPS (36), we investigated whether IL-12, like IFN-γ, has the same capacity. To test this, we incubated bone marrow-derived macrophages from WT, *Il12p40*$^{-/-}$ or *Ifnγ*$^{-/-}$ mice with rIL-12 or rIFN-γ. Then, we stimulated them with LPS. We found that pretreatment with IFN-γ, but not IL-12, is able to enhance production of TNF-α and IL-6 from WT, *Il12p40*$^{-/-}$ or *Ifnγ*$^{-/-}$ macrophages upon LPS stimulation (Supplementary Figure 3, available at *International Immunology*

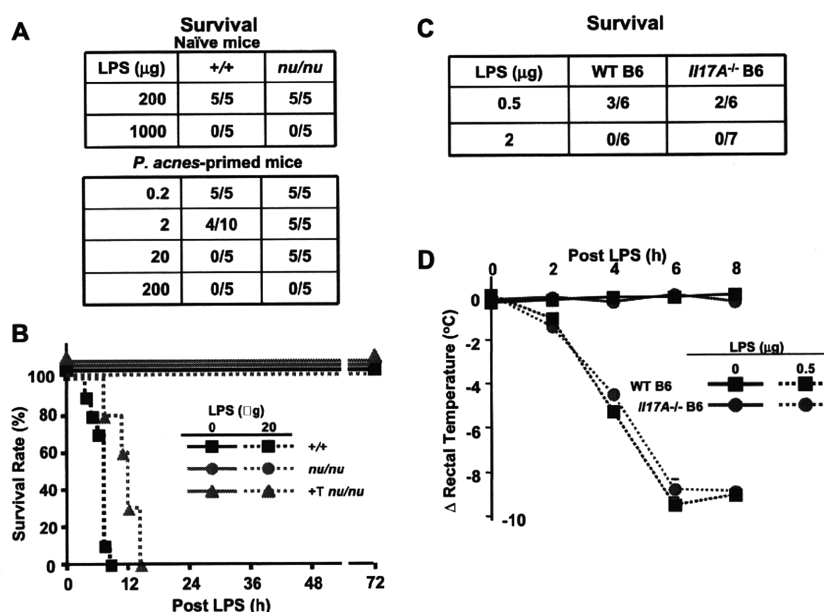

Fig. 3. Importance of T_h1 cells but not T_h17 cells for the LPS sensitization. Naive and *Propionibacterium acnes*-primed BALB/c WT (+/+) mice and nu/nu mice were administered with various doses of LPS, and mouse survival was monitored until 72 h (A). *nu/nu* mice reconstituted with T cells from WT mice were sequentially treated with *P. acnes* and 20 μg LPS (B). WT B6 mice (black symbols) and *Il17A*$^{-/-}$ B6 mice (blue symbols) were treated with *P. acnes* and subsequently challenged with LPS (C and D). The survival rate after 0.5 or 2 μg LPS (C) and body temperature reductions after challenge with 0.5 μg LPS (D) were monitored until 72 and 8 h, respectively.

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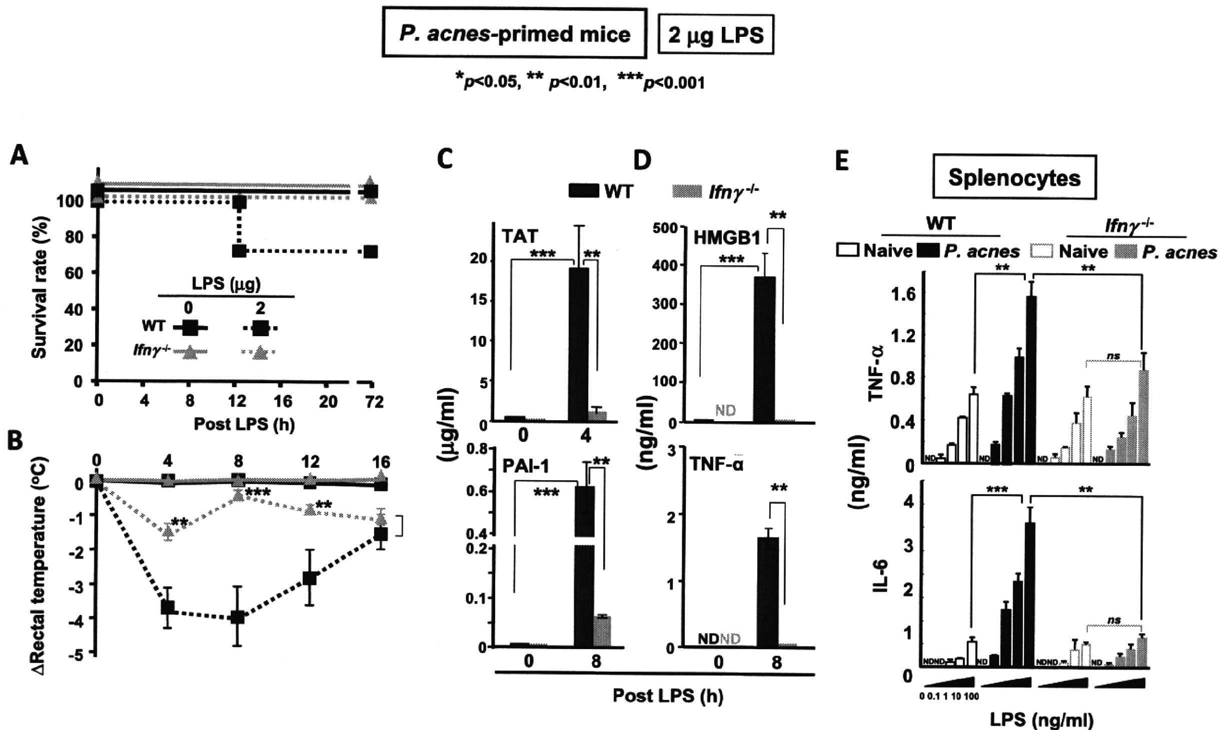

Fig. 4. *Propionibacterium acnes*-primed *Ifnγ*$^{-/-}$ mice were resistant to LPS. *Propionibacterium acnes*-primed WT mice (gray columns or symbols) and *Ifnγ*$^{-/-}$ mice (green columns or symbols) were administered intravenous with 2 μg LPS. Survival rates were monitored (A). Rectal temperatures were measured (B). Plasmas were sampled at 4 h post LPS for measurement of TAT (C), PAI-1(C), TNF-α (D) and HMGB1 (D). Splenocytes from naive or *P. acnes*-primed WT or *Ifnγ*$^{-/-}$ mice were incubated with various doses of LPS *in vitro*, and TNF-α and IL-6 concentrations in each supernatant were measured by ELISA. The data were shown as mean ± SD of those of splenocytes from four to five mice in each experimental group (E).

Online). Thus, IL-12 lacks the potential to directly sensitize macrophages to LPS either in the presence or in the absence of *Ifnγ*, while IFN-γ could fulfill the potential even in the absence of *Il12p40*. The results demonstrated prerequisite of IL-12-induced IFN-γ for the *P. acnes*-induced *in vivo* sensitization to LPS.

Requirement of IFN-γ for the sensitization to TNF-α

Since *P. acnes*-primed WT mice are susceptible to exogenous TNF-α (16), we next investigated whether IFN-γ is also critical for the sensitization to TNF-α. Consistent with our previous report (16), *P. acnes*-primed WT mice showed poor survival after treatment with a sub-lethal dose of TNF-α (Fig. 5A). Moreover, they developed all the symptoms observed in the *P. acnes*-primed mice with endotoxin shock syndrome (Fig. 5B–D), indicating that TNF-α is capable of replacing LPS in induction of each symptom. TNF-α blockade reportedly can protect against lethal outcome of *P. acnes*-primed mice after LPS challenge (37). This report together with our present results strongly suggested that TNF-α is a potent effector cytokine involved in the endotoxin shock syndrome. In sharp contrast, *P. acnes*-primed *Ifnγ*$^{-/-}$ mice were resistant to the lethal effects of TNF-α (Fig. 5A–D), indicating the importance of IFN-γ for the *in vivo* sensitization to TNF-α as well. Taken together, these results demonstrated a central role of IFN-γ in the development of the endotoxin shock syndromes via induction of *in vivo* sensitization to LPS and TNF-α.

IFN-γ also controls LPS challenge phase of endotoxin shock syndrome

We wanted to know whether IFN-γ is also necessary for the development of each symptom or sign during the excitation phase induced by LPS challenge. To test this, we administered neutralizing anti-IFN-γ mAb into *P. acnes*-primed WT mice at 30 min prior to challenge with 20 μg LPS. Neutralizing anti-IFN-γ mAb could rescue the lethal outcome and the serious hypothermia in a dose-dependent manner (Fig. 6A and B). Neutralizing anti-IFN-γ mAb also prevented the hyper-coagulation (Fig. 6C) and elevation of plasma levels of HMGB1 and TNF-α (Fig. 6D). Thus, IFN-γ is important for the development of each symptom during the excitation phase. Collectively, all the results demonstrated that IFN-γ is a master regulator of the endotoxin shock syndrome.

Discussion

Our present study demonstrated the importance of IL-12–IFN-γ axis for the development of endotoxin shock syndrome. In response to heat-killed *P. acnes*, macrophages and dendritic cells release IL-12, which induces and activates T_h1 cells (Supplementary Figure 2, available at

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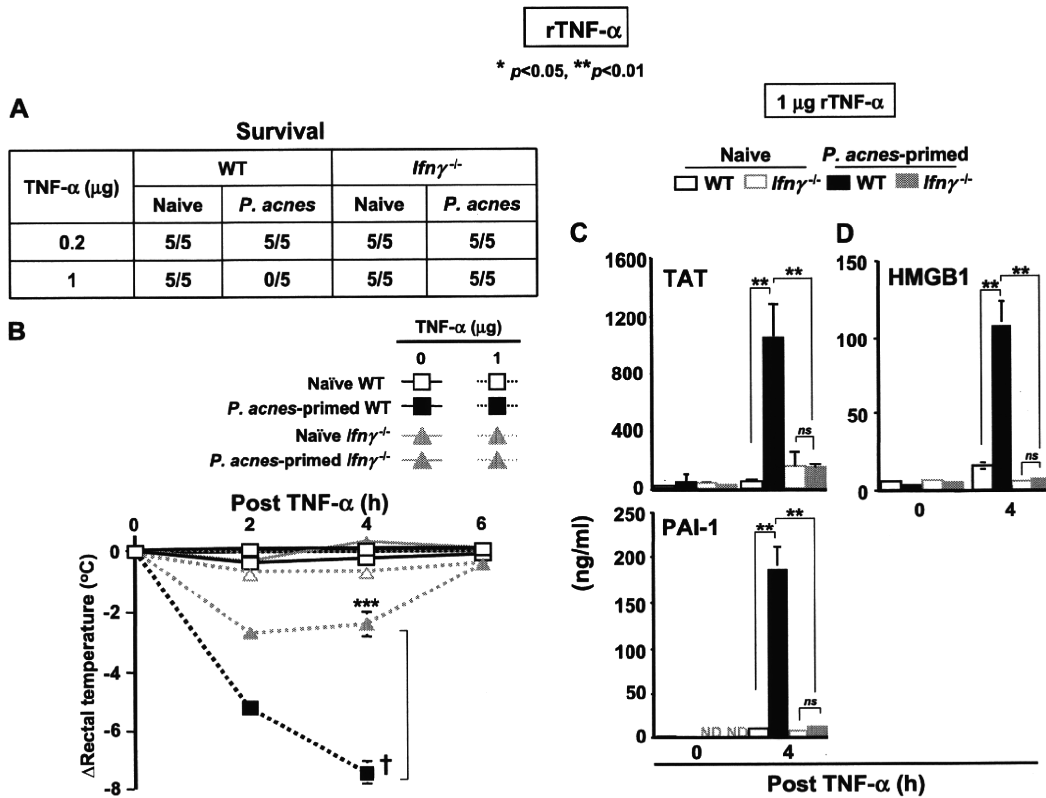

Fig. 5. Importance of IFN-γ for *in vivo* sensitization to TNF-α. *Propionibacterium acnes*-primed or naive WT mice and $Ifn\gamma^{-/-}$ mice were administered intravenous with 0.2 or 1 μg rTNF-α. Survival rates were monitored until 24 h after TNF-α challenge (A). Rectal temperatures were measured (B). Plasmas were sampled at 4 h for measurement of PAI-1 (C) and HMGB1 (D). A dagger indicates the time point at which all the mice die of shock.

International Immunology Online) to produce IFN-γ (38). Resultant IFN-γ prepares macrophages to be susceptible to LPS, which robustly induces TNF-α production (Fig. 7; Supplementary Figure 3, available at *International Immunology* Online). Furthermore, *P. acnes* pretreatment induces mice to be highly susceptible to TNF-α via induction of IFN-γ, which eventually results in their development of hypothermia, hyper-coagulation and lethal shock (Fig. 5). Thus, this IL-12–IFN-γ axis is critical for the *in vivo* sensitization to both LPS and TNF-α. After LPS challenge, IFN-γ-activated macrophages and perhaps dendritic cells produced large amounts of IL-12 and IL-18 (26), which synergistically activate T_h1 cells and NK cells to produce IFN-γ (39, 40) (Fig. 7). This IFN-γ positively regulates the development of lethal outcomes, hypothermia, systemic inflammation and hyper-coagulation by strongly increasing responsiveness to LPS and TNF-α and conceivably by synergistically cooperating with LPS and TNF-α (41) (Figs 6 and 7). Accordingly, IFN-γ is a central cytokine that initiates both the hypersensitization to LPS/TNF-α during *P. acnes* priming phase and the development of endotoxin shock syndrome after LPS challenge (Fig. 7).

This study does not exclude roles of NK cells as a cell source of IFN-γ during the priming and effector phases. However, we found that *P. acnes*-primed *nu/nu* mice are resistant to 20 μg of LPS, which kills 100% *P. acnes*-primed WT mice, and that reconstitution with WT T cells provided *nu/nu* mice with the capacity to develop LPS susceptibility after *P. acnes* treatment (Fig. 3A and B), indicating the importance of T_h1 cells for LPS sensitization. However, we also found that *P. acnes*-primed *nu/nu* mice died of endotoxin shock after challenge with high dose of LPS (200 μg per head) (Fig. 3A), suggesting possible contribution of NK cell production of IFN-γ to the LPS sensitization. Therefore, in the *nu/nu* mice NK cells might, at least partly, participate in the establishment of *P. acnes*-induced sensitization to LPS by production of IFN-γ in response to IL-12 and IL-18.

IL-18 is also a potent IFN-γ-inducing cytokine. However, in contrast to $Il12p40^{-/-}$ mice, $Il18^{-/-}$ mice shows normal susceptibility to the sequential treatment with *P. acnes* and LPS (19). This is partly due to the facts that IL-18 has little capability to induce T_h1 cell development (40) and that IL-18 does not affect IL-12 production (42). Thus, IL-12 is critically involved in the *P. acnes*-induced LPS sensitization via induction of production of IFN-γ principally from T_h1 cells.

Our present study revealed the importance of IFN-γ even during the excitation phase induced by LPS challenge. IFN-γ blockade 30 min prior to LPS challenge protected against all the endotoxin shock-associated alterations (Fig. 6). Several mechanistic possibilities might explain the involvement of IFN-γ in the development of TNF-α-mediated endotoxin

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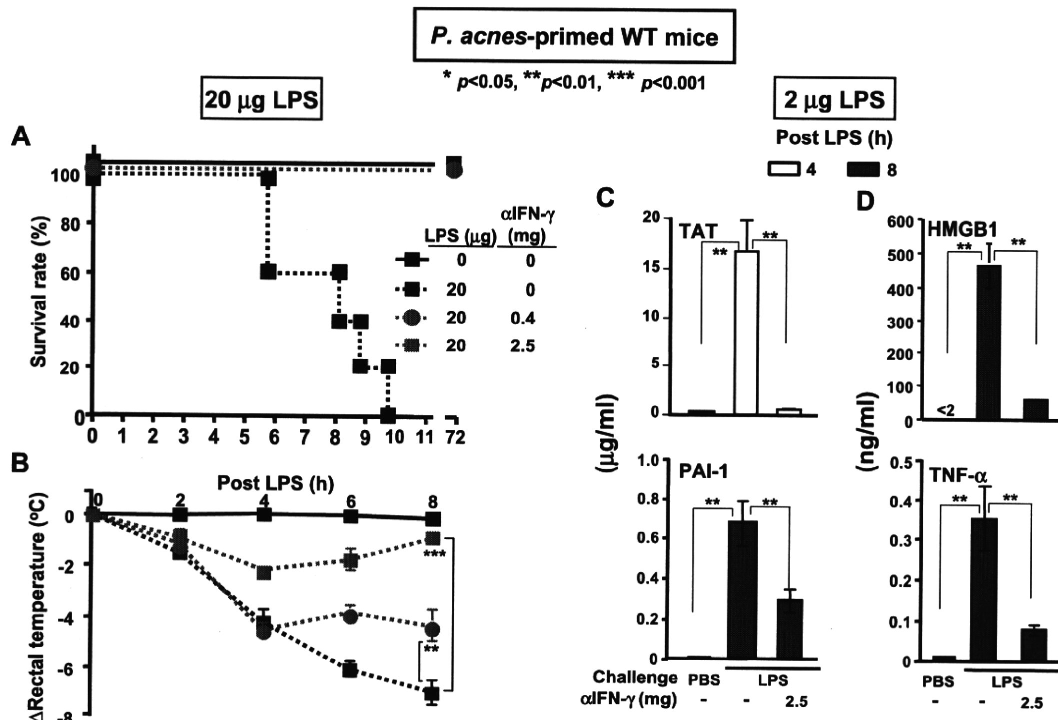

Fig. 6. IFN-γ profoundly controls endotoxin shock syndrome. *Propionibacterium acnes*-primed WT BALB/c mice were administered with various doses of neutralizing anti-IFN-γ 30 min prior to LPS challenge. Survival rate (A) and rectal temperature decrease (B) were monitored. At 4 h (open columns) or 8 h post LPS challenge, plasma was sampled for analysis of hyper-coagulation (TAT and PAI-1 concentrations) (C) and for measurement of HMGB1 and TNF-α concentrations (D).

shock syndrome. First, IFN-γ might modulate production of TNF-α after LPS challenge (Fig. 4E; Supplementary Figure 3, available at *International Immunology* Online). IFN-γ was reported to enhance TNF-α production induced by LPS (41). In fact, IFN-γ blockade significantly hampered plasma increase of TNF-α after LPS challenge (Fig. 6D). Second, although acting on the very late phase of *P. acnes* priming, this IFN-γ blockade may be able to desensitize the established sensitivity to LPS in *P. acnes*-primed WT mice. Third, IFN-γ and TNF-α might synergize for the development of each symptom. It is well established that IFN-γ and TNF-α synergize for production of various cytokines/chemokines, exemplified by IL-6 and CXCL10 (IP-10), via activating nuclear factors, such as STAT-1/IFN regulated factor 1 and NF-κB (41, 43). Likewise, the cooperative activation of these nuclear factors might control production of key factors involved in the development of each symptom.

HMGB1, originally discovered as a nuclear protein, was recently reevaluated as a potent late phase mediator of severe sepsis (32–34). HMGB1 levels are reported to elevate during severe sepsis in humans and animals. Furthermore, HMGB1 blockade prevents septic animals from lethality. We demonstrated that LPS induces an increase in the plasma level of HMGB1 in an IFN-γ-dependent manner (Figs 4D and 6D). As it is capable of inducing production of pro-inflammatory cytokines and chemokines in inflammatory cells, HMGB1 might be another potent target for the treatment of endotoxin shock syndrome.

PAI-1-induced hyper-coagulation seems to be beneficial for host defense against local bacterial invasion. Bacteria have unique proteolytic machinery for their successful invasion into mammalian host. Most important proteolytic proteins are plasminogen activators. Individual bacteria produce their own plasminogen activators, exemplified by streptokinase and staphylokinase produced by Group A *Streptococcus pyogenes* and *Staphylococcus aureus*, respectively. Bacterial plasminogen activators destroy host extracellular matrix barrier, allowing them to invade deeper into the host and finally to establish their infection (44). However, PAI-1-induced fibrin deposition surrounding the initial invasion sites might enclose the destroyed extracellular matrix and protect against bacterial translocation and dissemination by serving as a new barrier, eventually strengthening the efficient bacterial eradication. Therefore, local hyper-coagulation induced by IFN-γ might be regarded as a potent host defense weapon. In other word, immune cells produce IFN-γ, which protects host tissue from bacterial invasion by encapsulating them with thrombus. Thus, PAI-1 might be a potent host defense molecule induced by inflammatory and T_h1 responses. Indeed, $Pai1^{-/-}$ mice are highly susceptible to pneumonia induced by airway infection with *Klebsiella pneumoniae*, a Gram-negative bacterium (45).

IFN-γ and TNF-α are essential for host defense against various pathogens by activating phagocytes and inducing inflammation. Upon microbial infection, mammalian host produces appropriate amounts of these cytokines

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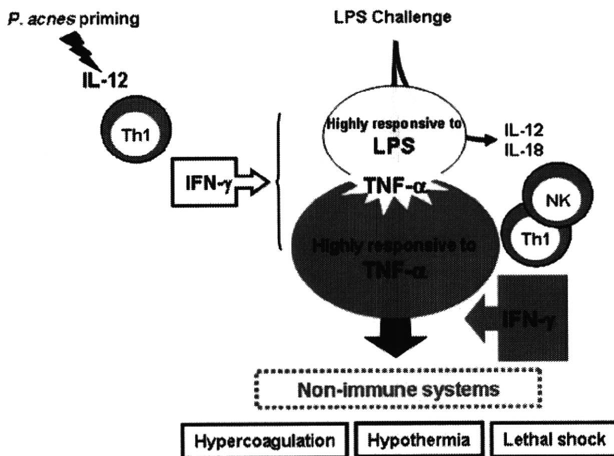

Fig. 7. A proposal model for the endotoxin shock in *Propionibacterium acnes*-primed mice. After priming with heat-killed *P. acnes*, macrophages produce IL-12, which causes T_h1 cell development. IFN-γ produced by the T_h1 cells prime macrophages to be highly susceptible to LPS. Besides, IFN-γ renders mice highly susceptible to TNF-α. After challenge of *P. acnes*-primed mice with LPS, IFN-γ-primed macrophages produce robust IL-12 and IL-18, which then activates T_h1 cells and NK cells to produce a large amount of IFN-γ. The IFN-γ-primed macrophages simultaneously produce enormous TNF-α. TNF-α, in turn, might act on the cells that become highly responsive to TNF-α after *P. acnes* priming to initiate the development of hypothermia, hyper-coagulation, systemic inflammatory responses and lethal shock. IFN-γ positively regulates the development of those clinical symptoms and signs. Thus, IFN-γ is a central factor that primarily controls both *P. acnes*-induced priming phase and the effector phase induced by LPS challenge.

under the proper control of cytokine activation cascades. For example, following *Listeria monocytogenes* infection, mice produce various pro-inflammatory cytokines, including TNF-α, IL-12 and IL-18, via recognizing listerial PAMPs by pattern recognition receptors such as TLR and Nod-like receptor (46, 47). IL-12 and IL-18 then induce production of IFN-γ, which in collaboration with TNF-α efficiently eliminates *L. monocytogenes*. Thus, appropriate amounts of IFN-γ and TNF-α are beneficial for the host. However, dysregulated production of or responsiveness to those cytokines often leads to diseases, exemplified by the endotoxin shock syndrome. Thus, IFN-γ might tip the balance of actions of TNF-α. Furthermore, IFN-γ primarily contributes to the development of severe liver injury induced by activation of a second cell death receptor Fas, as well. *Propionibacterium acnes*-primed mice, but not naive mice, develop massive liver injury after challenge with soluble Fas ligand (48), while *P. acnes*-primed *Ifnγ*$^{-/-}$ mice can evade this injury (our unpublished data). Thus, IFN-γ might play a central role in the development of severe illnesses and syndromes that are caused by activation of cell death receptors.

Endotoxin shock is a life-threatening condition. Thus, it is very important to determine the master regulator of endotoxin shock. Our present study could reveal that IFN-γ is a master regulator of endotoxin shock and neutralization of IFN-γ even just before LPS challenge could rescue animals from endotoxin shock. Many investigators revealed the molecular mechanisms how IFN-γ synergizes with LPS and/or TNF-α for induction of various gene expressions *in vitro* (41). However, it is still to be elucidated how endogenous IFN-γ synergizes with LPS and TNF-α for *in vivo* induction of hypothermia, hyper-coagulation and shock. Although we need extensive efforts to resolve this issue, we believe our data present key information on the treatment of endotoxin shock syndrome.

Supplementary data

Supplementary data are available at *International Immunology* Online.

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REVIEW ARTICLE

Importance of IL-18-Induced Super Th1 Cells for the Development of Allergic Inflammation

Kenji Nakanishi[1], Hiroko Tsutsui[2] and Tomohiro Yoshimoto[3]

ABSTRACT
Th1 cells, which express IL-18R, produce IFN-γ in response to Ag and IL-2 and increase further production of IFN-γ upon additional IL-18 stimulation. They simultaneously produce Th2 cytokines (IL-9 and IL-13), GM-CSF and chemokines (RANTES, MIP-1α). Human Th1 cells also produce IFN-γ and IL-13 in response to anti-CD3 and IL-18. Recently, we demonstrated Th1 cells induce intrinsic type atopic asthma and dermatitis by production of Th1- and Th2-cytokines and chemokines. Here, we review the pathological roles of Th1 cells, stimulated with Ag and IL-18 *in vivo*, in the pathogenesis of allergic disorders by production of Th1 and Th2 cytokines and chemokines. Based on this unique function of Ag- plus IL-18-stimulated Th1 cells, we proposed to designate them as "super Th1 cells".

KEY WORDS
allergic inflammation, atopic dermatitis, bronchial asthma, IL-18, super Th1

INTRODUCTION

Bronchial asthma is a complex syndrome characterized by airway hyperresponsiveness (AHR) and reversible airflow obstruction associated with airway inflammation and remodeling and occasional high serum level of IgE.[1-7] Th2 cells have been recognized as inducing bronchial asthma by production of Th2 cytokines.[1-10] Particularly, IL-13 is suggested to play a critical role in induction of AHR, eosinophilic infiltration, goblet cell metaplasia, and lung fibrosis.[9-11] In contrast, Th1 cells had been regarded to inhibit bronchial asthma by production of IFN-γ.[12-14] However, several studies have disclosed the disability of Th1 cell to suppress Th2 cell-induced AHR.[15-19] On the contrary, a combination of Th1 and Th2 cells or their products rather augment each activity to induce airway inflammation and AHR.[15,16,19]

We demonstrated recently that OVA (Ag) plus IL-18 acts on adoptively transferred OVA-specific memory type Th1 cells to induce airway inflammation and AHR in a naive host mouse.[20] Th1 cells, which express IL-18R, produce IFN-γ in response to OVA and increase further IFN-γ production in response to additional IL-18 stimulation.[21] Surprisingly, they simultaneously produce Th2 cytokines (e.g., IL-9 and IL-13), GM-CSF and chemokines (e.g., RANTES and MIP-1) when stimulated with OVA and IL-18.[20] Human Th1 cells also produce IFN-γ and IL-13 in response to anti-CD3 plus IL-18.[22] Recently, we demonstrated Th1 cells induce intrinsic atopic dermatitis by production of Th1 and Th2 cytokines and chemokines.[23] Thus, IL-18 has added its new function to its growing functional list.[24-26] Based on this unique function of Ag-plus IL-18-stimulated Th1 cells, we proposed to designate them as "super Th1 cells".[23]

THE MOLECULAR MECHANISM FOR IL-18 SECRETION

As *Il18*, like *Il1β*, lack leader sequence, *Il18* product pro-IL-18 cannot be secreted, but is stored intracellularly.[24,25,27,28] Many cell types exemplified by macrophages produce pro-IL-18 in the steady state.[24,27,28] Epithelial cells lining host body, such as respiratory epithelial cells, intestinal epithelial cells and keratinocytes can produce pro-IL-18 under normal conditions as well. Pro-IL-18 needs appropriate post-translational processing to become biologically active and to be ex-

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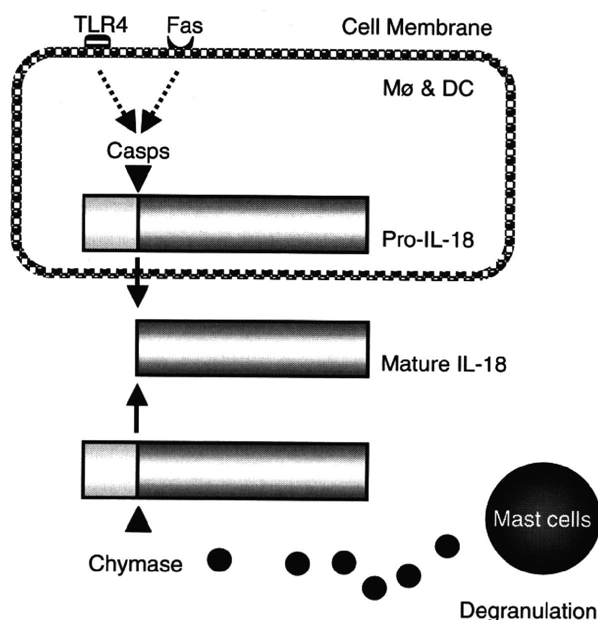

Fig. 1 Mechanisms involved in the processing and releasing IL-18. Macrophages (Mϕ) and dendritic cells are major cell sources of IL-18. The cells constitutively produce pro-IL-18. After stimulation through Toll-like receptor 4 (TLR4) and Fas, caspases (Casps) are activated for appropriately cleavage of proIL-18, resulting in the release of biologically active mature IL-18. Chymase degranulated from activated human mast cells can process pro-IL-18 into biologically active IL-18 as well.

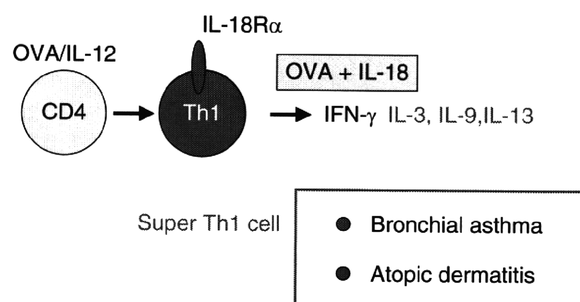

Fig. 2 Super Th1 cells. When they are activated with Ag together with IL-18, Th1 cells become to exert their actions as super Th1 cells by producing both Th1 and Th2 cytokines. Among the cytokines, IFN-γ and IL-13 are critical for the development of AHR and airway fibrosis, respectively.

tracellularly released (Fig.1).[27,28] Caspase 1 (Casp1) is an authentic processing enzyme for IL-18 and IL-1β.[27] Casp1 is also produced as enzymatically inactive zymogen in the cytoplasm and needs mutual cleavage to become active. Recently, the multiple protein complex named inflammasome is verified to be the platform for Casp1 activation.[29] Inflammasome is composed of Nod-like receptor (NLR), a cytoplasmic sensor, Casp1 activation adaptor ASC, pro-Casp1 and substrates such as pro-IL-18 and pro-IL-1β. Nalp3/NLRP3 is believed to senses extrinsic pathogen-associated molecular patterns (PAMPs). Indeed, after stimulation with LPS, Nalp3 inflammasome is promptly formed, followed by rapid processing of IL-1β and/or IL-18. Thus, microbial infection induces IL-18 and IL-1β release via activation of Nalp3 inflammasome. For example, in response to TLR4 agonist LPS, hepatic tissue macrophages secrete IL-18 and IL-1β in a manner dependent on Casp1, ASC and Nalp3.[30]

IL-18 processing might occur extracellularly as well. Recent report shows that chymase, an enzyme localized in the granules of mast cells, has capacity to cleave pro-IL-18 into biologically active IL-18 (Fig.1).[31] Since mast cells are accumulated into the skin lesion of mice with AD-like dermatitis,[23,26,32] chymase from the activated mast cells might exacerbate the skin inflammation by enhancing the local release of biologically active IL-18.

Epithelial cells are major cell source of various pro-allergic cytokines, such as IL-18, IL-25, TSLP and IL-33. As they are accumulated preferentially in the inflammatory sites of patients with Th2 type allergic diseases, IL-25, TSLP and IL-33 might be involved in the development of Th2 type allergy.[33-35] By contrast, epithelium-derived IL-18 might trigger infectious type of allergic diseases as described later, although the mechanism for secretion of IL-18 from the epithelial cells are still to be elucidated.[36,37]

SUPER TH1 CELLS AND ASTHMA

IL-18R expression levels determine the intensity of responsiveness to IL-18 and are quite distinct among Th cell subsets.[25,26] Naïve CD4+ T cells and Th2 cells express little IL-18R, while Th1 cells express high levels of it. Consistently, the amount of IL-4 produced by Th2 cells is not affected by additional stimulation with IL-18. In contrast, Th1 cells produce larger amounts of Th1 cytokines such as IFN-γ and TNF-α when additionally stimulated through their IL-18R (Fig.2). From these early studies IL-18 was regarded as the Th1 response-activating cytokine. Our resent study unveiled a second important property of IL-18 in the adaptive immunity. IL-18 has potential to render Th1 cells to produce Th2 cytokines.[20] Upon simultaneous engagement of TCR and IL-18R, Th1 cells become to produce abundant IL-3, IL-13 and IL-9, but still not IL-4, in addition to the Th1 cytokines. They also produce larger amounts of chemokines that can recruit various pro-atopic cells, including granulocytes, macrophages and lymphocytes. We designate these IL-13/IL-9/chemokine-producing Th1 cells as super Th1

cells (Fig.2).

What about *in vivo* role of super Th1 cells? Naïve mice transferred with OVA-specific Th2 cells that are generated from OVA-specific naïve DO11.10 CD4+ cells by *in vitro* incubation under Th2 condition, namely "Passive Th2 mice", expectedly develop asthmatic response upon intranasal OVA challenge.[20] They develop AHR, airway eosinophillia and goblet cell metaplasia of airway epithelial cells. Expectedly, IL-13 blockade can protect against the development of all of those manifestations. In contrast, "Passive Th1 mice", which are generated by the protocol similar to "Passive Th2 mice" except for *in vitro* incubation of naïve OVA-specific CD4+ cells under Th1 condition, do not show any asthmatic signs and/or symptoms after intranasal challenge with OVA alone. However, whenever challenged with OVA together with IL-18, "Passive Th1 mice" start to succumb to AHR, airway eosinophilia and peribronchial fibrosis, suggesting the possible activation of super Th1 cells. In contrast to Th2 type asthma observed in "Passive Th2 mice", IL-13 blockade prevents airway eosinophilic inflammation and peribronchial fibrosis, partly and profoundly, but entirely not AHR.[37] This AHR can be protected by IFN-γ blockade. Thus, super Th1 cells might be involved in the pathogenesis of certain types of allergic disorders by producing both IFN-γ and IL-13.

INFECTIOUS TYPE BRONCHIAL ASTHMA

It is well documented that microbial infection aggravates and/or triggers allergic diseases in human. For example, lower respiratory infection with rhinovirus, a common microbe relevant to cold, or with *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae*, common bacteria causative of community-acquired pneumonia, frequently provokes or exacerbates bronchial asthma in asthmatic patients.[38,39] Lesional skin infection with *Staphylococcus aureus* worsens the disease severity in patients with atopic dermatitis (AD). As microbial infection sometimes evokes IL-18 secretion,[25,26] we may assume that microbial products might cause local release of IL-18, which in turn triggers bronchial asthma by activation of super Th1 cells. As expected, murine bronchial epithelial cells can respond to LPS by releasing IL-18. "Passive Th1 mice" or wild-type mice immunized with OVA in Th1 adjuvant ("Active Th1 mice") show AHR, peribronchial eosinophilic inflammation upon intranasal challenge with OVA in combination with LPS, a cell-wall component of Gram-negative bacteria.[37] In sharp contrast, IL-18 blockade can rescue "Active Th1 mice" from these clinical manifestations after intranasal challenge with OVA and LPS. *Il18*-/- mice immunized with OVA in Th1 adjuvant can evade them after being similarly challenged.[37] Thus, endogenously produced IL-18 and exogenously administered OVA both might activate OVA-specific super Th1 cells, leading to the development of asthmatic manifestations in infectious type of asthma.

ATOPIC DERMATITIS INDUCED BY TOPICAL APPLICATION WITH STAPHYLOCOCCAL PRODUCT

Super Th1 cells are also highlighted in infectious type of AD in mice. Consecutive and topical application of protein A (SpA) purified from cell wall of *Staphylococcus aureus* induces AD-like pruritic dermatitis in mice with genetically impaired skin barrier function, NC/Nga mice.[23] CD4+ T cells purified from draining lymph nodes (DLN) of mice prior to the onset show the characteristics of Th1 cells. These cells produce Th1 cytokines (IFN-γ and TNF-α), but not Th2 cytokine (IL-4 and IL-13) upon TCR engagement. However, CD4+ DLN cells prepared from the mice post onset exhibit the feature as super Th1 cells. Keratinocytes freshly isolated from naive mice release IL-18 in response to SpA *in vitro*,[36] suggesting involvement of IL-18 in the in vivo development into super Th1 cells. In fact, IL-18 blockade and deletion of *Il18* rescue mice from the development of SpA-induced AD-like dermatitis, concomitant with prevention of their super Th1 cell development. Among cytokines produced by super Th1 cells IFN-γ and TNF-α are important. IFN-γ or TNF-α blockade prevents the development of this skin inflammation. Thus, IL-18-dependent super Th1 cell development is important for the development of this dermatitis.

CLINICAL EVIDENCE FOR IL-18

Accumulating evidence suggests positive relationship between IL-18 levels in the lesion or circulation and allergic diseases, such as asthma, allergic rhinitis and AD.[40-42] In particular, after inhalatory challenge test with flour allergens patients with occupational allergic asthma and/or rhinitis show a significant increase in IL-18 levels in nasal lavage fluid. Furthermore, *IL18* polymorphism that ensures higher production of IL-18 upon appropriate stimuli is preferentially accumulated in patients with allergic disorders.[43-45] Although no polymorphisms differed significantly in frequency between the control and adult asthma groups, functional polymorphism in IL-18 is associated with severity of adult bronchial asthma.[46] These results suggest association of IL-18 with allergic disorder in human. However, the molecular mechanism for IL-18 induction of differentiation from Th1 cells into Super Th1 cells is unclear. Nonetheless, possible therapeutics targeting IL-18 might be beneficial for inflammatory type of allergic disorders.

CONCLUDING REMARKS

One may accept that super Th1 cells are activated upon microbial infection of allergic lesion. What is a super Th1 cell subset? Do super Th1 cells, like Th1 cells, require the proper epigenetic regulation? If so,

what is a transcription factor essential for the differentiation into super Th1 cells, like T-bet/STAT4 for Th1 cells (Fig.2)?

Although we need further studies to settle those issues, targeting super Th1 cells and super Th1-associated cytokines might be of value in the therapy of severe, recurrent asthma and perhaps of infectious type allergic diseases. We previously generated human anti-human IL-18 mAb by the gene-manipulating technique.[47] This human-derived mAb targeting human IL-18 might be highlighted as a therapeutic agent against infectious type allergic diseases as well.

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Contribution of IL-18 to eosinophilic airway inflammation induced by immunization and challenge with *Staphylococcus aureus* proteins

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Abstract

We previously reported that intranasal challenge with ovalbumin (OVA) plus IL-18 induces airway hyperresponsiveness (AHR) and eosinophilic airway inflammation in mice with OVA-specific T_h1 cells. These two conditions can be prevented by neutralizing anti-IFN-γ and anti-IL-13 antibodies, respectively. The mice develop AHR and eosinophilic airway inflammation after challenge with OVA plus LPS instead of IL-18 and endogenous IL-18 is known to be involved. In contrast, IL-18 does not facilitate these changes in mice possessing OVA-specific T_h2 cells. Here, we investigated whether IL-18 is involved in the development of asthma in mice immunized and challenged with bacterial proteins. Upon intranasal exposure to protein A (SpA) derived from *Staphylococcus aureus*, mice immunized with SpA exhibited AHR and peribronchial eosinophilic inflammation if IFN-γ or IL-13 were present, respectively. The $CD4^+$ T cells from draining lymph nodes (DLNs) of the SpA-immunized and -challenged mice produced a robust IFN-γ and IL-13 in response to immobilized anti-CD3 antibodies. Treatment with neutralizing anti-IL-18 antibodies prevented asthmatic inflammation concomitant with their impaired potential to express IFN-γ and IL-13. Furthermore, naive mice that received the $CD4^+$ T cells from DLNs of SpA-immunized mice developed airway inflammation depending upon the presence of IL-18. Immunodeficient mice that received human PBMCs, which had been stimulated with SpA *in vitro*, developed dense peribronchial accumulation of human $CD4^+$ T cells upon SpA challenge. Neutralizing anti-human IL-18 antibodies protected against this airway inflammation. These results suggest the importance of IL-18 for the development of asthmatic inflammation associated with airway exposure to bacterial proteins.

Keywords: airway hyperresponsiveness, asthma, eosinophilic inflammation, IL-18, *Staphylococcus aureus*

Introduction

Bronchial asthma is complex syndrome characterized by airway hyperresponsiveness (AHR) and reversible airflow obstruction with airway inflammation and mucus formation (1–8). Bronchial asthma is believed to be mediated by T_h2 cells and their cytokines. IL-13 produced by the T_h2 cells can principally account for almost all the above pathogenic responses (2, 9). However, other subsets of $CD4^+$ T cells, such as T_h1, T_h17, regulatory T (Treg) and CD1d-restricted NKT cells, are now recognized to play a role in the modulation of airway allergic inflammation (10). T_h2 cell-directed therapy has limited

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efficacy (11), suggesting that bronchial asthma develops by diverse immunological mechanisms. Respiratory infections caused by bacteria frequently activate the T_h1-cell response through activation of Toll-like receptors (12, 13) and are associated with the initiation and/or exacerbation of bronchial asthma in humans (14, 15). These clinical studies strongly suggest that some types of bronchial asthma may be explained by the activation of T_h1-cell responses. However, intranasal challenge with ovalbumin (OVA) alone cannot evoke asthma in mice carrying OVA-specific T_h1 cells (16, 17), indicating that the T_h1-cell response alone is not sufficient enough to induce these pathological alterations. We have demonstrated that intranasal challenge with exogenous IL-18 or bacterial LPS induces IL-18 production in mice. In conjunction with OVA, this can induce robust asthma in mice immunized with OVA and the T_h1 adjuvant, CFA (16–18). OVA initiates OVA-specific T_h1 cells to produce IFN-γ but not IL-13, whereas OVA with IL-18 is capable of activating these T_h1 cells to produce larger amounts of IFN-γ, as well as IL-13, IL-9 and various chemokines that recruit eosinophils and other leukocytes (16). Persistent stimulation with IL-18 and the antigen alters the T_h1 cells, resulting in them producing both IFN-γ and IL-13 (19). With respect to their potential to produce both pro-inflammatory and pro-atopic cytokines/chemokines, we designated T_h1 cells that were re-stimulated with antigen and IL-18 as super T_h1 cells (16, 18, 19). In asthmatic mice possessing super T_h1 cells, IL-13 is responsible for the eosinophilic airway inflammation and remodeling. AHR is caused by IFN-γ, but not IL-13 (16, 17), which is in contrast to T_h2 cell-initiated asthmatic alterations where IL-13 plays a common and critical role (20, 21). Thus, IL-18 is likely to be involved in the development of bacterial infection-associated asthma. However, it is entirely unknown whether bacteria or their products by themselves can trigger super T_h1 cell type bronchial asthma.

The site of a pathogenic infection often determines the phenotype of infection-associated atopic diseases presumably by recruiting and activating pathogen-specific effector T cells and by inducing IL-18 release from the site. Infection with bacteria such as *Staphylococcus aureus* sometimes exacerbates atopic dermatitis in humans (22). We recently observed that consecutive and topical application of *S. aureus* protein A (SpA) (23) induces atopic dermatitis-like skin alterations in naive NC/Nga mice that have a genetically impaired skin barrier (19). The $CD4^+$ T cells prepared from the DLNs of mice with SpA-induced dermatitis express a cytokine profile characteristic of super T_h1 cells. Administration of neutralizing anti-IL-18 antibodies protects against dermatitis as well as super T_h1-cell development (19). Based on these observations, we assumed that the mice carrying SpA-specific T_h1 cells were highly vulnerable to asthma upon intranasal challenge with SpA. To test this hypothesis, we generated a novel asthmatic inflammation mouse model to determine the requirement of IL-18 in the development of SpA-induced asthma. Severely immunodeficient mice that had been inoculated with SpA-stimulated human PBMCs exhibited airway inflammation following intranasal challenge with SpA. Treatment with neutralizing anti-human IL-18 antibodies prevented this airway inflammation. Thus, IL-18 could be a potential target for the treatment of asthmatic inflammation associated with bacterial infection.

Methods

Animals and reagents

Female BALB/c mice and BALB/c *nu/nu* mice were purchased from CLEA Japan (Osaka, Japan). C57BL/6 background $Rag2^{-/-}C\gamma^{-/-}$ mice were from Taconic Farms (Hudson, NY, USA). All animals were bred and/or maintained in specific pathogen-free conditions at the animal facilities of Hyogo College of Medicine and were used at 6–10 weeks of age. Animal experiments were performed in accordance with the guidelines of the National Institutes of Health, as specified by the animal care policy of Hyogo College of Medicine. SpA from *S. aureus* Cowan I was purchased from CalbioChem (La Jolla, CA, USA). Recombinant murine IL-18 was purchased from MBL (Nagoya, Japan). Anti-mouse CD3ε mAb (2C11), anti-mouse CD4 mAb (GK1), anti-human CD4 mAb (RPA-T4) and anti-human CD45 mAb (HI30) were from BD Biosciences Pharmingen (San Diego, CA, USA). Neutralizing anti-IFN-γ mAb was partly purified from the ascites fluid collected from BALB/c *nu/nu* mice inoculated intraperitoneally with hybridoma 6A2 purchased from the American Type Culture Collection (Manassas, VA, USA) (17, 19). Soluble IL-13Rα2-Fc was purchased from R&D Systems (San Diego, CA, USA) (17, 19). Rabbit polyclonal anti-mouse IL-18 antibodies were prepared in our laboratory (19). We generated a neutralizing anti-human IL-18 mAb as described previously (24).

Induction of asthma

The experimental protocol for asthma induction was the same as described in our previous report except we used SpA instead of OVA (17) (Supplementary Figure 1 is available at *International Immunology* Online). Briefly, BALB/c mice were immunized with SpA (500 μg) in CFA, followed by a boost with SpA in incomplete Freund's adjuvant (IFA) at day 14. For the adoptive cell transfer study, $CD4^+$ T cells isolated from DLNs of the immunized and boosted mice were labeled with 5-carbozyfluorescein diacetate succinimidyl ester (CFSE) and 6-CFSE. The CFSE-labeled cells (1×10^7) were administered intravenously into naive BALB/c mice (17). Two weeks following the SpA boost or after $CD4^+$ T cell transfer, mice were exposed intranasally to 50 μl of SpA (250 μg) in PBS for three consecutive days. In some experiments, neutralizing anti-mouse IL-18 antibodies (500 μg) were injected intraperitoneally into the mice at 1 day before and 1 day after intranasal exposure to SpA (17, 19). Anti-IFN-γ mAb (100 μg) or IL-13R2α-Fc (20 μg) was intranasally administered as outlined previously (17). Mice were sacrificed at 24 h after the final intranasal exposure of SpA.

Invasive measurement of AHR

Invasive measurement of AHR was assessed as an increase in pulmonary resistance (RLung) in response to aerosolized β-methacholine as described previously (17). RLung was measured by Pulmos-II (MIPS, Osaka, Japan) hardware and software (MIPS).

Preparation of $CD4^+$ lymph node cells and lung homogenate

$CD4^+$ T cells from the DLN were purified by magnetic-activated cell sorting (17). Lungs were homogenized with

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1 ml of lysis buffer according to the method described previously (19, 25).

Cytoplasmic staining for IFN-γ and IL-13

Cells were isolated from mediastinal lymph nodes of SpA-immunized mice after consecutive 3-day challenge with SpA and were incubated with immobilized anti-CD3 mAb and 100 U ml^{-1} of IL-2 in the presence or absence of rmIL-18 (100 ng ml^{-1}) for 48 h. Cytoplasmic staining of the cells for IFN-γ and IL-13 were performed using antigen-presenting cells (APC)-anti-CD4 mAb (RM4-5), FITC-anti-IFN-γ mAb (XMG1.2) and PE-anti-IL-13 mAb (eBio13A).

Preparation of human PBMCs

PBMCs from healthy volunteers (26) were cultured with 100 μg ml^{-1} of SpA for 4 days. Experimental protocols for the use of human PBMCs were approved by the College Review Board of Hyogo College of Medicine.

Establishment of mice implanted with human PBMCs

SpA-stimulated human PBMCs (1×10^7) were transplanted intravenously into *Rag2*$^{-/-}$*Cγ*$^{-/-}$ mice (27). One-week post-transplantation, we isolated lymphocytes from the peripheral blood and spleen of the recipient mice and analyzed proportions of human $CD45^+$ cells in each preparation by flow cytometry. We used mice that contained >5% human $CD45^+$ cells in their peripheral blood because they also contained > 10% dual $CD45^+/CD4^+$ cells in their spleen (described below). The mice that received human SpA-stimulated PBMCs were then exposed intranasally to SpA for three consecutive days. In order to block the action of human IL-18, anti-human IL-18 mAb (300 μg) was intranasally administered 1 h before SpA exposure. Twenty-four hours following the final administration of SpA, lungs were sampled for histological and confocal microscopic studies.

Preparation of bronchoalveolar lavage fluid

Bronchoalveolar lavage fluid (BALF) was collected (17) and total cell number was determined in each sample. Cytospin preparations of BALF were stained with Dif-Quik (Baxter Healthcare Corp., Miami, FL, USA). Eosinophils and neutrophils were distinguished from each other by their difference in staining.

Histology

Lung specimens were fixed in 10% buffered formalin and sections were stained with hematoxylin and eosin (17). Fields of view on a microscope were selected at random and printed in large scale to distinguish eosinophils from other cell types. Eosinophils and the total number of nucleated cells in each field of view were counted. The mean ± SD of 10 fields of view per sample were calculated.

Confocal laser microscopic analysis

Frozen sections were fixed and incubated with FITC- or PE-conjugated mAb, followed by evaluation using a laser confocal microscope (model IX81; Olympus, Tokyo, Japan) (19).

Detection of cytokines and chemokines

Concentrations of IL-4, IL-13, tumor necrosis factor-α and IFN-γ in culture supernatants were determined with appropriate ELISA kits (Genzyme, Cambridge, MA, USA). Mouse IL-18 was measured by an ELISA kit from MBL. The concentrations of various mouse chemokines were measured with a Bio-Plex Cytokine assay kit (Bio-Rad, Hercules, CA, USA).

Statistics

Three to five mice were used for each experimental group. Data are expressed as the mean ± SD of triplicate samples. Significance between experimental and control groups was determined via an unpaired Student's *t*-test. A *P* value <0.05 was considered significant. Two to three experiments were performed per assay, and the representative data were shown.

Results

SpA-induced asthmatic inflammation

We examined whether intranasal challenge with SpA induces asthma-like airway inflammation in SpA-immunized mice. We immunized BALB/c mice subcutaneously with SpA in the T_h1 adjuvant, CFA, followed by a booster with SpA in IFA 2 weeks later. Twenty-eight days after the initial immunization, we administered SpA through a nasal tract for three consecutive days and examined the severity of asthmatic inflammation by measuring AHR, analyzing BALF preparations and lung histology (Supplemental Figure 1 is available at *International Immunology* Online). Invasive measurement of AHR revealed that SpA-immunized mice exhibited substantial AHR upon intranasal SpA challenge (Fig. 1A). None of the mice exhibited AHR after treatment with PBS (Fig. 1A) and naive mice were free from AHR even after SpA challenge (Fig. 1A). Thus, SpA immunization and SpA challenge are both required for the development of AHR. Following intranasal challenge with SpA, SpA-immunized mice demonstrated an increase in the number of eosinophils and neutrophils in BALF (Fig. 1B). These increases were not observed after treatment with PBS (Fig. 1B). The severity of AHR, consistent with our previous observations (16, 17), coincided with the cell numbers of eosinophils in BALF. This was also the case for the density of eosinophilic inflammation around the airway. Histological analysis revealed that only SpA-immunized mice developed severe inflammation around the airway following challenge with SpA, but not with PBS (Fig. 1C, E and F). Furthermore, intranasal challenge with SpA, but not PBS or OVA, induced eosinophilia (Supplementary Figure 2 is available at *International Immunology* Online), suggesting that SpA works in an antigen-specific manner. Naive mice exhibited only modest lung inflammation, if any, after intranasal SpA challenge (Fig. 1D). Eosinophils accumulated around the airway of the SpA-immunized and -challenged mice but not in mice treated with the other combinations of immunogens (Fig. 1J). Taken together, these results indicate that SpA-immunized and -challenged mice fulfill the clinical signs of asthmatic inflammation and are a suitable mouse model for bacterial infection-associated asthma-like inflammatory illnesses.

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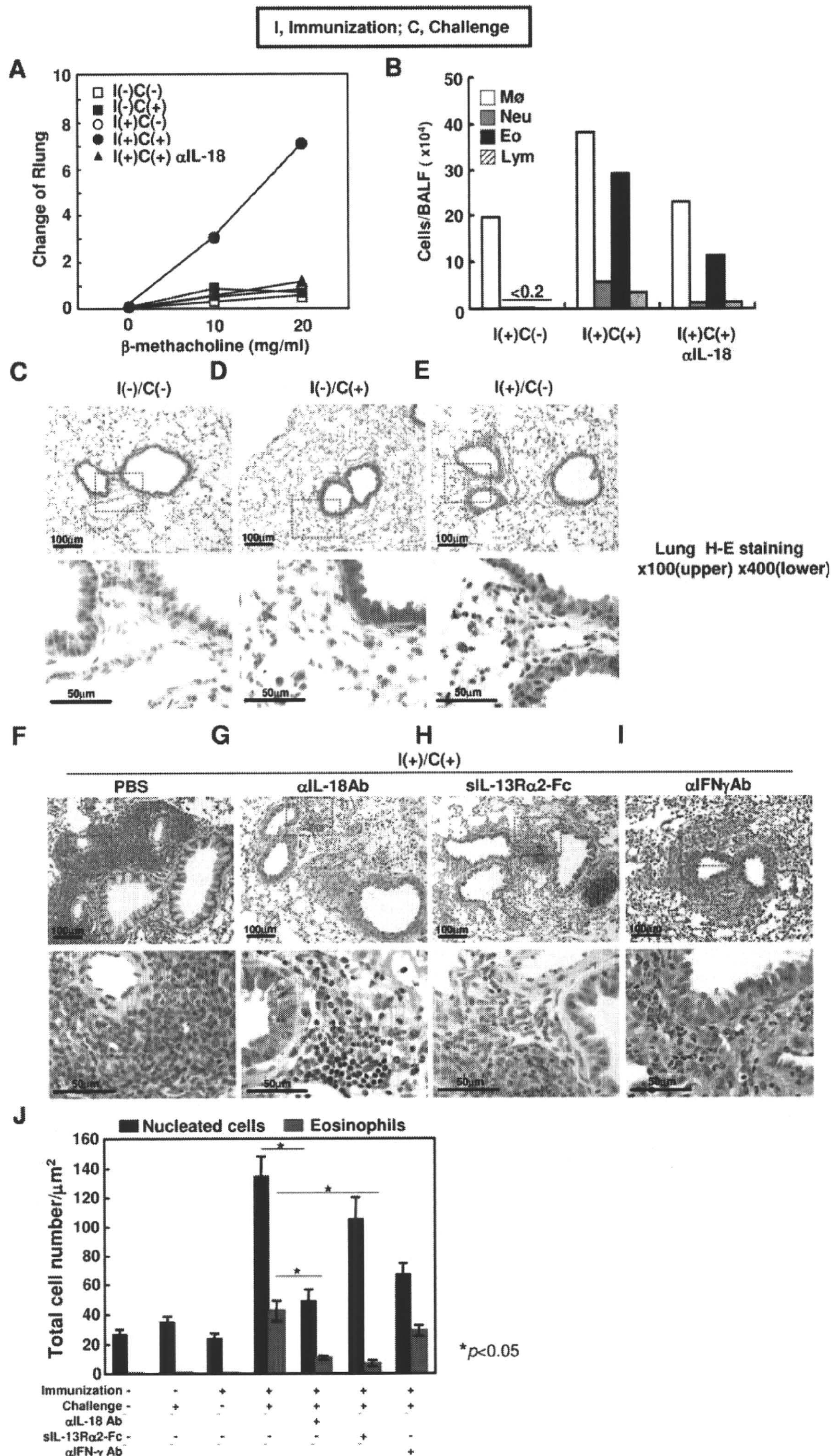

Fig. 1. Requirement of endogenous IL-18 for SpA-induced bronchial asthma. SpA-immunized mice 'I(+)' or naive mice 'I(−)' were intranasally challenged with SpA (250 μg/50 μl) 'C(+)' or PBS (50 μl) 'C(−)'. Neutralizing anti-IL-18 antibodies (500 μg) (A, B, G and J), soluble IL-13R2α-Fc (sIL-13Rα-Fc) (20 μg) (H) or neutralizing anti-IFN-γ (αIFNγ antibody) (100 μg) was administered twice into SpA-immunized mice intravenously, (I) 1 day before or after 3-day intranasal challenge with SpA (αIL-18 antibody). Twenty-four hours after the last SpA challenge, invasive measurement of AHR (A), cellular analysis of BALF (B) and histological analysis of lung specimens (C–I) were performed. (C–I) Upper panels are at low magnifications, and the lower panels are high magnification images of the areas indicated by a red-dotted square in the corresponding

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Requirement of IL-18 for SpA-induced asthma

As previously reported, SpA-induced atopic dermatitis develops in an IL-18-dependent manner (19). We investigated whether IL-18 plays a pivotal role in the development of SpA-induced asthmatic inflammation. To test this, we administered neutralizing anti-IL-18 antibodies into the SpA-immunized mice 1 day before and 1 day after the initial intranasal SpA challenge (Supplementary Figure 1 is available at *International Immunology* Online). Administration of neutralizing anti-IL-18 antibodies profoundly reduced AHR (Fig. 1A) and significantly hampered respiratory inflammation and eosinophilia (Fig. 1B, G and J). Consistently (2, 9, 16), blocking the action of IL-13, but not IFN-γ, protected against eosinophilia in the airway (Fig. 1H–J). Conversely and consistently, blockade of IFN-γ, but not of IL-13, prevented AHR (16) (Supplementary Figure 3 is available at *International Immunology* Online). It would appear that endogenous IL-18 seems to be important in the development of SpA-induced asthmatic airway inflammation due to IL-13 production.

Super T_h1-cell differentiation

As IL-13 was profoundly involved in inflammation of the airways (Fig. 1H and J), we examined whether this experimental immunization/challenge protocol induces the development of $CD4^+$ DLN cells into super T_h1 cells or into IL-13-secreting T_h2 cells. We stimulated $CD4^+$ DLN cells with immobilized anti-CD3 mAb and measured the concentrations of T_h2 and super T_h1 cytokines. $CD4^+$ DLN cells from SpA-immunized and -challenged mice produced larger amounts of IFN-γ and IL-13, but little IL-4, compared with SpA-immunized mice without SpA challenge (Fig. 2A), indicating their development into super T_h1 cells, but not T_h2 cells, during intranasal SpA challenge. Furthermore, we examined whether both IL-13 and IFN-γ are produced by a single $CD4^+$ cell isolated from SpA-challenged and SpA-immunized mice. We isolated cells from mediastinal lymph nodes of SpA-challenged and SpA-immunized mice and incubated the cells with plate-bound anti-CD3 in the presence or absence of exogenous IL-18. We found a very small proportion of $IL\text{-}13^+IFN\text{-}\gamma^+$ $CD4^+$ T cells after TCR stimulation alone (Fig. 2B). However, upon TCR and IL-18 stimulation, the proportion of $IL\text{-}13^+IFN\text{-}\gamma^+$ $CD4^+$ T cells was significantly elevated (Fig. 2B), suggesting that super T_h1-cell differentiation occurs in SpA-immunized mice after intranasal challenge with SpA. At the same, this stimulation induced an increase in two other populations: IL-13-produicng cells and IFN-γ-producing cells (Fig. 2B). Thus, three populations, consisting of IL-13-produicng cells, IFN-γ-producing cells and IL-13 plus IFN-γ-producing cells, contribute to induction of SpA-induced bronchial asthma.

Next, we investigated the roles of endogenous IL-18 in super T_h1-cell development. $CD4^+$ DLN cells prepared from the mice additionally treated with anti-IL-18 antibodies produced much less IFN-γ and IL-13 than those from SpA-induced asthmatic mice (Fig. 2A), suggesting the possibility that IL-18 release during SpA challenge participates in super T_h1-cell differentiation. To test this possibility, we examined whether IL-18 is produced in the asthmatic lung. SpA immunization alone failed to induce significant increase in IL-18 concentration within the lung tissue (Fig. 2C). SpA immunization and challenge seemed to increase IL-18 levels significantly in the lung (Fig. 2C). These results suggest that airway constituents such as respiratory epithelial cells and/or alveolar macrophages might release IL-18 in response to SpA.

Induction of chemokines attracting eosinophils and neutrophils in the lungs

As IL-18 is capable of inducing chemokine production from epithelial cells, T_h1 cells and super T_h1 cells (16, 18), we examined whether IL-18 could induce expression of chemokines in the lung, particularly chemokines recruiting eosinophils and neutrophils, during intranasal SpA challenge. Lung homogenates from mice immunized with SpA only contained almost basal amounts of chemokines attracting eosinophils, including CCL5 (RANTES) and CCL11 (Eotaxin), and neutrophils, such as CXCL1 (KC) and CCL2 (MCP-1), when compared with naive mice (Fig. 3). However, it was only after SpA challenge that pulmonary levels of CCL5, CCL11, CCL2 and CXCL1 were significantly elevated (Fig. 3). This was also the case for the chemokines attracting diverse types of leukocytes, such as, CCL3 (MIP-1α) and CCL4 (MIP-1β), as well as the pro-inflammatory cytokines, IL-1β and IL-6 (Fig. 3). In contrast, T_h2 cytokines, IL-4 and IL-5 were not induced after SpA challenge (Fig. 3). As expected, treatment with neutralizing anti-IL-18 antibodies during intranasal exposure to SpA significantly reduced chemokine expression levels (Fig. 3). Thus, the expression of these chemokines could be induced by IL-18.

Importance of SpA-activated super T_h1 cells in the development of airway inflammation

We examined whether super T_h1 cells are effector cells of SpA-induced asthmatic inflammation. To test this, we transferred $CD4^+$ DLN cells from SpA-immunized mice into naive mice, followed by intranasal administration of SpA for three consecutive days. Upon daily exposure to PBS, mice receiving the $CD4^+$ DLN cells demonstrated an intact response to methacholine treatment and evaded airway inflammation (Figs 1A and C and 4A and C). Upon exposure to SpA, these mice exhibited obvious AHR (Fig. 4A) and airway inflammation (Fig. 4C), prompting us to investigate whether donor $CD4^+$ DLN cells migrated into the airway as a response to SpA challenge in order to exert their effector functions. We labeled the donor cells with CFSE, injected them into naive recipient mice and analyzed their localization in the recipient lung after SpA challenge. Many CFSE-labeled cells had migrated into the lung (Fig. 4B). Most of the

upper panels. Upper scale bars indicate 100 μm, while the lower ones represent 50 μm. Lung sections were stained with hematoxylin and eosin (H&E). The mean ± SD of total nucleated cells per square micrometer (black bars) and eosinophils per square micrometer (red bars) in 10 fields of view selected at random are shown (J). Mø, macrophages; Neu, neutrophils; Eo, eosinophils; Lym, lymphocytes. Data are representative of three independent experiments with five mice per group.

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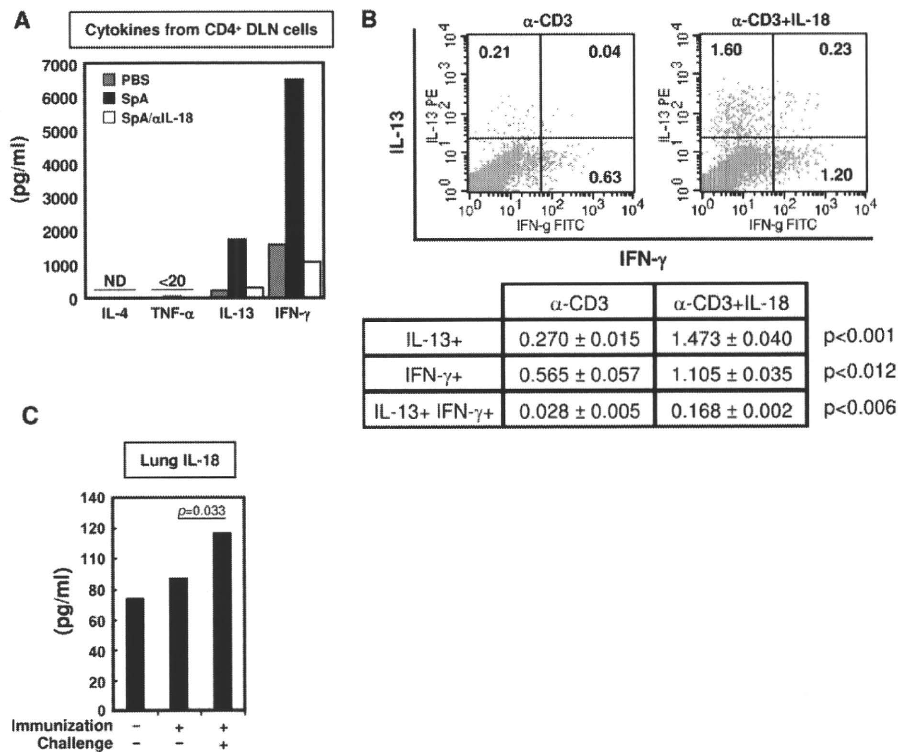

	α-CD3	α-CD3+IL-18	
IL-13+	0.270 ± 0.015	1.473 ± 0.040	p<0.001
IFN-γ+	0.565 ± 0.057	1.105 ± 0.035	p<0.012
IL-13+ IFN-γ+	0.028 ± 0.005	0.168 ± 0.002	p<0.006

Fig. 2. IL-18-dependent differentiation toward super T_h1 cells. (A) DLN cells were prepared from variously treated mice as shown in the legend to Fig. 1A, and CD4$^+$ T cells (1×10^5) were incubated on immobilized anti-CD3ε mAb. Various cytokine concentrations in each supernatant were measured by ELISA. (B) Mediastinal lymph node cells were prepared from SpA-challenged SpA-immunized mice and were incubated with immobilized anti-CD3ε mAb alone (α-CD3) or anti-CD3ε mAb plus IL-18 for 48 h, followed by cytoplasmic staining for IFN-γ and IL-13. (C) Lung homogenates were prepared from naive mice, SpA-immunized mice and SpA-immunized and -challenged mice, for the measurement of IL-18 by ELISA. Data are representative of three independent experiments with five mice in each group.

pulmonary CD4$^+$ T cells co-expressed CFSE (Fig. 4B), indicating that the donor CD4$^+$ T cells but few of the recipient cells, accumulated in the lung. Upon exposure to PBS, few CFSE-labeled cells or CD4$^+$ T cells were observed in the recipient lung (data not shown). These results demonstrate that SpA-specific CD4$^+$ DLN cells migrate and are fully activated after being exposed to SpA, eventually leading to the development of asthmatic inflammation. Blocking the action of IL-18 protected the mice from AHR and airway inflammation (Fig. 4A and C), suggesting that SpA-induced IL-18 in the airway in combination with administered SpA might differentiate the donor cells toward super T_h1 cells, thereby becoming highly pathogenic effector cells.

Involvement of human IL-18 in SpA-induced airway inflammation in transiently humanized mice

Finally, we investigated whether IL-18 is a therapeutic target for the treatment of airway inflammation in humans, associated with bacterial infection. First, we tried to generate mice transiently carrying human immune competent cells. We incubated PBMCs from healthy donors with SpA *in vitro*, transferred them into immunodeficient mice without T cells, B cells and NK cells and investigated whether human PBMCs settled in the recipient mice by calculating proportions of human CD45$^+$ hematopoietic cells (27) in peripheral immune tissues. Human CD45$^+$ cells were robustly observed in the spleen and peripheral blood of the recipient mice at day 7 after PBMC transfer (Fig. 5). About one-third of human CD45$^+$ cells co-expressed the human CD4 marker (Fig. 5).

Like the CD4$^+$ DLN cell-transplanted mice (Fig. 4), the SpA-stimulated humanized mice developed airway inflammation upon intranasal challenge with SpA, concomitant with dense accumulation of human CD4$^+$ T cells around the airway (Fig. 6C and D). This result indicated that SpA-stimulated human CD4$^+$ T cells migrated into the airway and presumably evoked pulmonary inflammation in response to exogenous SpA and endogenous IL-18. Upon PBS exposure, however, the host mice showed weak airway inflammation with modest but apparent accumulation of human CD4$^+$ cells (Fig. 6A and B). We investigated the role of human IL-18 and confirmed that anti-human IL-18 mAb (24) potently neutralized human IL-18 (Supplementary Figure 4 is available at *International Immunology* Online). This mAb prevented SpA-induced airway inflammation in these mice by attenuating airway accumulation of human CD4$^+$ T cells (Fig. 6E and F). Therefore, human cell-derived IL-18 might fully activate human SpA-specific T_h1 cells to become pathogenic effector cells. Indeed, human PBMCs could release super T_h1 cell-inducing cytokines, such as IL-12 and IL-18, and super T_h1 cytokines, such as IL-13 and IFN-γ, in response to SpA *in vitro* (Supplementary Figure 5 is

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