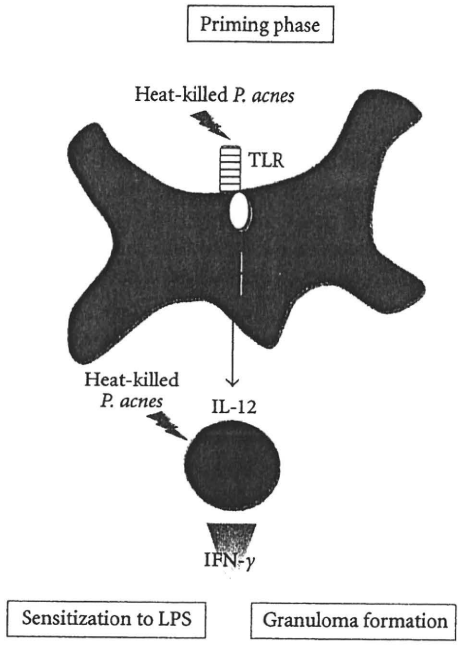

FIGURE 5: Molecular and cellular mechanisms underlying *P. acnes*-induced sensitization to LPS. After recognition of heat-killed *P. acnes*, cytoplasmic domain of TLR9 recruits MyD88 and relays a signal for nuclear translocation of NF-κB, eventually resulting in various gene expressions including IL-12 production. IL-12 is involved in the development of *P. acnes*-specific Th1 cells, which produce robust IFN-γ in response to *P. acnes*. IL-12 also activates hepatic NK cells to release IFN-γ. IFN-γ derived from Th1 cells and NK cells sensitize mice to LPS and induce their dense hepatic granuloma formation. Administration of NF-κB decoy profoundly inhibits both the LPS sensitization and the hepatic granuloma formations [16].

clodronate liposome depletes macrophages in mice, while control PBS liposome do not affect them [41]. These two groups of mice are treated with *P. acnes*, followed by LPS challenge at day 7. The *P. acnes*-primed mice depleted of macrophages show phenotypes similar to naïve mice after LPS challenge [38]. They lack liver injury and 100% survive (Table 1). *P. acnes*-primed mice receiving PBS liposome, however, show the susceptibility to LPS similar to that in *P. acnes*-primed mice [38]. Thus, macrophages are necessarily required for the *P. acnes*-induced sensitization to LPS.

5. Importance of MyD88-IL-12-IFN-γ Axis for the Sensitization to LPS

It is well established that IFN-γ can potently prime macrophages to efficiently respond to LPS [42]. IFN-γ-primed macrophages produce much larger amounts of TNF-α and IL-6 than naïve cells [32]. Furthermore, Th1 cell differentiation occurs both in the liver and spleen after *P. acnes* treatment in a manner dependent on IL-12, a prototype cytokine for Th1 cell differentiation [32, 43]. Splenocytes and splenic $CD4^+$ T cells from *P. acnes*-primed WT mice produce a large amount of IFN-γ but entirely not IL-4 in response to heat-killed *P. acnes* and immobilized anti-CD3 mAb, respectively [18, 32]. Besides, splenic $CD4^+$ T cells from *P. acnes*-primed $Il12p40^{-/-}$ mice do not differentiate into Th1 cells [18, 44]. Hepatic $CD4^+$ T cells differentiate toward Th1 cells as well, which is totally inhibited by the treatment with neutralizing anti-IL-12 monoclonal antibody (mAb) [45]. IL-12 directly activates hepatic NK cells to produce IFN-γ [46, 47]. Furthermore, hepatic NK cells are numerically increased and acquire the high responsiveness to LPS during *P. acnes* priming phase [48]. From these observations together, one may assume the importance of IL-12-IFN-γ axis for the development of LPS sensitization via induction of Th1 cells. Expectedly, *P. acnes*-primed $Ifny^{-/-}$ mice, $Il12p40^{-/-}$ mice or mice with inherited unresponsiveness to IL-12 are resistant to LPS, in terms of lack of hypothermia, hypercoagulation or high mortality [32, 49] (Table 2). In addition, neither $Ifny^{-/-}$ nor $Il12p40^{-/-}$ mice form dense hepatic granulomas after *P. acnes* treatment [18, 50] (Table 2). Thus, IL-12-IFN-γ axis is critical for the LPS sensitization.

As they cannot actively enter into inside of cells, heat-killed *P. acnes* are likely to be recognized by extracellular sensor TLR. As expected, MyD88, which is a key signal adapter molecule of the major TLR signal pathway [2], is essentially required for the development of hepatic granulomas after *P. acnes* priming, strongly suggesting critical role of TLR/MyD88 pathway in the development of *P. acnes*-induced LPS sensitization. $Myd88^{-/-}$ mice lack hepatic granuloma formation after *P. acnes* treatment, and after LPS challenge *P. acnes*-primed $Myd88^{-/-}$ mice do not suffer from the mortality or liver injuries [38, 51] (Table 2, Figure 5). The MyD88-mediated pathway activates nuclear translocation of NF-κB [2]. It is intriguingly to note that administration of NF-κB decoy during *P. acnes* priming phase completely abrogates the hepatic granuloma formation and the sensitization to LPS in WT mice [16]. This strengthens further the importance of the MyD88-mediated pathway for the LPS sensitization. Among TLR members, TLR9 that senses bacterial unmethylated CpG DNA, but not TLR2 that recognize bacterial cell wall product peptidoglycan, was clearly verified to be required for the LPS sensitization by *P. acnes* priming [52, 53]. Indeed, *P. acnes*-primed $Tlr2^{-/-}$ mice are comparably susceptible to LPS as *P. acnes*-primed WT mice, although *P. acnes* possess abundant TLR2 ligands in their cell walls (52). In contrast, *P. acnes*-primed $Tlr9^{-/-}$ mice, like $Myd88^{-/-}$mice, fail to develop hepatic granulomas and become susceptible to LPS [53]. This suggests that unmethylated CpG-DNA of *P. acnes* is pivotal for the sensitization to LPS at least by *P. acnes*-priming. Taken together, these observations strongly suggest that the MyD88-IL-12-IFN-γ axis plays a pivotal role in the hepatic granuloma formation and sensitization to LPS (Figure 5).

Upon challenge with TNF-α instead of LPS, *P. acnes*-primed WT mice show the manifestations/signs similar to those of endotoxin shock syndrome [29, 30, 32], indicating that TNF-α is an effector cytokine and that *P. acnes* treatment tremendously facilitates responsiveness to TNF-α. TNF-α-challenged, *P. acnes*-primed mice, but not naïve mice,

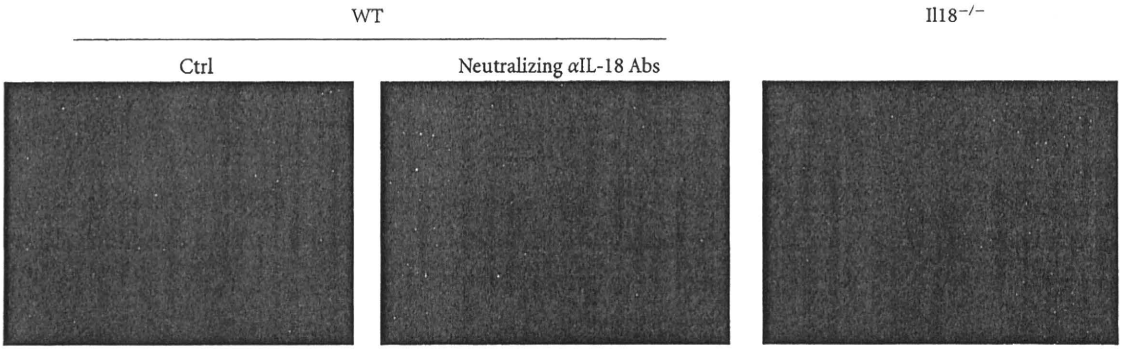

FIGURE 6: Importance of IL-18 for the development of *P. acnes*/LPS-induced liver injury. *P. acnes*-primed wild-type (WT) showed liver necrosis after LPS challenge. However, treatment with neutralizing anti-IL-18 just before LPS challenge could protect against this liver injuries [17]. Furthermore, *Il18*$^{-/-}$ mice were resistant to the sequential treatment with *P. acnes* and LPS [18].

TABLE 1: Clinical manifestations upon LPS challenge. Naïve mice, *P. acnes*-primed mice, and *P. acnes*-primed mice depleted of macrophages (Mø) were challenged with LPS, and hypothermia, hypercoagulation, lethality and liver injuries were monitored by measurement of rectal temperature[a], measurement of plasma TAT/PAI-1 levels[b] and histological analyses[d].

Mice		Systemic alterations			Liver injury[d]
		Hypothermia[a]	Hypercoagulation[b]	Lethality (%)[c]	
Naïve		−	±	0	−
P. acnes-primed	Mø-sufficient[e]	+++	+++	100	+++
	Mø-ablated[f]	ND	ND	0	−

[a]+++ indicates more than 5°C reduction of rectal temperature after LPS challenge; − indicates less than 1°C of it.
[b]+++ indicates more than 10 μg/ml of plasma TAT levels; − indicates normal range of them (< 50 ng/ml); ± indicates less than 200 ng/ml.
[c]Mice were monitored for 48 h after LPS challenge.
[d]+++ indicates more than 300 IU of serum ALT levels; − indicates normal range of them (< 50 IU).
[e] *P. acnes*-primed mice were treated twice with PBS liposome.
[f] *P. acnes*-primed mice were treated twice with clodronate liposome to deplete macrophages.
ND; not done

suffer from hypothermia with exceptionally high plasma levels of plasma TAT and PAI-1 and show high mortality. Consistently, *P. acnes*-primed *Ifnγ*$^{-/-}$ mice are resistant to TNF-α as well [32]. Thus, *P. acnes* treatment renders mice highly susceptible to LPS for TNF-α production and also to TNF-α itself via induction of IFN-γ production.

6. Importance of IFN-γ for the Systemic Endotoxin Shock Manifestations after LPS Challenge

Administration of neutralizing anti-IFN-γ mAb just before LPS challenge could partly rescue *P. acnes*-primed mice from hypothermia, hypercoagulation, and high mortality [32], demonstrating the importance of endogenous IFN-γ for the accomplishment of LPS phase as well. Taken together, IFN-γ is a master regulator of the systemic endotoxin shock syndrome by induction of the sensitization to LPS and activation of the LPS phase.

7. IL-18 Is Necessary and Sufficient for the Development of Liver Injuries

Upon LPS challenge many *P. acnes*-primed WT mice shortly died, and the surviving mice suffer from liver injuries later (Figure 6). Blockade of IL-18 or genetic depletion of *Il18* can protect against the liver damages [17, 54] (Figure 6). Upon LPS challenge *P. acnes*-primed *Il18*$^{-/-}$ mice having normally dense hepatic granulomas develop the endotoxin shock syndrome comparably as *P. acnes*-primed WT mice [18]. In contrast, the surviving *Il18*$^{-/-}$ mice evade the liver injuries [18] (Figure 6). These results indicate that IL-18 is necessary for the development of this liver injury. Furthermore, administration of IL-18 causes liver injuries in *P. acnes*-primed WT mice but not naïve mice [55]. Therefore, IL-18 is necessary and sufficient for *P. acnes*/LPS-induced liver injury.

IL-18 is capable of inducing hepatocytotoxic TNF-α directly in many cell types [13]. NK cells and Th1 cells, but not naïve CD4$^+$ T cells, express IL-18R [47, 56]. During *P. acnes* priming phase, naïve CD4$^+$ T cells differentiate into *P. acnes*-specific Th1 cells as described above. Therefore, IL-18 activates both NK cells and *P. acnes*-specific Th1 cells to produce robust IFN-γ, which in turn might fully activate Kupffer cells and hepatic macrophages to further produce TNF-α [54]. In addition, IL-18 has potent capacity to induce and upregulate Fas ligand expression on NK cells enough to kill Fas-expressing hepatocytes [54]. Thus, endogenous IL-18 participates in the liver injuries through induction of proinflammatory cytokines and cell death-inducing protein.

Table 2: Importance of IL-12-IFN-γ axis for in vivo LPS sensitization by *P. acnes* treatment. Mice with various genotypes were sequentially administered with *P. acnes* and LPS. At day 7 after *P. acnes* priming, hepatic granuloma formation was determined by histological analyses.

P. acnes-primed mice	Sensitization phase	LPS phase				
	Granuloma formation[a]	Hypothermia[b]	Hypercoagulation[c]	Serum TNF-α[d]	Lethality[e]	Liver injury[f]
WT	+++	+++	+++	+++	100	+++
Il12p40$^{-/-}$	–	–	–	–	0	–
Ifnγ$^{-/-}$	–	–	–	–	0	–

[a]+++ indicates that 20% and more area of the liver section is occupied by granulomas; – indicates no granulomas.
[b]+++ indicates more than 5°C reduction of rectal temperature after LPS challenge; – indicates less than 1°C of it.
[c]+++ indicates more than 10 μg/mL of plasma TAT levels; – indicates normal range of them (< 50 ng/mL); ± indicates < 50 ng/mL and > 200 ng/mL.
[d]+++ indicates more than 5 ng/mL; – indicates less than 0.1 ng/mL.
[e]Mice were monitored for 48 h after LPS challenge.
[f]+++ indicates more than 300 IU of serum ALT levels; – indicates normal range of them (< 50 IU).

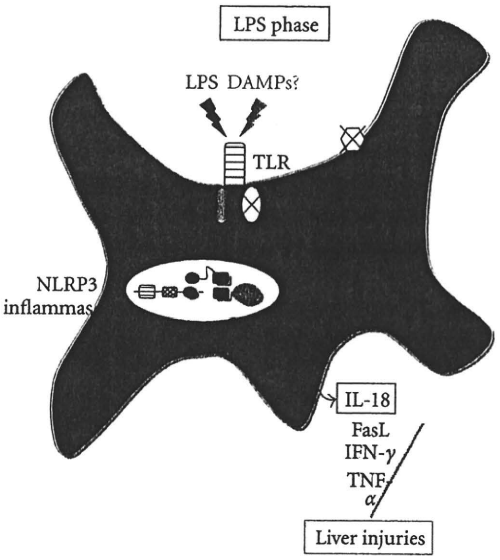

Figure 7: Molecular and cellular mechanisms for LPS phase. After challenge of *P. acnes*-primed wild-type mice with LPS, TLR4 is activated via TRIF for activation of NLRP3 inflammasome, which eventually leads to cleavage of procaspase-1 into its active form caspase-1. Active caspase-1, then, processes pro-IL-18 into active IL-18 for extracellular release. IL-18 upregulates both hepatotoxic Fas ligand (FasL) expression and TNF-α production. DAMPs and alarmin released from the injured hepatocytes might further activate the Kupffer cells, eventually resulting in the acceleration of inflammatory responses. DAMPs, damage-associated molecular patterns.

8. Kupffer Cells Secrete IL-18 in a Manner Dependent on TRIF and the NLRP3 Inflammasome

Many investigators use peritoneal exudate cells (PEC) prepared from the mice administered intraperitoneally with thioglycorate or bone marrow-derived macrophages (BMM) by incubation of bone marrow cells with recombinant monocyte colony-stimulating factor as conventional murine macrophages. These two types of macrophages cannot secrete IL-1β or IL-18 after stimulation with LPS alone. However, LPS-primed PEC or BMM can secrete robust IL-1β and IL-18 upon subsequent stimulation with exogenous ATP in a TLR4- and caspase-1-dependent fashion [57, 58]. From these observations the following possibilities have been believed. First, TLR4-mediated signal pathway cannot activate caspase-1. Second, ATP signaling via its cell surface receptor $P2x_7R$ is a central event required for the caspase-1 activation in LPS-stimulated macrophages. Third, the TLR4-mediated signal pathway is only required for induction of proIL-1β production. We also confirmed the absence of IL-1β or IL-18 release from LPS-activated PEC or BMM. In contrast to these PEC and BMM, WT Kupffer cells can release substantial amounts of IL-1β and IL-18 in response to LPS or synthetic lipid A (active center of LPS) alone [15, 38, 54, 59] (Table 3), strongly suggesting involvement of the TLR4 signaling in release of IL-1β and IL-18. As LPS-stimulated caspase-1-deficient Kupffer cells do not secrete IL-1β or IL-18 [11, 12], caspase-1 is an essential processing enzyme of such IL-1β and IL-18. In fact, western blotting analyses reveal active form of caspase-1 in the lipid A-stimulated WT Kupffer cells [38] (Table 3). Expectedly, *Tlr4*$^{-/-}$ Kupffer cells fail to activate caspase-1 upon stimulation with lipid A [38]. Thus, Kupffer cells seem to be different from PEC or BMM in the ability to activate caspase-1 upon TLR4 engagement. However, it is still to be elucidated how Kupffer cells acquire the potential to activate caspase-1 in response to TLR4 agonists alone.

The TLR4 signaling is relayed by the MyD88- and TRIF-mediated pathways [2]. *Myd88*$^{-/-}$ Kupffer cells stimulated with TLR4 agonists show normal caspase-1 activation [38]. As the *Myd88*$^{-/-}$ Kupffer cells cannot produce pro-IL-1β, eventually resulting in lack of mature IL-1β secretion [38] (Table 3). In contrast to proIL-1β, pro-IL-18 is constitutively stored in *Myd88*$^{-/-}$ Kupffer cells as well as WT cells [15]. Therefore, it is convincing that *Myd88*$^{-/-}$ Kupffer cells cultured with LPS can secrete IL-18 [15, 38] (Table 3). *Trif*$^{-/-}$ Kupffer cells show the reverse phenomena. Despite of their normal production of pro-IL-1β and pro-IL-18, *Trif*$^{-/-}$ Kupffer cells cannot release IL-1β or IL-18 due to their inability to activate caspase-1 [38]. These results demonstrate a pivotal role of TRIF but not

Table 3: Requirement of MyD88 and TRIF for LPS sensitization and Caspase-1 activation, respectively. Mice with various genotypes were sequentially treated with *P. acnes* and LPS, and liver specimens and sera were sampled for histological analyses and measurement of IL-18/IL-1β levels by ELISA, respectively. Kupffer cells were incubated with LPS for 4 h, and each supernatant was collected for western blotting analyses and ELISA. Naïve mice have no hepatic granulomas or injuries, and their serum IL-18 and iL-1β were undetectable. Naïve Kupffer cells released no IL-18 and IL-1β.

Genotype	Sensitization phase	Response to LPS				
		In vivo		LPS-stimulated Kupffer cells		
				Western blotting analyses		ELISA
	Granuloma formation[a]	Liver injury[b]	Serum IL-18/IL-1β[c](ELISA)	ProIL-1β production[d]	Active Casp1[e]	IL-1β/IL-18 release[f]
WT	+++	+++	+++	+++	+++	+++
Tlr4$^{-/-}$	+++	–	–	–	–	–
Myd88$^{-/-}$	–	–	–	–	+++	++[g]
Trif$^{-/-}$	+++	–	–	+++	–	–
Casp1$^{-/-}$	+++	–	–	+++	–	–
Asc$^{-/-}$	+++	–	–	+++	–	–
Nlrp3$^{-/-}$	+++	–	–	ND	ND	–
P2x7r$^{-/-}$	+++	+++	+++	+++	+++	+++

[a]+++ indicates that 20% and more area of the liver section is occupied by granulomas; – indicates no granulomas.
[b]+++ indicates more than 300 IU of serum ALT levels; – indicates normal range of them (< 50 IU).
[c]+++ indicates more than 1000 and 50 pg/mL of serum IL-18 and IL-1β levels, respectively; – indicates normal range of them.
[d]+++ indicates 10 times and more pro-IL-1β density in cell lysates; – indicates the absence of pro-IL-1β.
[e]+++ indicates the presence of active Caspase-1 (Casp1) in supernatant; – indicates the absence of it.
[f]+++ indicates more than 100 pg/mL; – indicates undetectable levels.
[g]++ indicates more than 50 pg/mL of IL-18, but undetectable IL-1β.
ND; not done

MyD88 in the TLR4-mediated caspase-1 activation (Table 3, Figure 7).

Asc$^{-/-}$ Kupffer cells have the phenotype similar to *Caspase1*$^{-/-}$ cells [38, 60], suggesting that NLRP3 or AIM2 inflammasome or unidentified one that needs ASC protein (Figure 1) is involved in the caspase-1 activation. Lipid A-stimulated *Nlrp3*$^{-/-}$ Kupffer cells fail to secrete IL-18 or IL-1β [38]. Therefore, the NLRP3 inflammasome activation is necessary for the TLR4-mediated casapse-1 activation (Table 3, Figure 7).

These results cannot exclude the possibility that the TRIF-mediated pathway might cause extracellular release of ATP and that this self-derived ATP might activate the NLRP3 inflammasome in LPS-stimulated Kupffer cells [57, 58]. Unexpectedly, *P2x7r*$^{-/-}$ Kupffer cells show normal caspase-1 activation and normal release of IL-1β and IL-18 [38]. These results demonstrate the dispensability of endogenous ATP/P2x$_7$R-mediated pathway for the TLR4/TRIF/NLRP3-mediated caspase-1 activation (Table 3, Figure 7).

Although we now know the importance of the NLRP3 inflammasome, the precise mechanisms by which the TRIF-mediated signal pathway activates the NLRP3 inflammasome is unclear. It is also unknown whether NLRP3 protein directly recognizes TLR4 agonists. If so, how do the TLR4 agonists translocate into the inside of Kupffer cells? Alternatively, does TRIF-mediated pathway trigger synthesis of cytoplasmic NLRP3 agonist? If so, what is the NLRP3 agonist? And, how about the molecular mechanisms for the TRIF-induced NLRP3 agonist? We need further extensive studies to address these key queries.

9. Requirement of NLRP3 Inflammasome Activation for the Liver Injury

The capacity to activate caspase-1 reflects on the development of liver injury [38, 60]. Expectedly, *P. acnes*-primed *Tlr4*$^{-/-}$ mice, although manifesting normal levels of hepatic granuloma formation, can avoid the liver injury after LPS challenge accompanied by lack of serum elevation of IL-18 (Table 3, Figure 7). Since they fail to develop hepatic granulomas, *P. acnes*-primed *Myd88*$^{-/-}$ mice lack the production of robust IL-18 after *P. acnes* priming, presumably resulting in escape from the liver injury (Table 3, Figure 7). This demonstrates again requirement of MyD88 for the *P. acnes*-induced LPS sensitization. Conversely, *P. acnes*-primed *Trif*$^{-/-}$ mice, *Caspase1*$^{-/-}$ mice, *Asc*$^{-/-}$ mice and *Nlrp3*$^{-/-}$ mice all have normal dense granulomas in their livers, but fail to develop liver injury after LPS challenge, concomitant with the absence of the serum IL-18 increase (Table 3, Figure 7). *P2x7r*$^{-/-}$ mice have comparable phenotype as WT mice (Table 3, Figure 7), demonstrating dispensability of endogenous ATP/P2x$_7$R pathway for the liver injuries. Thus, the TLR4/TRIF-mediated activation of NLRP3 inflammasome is critical for the development of the *P. acnes*/LPS-induced liver injuries via activation of caspase-1 for maturation and release of IL-18.

10. Methods

10.1. Mice. C57BL/6 mice were purchased from Clea Japan (Osaka, Japan). Female mice (8–12-week-old) were used for this study. Mice were maintained under specific pathogen-free conditions, and received humane care as outlined in the Guide for the Care and Use of Experimental Animals in Hyogo College of Medicine.

10.2. Reagents. Monoclonal antibody (mA) against F4/80 of mouse macrophage was purchased from BMA (Augst, Switzerland). DAPI was from KPL (Gaitherburg, MD).

10.3. Treatment with P. acnes. Heat-killed *P. acnes* were labeled with or without Cy3 (GE, Buchinghamshire, UK) according to the manufacture's instruction and were injected into mice through a tail vein. At the indicated time points various tissues and tissue specimens were sampled for weighing and analysis of the cellularity by confocal microscopy, respectively.

10.4. Confocal Microscopic Analysis. Frozen sections of various tissues were incubated with mAb against F4/80, biotinylated antirat IgG, and then Alexa Fluor 488-conjugated streptavidin (Molecular Probes). Nuclei were stained by DAPI. The immunostaining of each section was evaluated using a laser scanning confocal microscopy [61, 62].

10.5. Flowcytometry [61]. Spleen cells and Kupffer cells were isolated from variously treated WT B6 mice [55]. Cells were incubated with APC-conjugated anti-F4/80 mAb.

11. Closing Remarks

PAMPs evoke innate immune responses by activating pattern recognition receptors (PRRs), such as TLR, NLR, and RLR. Similarly, injured host cells release endogenous "damage"-associated molecular patterns (DAMPs) that induce similar responses via recognition by PRRs [63–65]. For example, high-mobility group box1 protein (HMGB1) that is localized in the nuclei of various cell types in the steady state becomes to be extracellularly released upon stimulation of the cells with death stress. HMGB1, then, initiates innate immune responses via activating TLR4 [66]. Mitochondria are endosymbionts derived from certain bacteria during the evolution of life. Therefore, it is plausible that mitochondria possess DAMPs homologous to its ancestral PAMPs. Very recently, this was verified [67]. Mitochondrion possesses unmethylated CpG-DNA and formyl peptides similar to bacterial N-formylated proteins, which are recognized by PRRs expressed on neutrophils, TLR9, and formyl peptide receptor, respectively. Intravenous injection of the mitochondrial DAMPs causes systemic inflammatory responses and lung injuries [67]. As trauma patients have elevated serum levels of these mitochondrial DAMPs, sterile injury-induced systemic inflammatory response syndrome (SIRS), often occurring after severe trauma, might undergo in response to endogenous mitochondrial DAMPs derived from the injured cells [67]. In addition to DAMPs, self-derived "alarmin" is proposed as another potent inflaming molecules. "Alarmin" is compartmentalized in certain organelle in the steady state. Once damaged, cells begin to actively secrete "alarmin", which in turn triggers inflammatory responses. Intraperitoneal injection of dying cells was reported to be able to trigger peritonitis with dense neutrophil recruitment in an IL-1α/IL-1R-dependent manner [68]. Furthermore, administration of acetoaminophen, a common antipyretic, causes massive liver injury with sterile neutrophilic inflammation in a manner dependent on IL-1α presumably derived from the damaged hepatocytes as well [68]. Thus, dying cell-derived IL-1α is regarded as alarmin. Like IL-1α, IL-33 is localized in the cell nuclei in the steady state and is believed to be secreted after stimulation of the cells with death stress [69]. Histone proteins derived from cell nuclei play a role as alarmin as well [70]. These endogenous DAMPs and alarmin might accelerate liver injuries induced by exogenous PAMPs and might become novel therapeutic targets for severe sepsis with organ failures.

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The Jmjd3-*Irf4* axis regulates M2 macrophage polarization and host responses against helminth infection

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Polarization of macrophages to M1 or M2 cells is important for mounting responses against bacterial and helminth infections, respectively. Jumonji domain containing-3 (Jmjd3), a histone 3 Lys27 (H3K27) demethylase, has been implicated in the activation of macrophages. Here we show that Jmjd3 is essential for M2 macrophage polarization in response to helminth infection and chitin, though Jmjd3 is dispensable for M1 responses. Furthermore, *Jmjd3* (also known as *Kdm6b*) is essential for proper bone marrow macrophage differentiation, and this function depends on demethylase activity of Jmjd3. *Jmjd3* deficiency affected trimethylation of H3K27 in only a limited number of genes. Among them, we identified *Irf4* as encoding a key transcription factor that controls M2 macrophage polarization. Collectively, these results show that Jmjd3-mediated H3K27 demethylation is crucial for regulating M2 macrophage development leading to anti-helminth host responses.

Innate immune cells such as macrophages sense the presence of microbial infection through pattern-recognition receptors and mount anti-microbial responses[1–3]. The Toll-like receptor (TLR) family is one of the best-characterized PRR families recognizing various pathogens such as bacteria, viruses, protozoa and fungi. TLRs are crucial in evoking innate immune responses to infection by various pathogens, leading to production of inflammatory mediators, including proinflammatory cytokines, chemokines and interferons.

Macrophages are functionally polarized into M1 and M2 cells in response to infection with microorganisms and host mediators[4,5]. M1 macrophages produce large amounts of nitric oxide by expressing inducible nitric oxide synthase (iNOS) and tumor necrosis factor (TNF), and are essential for clearing bacterial, viral and fungal infections[6]. Other macrophages, called alternatively activated macrophages or M2 macrophages, have an important role in responses to parasite infection, tissue remodeling, angiogenesis and tumor progression. M2 macrophages are characterized by their high expression of arginase-1 (*Arg1*), chitinase-like Ym1 (*Chi3l3*), found in inflammatory zone-1 (*Fizz1*, also called *Retnla*) mannose receptor (*Mrc1* encoding MR, also known as CD206), and chemokines such as CCL17 and CCL24 (refs. 7–11). The PRR system responsible for the recognition of helminth infection and M2 polarization has yet to be identified.

Cytokines and growth factors have been implicated in the reprogramming of M1 and M2 macrophages. Whereas interferon-γ (IFN-γ), produced by activated T cells and TLR ligands, induces M1 macrophage generation, stimulation of macrophages with interleukin-4 (IL-4) or IL-13 induces M2-type macrophages[4,5]. In addition, immune complexes, IL-10 and glucocorticoid or secosteroid hormones are also known to generate M2 macrophages. Among growth factors, treatment of bone marrow cells with granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) leads to generation of M1 and M2 cells, respectively[12–15].

TLRs trigger intracellular signaling pathways, inducing activation of a set of transcription factors, such as NF-κB, AP-1, C/EBPβ, PU.1 and interferon-regulatory factors (IRFs)[1,16]. These transcription factors cooperatively upregulate the expression of multiple genes such as proinflammatory cytokines, leading to M1 macrophage polarization. Proteins induced by TLR signaling are known to modulate inflammatory responses. IκBζ, an IκB family member, positively regulates certain genes such as *Il6* by interacting with NF-κBp50 or inducing histone modification[17,18], whereas ATF3 negatively regulates expression[19]. In contrast, transcription factors such as STAT6 and peroxisome proliferator-activated receptor-γ (PPARγ) are involved in polarization of M2 macrophages[5,20].

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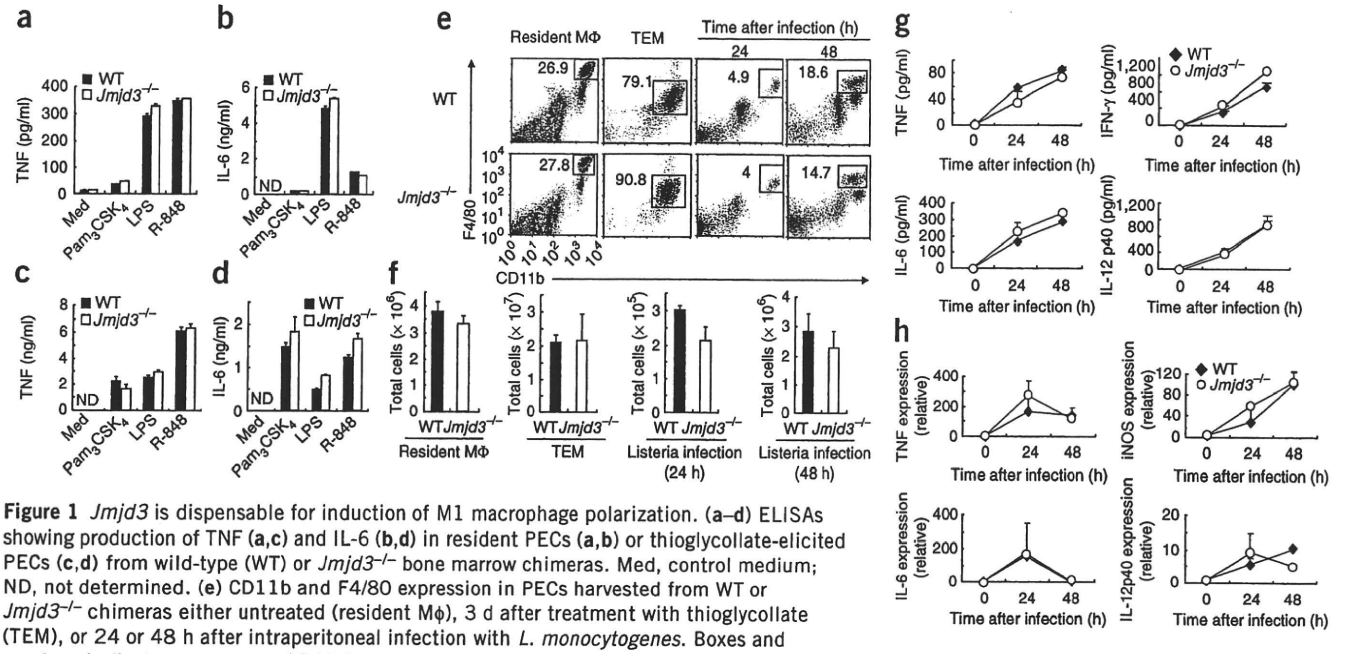

Figure 1 *Jmjd3* is dispensable for induction of M1 macrophage polarization. (**a–d**) ELISAs showing production of TNF (**a,c**) and IL-6 (**b,d**) in resident PECs (**a,b**) or thioglycollate-elicited PECs (**c,d**) from wild-type (WT) or *Jmjd3*$^{-/-}$ bone marrow chimeras. Med, control medium; ND, not determined. (**e**) CD11b and F4/80 expression in PECs harvested from WT or *Jmjd3*$^{-/-}$ chimeras either untreated (resident Mϕ), 3 d after treatment with thioglycollate (TEM), or 24 or 48 h after intraperitoneal infection with *L. monocytogenes*. Boxes and numbers indicate percentage of F4/80^{+}CD11b^{+} cells in total PECs. (**f**) Numbers of F4/80^{+}CD11b^{+} macrophages in PECs harvested from WT or *Jmjd3*$^{-/-}$ chimeras either untreated, 3 d after thioglycollate treatment or 24 or 48 h after intraperitoneal infection with *L. monocytogenes*. (**g**) ELISAs showing concentrations of TNF, IL-6, IFN-γ and IL-12p40 in the sera of WT or *Jmjd3*$^{-/-}$ chimeras infected with *L. monocytogenes*. (**h**) Quantitative PCR analysis showing expression of TNF, IL-6, IL-12p40 and iNOS mRNAs (relative to 18S rRNA) in PECs in mice infected with *L. monocytogenes*. Results are representative of four (**c–f**) or two (**a,b,g,h**) independent experiments (error bars indicate s.d.).

In addition to regulation by transcription factors, epigenetic regulation is essential for controlling proper gene expression[16]. Histone modifications, particularly at the N-terminal tails, and dynamic chromatin remodeling have been shown to be important for controlling sets of genes. In the case of histone modification, trimethylation of H3K4 is associated with active gene transcription, whereas trimethylation of H3K9, H3K27 and H4K20 is linked to silencing of gene expression[16,21,22]. The methylation of H3K27 is mediated by the Polycomb repressive complex-2 (PRC2), composed of Ezh2, Suz12 and Eed (ref. 23). Proteins harboring a Jumonji-C (JmjC) domain, such as Jmjd3, Utx and Uty, are known to act as H3K27 demethylases, catalyzing the conversion of H3K27me3 (trimethylated) to H3K27me1 (monomethylated)[24–26].

It has been reported that the expression of *Jmjd3* is induced in macrophages by TLR stimuli in an NF-κB–dependent fashion[26]. *Jmjd3* has also been identified as an early TLR-inducible gene in mouse macrophages by microarray analysis[27]. H3K27 trimethylation is implicated in the silencing of gene expression, and it has been shown that Jmjd3 is recruited to transcription start sites (TSSs) that have abundant RNA polymerase II and H3K4me3 (ref. 28). Jmjd3 is reported to fine-tune macrophage activation by controlling *Bmp2* and *Hox* expression[26]. Furthermore, Jmjd3 has been linked to the control of development through the regulation of *Hox*, and to oncogenesis through promotion of the expression of *Ink4a*[29,30]. Here we report the role of Jmjd3 *in vivo* in controlling M2 macrophage polarization and identify *Irf4* as a Jmjd3 target gene crucial for the regulation of macrophages.

RESULTS

Generation of *Jmjd3*$^{-/-}$ mice

To investigate the functional roles of Jmjd3 in immune responses *in vivo*, we generated *Jmjd3*$^{-/-}$ mice (**Supplementary Fig. 1a,b**). Reverse-transcription PCR analysis showed that the expression of *Jmjd3* was abrogated in *Jmjd3*$^{-/-}$ mouse embryonic fibroblasts (**Supplementary Fig. 1c**). *Jmjd3*$^{-/-}$ mice died perinatally, and adult *Jmjd3*$^{-/-}$ mice were not obtained (**Supplementary Fig. 2a**). Histological examination revealed that alveolar cell walls were thickened with tissues, and almost no air space was observed in lungs of *Jmjd3*$^{-/-}$ neonates (**Supplementary Fig. 2b**), suggesting that the postnatal lethal phenotype of *Jmjd3*$^{-/-}$ mice is due to premature development of lung tissues. To analyze the role of *Jmjd3* in hematopoietic cells, we obtained fetal liver cells from *Jmjd3*$^{-/-}$ E15.5 embryos and established bone marrow–chimeric mice. Flow cytometry revealed that populations of T cells, B cells, conventional dendritic cells, plasmacytoid dendritic cells, natural killer cells, neutrophils, F4/80^{+}CD11b^{+} macrophages and Ly6C^{hi}CD11b^{+} inflammatory monocytes in the spleen were similar between wild-type and *Jmjd3*$^{-/-}$ chimeras (**Supplementary Fig. 3**). Proliferative responses of splenic T and B cells to mitogens and antigen receptor stimuli were also not altered (**Supplementary Fig. 4**). Furthermore, *Jmjd3*$^{-/-}$ splenic T cells differentiated into either type 1 or type 2 helper T cells (T_H1 and T_H2) produced normal amounts of IFN-γ or IL-4, respectively (**Supplementary Fig. 5**). These results indicate that T cells lacking *Jmjd3* are capable of differentiating into T_H1 and T_H2 cells in response to cytokines.

Jmjd3 is dispensable for M1 macrophages

As *Jmjd3* is a TLR-inducible gene in macrophages, we first examined cytokine production of peritoneally resident and thioglycollate-elicited peritoneal exudate cells (PECs) in response to TLR ligands including lipopeptide (Pam_3CSK_4, a TLR2 ligand), lipopolysaccharide (LPS, a TLR4 ligand), imidazoquinoline analog (R-848, a TLR7 ligand) and oligonucleotide with a CpG motif (CpG-DNA, a TLR9 ligand). Production of TNF and IL-6 in response to TLR ligands did not differ between wild-type and *Jmjd3*$^{-/-}$ tissue-resident macrophages and thioglycollate-elicited PECs (**Fig. 1a–d**). PECs elicited

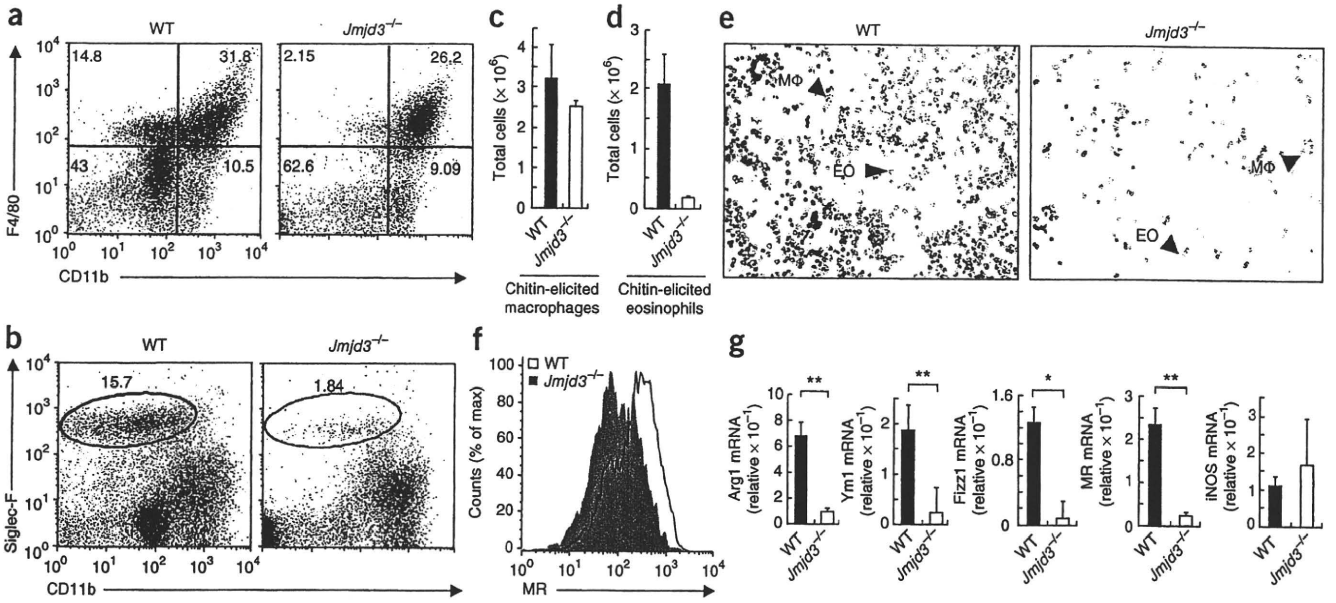

Figure 2 Crucial role of *Jmjd3* in M2 macrophage polarization in response to chitin administration. (**a**,**b**) Expression of CD11b and either F4/80 (**a**) or Siglec-F (**b**) in PECs harvested from wild-type (WT) or *Jmjd3*$^{-/-}$ chimeric mice 2 d after peritoneal injection with chitin. Quadrants and numbers in **a** indicate percentage of cells in each gate (F4/80$^+$CD11b$^+$, F4/80$^+$CD11b$^-$, F4/80$^-$CD11b$^+$ and F4/80$^-$CD11b$^-$); circles and numbers in **b** indicate percentage of Siglec-F$^+$ eosinophils in total PECs. (**c**,**d**) Numbers of F4/80$^+$CD11b$^+$ macrophages (**c**) or Siglec-F$^+$ eosinophils (**d**) in PECs harvested from WT or *Jmjd3*$^{-/-}$ chimeric mice 2 d after peritoneal injection with chitin. (**e**) Cell types within PEC population from chitin-treated mice, stained with Diff-Quick in cytospin centrifuges. Mϕ, macrophages; EO, eosinophils. (**f**) Surface MR expression on peritoneal F4/80$^+$CD11b$^+$ macrophages from chitin-treated mice. Graph shows MR expression on macrophages on horizontal axis. (**g**) Quantitative PCR showing expression of Arg1, Ym1, Fizz1, MR and iNOS mRNAs (relative to 18S rRNA). Total RNA was prepared from PECs 48 h after administration of chitin. $^*P < 0.05$; $^{**}P < 0.01$ (two-tailed Student's *t*-test). Results are representative of five (**a**–**d**), two (**e**) or three (**f**,**g**) independent experiments (error bars indicate s.d.).

by peptone treatment of wild-type or *Jmjd3*$^{-/-}$ chimeras also produced similar amounts of TNF and IL-6 in response to TLR ligand stimulation (**Supplementary Fig. 6a**). Flow cytometry revealed that the proportions of F4/80$^+$CD11b$^+$Gr1$^-$ cells among resident and thioglycollate-elicited PECs did not differ between wild-type and *Jmjd3*$^{-/-}$ chimeras (**Fig. 1e** and **Supplementary Fig. 7**). The total number of peritoneally resident macrophages, thioglycollate-elicited macrophages and peptone-elicited macrophages was not changed in *Jmjd3*$^{-/-}$ chimeras (**Fig. 1f** and **Supplementary Fig. 6b**). We then examined the role of Jmjd3 in M1 macrophage polarization in response to *Listeria monocytogenes* infection. When *L. monocytogenes* was inoculated intraperitoneally, production of proinflammatory cytokines in the sera was similar in wild-type and *Jmjd3*$^{-/-}$ chimeras (**Fig. 1g**). F4/80$^+$CD11b$^+$Gr1$^+$ macrophages, F4/80^{int}CD11b$^+$Gr1^{hi} neutrophils, and F4/80$^-$CD11b^{int}B220$^+$ and F4/80$^-$CD11b$^-$B220$^+$ B cells were examined in PECs prepared from *L. monocytogenes*–infected mice (**Supplementary Fig. 8**). The number of macrophages recruited to the peritoneal cavity was similar in wild-type and *Jmjd3*$^{-/-}$ chimeras (**Fig. 1e**,**f**). Furthermore, the expression of genes encoding TNF, IL-6, IL-12p40 and iNOS in PECs were similarly upregulated (**Fig. 1h**). Collectively, these data suggest that Jmjd3 is not involved in the generation and recruitment of M1 macrophages in response to inflammatory reagents and bacterial infection *in vivo*.

Jmjd3 is needed for M2 polarization to chitin

Chitin is a polymerized sugar and a structural component of helminths, arthropods and fungi[31]. It has been shown that chitin administration recruits macrophages with the M2 phenotype to the site of administration, which are important for subsequent recruitment of eosinophils[32,33]. Indeed, we found that intraperitoneal administration of chitin recruited F4/80$^+$CD11b$^+$ macrophages (**Fig. 2a**), Siglec-F$^+$CCR3$^+$CD4$^-$ eosinophils and CD11b^{int}B220$^+$ B cells to the peritoneal cavity after 48 h in wild-type mice (**Fig. 2b** and **Supplementary Fig. 9**). Whereas the number of chitin-elicited F4/80$^+$CD11b$^+$ macrophages was similar between wild-type and *Jmjd3*$^{-/-}$ chimeras (**Fig. 2c**), the recruitment of eosinophils was severely impaired in *Jmjd3*$^{-/-}$ chimeras (**Fig. 2b–e**). Furthermore, the expression of MR on chitin-elicited macrophages was severely impaired in *Jmjd3*$^{-/-}$ chimeras (**Fig. 2f**). Chitin-elicited macrophages, but not eosinophils or B cells, expressed high levels of mRNA encoding Arg1, Ym1, Fizz1 and MR, the hallmarks of M2 macrophages (**Supplementary Fig. 10**). The expression of genes encoding Arg1, Ym1, Fizz1 and MR was considerably lower in chitin-induced PECs obtained from *Jmjd3*$^{-/-}$ chimeras compared with wild-type controls, whereas expression of the gene encoding iNOS, associated with M1 macrophages, was not altered (**Fig. 2g**). Of note, the numbers of eosinophils circulating in the blood were similar in wild-type and *Jmjd3*$^{-/-}$ chimeric mice, suggesting that eosinophil development was not impaired by *Jmjd3* deficiency (data not shown). Together, these results suggest Jmjd3 is necessary for M2 macrophage polarization in response to chitin administration.

Role of *Jmjd3* in helminth infection

Next we investigated whether Jmjd3 contributes to the responses against helminth infection *in vivo*. We used the *Nippostrongylus brasiliensis* infection model, which induces a strong type 2 immune response in the lung. Bronchoalveolar lavage (BAL) staining 5 and 13 d after infection revealed that macrophages were similarly recruited to the lung in wild-type and *Jmjd3*$^{-/-}$ bone marrow chimeras (**Fig. 3a**,**b**).

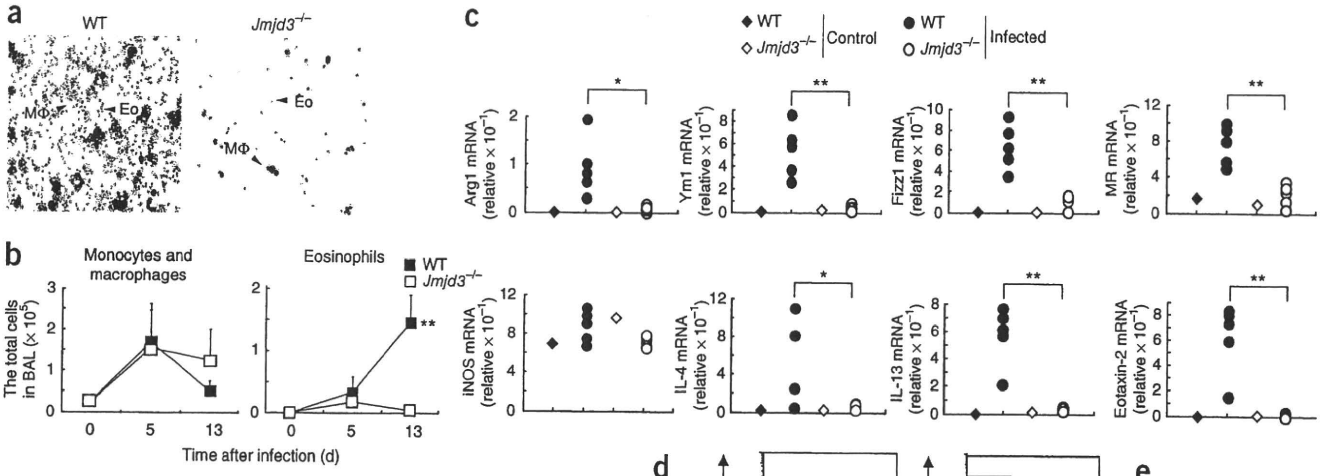

Figure 3 Crucial role of *Jmjd3* in the responses to *N. brasiliensis* infection. (**a**) Macrophages and eosinophils from WT and *Jmjd3*$^{-/-}$ chimeric mice inoculated with 300 third-stage larvae of *N. brasiliensis*. BAL fluid was extracted 13 d after *N. brasiliensis* infection, and cells were stained with Diff-Quik. (**b**) Total numbers of monocytes and macrophages and eosinophils in the BAL fluid from wild-type (WT; $n = 7$) and *Jmjd3*$^{-/-}$ ($n = 7$) chimeras 0, 5 and 13 d after infection. (**c**) Expression of indicated mRNAs (vertical axes; relative to 18S rRNA) determined by quantitative PCR in *N. brasiliensis*–infected or uninfected mice (WT or *Jmjd3*$^{-/-}$). Infected, $n = 7$; uninfected, $n = 2$. (**d**,**e**) Representative result of intracellular IL-4 and IFN-γ staining in T cells from hilar lymph nodes (**d**), and number of IL-4–producing cells in a hilar lymph node (**e**), harvested from WT and *Jmjd3*$^{-/-}$ chimeras 9 d after *N. brasiliensis* inoculation. After harvesting, cells were stimulated with CD3 and CD28 for 4 h, and CD4, CD8, IL-4 and IFN-γ expression were determined. Boxes and numbers indicate percentages of CD4$^+$ cells in hilar lymph node cells and IL-4–producing cells in CD4$^+$ cells. $^*P < 0.05$; $^{**}P < 0.01$ (two-tailed Student's *t*-test). Results are representative of two experiments with four mice per group (**a**,**b**), a single experiment with seven mice per group (**c**) or a single experiment with five mice (**d**,**e**) (error bars indicate s.d.).

However, the recruitment of eosinophils was severely impaired in *Jmjd3*$^{-/-}$ chimeric mice (**Fig. 3b**). To investigate the characteristics of recruited macrophages, we extracted RNA from lung tissue 5 d after *N. brasiliensis* infection. M2 markers such as Arg1, Ym1, Fizz1 and MR were barely expressed in *Jmjd3*$^{-/-}$ chimeras (**Fig. 3c**). Furthermore, induction of genes encoding the eosinophil-recruiting chemokine eotaxin-2 and the T_H2-inducing cytokines IL-4 and IL-13 was severely impaired in *Jmjd3*$^{-/-}$ chimeric mice (**Fig. 3c**). Thus, we

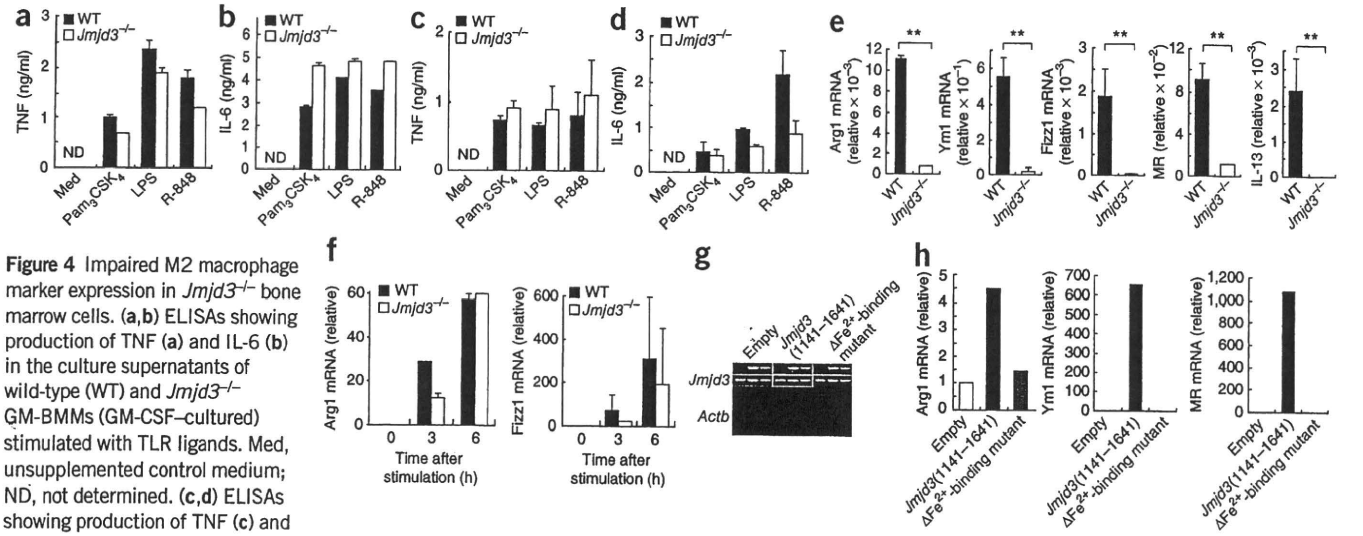

Figure 4 Impaired M2 macrophage marker expression in *Jmjd3*$^{-/-}$ bone marrow cells. (**a**,**b**) ELISAs showing production of TNF (**a**) and IL-6 (**b**) in the culture supernatants of wild-type (WT) and *Jmjd3*$^{-/-}$ GM-BMMs (GM-CSF–cultured) stimulated with TLR ligands. Med, unsupplemented control medium; ND, not determined. (**c**,**d**) ELISAs showing production of TNF (**c**) and IL-6 (**d**) in the culture supernatants of WT and *Jmjd3*$^{-/-}$ M-BMMs (M-CSF–cultured) stimulated with TLR ligands. (**e**) Quantitative PCR analysis showing expression of Arg1, Ym1, Fizz1, MR and IL-13 mRNAs (relative to 18S rRNA) in total RNA prepared from WT and *Jmjd3*$^{-/-}$ M-BMMs. (**f**) Quantitative PCR analysis showing expression of Arg1 and Fizz1 mRNAs (relative to 18S rRNA) in total RNA prepared from WT and *Jmjd3*$^{-/-}$ M-BMMs stimulated with IL-4 (10 ng/ml). (**g**,**h**) Reverse-transcription PCR showing expression of *Jmjd3* cDNA, with *Actb* cDNA as an expression control (**g**), and quantitative PCR showing expression of Arg1, Ym1 and MR mRNAs (relative to empty-vector control; **h**), in total RNA of *Jmjd3*$^{-/-}$ M-BMMs generated from bone marrow cells infected with retroviruses expressing WT Jmjd3 (amino acid residues 1141–1641) or its iron binding–deficient mutant. Results are representative of four (**a**–**d**), three (**e**,**f**) or two (**g**,**h**) independent experiments (error bars indicate s.d.).

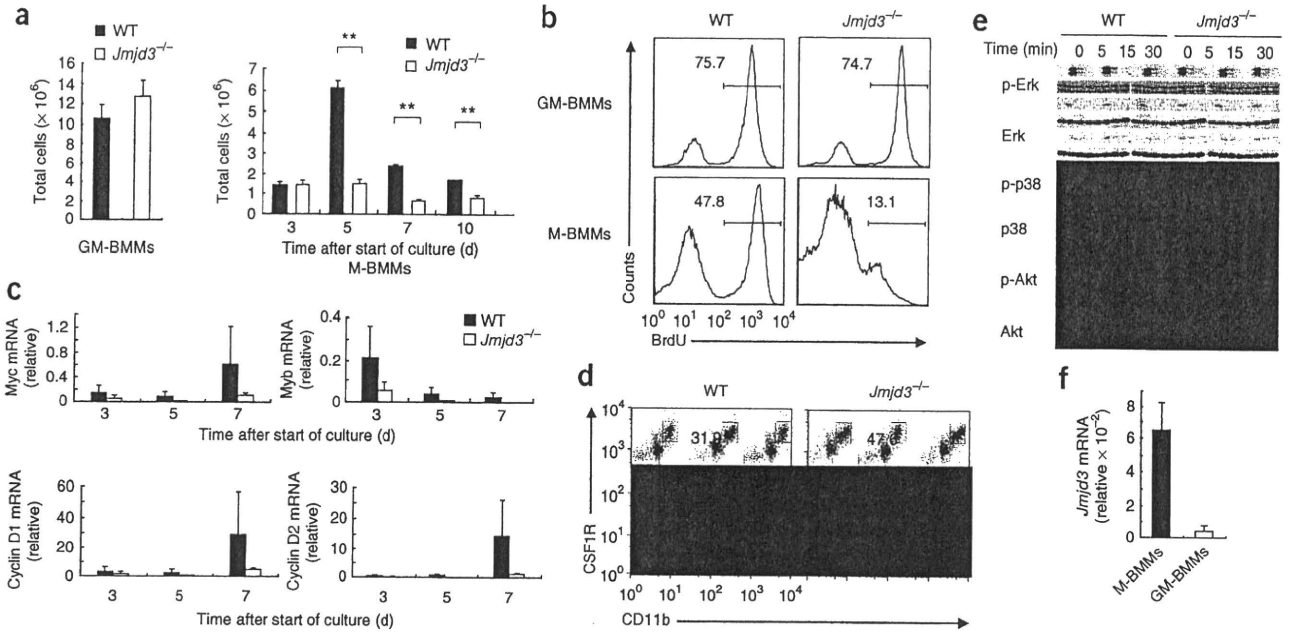

Figure 5 *Jmjd3* is required for the cell-cycle progression of M-BMMs. (**a**) Numbers of GM-BMMs and M-BMMs generated from wild-type (WT) and *Jmjd3*$^{-/-}$ bone marrow cells. Bone marrow cells from WT and *Jmjd3*$^{-/-}$ chimeras were cultured in the presence of GM-CSF for 5 d or M-CSF for time indicated (horizontal axis), and the number of adherent CD11b^{+} cells was counted. (**b**) Incorporation of BrdU in WT and *Jmjd3*$^{-/-}$ GM-BMMs and M-BMMs incubated in the presence of BrdU for 24 h. Incorporation was examined by intracellular staining with anti-BrdU. (**c**) Quantitative PCR showing expression of mRNAs encoding c-Myc, c-Myb, cyclin D1 and cyclin D2 (relative to 18S rRNA) in WT and *Jmjd3*$^{-/-}$ M-BMMs. (**d**) Surface expression of colony stimulating receptor (CSF1R) and CD11b on WT and *Jmjd3*$^{-/-}$ M-BMMs. Boxes and numbers indicate percentages of CSF1R-expressing M-BMMs. (**e**) Expression of phosphorylated (p-) and unphosphorylated Erk, p38 and Akt in WT and *Jmjd3*$^{-/-}$ M-BMMs stimulated with M-CSF (50 ng/ml). Cells were starved for 4 h before stimulation, and cell lysates were subjected to immunoblot analysis with antibodies to p-Erk, p-p38 and p-Akt. The membrane was stripped and reprobed for expression of Erk, p38 and Akt. (**f**) Quantitative PCR showing Jmjd3 mRNA expression (relative to 18S rRNA) by M-BMMs and GM-BMMs. $*P < 0.05$; $**P < 0.01$ (two-tailed Student's *t*-test). Results are representative of four (**a**), three (**b**,**f**) or two (**c**–**e**) independent experiments (error bars indicate s.d.).

investigated the activation of T cells in the pulmonary lymph nodes 9 d after *N. brasiliensis* infection. Whereas CD4^{+} T cells prepared from wild-type pulmonary lymph nodes expressed IL-4, but not IFN-γ, the frequency of IL-4–producing CD4^{+} T cells was severely impaired in pulmonary T cells prepared from *Jmjd3*$^{-/-}$ chimeric mice, suggesting that Jmjd3-mediated M2 macrophage activation is crucial for *N. brasiliensis* to induce T_H2 responses in the lung (**Fig. 3d,e**). However, histological changes in the intestine were not severely impaired in *Jmjd3*$^{-/-}$ chimeric mice (data not shown). Collectively, our results demonstrate that Jmjd3 is essential for mounting immune responses to helminth infection, directing M2 macrophage polarization in the lung but not in the small intestine.

Role of *Jmjd3* in M2 macrophage generation in response to M-CSF

Numerous studies have shown that GM-CSF induces M1 macrophages from bone marrow cells and M-CSF induces M2 macrophages from bone marrow cells[12–15]. When we used GM-CSF to generate macrophages, similar amounts of TNF and IL-6 were produced in wild-type and *Jmjd3*$^{-/-}$ chimeras in response to TLR ligands from adherent CD11b^{+} macrophages (termed GM-BMMs, for GM-CSF–induced bone marrow–derived macrophages; **Fig. 4a,b**). In contrast, production of IL-6, but not TNF, in response to TLR ligand stimulation was partially impaired in *Jmjd3*$^{-/-}$ bone marrow cultured in the presence of M-CSF (M-BMMs; **Fig. 4c,d**). Furthermore, the expression of genes encoding Arg1, Ym1, Fizz1, MR and IL-13 was severely impaired in M-BMMs from *Jmjd3*$^{-/-}$ chimeras (**Fig. 4e**), which indicates that *Jmjd3* is crucial for expression of hallmark M2 genes in M-BMMs. Jmjd3 is involved in the response of macrophages to IL-4 stimulation[34]; nevertheless, Arg1 and Fizz1 gene expression were similar after IL-4 stimulation in wild-type and *Jmjd3*$^{-/-}$ M-BMMs (**Fig. 4f**), suggesting that the responses to IL-4 were not impaired in *Jmjd3*$^{-/-}$ M-BMMs. We then used microarray analysis to examine the gene expression profiles in wild-type and *Jmjd3*$^{-/-}$ M-BMMs with or without LPS stimulation. The expression of 1,371 genes was more than 50% lower in unstimulated *Jmjd3*$^{-/-}$ M-BMMs compared with wild-type (**Supplementary Table 1**). In addition to *Arg1*, *Chi3l3* and *Fizz1*, the expression of cytokine genes such as *Il2*, *Il3*, *Il4*, *Il5* and *Il13*, as well as that of chemokine genes such as *Ccl1*, *Ccl17*, *Ccl22* and *Ccl24*, was severely impaired in *Jmjd3*$^{-/-}$ M-BMMs (**Supplementary Table 1**). The expression of 2,188 genes was more than twofold higher in wild-type M-BMMs in response to LPS stimulation, and that of 436 genes was reduced by over 50% in LPS-stimulated *Jmjd3*$^{-/-}$ M-BMMs (**Supplementary Table 2**). For example, the expression of *Il6* and *Il12b* was partially impaired in *Jmjd3*$^{-/-}$ M-BMMs, consistent with a previous report (**Supplementary Table 2**). Therefore, Jmjd3 is important for inducing expression of a large set of genes, and some of these are related to M2 macrophage polarization in M-BMMs.

In addition to its JmjC domain, Jmjd3 contains a putative tetratricopeptide repeat domain in the N terminus. We therefore examined whether the demethylase activity of Jmjd3 is needed for the defect in M2 macrophage marker expression. We retrovirally expressed the C-terminal part of Jmjd3, containing the JmjC domain (amino acid residues 1141–1641), or its iron binding–deficient mutant (A1388H) in *Jmjd3*$^{-/-}$ bone marrow cells and then induced M-BMMs (**Fig. 4g**).

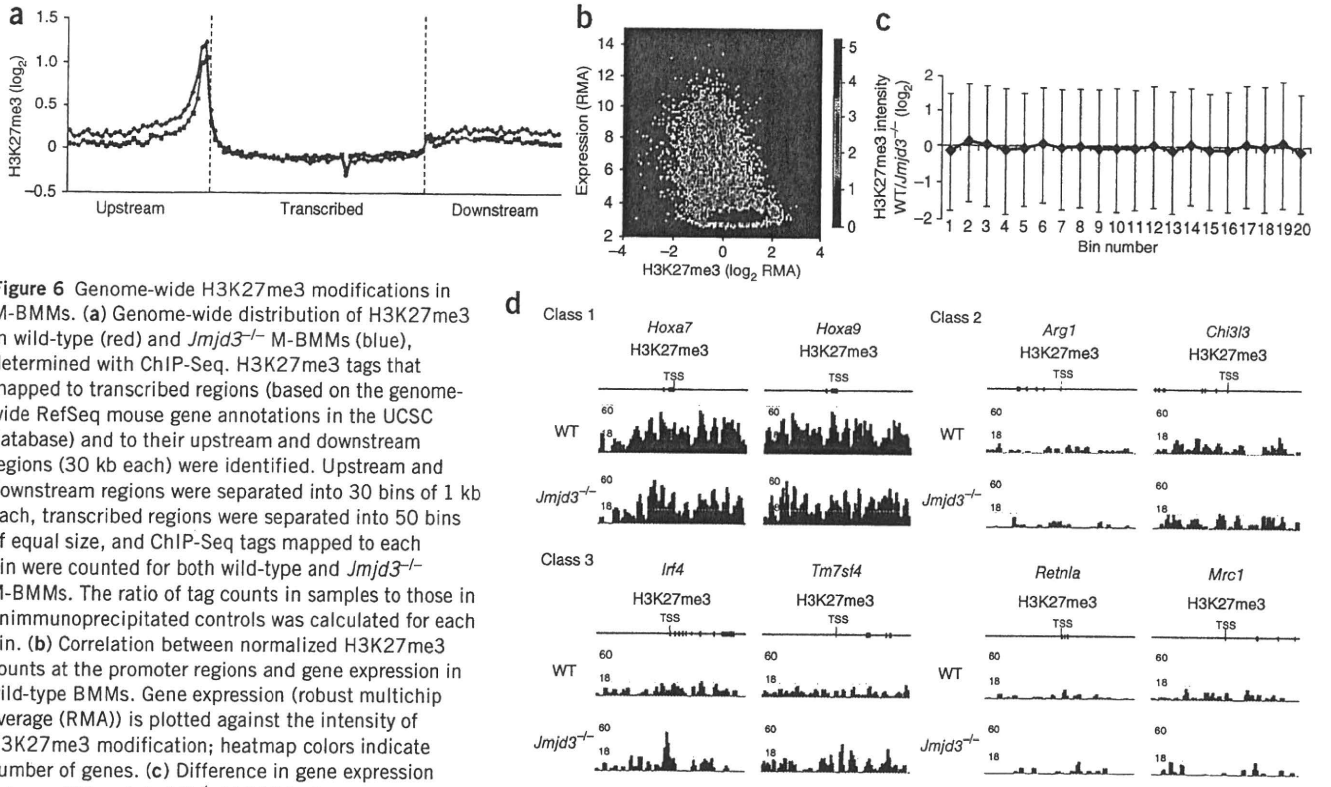

Figure 6 Genome-wide H3K27me3 modifications in M-BMMs. (**a**) Genome-wide distribution of H3K27me3 in wild-type (red) and *Jmjd3*$^{-/-}$ M-BMMs (blue), determined with ChIP-Seq. H3K27me3 tags that mapped to transcribed regions (based on the genome-wide RefSeq mouse gene annotations in the UCSC database) and to their upstream and downstream regions (30 kb each) were identified. Upstream and downstream regions were separated into 30 bins of 1 kb each, transcribed regions were separated into 50 bins of equal size, and ChIP-Seq tags mapped to each bin were counted for both wild-type and *Jmjd3*$^{-/-}$ M-BMMs. The ratio of tag counts in samples to those in unimmunoprecipitated controls was calculated for each bin. (**b**) Correlation between normalized H3K27me3 counts at the promoter regions and gene expression in wild-type BMMs. Gene expression (robust multichip average (RMA)) is plotted against the intensity of H3K27me3 modification; heatmap colors indicate number of genes. (**c**) Difference in gene expression between WT and *Jmjd3*$^{-/-}$ M-BMMs does not correlate with H3K27me3 levels. We classified RefSeq genes into 20 bins sorted by their expression difference between WT and *Jmjd3*$^{-/-}$ cells. Bins are numbered from low to high WT/*Jmjd3*$^{-/-}$ expression ratio. The average H3K27 modification intensity was calculated in each bin. (**d**) H3K27me3 modifications of wild-type (WT, blue) and *Jmjd3*$^{-/-}$ (red) cells on class 1 genes (*Hoxa7* and *Hoxa9*), class 2 genes (*Arg1*, *Chi3l3* for Ym1, *Fizz1*, *Mrc1* for MR) and class 3 genes (*Irf4* and *Tm7sf4*). Gray lines indicate the threshold tag counts for WT (18 tags) and *Jmjd3*$^{-/-}$ M-BMMs (18 tags) corresponding to a false discovery rate of 1×10^{-6}.

The A1388H mutation has been shown to abrogate the H3K27 demethylase activity of Jmjd3 (ref. 26). Expression of the C-terminal part of Jmjd3 was sufficient to upregulate M2 marker genes such as *Arg1*, *Chi3l3* and *Mrc1* (**Fig. 4h**). In contrast, the expression of *Jmjd3* (A1388H) did not increase the expression of M2 marker genes, which indicates that the H3K27me3 demethylase activity of Jmjd3 is necessary and sufficient for expression of M2 marker genes in M-BMMs.

M-BMMs require *Jmjd3* for cell cycle progression

In addition to the impaired M2 marker expression, the total number of M-BMM cells in *Jmjd3*$^{-/-}$ chimeras was considerably lower than in the wild type at days 5 and 7 of culture with M-CSF (**Fig. 5a**), although the number of GM-BMM cells (M1) was not altered. We found 5-bromodeoxyuridine (BrdU) incorporation, a measure of cell division, was severely impaired in *Jmjd3*$^{-/-}$ M-BMMs compared with wild-type cells (**Fig. 5b**), whereas wild-type and *Jmjd3*$^{-/-}$ GM-BMMs incorporated BrdU similarly. These results indicate that Jmjd3 controls cell-cycle progression in response to M-CSF stimulation. Expression of cell cycle–regulatory proteins (c-Myc, c-Myb, cyclin D1 and cyclin D2) was impaired in *Jmjd3*$^{-/-}$ M-BMMs at day 5 of culture (**Fig. 5c**); however, the surface expression of M-CSF receptor (CSF-1R) was normal in *Jmjd3*$^{-/-}$ M-BMMs (**Fig. 5d**). Furthermore, *Jmjd3* deficiency did not affect activation of the intracellular signaling molecules Erk, p38 and Akt, as indicated by their phosphorylation in M-BMMs (**Fig. 5e**), implying that the cell proliferation defects in *Jmjd3*$^{-/-}$ M-BMMs are not due to less activation of MAP kinases or Akt. The expression of *Jmjd3* in M-BMMs was much higher than that in GM-BMMs (**Fig. 5f**), suggesting that differential expression of *Jmjd3* in M-BMMs and GM-BMMs determines the contribution of Jmjd3 to their proliferation. Together, these data indicate that Jmjd3 performs a key step in the generation of M-BMMs, but not GM-BMMs, by controlling cell proliferation downstream of CSF-1R signaling.

Genome-wide analysis of H3K27me3 controlled by Jmjd3

Next we analyzed the genome-wide distribution of H3K27 trimethylation in wild-type and *Jmjd3*$^{-/-}$ M-BMMs by chromatin immunoprecipitation–sequencing (ChIP-Seq) analysis. We obtained an overall picture of the H3K27me3 distribution in transcribed regions (based on the genome-wide RefSeq mouse gene annotations in the University of California, Santa Cruz database) and in regions 30 kb upstream and 30 kb downstream. High levels of H3K27me3 tags were detected surrounding TSSs in M-BMMs from wild-type and *Jmjd3*$^{-/-}$ chimeras (**Fig. 6a**). In contrast, H3K27me3 levels were low in transcribed loci compared with upstream and downstream regions (**Fig. 6a**). Notably, H3K27me3 signals at the promoter and downstream regions were higher in *Jmjd3*$^{-/-}$ M-BMMs compared with wild-type cells.

We then compared the gene expression data obtained by microarray experiments with H3K27 methylation status. Overall, H3K27me3 levels in regions close to the TSS (−5 to +1 kb) correlated negatively with gene expression in M-BMMs (correlation coefficient −0.441; **Fig. 6b**). Next, we sorted genes by their ratio of expression in wild-type and *Jmjd3*$^{-/-}$ M-BMMs and examined H3K27me3 levels. However, we did not detect a correlation between H3K27me3 status and the difference in gene expression in wild-type compared with *Jmjd3*$^{-/-}$ M-BMMs (**Fig. 6c**). These data suggest that only small numbers of genes were affected by the absence of *Jmjd3*, and most loci are regulated by Utx or by both Jmjd3 and Utx.

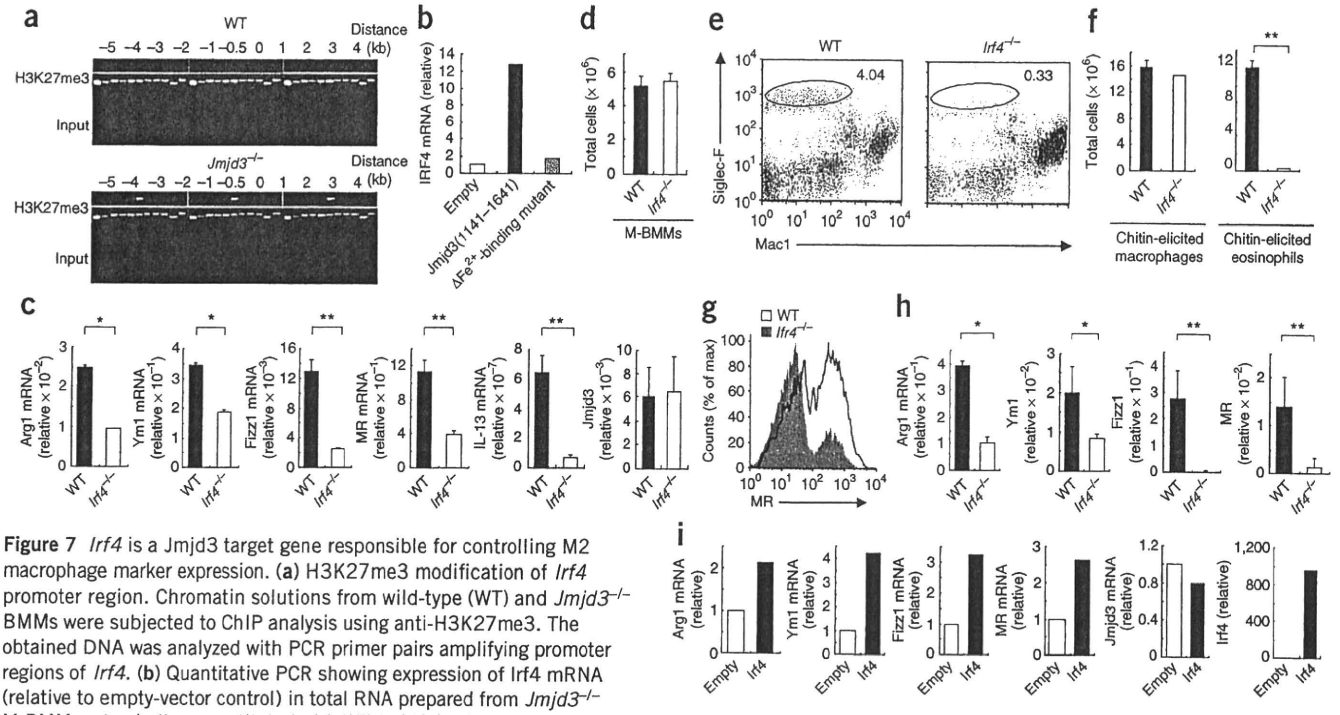

Figure 7 *Irf4* is a Jmjd3 target gene responsible for controlling M2 macrophage marker expression. (**a**) H3K27me3 modification of *Irf4* promoter region. Chromatin solutions from wild-type (WT) and *Jmjd3*$^{-/-}$ BMMs were subjected to ChIP analysis using anti-H3K27me3. The obtained DNA was analyzed with PCR primer pairs amplifying promoter regions of *Irf4*. (**b**) Quantitative PCR showing expression of Irf4 mRNA (relative to empty-vector control) in total RNA prepared from *Jmjd3*$^{-/-}$ M-BMMs retrovirally reconstituted with WT Jmjd3 (amino acid residues 1141–1641) or its iron binding–deficient mutant. (**c**) Quantitative PCR showing expression of M2 markers and Jmjd3 in bone marrow cells from WT and *Irf4*$^{-/-}$ mice cultured in the presence of M-CSF for 5 d. (**d**) Numbers of M-BMMs obtained from wild-type and *Irf4*$^{-/-}$ mice. (**e–g**) Macrophage and eosinophil recruitment (**e**), numbers of macrophages and eosinophils (**f**) and expression of MR (**g**) in macrophages from chitin-elicited PECs. WT and *Irf4*$^{-/-}$ mice were intraperitoneally treated with chitin, and PECs were prepared 48 h after treatment. Graph in **g** shows percentage of macrophages with the MR expression levels indicated on horizontal axis. (**h**) Quantitative PCR showing expression of M2 markers (relative to 18S rRNA) in total RNA prepared from PECs obtained from chitin-treated WT and *Irf4*$^{-/-}$ mice. (**i**) Quantitative PCR showing expression of M2 markers and Jmjd3 (relative to empty-vector control) in *Jmjd3*$^{-/-}$ M-BMMs in which Irf4 was ectopically expressed using retrovirus. $^{*}P < 0.05$; $^{**}P < 0.01$ (two-tailed Student's *t*-test). Results are representative of two (**a**,**b**,**i**) or three (**c–h**) independent experiments (error bars indicate s.d.).

Given the higher concentration of H3K27me3 tags in the region near the TSS, and the lack of overall correlation between expression changes and tag numbers, we focused on the promoter regions of individual genes that showed H3K27me3 peaks. We looked for peaks in wild-type and *Jmjd3*$^{-/-}$ M-BMM samples and divided the genome-wide set of genes into three different classes depending on H3K27me3 status (**Fig. 6d**). Class 1 genes harbored an H3K27me3 peak in wild-type M-BMMs. Class 2 genes did not have an H3K27me3 peak in either wild-type or *Jmjd3*$^{-/-}$ M-BMMs. Class 3 genes such as *Irf4* and *Tm7sf4* had an H3K27me3 peak in *Jmjd3*$^{-/-}$ but not in wild-type M-BMMs. We generated a table of 500 genes differentially expressed in wild-type and *Jmjd3*$^{-/-}$ M-BMMs, comparing H3K27 methylation status and gene expression from the microarray data (**Supplementary Table 3**).

Although *Hox* genes, such as *Hoxa7*, *Hoxa9* and *Tlx1* (also called *Hox11*), and *Bmp2* have been reported to be regulated by Jmjd3 (ref. 26), the H3K27 of their loci were highly trimethylated in M-BMMs both in the presence and absence of *Jmjd3*, and they therefore were assigned to class 1 (**Fig. 6d** and **Supplementary Table 3**). Furthermore, *Hox* and *Bmp2* gene expression was not lower in *Jmjd3*$^{-/-}$ M-BMMs (**Supplementary Table 3**), which indicates that these genes are not crucially regulated by Jmjd3 in M-BMMs. Furthermore, M2 marker genes, such as *Arg1*, *Chi3l3*, *Rentla* and *Mrc1*, were all in class 2, which indicates that their expression is not directly regulated by Jmjd3 (**Fig. 6d** and **Supplementary Table 3**). Thus, we proposed that transcription factors directly regulated by Jmjd3-mediated demethylation are responsible for the polarization of macrophages. When we searched genes categorized in class 3, we found *Irf4* and *Cebpb*. In particular, the promoter region close to the TSS of *Irf4* had a high H3K27me3 signal in *Jmjd3*$^{-/-}$ but not in wild-type M-BMMs (**Fig. 6d**).

Identification of *Irf4* as a Jmjd3 target gene

We confirmed by ChIP analysis that H3K27 at the promoter region of *Irf4* is differentially methylated in wild-type and *Jmjd3*$^{-/-}$ macrophages (**Fig. 7a**). Furthermore, when we retrovirally expressed the C-terminal region of Jmjd3 or its mutant in *Jmjd3*$^{-/-}$ macrophages, we found the expression of *Irf4* was demethylase activity dependent (**Fig. 7b**). These results demonstrate that *Irf4* is one of the Jmjd3 target genes in M-BMMs. Therefore, we examined the contribution of Irf4 to expression of mRNAs encoding Arg1, Ym1, Fizz1 and MR by using *Irf4*$^{-/-}$ mice. Induction of M2-related genes was severely impaired in *Irf4*$^{-/-}$ M-BMMs; in contrast, the expression of *Jmjd3* was similar between wild-type and *Irf4*$^{-/-}$ M-BMMs (**Fig. 7c**). Notably, the number of M-BMM cells was not lower in *Irf4*$^{-/-}$ mice (**Fig. 7d**). When chitin was administered peritoneally, recruitment of eosinophils, but not macrophages, was severely impaired in *Irf4*$^{-/-}$ mice (**Fig. 7e,f**). MR expression in chitin-elicited peritoneal macrophages was greatly impaired in *Irf4*$^{-/-}$ mice (**Fig. 7g**). In addition, the mRNA expression of the M2 macrophage markers encoding Arg1, Ym1, Fizz1 and MR was severely impaired in chitin-induced macrophages from *Irf4*$^{-/-}$ mice (**Fig. 7h**). These results demonstrate that Irf4 is crucial for the polarization of macrophages to M2 in M-BMMs and *in vivo* in response to chitin administration.

We then retrovirally expressed Irf4 in *Jmjd3*$^{-/-}$ M-BMMs and examined the expression of M2 marker genes (**Fig. 7i**). The expression

of Irf4 upregulated mRNAs encoding Arg1, Ym1, Fizz1 and MR in *Jmjd3*$^{-/-}$ M-BMMs, though the expression of Jmjd3 was unaltered (**Fig. 7i**). These results suggest that Irf4 contributes to the expression of M2 marker genes downstream of Jmjd3.

DISCUSSION

Here we focused on the role of Jmjd3 in macrophages mounting antibacterial and anti-parasitic responses. Whereas Jmjd3 was dispensable for M1 macrophage polarization, mice lacking *Jmjd3* did not mount proper M2 responses against helminth infection or chitin administration. Furthermore, bone marrow macrophages induced by M-CSF showed demethylase activity–dependent defects in expressing various genes, including M2 macrophage markers. Nevertheless, only a subset of genes had H3K27me3 levels differentially regulated by the presence or absence of *Jmjd3*. Among these genes, we found *Irf4* to be one of the direct targets of Jmjd3-mediated demethylation. Finally, we found that Irf4 is a transcription factor crucial for the induction of M2 macrophage responses.

Although *Jmjd3* is a TLR-inducible gene, *Jmjd3*$^{-/-}$ mice showed vigorous M1 macrophage activation in response to *Listeria* inoculation. These results suggest that *Jmjd3* is not essential for generating and recruiting M1 macrophages to bacterial infections. Our data are consistent with a previous report showing that gene expression in response to LPS stimulation is only modestly changed in macrophages lacking *Jmjd3* and that Jmjd3 in this case fine-tunes the transcriptional output[28]. TLR signaling upregulates genes involved not only in the promotion of inflammation, but also in termination or tissue remodeling. For instance, ATF3 and Zc3h12a are rapidly induced in response to TLR stimulation and inhibit inflammatory cytokine production[19,27]. It has been shown that M2 macrophages promote tissue remodeling as well as T_H2 responses. Thus, it is possible that *Jmjd3* induction functions as part of a feedback mechanism acting to repair inflammatory damage caused by TLR stimulation.

Chitin is an abundant structural component of helminths, crustaceans and fungi, and administration of chitin strongly induces M2 macrophage activation. Intraperitoneal administration of chitin recruited M2 macrophages and eosinophils in a Jmjd3- and Irf4-dependent fashion. These results indicate that the Jmjd3-*Irf4* axis is essential for M2 macrophage polarization to helminth infection.

However, addition of chitin to the macrophage culture did not stimulate the cells to upregulate M2 marker gene expression in our experiments (data not shown). Although TLR2 has been reported to mediate acute inflammation in response to chitin, another study has shown that chitin-mediated M2 macrophage activation is independent of MyD88, an adaptor molecule used by all TLRs (refs. 32,35). Currently, the mechanism by which chitin activates macrophages is not well understood. Moreover, it is still not clear what unique role Jmjd3 carries out in the generation of M2 macrophages in response to chitin and helminth infection. The identification of the chitin receptor(s) in the future will be vital for clarifying mechanisms of innate immune activation in response to helminth infection.

Jmjd3$^{-/-}$ mice also showed severe defects in recruiting M2 macrophages in response to *N. brasiliensis* infection. Although it is unknown which components of *N. brasiliensis* activate innate immune cells, Jmjd3-mediated H3K27me3 demethylation seems to be essential for macrophage responses to this parasite. Further studies are needed to identify the role of Jmjd3 in controlling infection with other helminths pathogenic to humans. M2 macrophages are known to be important for tumor cell survival and tissue remodeling in response to inflammation, in addition to the response against helminth infection[36]. Thus, it would be interesting to use this mouse model to explore how epigenetic regulation in macrophages promotes cancer progression or wound healing.

A previous report has found that *Jmjd3* expression is upregulated in response to IL-4 and that H3K27me3 levels decrease in response to IL-4 stimulation[34]. We observed that although M-BMMs and chitin-induced peritoneal macrophages showed severe defects in M2 macrophage marker expression in the absence of *Jmjd3*, *Jmjd3*$^{-/-}$ M-BMMs were capable of upregulating expression of genes representative of M2 macrophages in response to IL-4 stimulation. These findings suggest that IL-4 acts independently of Jmjd3-mediated H3K27 demethylation to promote M2 polarization. The same report[34] showed that H3K27me3 levels of various M2 marker genes were directly controlled by Jmjd3 to activate transcription. In contrast, our ChIP-Seq data demonstrate that H3K27me3 levels of most M2 marker genes, such as *Arg1*, are not changed in the absence of *Jmjd3*. Furthermore, deficiency in *Irf4*, one of the Jmjd3 target genes, resulted in defective M2 responses to chitin administration or M-CSF culture. Thus, it is more likely that Jmjd3 secondarily regulates M2 macrophage polarization by controlling expression of a set of transcription factors.

In addition to M2 marker gene expression, M-BMMs lacking *Jmjd3* showed proliferation defects in response to M-CSF stimulation. This is not due to impaired M-CSF receptor expression or defective activation of initial signaling molecules. Although the expression of genes involved in cell-cycle progression, such as those encoding c-Myc, cyclin D1 and cyclin D2, was impaired in *Jmjd3*$^{-/-}$ M-BMMs, H3K27me3 levels of these genes did not differ between wild-type and *Jmjd3*$^{-/-}$ M-BMMs. Furthermore, *Irf4*$^{-/-}$ M-BMMs did not show a defect in cell cycling (data not shown). Thus, it is possible that other Jmjd3 target genes are responsible for controlling the proliferation of M-BMMs.

ChIP-Seq analysis revealed that, in general, differences between wild-type and *Jmjd3*$^{-/-}$ M-BMM H3K27me3 levels at gene promoter regions were subtle. Nevertheless, gene expression profiles examined by microarray analysis were substantially different in wild-type and *Jmjd3*$^{-/-}$ M-BMMs, and the responses to chitin or helminth infection *in vivo* were severely impaired in *Jmjd3*$^{-/-}$ mice. Although it has been shown that *Hoxa* and *Bmp2* genes are potential targets of Jmjd3 (ref. 26), the expression of these genes was not lower in *Jmjd3*$^{-/-}$ cells, and the H3K27me3 levels were similar between wild-type and *Jmjd3*$^{-/-}$ M-BMMs. These results suggest that other H3K27 demethylases such as Utx and Uty compensate for the lack of *Jmjd3* in macrophages.

However, we identified Irf4 as one direct Jmjd3-specific target transcription factor. Irf4 has been shown to be involved in T_H2 cell polarization as well as in plasma cell differentiation and class-switch recombination in B cells[37,38]. It has also been reported that Irf4 functions in regulatory T cells to regulate T_H2 responses[39]. Indeed, *Irf4*$^{-/-}$ mice have been found to show defective T_H2 responses to *N. brasiliensis* infection[40]. Given that *Irf4*$^{-/-}$ mice did not induce M2 macrophages in response to chitin administration in our experiments, it is likely that the defects of macrophages in *Irf4*$^{-/-}$ mice also contribute to their abnormal responses to *N. brasiliensis* infection. In macrophages, Irf4 functions as a negative regulator of TLR signaling by associating with MyD88 (refs. 41,42).

Jmjd3$^{-/-}$ mice showed neonatal death due to a developmental defect in lung tissue. Although Jmjd3 directly regulated the expression of *Irf4* in macrophages, *Irf4*$^{-/-}$ mice did not show a developmental defect. Thus, Jmjd3 controls genes other than *Irf4* in the lung tissues for proper tissue development, and we focused solely on the role of this molecule in macrophages.

It is tempting to speculate that the change in epigenetic status is crucial for determining macrophage polarization. Future development of procedures to specifically regulate Jmjd3 demethylase activity might be useful for manipulating macrophages to mount anti-helminth host defenses and tissue repair.

METHODS

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

Accession codes. GEO: microarray data, GSE23180; ChIP-Seq data, GSE23297.

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

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AUTHOR CONTRIBUTIONS

T. Satoh and O.T. designed and performed experiments. Y.K., T. Miyake, K.M., T.O. and T. Saitoh performed experiments. A.V., Y.T., D.M.S. and K. Nakai analyzed ChIP-Seq data. K. Yasuda and K. Nakanishi performed *N. brasiliensis* infection experiments. K.H., T. Matsuyama and K. Yui provided *Irf4*$^{-/-}$ mice. T.T. performed histological examination. O.T., T. Satoh and S.A. wrote the manuscript. S.A. supervised the project. A.V. and K.Y. contributed equally to this work.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interest.

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ONLINE METHODS

Generation of *Jmjd3*$^{-/-}$ mice. The *Jmjd3* gene was isolated from genomic DNA extracted from embryonic stem cells (GSI-I) by PCR. The targeting vector was constructed by replacing a 4-kb fragment encoding the *Jmjd3* open reading frame (exons 14–21, including exons encoding the JmjC domain) with a neomycin-resistance gene cassette (*neo*), and herpes simplex virus thymidine kinase was inserted into the genomic fragment for negative selection. After the targeting vector was transfected into embryonic stem cells, G418 and gancyclovir doubly-resistant colonies were selected and screened by PCR; recombination was further confirmed by Southern blotting. These homologous-recombinant clones were microinjected into blastocysts derived from C57BL/6 mice and were transferred to pseudopregnant females. Matings of chimeric male mice to C57BL/6 female mice resulted in transmission of the mutant allele to the germline. Resulting *Jmjd3*$^{+/-}$ mice were intercrossed to generate *Jmjd3*$^{-/-}$ mice. All animal experiments were done with the approval of the Animal Research Committee of the Research Institute for Microbial Diseases, Osaka University.

Mice, cells and reagents. *Irf4*$^{-/-}$ mice were prepared as described[41]. Bone marrow–derived macrophages were generated in RPMI-1640 medium containing 10% (vol/vol) FCS, 50 μM 2-mercaptoethanol and 10 ng/ml GM-CSF (PeproTech) or 10 ng/ml M-CSF (PeproTech). Pam_3CSK_4 and R-848 were prepared as described[27]. LPS (*Salmonella minnesota* Re595) was from Sigma.

Generation of bone marrow–chimeric mice. Fetal liver cells were prepared from wild-type and *Jmjd3*$^{-/-}$ embryos (embryonic day 15.5). The cell suspensions were intravenously injected into lethally irradiated CD45.1 C57BL/6 mice. The chimeric mice were given neomycin and ampicillin in their drinking water for 4 weeks. The mice were analyzed at least 8 weeks after reconstitution. More than 90% of splenocytes from chimeric mice were CD45.2 positive.

Quantitative PCR analysis. Total RNA was isolated with TRIzol (Invitrogen), and reverse transcription was performed with ReverTra Ace (Toyobo) according to the manufacturer's instructions. For quantitative PCR, cDNA fragments were amplified by Realtime PCR Master Mix (Toyobo); fluorescence from the TaqMan probe for each cytokine was detected by a 7500 real-time PCR system (Applied Biosystems). To determine the relative induction of cytokine mRNA in response to various stimuli, the mRNA expression level of each gene was normalized to the expression level of 18S rRNA. The experiments were repeated at least twice.

Immunoblot analysis. M-BMMs were cultured for 4 h in medium without M-CSF (PeproTech) and then were collected and replated. M-BMMs were stimulated with M-CSF for times indicated in **Figure 5e** and were lysed with lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA and 1% (vol/vol) Nonidet P-40) containing complete mini protease inhibitor cocktail (Roche). Cell lysates were separated by standard SDS-PAGE and analyzed by immunoblot. Antibodies to the following proteins were used: phosphorylated Erk (Cell Signaling no. 9101), phosphorylated Akt (Cell Signaling 9271), phosphorylated p38 (Cell Signaling 9211), Akt (Cell Signaling 9272), p38 (Santa Cruz C-20), Erk (Santa Cruz K-23) and β-actin (Santa Cruz C-11).

Flow cytometry. Antibodies for flow cytometry were purchased from BD Biosciences and eBioscience. Cells were washed in ice-cold flow-cytometry buffer (2% (vol/vol) FCS and 2 mM EDTA in PBS, pH 7.5), then incubated with each antibody for 15 min and washed twice with flow-cytometry buffer. Intracellular cytokines were stained with Cytofix/Cytoperm Plus Fixation/Permeabilization Kit (BD Biosciences) according to the manufacturer's instructions. Data were acquired on a FACSCalibur flow cytometer (BD Biosciences) and analyzed with FlowJo (Tree Star).

Construction of *Jmjd3* expression plasmids. *Jmjd3* cDNA (corresponding to amino acid residues 1141–1641) was obtained by PCR from a mouse cDNA library, and a point mutation resulting in the A1388H substitution in the JmjC domain was introduced by site-directed mutagenesis (Stratagene). The full or mutated *Jmjd3* cDNAs were cloned into the pMRX-ires-puro vector for retrovirus production[43].

Retroviral transduction. Bone marrow cells were isolated from *Jmjd3*$^{-/-}$ mice that had been injected intraperitoneally 4 d earlier with 5 mg of 5-fluorouracil (Nacalai Tesque). Cells were cultured in stem cell medium (RPMI supplemented with 15% (vol/vol) FCS, 10 mM sodium pyruvate, 2 μM L-glutamine, 50 μM β-mercaptoethanol, 100 U/ml penicillin, 100 g/ml streptomycin, 100 ng/ml stem cell factor, 10 ng/ml IL-6 and 10 ng/ml IL-3). Then, 48 h later, these cells were transduced with retroviral supernatant (supplemented with stem cell factor, IL-6, IL-3 and 10 ng/ml of polybrene) on two successive days. Virus was produced by PlatE packaging cells transfected with various plasmids. After the second transduction, cells were washed and resuspended in macrophage growth medium (RPMI-1640 medium supplemented with 10% (vol/vol) FCS, 50 μM β-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin and 20 ng/ml M-CSF). After 3.5 d in culture, cells were washed once and macrophage growth medium with 2.5 μg/ml puromycin (InvivoGen) was added. The cells were cultivated for 2 d after changing of the medium and then were analyzed.

Chitin administration. Chitin (Sigma) was washed three times in PBS and then sonicated with a UR-20P device (Tomy) for 30 min on ice. After filtration with 100 μM cell strainer, chitin was diluted in 50 ml PBS. About 800 ng chitin was intraperitoneally injected, and PECs were collected 2 d after administration.

Responses to *N. brasiliensis* infection. Wild-type and *Jmjd3*$^{-/-}$ fetal liver–chimeric mice were subcutaneously inoculated with 300 third-stage larvae of *N. brasiliensis* 8 weeks after fetal liver transfer. On day 5 after infection, *N. brasiliensis*–inoculated mice were killed and perfused with PBS, and total RNAs from lungs were extracted. RNA was subjected to quantitative PCR for the analysis of expression of various genes. Nine days after infection, hilar lymph nodes were harvested, a single-cell suspension was prepared and cell numbers were counted. The lymph node cells were stimulated with anti-CD3 and anti-CD28. They were stained with CD4 and treated with cytofix (BD Biosciences), then stained with anti–IL-4 and anti–IFN-γ. Next, the cells were examined by flow cytometry. BAL was performed at 5 and 13 d after *N. brasiliensis* infection, and macrophages and eosinophils were enumerated on cytospin smears stained with Diff-Quick (Baxter Healthcare).

Microarray and chromatin immunoprecipitation–sequencing analysis. Microarray and ChIP-Seq protocols and data analysis are described in **Supplementary Methods.**

Statistics. Statistical significance was calculated with the two-tailed Student's *t*-test.

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Current Opinion in Immunology

Basophils as APC in Th2 response in allergic inflammation and parasite infection

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Basophils are important effector cells, which contribute to protection against helminths and execute proinflammatory effector function during allergic inflammation. Basophils are also regulators of Th2 responses in helminth-infected hosts and in allergen-injected animals. Recently, three groups using different experimental systems have shown that basophils are antigen-presenting cells (APC), which induce Th2 cells both *in vitro* and *in vivo*. Basophils express MHC class II and CD80/86, have the potential to take-up and process protein antigen (Ag), particularly Ag-IgE complexes, and to present peptide with MHC class II and produce IL-4. However, relevance of basophils as Th2 cell-inducing APC *in vivo* has been challenged by several recent reports that favor the concept that basophils and DC cooperate or basophils merely amplify DC-driven Th2 cell differentiation. In this review, I summarize and discuss the data on the role of basophils as Th2 cell-inducing APC in allergy and parasite infection.

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Introduction

Mast cells, basophils, and eosinophils are important effector cells in helminth infection and allergic inflammation [1,2,3•,4•,5]. Basophils are rare circulating cells, accounting for less than 1% of total circulating granulocytes. Basophils, mast cells, and eosinophils arise from the same progenitor. Basophils and eosinophils complete their terminal differentiation in bone marrow. By contrast, mast cells migrate as immature cells from the bone marrow to the peripheral tissues, where they undergo their terminal differentiation [1,2]. Mast cells that complete their differentiation in the skin or in the intestine develop into connective tissue mast cells (CTMCs) and mucosal mast cells (MMCs), respectively. Circulating basophils share several features with tissue-resident mast cells. Both cell types constitutively express FcεR1 and contain basophilic granules in the cytoplasm, and upon cross-linking of FcεR1-bound IgE with multivalent antigens, immediately release various kinds of effector molecules such as histamine and lipid mediators, and Th2-associated cytokines such as IL-4, IL-5, and IL-13, causing immediate type hypersensitivity [1,2,3•,4•,5]. Eosinophils also express FcεR1 and induce allergic inflammation by production of chemical mediators when stimulated with cross-linking of FcεR1-bound IgE with antigen. Owing to scarcity of cell number, paucity of specific basophil markers, and their functional similarity to mast cells, basophils have been simply regarded as effector cells of the Th2 immune response [3•,4•,5].

IL-3 is a growth factor for basophils. Furthermore, IL-3 is required for optimal basophil IL-4 and IL-13 production. FcR common γ-chain (FcRγ) is a widely expressed adapter, which bears an immunoreceptor tyrosine-based activation motif (ITAM) [6]. This adapter protein is known to associate with various FcR including FcεR1. Recent study by Hida and colleagues demonstrated that basophils lacking FcRγ could proliferate normally but failed to produce IL-4 in response to IL-3, suggesting that FcRγ-mediated IL-3 signal is crucially involved in induction of IL-4 production by basophils [7]. Now it is well recognized that basophils strongly produce IL-4 and IL-13 when stimulated with cross-linking of FcεR1 and IL-3.

The effect of IL-3 is also found in IgE-independent basophil IL-4 production [5,8,9•]. Basophils express IL-18R and IL-33R and very strongly produce IL-4 and IL-13 when stimulated with IL-18 or IL-33 in the presence of IL-3 *in vitro* [8,9•]. Basophils also express Toll-like receptor (TLR)1, TLR2, TLR4, and TLR6 and produce Th2 cytokines in response to IL-3 plus corresponding TLR ligands[10]. Thus, there are at least two major activation pathways for basophil IL-4 production. One is the Ag/IgE complex-induced activation pathway responsible for 'acquired-type allergic inflammation' and the other is the IL-18, IL-33, or pathogen associated molecular pattern (PAMP)-induced activation pathway responsible for 'innate type allergic inflammation'. Here, I review the major function of basophils as effectors cells in the development of allergic inflammation and the recently found novel function of basophils as Th2 cell-inducing APC in allergy and helminth infection.

Development of basophils, mast cells and eosinophils

Blood circulating basophils are mature cells with a life span of about 60 h. Injection of IL-3 increases basophil generation in bone marrow, resulting in an increase in the number of circulating basophils. By contrast, mast cells in the peripheral tissue have the potential to proliferate in response to IL-3 and IL-9 *in situ*. Thus, intestinal helminth infection induces intestinal mast cell hyperplasia in an IL-3-dependent manner. A study of the development of mouse eosinophils, basophils, and mast cells has identified that granulocyte/monocyte progenitor (GMP) is a common progenitor of basophils, mast cells, and eosinophils [11]. Both basophil/mast cell precursors (BMCP) and eosinophil precursor (EoP) arise from GMP. Development from GMP to EoP and BMCP, and from BMCP to basophil precursors (BaP) and mast cell precursors (MCP), are regulated by the level and order of expression of transcription factors, C/EBPα, and, GATA-2 [11]. However, in the case of development of human granulocytes, common myeloid progenitor (CMP) develops into GMP and EoP, which then develop into basophils/mast cells and eosinophils, respectively [11].

The major function of basophils as effector cells

Although basophils and mast cells are regarded as important effector cells in allergic response by their potential to promptly produce chemical mediators and cytokines after FcεR1 cross-linkage [1,2,3•,4•,5], recent studies suggest that basophils also induce IgE-mediated chronic allergic inflammation and IgG1-mediated systemic anaphylactic shock [4•,12,13•]. Karasuyama and colleagues demonstrated that a single injection of multivalent antigen in the ear of mice passively sensitized with antigen-specific IgE, elicits immediate-phase, late-phase, and delayed onset of ear swelling characterized by infiltration with basophils and eosinophils (chronic allergic inflammation) [12]. They showed that mast cell-deficient mice only developed chronic allergic inflammation, while basophil-depleted mice failed to develop it, suggesting that basophils are responsible for inducing chronic allergic inflammation. Very recently, a study by Voehringer and colleagues has confirmed this observation by using basophil-deficient mice (*Mcp8Cre*), which constitutively lack more than 90% of basophils [14••]. Furthermore, they demonstrated that reconstitution of *Mcpt8Cre* mice with bone marrow basophils restored this IgE-mediated chronic allergic inflammation response [14••]. These results strongly indicate that basophils are required and sufficient to induce IgE-mediated chronic allergic inflammation by recruiting eosinophils [12,14••].

It is well documented that mast cells and IgE are crucially involved in the development of systemic anaphylaxis. However, interestingly, mice deficient for mast cells, IgE, or FcεR1α chain still develop systemic anaphylaxis, indicating involvement of an alternative pathway. FcRγ-deficient mice that lack the expression of FcεR1 and stimulatory FcγR do not develop systemic anaphylaxis, suggesting that IgG also plays a crucial role in induction of systemic anaphylaxis. Karasuyama and colleagues demonstrated that basophils and IgG1 contribute to certain type of mast cell-independent systemic anaphylaxis [13•]. They demonstrated that basophils released a large amount of platelet-activating fator (PAF) when stimulated with allergen-IgG1 immune complexes. Based on this result, they speculated that basophils induce systemic anaphylaxis through the release of PAF that is 30,000 times more potent than histamine [13•]. However, basophil-ablated *Mcpt8Cre* mice are shown to normally develop IgE or IgG1-dependent systemic anaphylaxis, suggesting the possibility that basophils play a minor role in an induction of systemic anaphylaxis [14••]. In a previous study, Finkelman and colleagues reported that macrophages play a major role in IgG-mediated systemic anaphylaxis through the release of PAF [15]. These results suggest that both basophils and macrophage contribute to IgG1-mediated systemic anaphylaxis. However, further research is needed to determine which cell contributes most strongly to this IgG-mediated anaphylaxis.

Th subset

Naïve $CD4^+$ T cells develop into Th1, Th2, Th17, and Treg cells, when they are given antigenic signals, co-stimulatory signals, and appropriate cytokine signals by APC and accessory cells [16]. IFN-γ, IL-12, and T-bet control development of Th1 cells, which are highly effective in clearance of intracellular pathogens by the production of IFN-γ [16,17]. IL-4 and GATA-3 control development of Th2 cells [16,18], which produce IL-4, IL-5, IL-6, IL-9, and IL-13. These Th2 cytokines are important for the development of allergic inflammation and clearance of helminth infections via the induction of IgE production, activation of mast cells, basophils, and eosinophils. Th17 cell subset is important for the development of autoimmune diseases and for the clearance of extracellular pathogens and fungi by producing IL-17 [19]. Differentiation of Th17 cells is induced by TGFβ and IL-6 in the mouse and by TGFβ and IL-6/IL-21 in the human. Treg cells are induced by TGFβ and are essential for immune tolerance and regulation of allergy and autoimmunity [20].

It is well recognized that DCs play a central role in initiation of activation and differentiation of Th subsets. DCs sense microbes through TLRs and mature to express co-stimulatory molecules CD80/86 and to produce the cytokines that provide the appropiate instructive signal for the development of Th1, Th17, and Treg cells [19–21]. Antigen-pulsed DC also induce the development of Th2 cells under the influence of IL-4 *in vitro* [16]. Furthermore, several reports indicate the presence of

other pathways for the differentiation of naïve CD4+ T cells into Th2 cells [22–26]. DCs cannot produce IL-4, however DCs have the potential to induce Th2 cells via expression of the Notch ligand Jagged 1 and Jagged2 [22]. DCs also induce Th2 cells by expressing OX-40L after being stimulated with thymic stromal lymphopoetin (TSLP) [23]. Aluminum adjuvant induces Th2 cell differentiation, although the exact mechanism still remains uncertain [24]. In addition, M2 macrophages (also known as alternatively activated macrophages), as well as eosinophils and mast cells are also important for the development of Th2 cells [25,26]. Thus, it is important to determine which cell types help DCs to induce the development of Th2 cells.

Th2 development: basophils as accessory cells that produce early IL-4

Min *et al.* reported that naïve CD4+ T cells stimulated with peptide-pulsed DCs could develop into Th2 cells when co-cultured with basophils from wild type mice but not from IL-4-deficient mice [27••]. As DCs and basophils are added to the same culture, it was initially interpreted that DC deliver antigenic-specific signal, and basophils provide IL-4 for the development of Th2 cells. In *in vivo* studies, mice deficient in interferon-regulatory factor 2 (IRF2) or Lyn have increased numbers of basophils and exhibit spontaneous Th2 differentiation under steady state conditions [28,29]. However, introduction of mutation in the gene encoding c-Kit inhibits this spontaneous Th2 differentiation by reducing the number of basophils [28]. Thus, basophils might be required for the development of naïve CD4+ T cells into Th2 cells *in vivo*. Medzhitov and colleagues showed that basophils are important in initiation of the development of Th2 cells in response to the protease allergen, papain [30••,31]. At day 3 after subcutaneous papain injection, basophils enter and transiently reside in the T cell zones of the draining lymph nodes, where basophils are stimulated to produce IL-4 and/or TSLP, which promote Th2 differentiation *in vivo*. Basophils are also necessary for Th2 differentiation in the mice infected with *T. muris* [32]. Depletion of basophils with antibody against FcεR1 diminished the development of Th2 cells in both models of Th2 cell differentiation, suggesting that basophils are involved in Th2 cell differentiation by production of 'early IL-4' [31,32]. Furthermore, basophil production of IL-4 and IL-6 promotes the development of IL-10-producing CD8+ T cells *in vivo* [33], suggesting that basophils play important roles for the functional differentiation of CD4+ T cells and CD8+ T cells.

Induction of Th2 cells by basophils pulsed with Ag/IgE complex

Basophils promptly produce IL-4 and IL-13 when they are stimulated with Ag/IgE complex or with IL-18 and/or IL-33 in the presence of IL-3 [5,8,9•,10]. Thus, if basophils express MHC class II and CD80/86, we could speculate that basophils also have the potential to induce the development of Th2 cells. Three groups independently demonstrated that basophils constitutively express MHC class II, as well as co-stimulatory molecules such as CD40, CD80, and CD86 [31,32,34]. These groups further demonstrated that basophils are potent APCs [31,32,34]. We have reported that basophils have the capacity to induce Th2 differentiation both *in vitro* and *in vivo* [34].

We prepared splenic basophils from mice inoculated with *S. venezuelensis*, as helminth infection markedly induces an increase in the number of basophils in the spleen and liver [35]. Basophils from infected mice have the capacity to strongly produce IL-4, IL-6, and IL-13 in medium alone, even without IL-3 [34]. Furthermore, they express MHC class II and CD80/86 and induce the development of OVA-specific naïve CD4+ T cells into Th2 cells *in vitro* in the presence of OVA peptide, IL-2 and IL-3 without IL-4 (neutral culture condition) [34]. Thus, we initially regarded only those basophils derived from infected mice to be potent Th2 cell-inducing APC. However, we quickly learned that splenic basophils derived from naïve mice also produce IL-4, IL-6, and IL-13 in IL-3-containing medium [34]. Compared to the amount of cytokines produced by basophils from infected animals, the amount of cytokines produced by basophils from naïve mice is relatively low. Furthermore, they need the presence of IL-3 in the culture medium to produce these cytokine [7,34]. Nevertheless, splenic basophils from naïve mice express comparable levels of MHC class II and CD80/86 and have the capacity to induce the development of Th2 cells *in vitro* under neutral conditions. Thus, both types of basophils are potent APC that strongly induce Th2 cells *in vitro*.

We next tested whether bone marrow basophils also have the potential to induce the development of Th2 cells. We found that they express MHC class II, CD80, CD86, and CD62L, and take-up allergen such as OVA, and process them into small peptides [34]. Since they express FcεR1 abundantly, we speculated that they can take up a low dose of Ag/IgE complex, present Ag/MHC class II, and produce IL-4. Thus, we examined whether basophils become very potent Th2 cell-inducing APC, when Ag is provided as Ag/IgE complexes [34].

We prepared OVA-pulsed basophils by culturing basophils with DNP-OVA and anti-DNP IgE complexes. Then, we intravenously (iv) administered 0.25 or 0.5 million OVA-pulsed basophils to naïve mice. This treatment strongly induced OVA-specific Th2 cells in the spleen of naïve mice. By contrast, iv administration of OVA-pulsed DCs or mast cells failed to induce Th2 cells, although OVA-pulsed DCs induced IFN-γ producing Th1 cells. Thus, basophils are very potent Th2 cell-inducing APC even *in vivo*. We next tested whether