(NUMTs) (Richly and Leister, 2004). The contigs (File version; assembly 2007_05_08.gz) of *E. tenella* were retrieved from the Wellcome Trust Sanger Institute, and the whole mt sequence obtained in this study was used as query under cut-off conditions of >50 nucleotides and >95% identity. We identified 21 NUMTs and confirmed these by direct sequencing (Supplementary Table 3). Two NUMTs, Emt3 and Emt4 occurring in contigs 00028951 and 00029260, respectively (Supplementary Table 1B), were used for Southern blot hybridization analysis against *E. tenella* DNA. Copy number of *E. tenella* mt genome was estimated using dot blot hybridization. Briefly, DNA fragments of the mt genome and the contig (contig_00029260) were amplified by PCR using specific primers (Supplementary Table 1C), and DNA amount was measured. Serial known dilutions of control PCR products were dot-blotted onto a nylon membrane, following heat denaturation (99 °C, 10 min). Genomic DNA were electrophoresed on agarose gels and then transferred to a nylon membrane. A PCR product specifically amplified from target regions of the *E. tenella* mt genome (Supplementary Table 1B) was labeled as described. Chemiluminescence signals were quantitated using LAS-4000mini.

3. Results and discussion

3.1. Mitochondrial genome organization

We obtained a mt genome sequence (6213 bp) from *E. tenella*, in which three protein-coding genes, *cob*, *cox1* and *cox3*, and 12 fragments of the large subunit (LSU) rRNA gene and 7 fragments of the small subunit (SSU) rRNA gene were identified (Fig. 1A). These genes and rRNA gene fragments are also present in the *P. falciparum* (Fig. 1B) (Feagin et al., 1997), although gene arrangements greatly differ between the two mt genomes.

Southern blot hybridization with a *cox3* probe (Emt1) against undigested DNA produced a smeared signal from around 4 kb to 20 kb. Hybridization against DNA digested with *Hind*III gave a major band at 6.2 kb which tailed-off to lower contiguous fragments. Hybridization against DNA digested with *Pvu*II yielded a clear signal at 2.6 kb. These signal sizes matched to those predicted from the *E. tenella* mt sequence (Fig. 2A and C). A *cox1* probe (Emt2) gave similar results (not shown). Southern blot hybridization using a *P. falciparum* probe (Pmt1) revealed a smeared signal from 6 kb to 23 kb against *P. falciparum* undigested DNA, a distinct band at 1.3 kb against *Hind*III-digested DNA, and a predominant 6.0 kb single band which tailed-off to a smear against *Pvu*II-digested DNA (Fig. 2B and D); yielding a similar hybridization pattern to *E. tenella*. This suggests that the *E. tenella* mt genome structure is similar to that of *P. falciparum*. The long tailing-off smears observed in both *E. tenella* and *P. falciparum* were not found in the mt genomes of *Babesia* and *Theileria* (Hikosaka et al., 2010), which have monomeric linear structures. The tailing-off smears probably reflect DNA fragments of various sizes branching off from polydispersed linear DNA molecules with various length termini (Preiser et al., 1996), which seems to be characteristics of a polydispersed concatenated mtDNA. In *E. tenella*, this tailing was somewhat longer than in *P. falciparum*, probably due to fragmentation caused by repeated freeze–thawing to disrupt the oocyst wall, which is highly rigid and not permeable to common solvents used for disruption. The absence of specific restriction fragments smaller than 6.2 kb after digestion with single-site enzymes suggests that the ends of the linear concatemers are not defined by telomere-like unique sequences, as seen in *Babesia* and *Theileria* (Kairo et al., 1994; Hikosaka et al., 2010). These results strongly suggest that the bulk of *E. tenella* mtDNA consist of polydisperse head-to-tail tandem arrays of the 6.2 kb element as in *P. falciparum*.

3.2. Phylogeny

The ML tree of concatenated COX1 and COB amino acid sequences revealed monophyly of the genera *Babesia* and *Theileria*, and of the genus *Plasmodium* with high BP values of 98 and 89%, respectively (Fig. 3A). *E. tenella* was positioned close to *Plasmodium* with a moderate BP value (75%). The ML tree of LSU sequences showed the same topology to that of the *cox1*+*cob* tree (Supplementary Fig. 1): *E. tenella* positioned close to *Plasmodium* with a low BP value (55%). ML tree using SSU and LSU sequences of the apicoplast genome, however, yielded a topology with *E. tenella* and *T. gondii* branching off from a common ancestor of *Plasmodium* and *Babesia/Theileria* with 100% BP (Fig. 3B). Thus, topologies of the mt trees and the apicoplast tree are not consistent. The inconsistency was not due to differences in the number of taxa used for tree construction because BP value changed little (76%) even when the number of taxa in the *cox1*+*cob* tree was reduced to the same as the apicoplast tree (data not shown).

The two mt tree topologies are also not consistent with phylogenetic trees constructed using 18S rRNA gene or hundreds of protein-coding nuclear genes (Morrison and Ellis, 1997; Philippe et al., 2004; Kuo et al., 2008), whereas the apicoplast tree is consistent with trees of nuclear genes. Since the positions of *E. tenella* in the two mt trees were not well supported with high BP values, we tested other possibilities of *E. tenella* position. The KH, the SH or the AU tests did not reject these alternative positions of *E. tenella* placed at a common ancestor of *Plasmodium* and *Babesia/Theileria* (arrow a in Fig. 3A) or at a common ancestor of *Babesia/Theileria* (arrow b in Fig. 3A) (Supplementary Table 4). Phylogenetic position of *E. tenella*, thus, remains unresolved with the mt dataset. *Eimeria* and other intestinal coccidians belong to the class Coccidea, and *Plasmodium* and *Babesia/Theileria* belong to the class Haematozoea. The two classes show remarkably different life cycles (Hausmann and Hülsmann, 1996). This taxonomical classification is consistent with phylogenetic trees

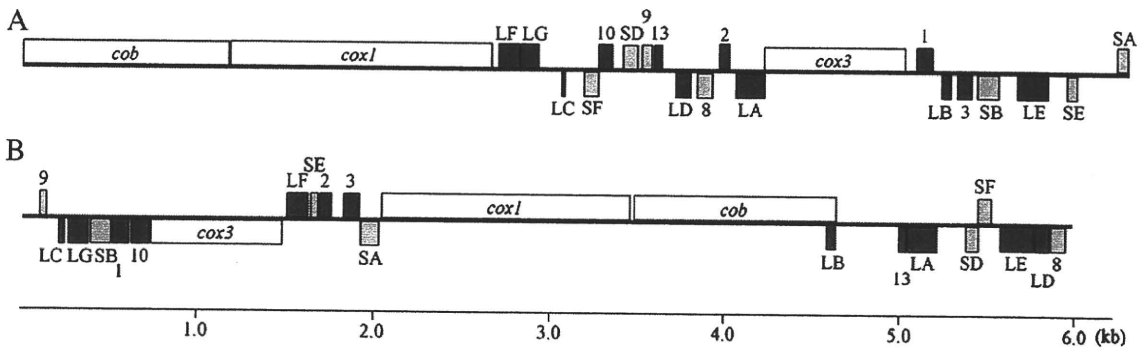

Fig. 1. Mitochondrial genome structure of *Eimeria tenella* (A) and *Plasmodium falciparum* (B). Genes shown above the bold line in each genome have predicted transcriptional directions from left to right; and those below, from right to left. Because the 6.2 kb element of *E. tenella* mt genome is tandemly repeated, both termini are arbitrary. For details refer to GenBank accession numbers AB564272 and M76611. White boxes indicate protein-coding genes (*cox1*, *cox3* and *cob*); fragments of LSU (LA–LG, 1, 2, 3, 10 and 13) and SSU (SA, SB, SD–SF, 8 and 9) rRNA genes are shown by dark and light gray boxes, respectively.

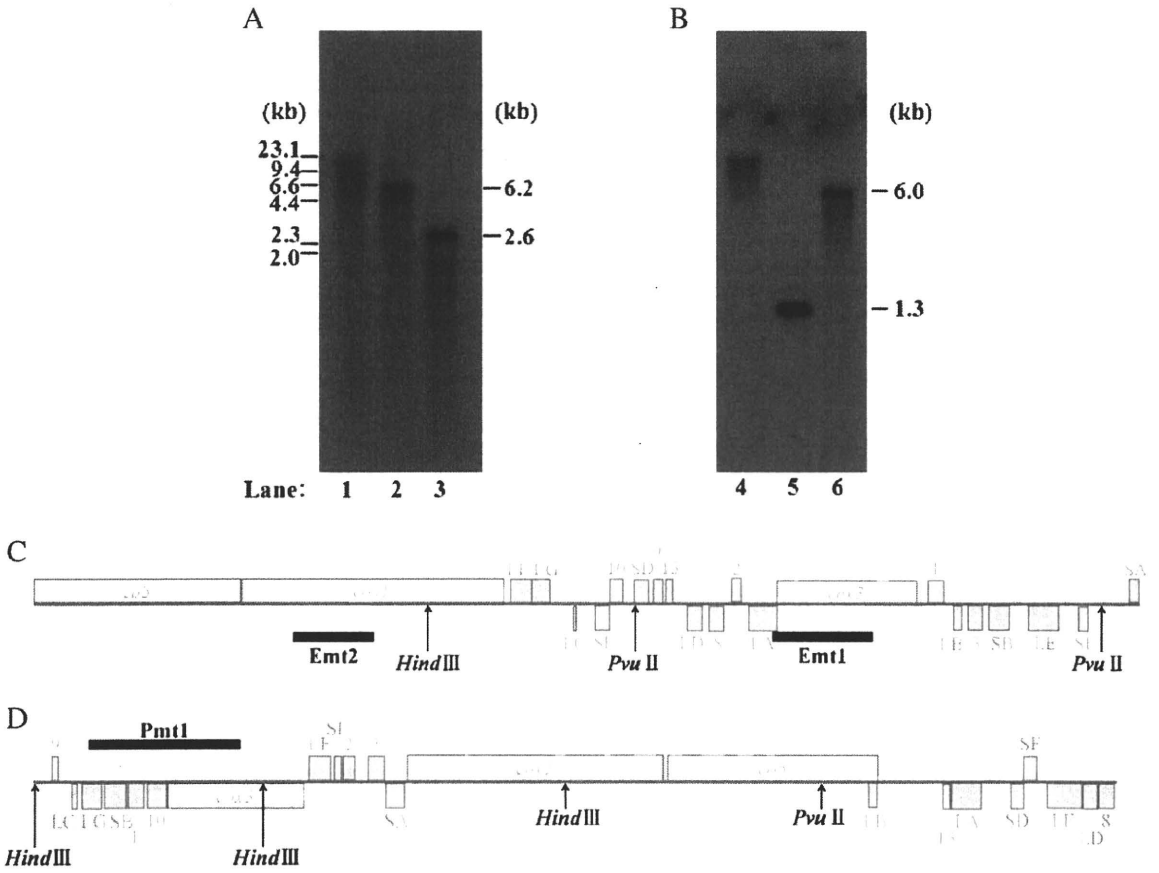

Fig. 2. Southern blot hybridization showing the mitochondrial (mt) genomes of *Eimeria tenella* (A) and *Plasmodium falciparum* (B). *E. tenella* probes (Emt1 and Emt2) and a *P. falciparum* probe (Pmt1), whose positions are shown in (C) and (D), were hybridized against undigested DNA of *E. tenella* and *P. falciparum*, respectively (lanes 1 and 4) and DNA digested with *Hind*III (lanes 2 and 5) or *Pvu*II (lanes 3 and 6).

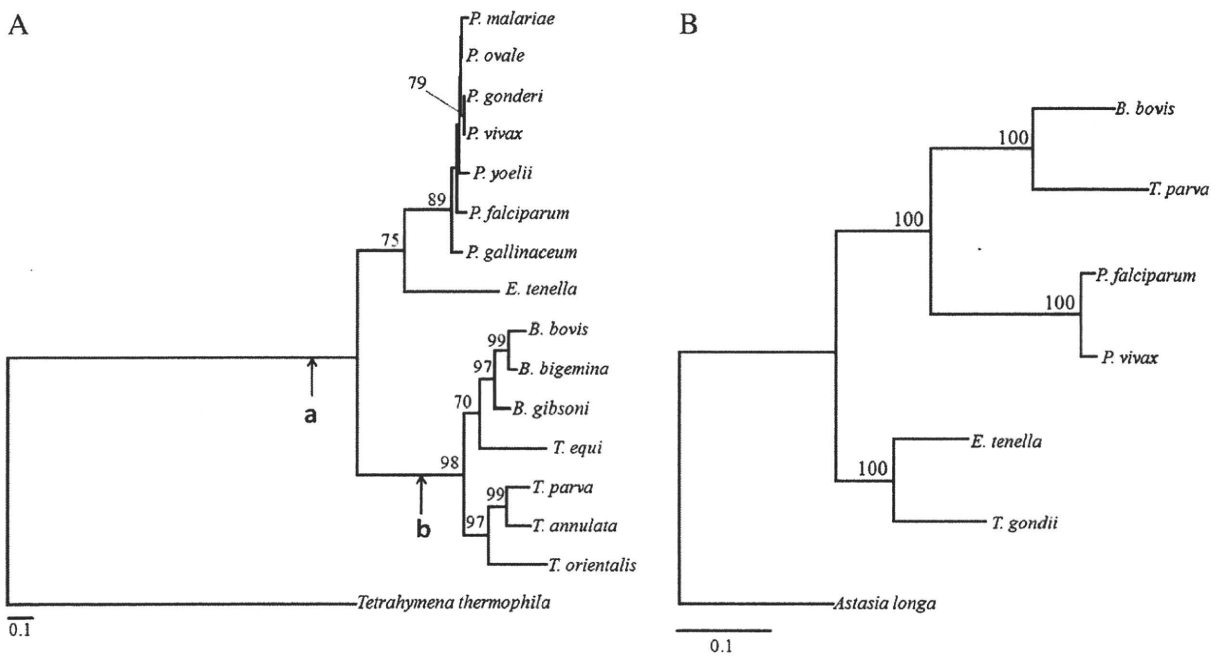

Fig. 3. Maximum likelihood phylogenetic trees of mitochondrial genes, *cox1* and *cob*, from *Plasmodium*, *Eimeria tenella*, *Babesia* and *Theileria* with *Tetrahymena thermophila* as an outgroup (A); and of apicoplast small and large subunits (SSU and LSU) of rRNA genes from six apicomplexan species with *Astasia longa* as an outgroup (B). For *cox1*+*cob* tree, concatenated amino acid sequences (755 sites) were used with 1000 heuristic replicates under a Jones, Taylor, and Thornton model (Jones et al., 1992) ($\alpha = 0.86$). For apicoplast SSU+LSU tree, concatenated nucleotide sequences (3045 sites in total: 1037 bp for SSU; 2008 bp for LSU) were used with 1000 heuristic replicates under a GTR+Γ model ($\alpha = 1.22$). Numbers shown along nodes represent bootstrap values. Arrows a and b indicate alternative positions of *E. tenella*. Both possibilities were statistically compared by the SH, KH and AU tests.

constructed using nuclear genes (Morrison and Ellis, 1997; Philippe et al., 2004; Kuo et al., 2008) and the apicoplast genome tree (Fig. 3B). We therefore consider it likely that *E. tenella* branched off from a common ancestor of *Plasmodium* clade and *Babesia/Theileria* clade.

Adopting this phylogenetic relationship allows us to infer a scenario for evolutionary trajectory of the mt genome structure of apicomplexans. Since both *Eimeria* and *Plasmodium* possess concatenated mtDNA, a common ancestor of these two parasites might have had a concatenated form of the mt genome, and the monomeric linear mt genomes of *Babesia/Theileria* were generated in the lineage. The present finding that *Eimeria* has the same 19 rRNA gene fragments as seen in the *Plasmodium* mt genome supports this scenario. Although we favor this scenario, we cannot completely rule out the possibility that a monomeric linear structure was an ancestral form and concatenated genome structures of *Eimeria* and *Plasmodium* evolved independently in each lineage. The likelihood is supported by *Tetrahymena* which has linear mt genomes, similar to those found in mt genomes of *Babesia/Theileria*. However, evolutionary distance between *Tetrahymena* and Apicomplexa is too far to gain insights into an ancestral form of the apicomplexan mt genome and, likewise, changes in molecular architecture of mt genomes are very frequent (Nosek and Tomaska, 2003). Nevertheless, it should be noted that mt genome architecture is conserved and does not change frequently within genus of the phylum: thus, in apicomplexan mt genome sequences available to date, all eight *Babesia/Theileria* species have the form of linear structure (Hikosaka et al., 2010), and all 23 *Plasmodium* species have the form of concatemer structure (Hikosaka et al., unpublished data). This within-genus stability of mt genome structure should allow us to infer an ancestral form of the apicomplexan mt genomes. In order to clarify evolutionary trajectory of the mt genome of Apicomplexa, further analysis of mt genomes of algae, closely related to apicomplexans such as *Chromera velia* and CCMP3155 (an undescribed species) would be required (Janouskovec et al., 2010).

3.3. Nuclear mitochondrial DNAs (NUMTs) in E. tenella

Blast search identified 21 sequence segments similar to the *E. tenella* mtDNA with lengths from 51 to 146 nucleotides in the *E. tenella* contigs (Supplementary Table 5). In contigs containing multiple NUMTs, several NUMTs were found arrayed in direct junction or in close proximity. Southern blot hybridization using probes Emt3 and Emt4, which contain NUMTs, gave signals derived from the *E. tenella* mt genome. We were, however, unable to detect signals derived from the nuclear genome by a similar procedure (Supplementary Fig. 2). In contrast, a probe specific to the nuclear genome (Enu1) hybridized at predicted sizes against either undigested DNA or DNA digested with *HindIII* or *EcoRI*, when a large amount of gDNA was used (data not shown). Copy number estimation analysis using Southern hybridization showed that *E. tenella* cells contained around 50 copies of the 6.2 kb element per haploid nuclear genome. The failure of detecting NUMTs in the nuclear genome with Emt3 and Emt4 was thus likely due to this copy number difference, with potential signals from the nuclear genome being masked in a smear of mtDNA.

4. Conclusion

This study suggests that the mt genome of ancestral apicomplexan parasites had a concatenated structure containing 19 rRNA gene fragments as well as three protein-coding genes and that the monomeric linear mt genome of *Babesia/Theileria* was generated in the lineage of the genera. Elucidation of a molecular mechanism, by which a linear mt genome with terminal inverted repeats on both ends was established, should help to further understand the evolution and divergence of mt genomes.

Supplementary data to this article can be found online at doi:10.1016/j.mito.2010.10.003.

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IFN-γ is a master regulator of endotoxin shock syndrome in mice primed with heat-killed *Propionibacterium acnes*

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Abstract

Hyper-coagulation, hypothermia, systemic inflammatory responses and shock are major clinical manifestations of endotoxin shock syndrome in human. As previously reported, mice primed with heat-killed *Propionibacterium acnes* are highly susceptible to the action of LPS to induce tumour necrosis factor (TNF)-α and to that of TNF-α to trigger lethal shock. Here we investigated the mechanisms underlying the *P. acnes*-induced sensitization to LPS and TNF-α and the development of individual symptoms after subsequent challenge with LPS or TNF-α. *Propionibacterium acnes*-primed wild-type (WT) mice, but not naive mice, exhibited hyper-coagulation with elevated levels of thrombin–antithrombin complexes and anti-fibrinolytic plasminogen activator inhibitor 1 in their plasma, hypothermia, systemic inflammatory responses and high mortality rate after LPS or TNF-α challenge. *Propionibacterium acnes* treatment reportedly induces both T_h1 and T_h17 cell development. *Propionibacterium acnes*-primed *Il12p40*$^{-/-}$ and *Ifnγ*$^{-/-}$ mice, while not *Il17A*$^{-/-}$ mice, evaded all these symptoms/signs upon LPS or TNF-α challenge, indicating essential requirement of IL-12–IFN-γ axis for the sensitization to LPS and TNF-α. Furthermore, IFN-γ blockade just before LPS challenge could prevent *P. acnes*-primed WT mice from endotoxin shock syndrome. These results demonstrated requirement of IFN-γ to the development of endotoxin shock and suggested it as a potent therapeutic target for the treatment of septic shock.

Keywords: hyper-coagulation, hypothermia, sepsis, T_h1 cells, TNF-α

Introduction

Disseminated intravascular coagulation (DIC), a status of hyper-coagulation, is commonly associated with septic shock syndrome (1, 2), in which tumour necrosis factor (TNF)-α produced by innate immune cells stimulated with pathogen-associated molecular pattern (PAMP) plays a critical role. Intravenous injection of recombinant human TNF-α activates coagulation system in healthy human and baboon, which is monitored by the elevation of plasma levels of thrombin–antithrombin complexes (TAT) (3, 4). Furthermore, this treatment increases levels of plasminogen activator inhibitor 1 (PAI-1), which promotes a procoagulant status by inhibiting the action of tissue-type plasminogen activator (tPA) to convert plasminogen into fibrinolytic plasmin (3–5). Thus, the balance between PAI-1 and tPA regulates coagulation, and induction of PAI-1 is crucial for the development of prothrombotic state by diminishing plasmin-dependent fibrinolysis. Indeed, plasma PAI-1 levels have been reported to correlate well with the disease severity of septic shock patients with DIC (6–8). We and other investigators reported that formation of intestinal adhesion is also regulated by the balance between PAI-1 and tPA (9, 10). We recently found that the PAI-1 induction is up-regulated by the action of IFN-γ from intestinal NKT cells in post-operative adhesion formation (10). Furthermore, IFN-γ from T_h1 cells was reported to be essential for the formation

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

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

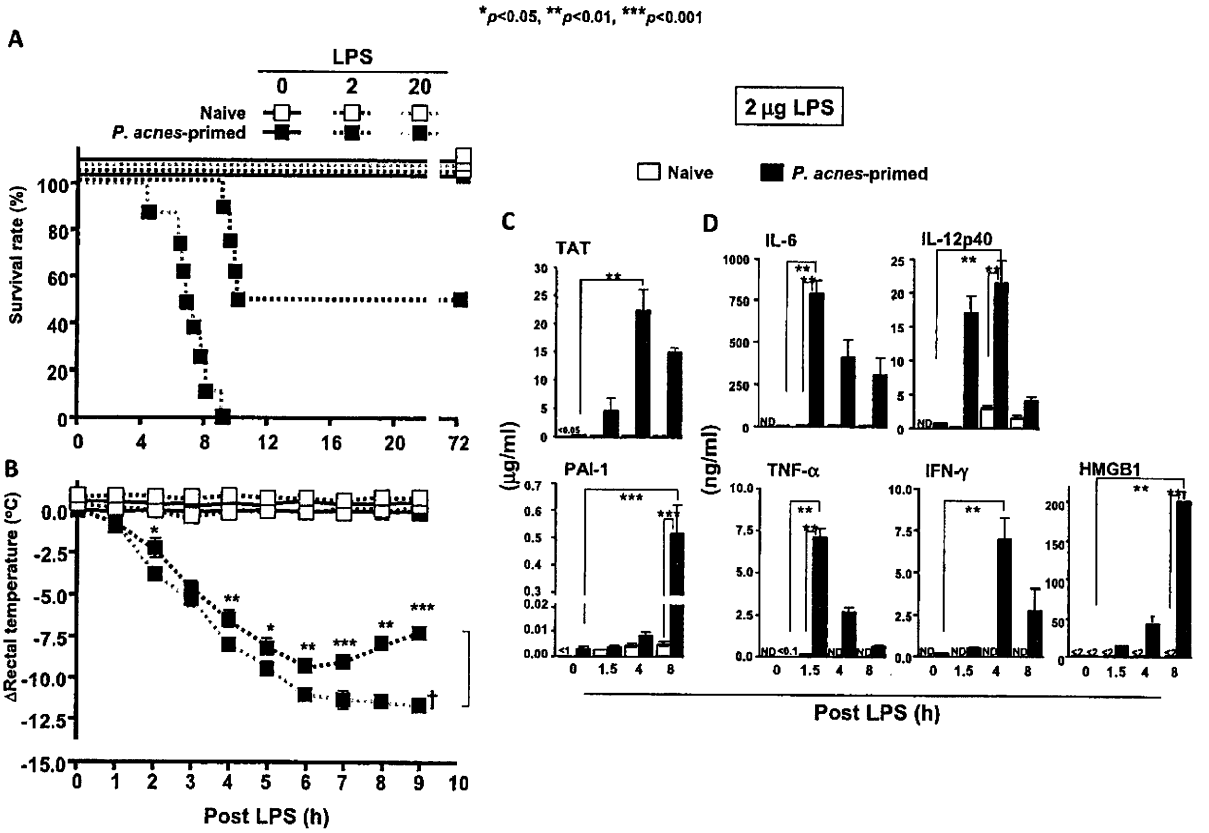

Fig. 1. Increase of the *in vivo* susceptibility to LPS by priming with heat-killed *Propionibacterium acnes*. WT BALB/c mice were administered with heat-killed *P. acnes*. *Propionibacterium acnes*-primed mice were challenged with 20 or 2 μg of LPS. Survival was monitored until 72 h (A). Rectal temperatures 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.

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

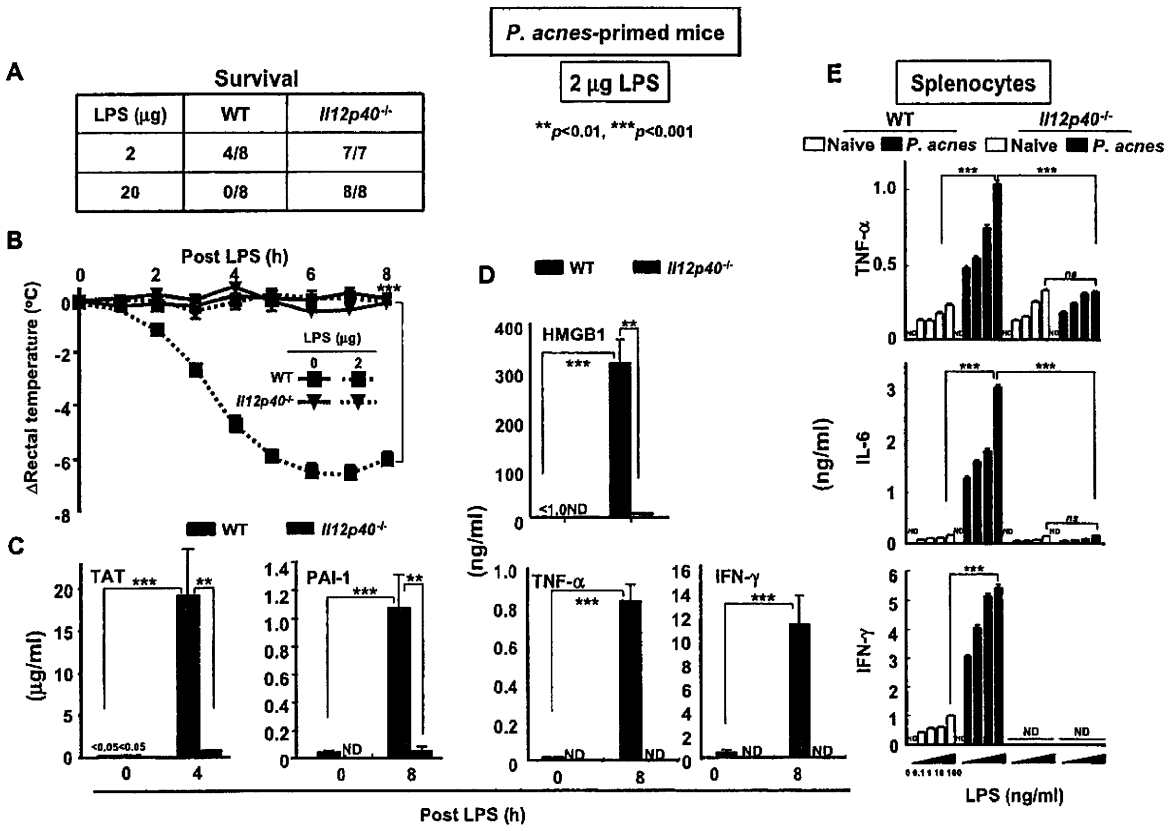

Fig. 2. Requirement of IL-12p40 for *Propionibacterium acnes*-induced LPS sensitization. *Propionibacterium acnes*-primed WT BALB/c mice or Il12p40$^{-/-}$ mice were challenged with 2 μg LPS. Survival rate (A) and rectal temperatures (B) were monitored until 72 and 8 h, respectively. At the indicated 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).

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*

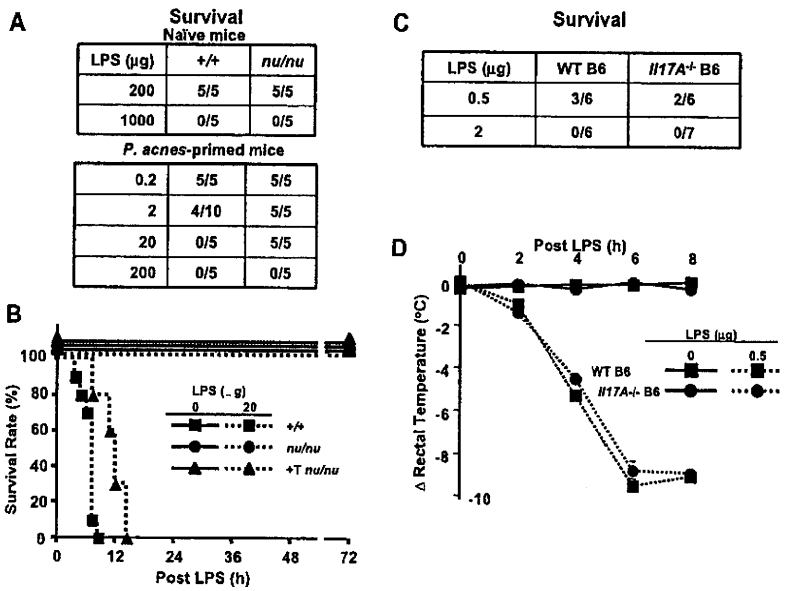

Fig. 3. Importance of T_h1 cells but not T_h17 cells for the LPS sensitization. Naive and *Propionibacterium acnes*-primed BALB/c WT (+/+) mice and nu/nu mice were administered with various doses of LPS, and mouse survival was monitored until 72 h (A). *nu/nu* mice reconstituted with T cells from WT 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.

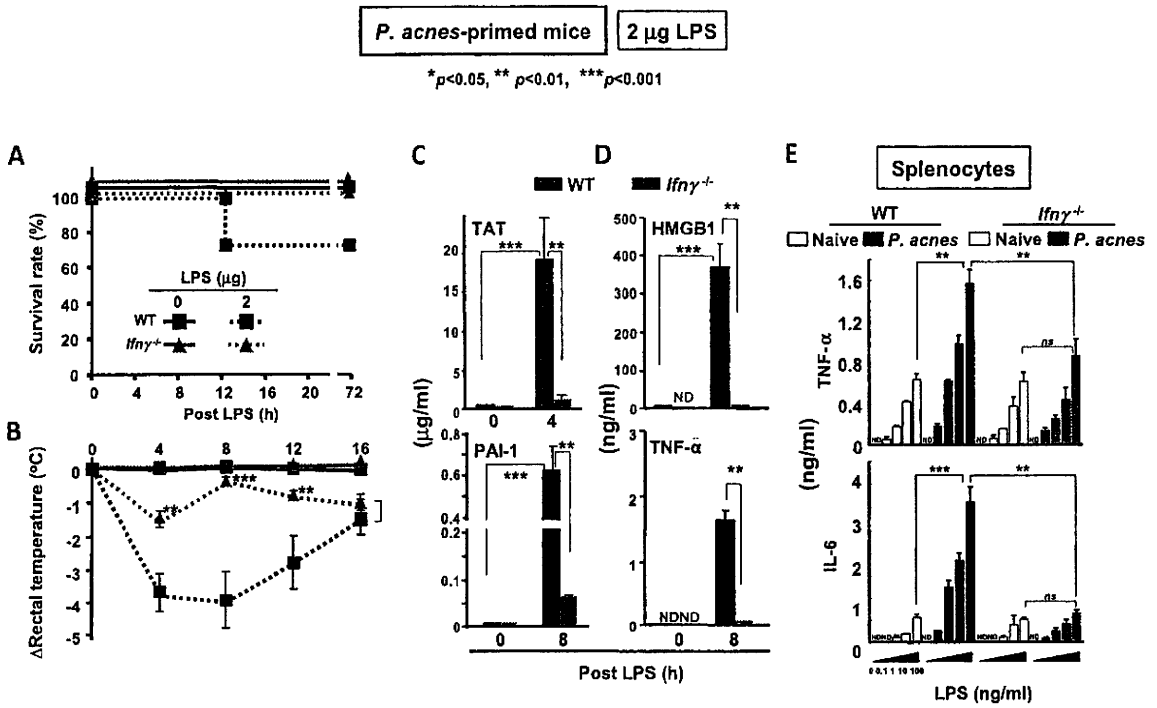

Fig. 4. *Propionibacterium acnes*-primed *Ifnγ*$^{-/-}$ mice were resistant to LPS. *Propionibacterium acnes*-primed WT mice (gray columns or symbols) and *Ifnγ*$^{-/-}$ mice (green columns or symbols) were administered intravenous with 2 μg LPS. Survival rates were monitored (A). Rectal temperatures 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

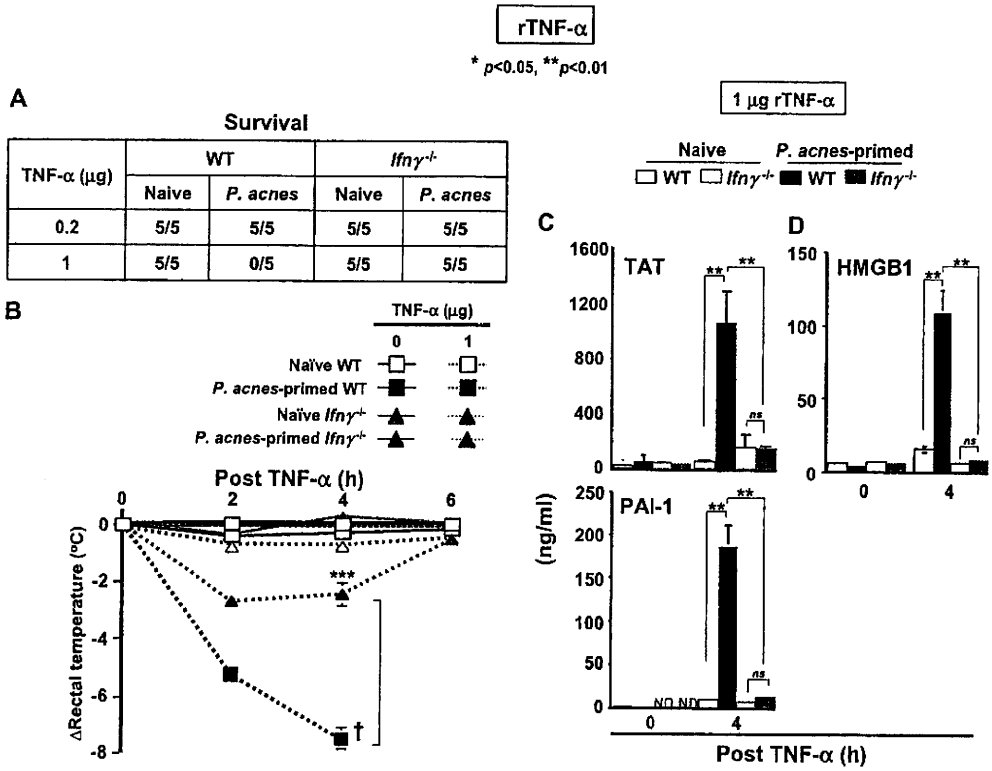

Fig. 5. Importance of IFN-γ for *in vivo* sensitization to TNF-α. *Propionibacterium acnes*-primed or naive WT mice and *Ifnγ*$^{-/-}$ mice were administered intravenous with 0.2 or 1 μg rTNF-α. Survival rates were monitored until 24 h after TNF-α challenge (A). Rectal temperatures were measured (B). 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

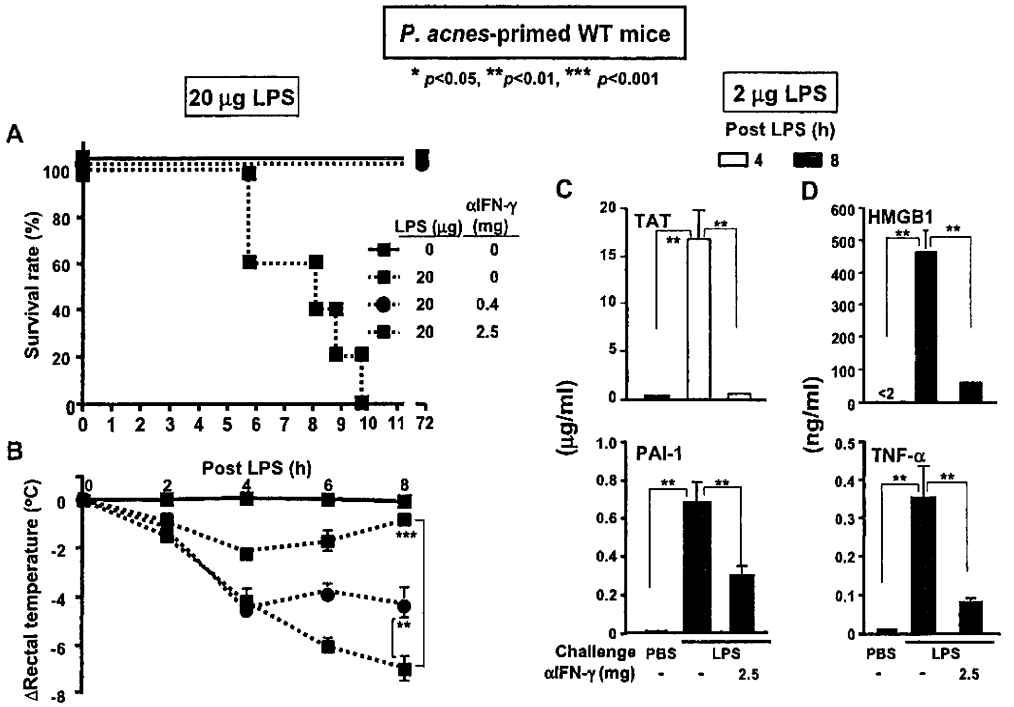

Fig. 6. IFN-γ profoundly controls endotoxin shock syndrome. *Propionibacterium acnes*-primed WT BALB/c mice were administered with various doses of neutralizing anti-IFN-γ 30 min prior to LPS challenge. Survival rate (A) and rectal temperature decrease (B) were monitored. At 4 h (open columns) or 8 h 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

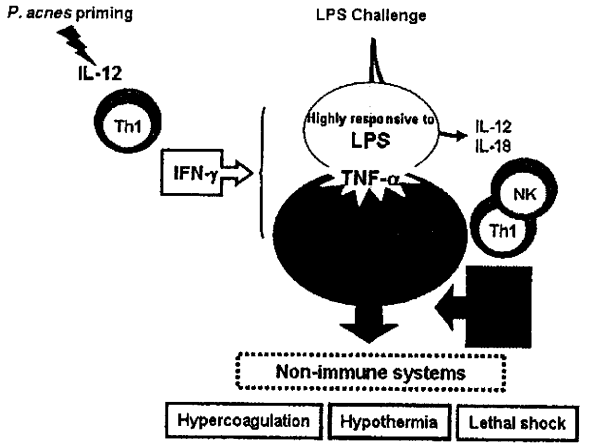

Fig. 7. A proposal model for the endotoxin shock in *Propionibacterium acnes*-primed mice. After priming with heat-killed *P. acnes*, macrophages produce IL-12, which causes T_h1 cell development. IFN-γ produced by the T_h1 cells prime macrophages to be highly susceptible to LPS. Besides, IFN-γ renders 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

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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 *Il1*β, 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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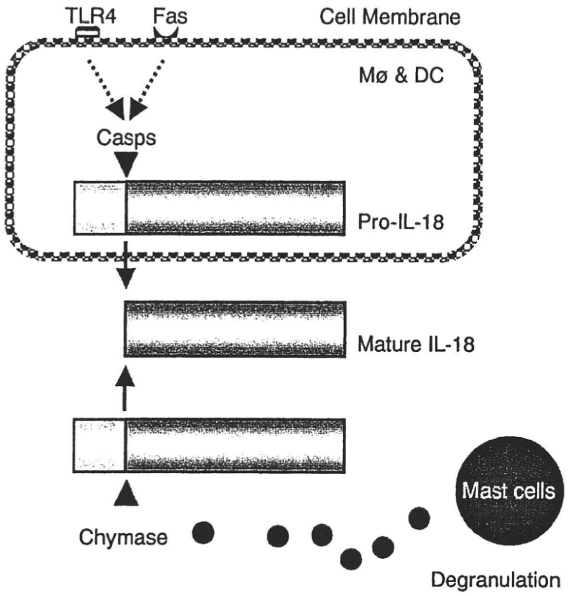

Fig. 1 Mechanisms involved in the processing and releasing IL-18. Macrophages (Mϕ) and dendritic cells are major cell sources of IL-18. The cells constitutively produce pro-IL-18. After stimulation through Toll-like receptor 4 (TLR4) and Fas, caspases (Casps) are activated for appropriately cleavage 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.

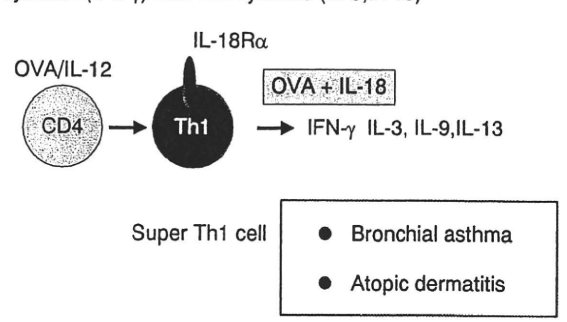

Fig. 2 Super Th1 cells. When they are activated with Ag together with IL-18, Th1 cells become to exert their actions as super Th1 cells by producing both Th1 and Th2 cytokines. Among the cytokines, IFN-γ and IL-13 are critical for the development of AHR and airway fibrosis, respectively.

tracellularly released (Fig.1).[27,28] Caspase 1 (Casp1) is an authentic processing enzyme for IL-18 and IL-1 β.[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-1 β 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