

- [10] K. Kuida, J. A. Lippke, G. Ku, et al., "Altered cytokine export and apoptosis in mice deficient in interleukin-1 β converting enzyme," *Science*, vol. 267, no. 5206, pp. 2000–2003, 1995.
- [11] Y. Gu, K. Kuida, H. Tsutsui, et al., "Activation of interferon- γ inducing factor mediated by interleukin-1 β converting enzyme," *Science*, vol. 275, no. 5297, pp. 206–209, 1997.
- [12] T. Ghayur, S. Banerjee, M. Hugunin, et al., "Caspase-1 processes IFN- γ -inducing factor and regulates LPS-induced IFN- γ production," *Nature*, vol. 386, no. 6625, pp. 619–623, 1997.
- [13] K. Nakanishi, T. Yoshimoto, H. Tsutsui, and H. Okamura, "Interleukin-18 regulates both Th1 and Th2 responses," *Annual Review of Immunology*, vol. 19, pp. 423–474, 2001.
- [14] H. Tsutsui, T. Yoshimoto, N. Hayashi, H. Mizutani, and K. Nakanishi, "Induction of allergic inflammation by interleukin-18 in experimental animal models," *Immunological Reviews*, vol. 202, pp. 115–138, 2004.
- [15] E. Seki, H. Tsutsui, H. Nakano, et al., "Lipopolysaccharide-induced IL-18 secretion from murine Kupffer cells independently of myeloid differentiation factor 88 that is critically involved in induction of production of IL-12 and IL-1 β ," *Journal of Immunology*, vol. 166, no. 4, pp. 2651–2657, 2001.
- [16] I. Ogushi, Y. Iimuro, E. Seki, et al., "Nuclear factor κ B decoy oligodeoxynucleotides prevent endotoxin-induced fatal liver failure in a murine model," *Hepatology*, vol. 38, no. 2, pp. 335–344, 2003.
- [17] H. Okamura, H. Tsutsui, T. Komatsu, et al., "Cloning of a new cytokine that induces IFN- γ production by T cells," *Nature*, vol. 378, no. 6552, pp. 88–91, 1995.
- [18] Y. Sakao, K. Takeda, H. Tsutsui, et al., "IL-18-deficient mice are resistant to endotoxin-induced liver injury but highly susceptible to endotoxin shock," *International Immunology*, vol. 11, no. 3, pp. 471–480, 1999.
- [19] J. P.-Y. Ting, R. C. Lovering, E. S. Alnemri, et al., "The NLR gene family: a standard nomenclature," *Immunity*, vol. 28, no. 3, pp. 285–287, 2008.
- [20] T.-D. Kanneganti, M. Lamkanfi, Y.-G. Kim, et al., "Pannexin-1-mediated recognition of bacterial molecules activates the cryopyrin inflammasome independent of Toll-like receptor signaling," *Immunity*, vol. 26, no. 4, pp. 433–443, 2007.
- [21] D. A. Muruve, V. Pétrilli, A. K. Zaiss, et al., "The inflammasome recognizes cytosolic microbial and host DNA and triggers an innate immune response," *Nature*, vol. 452, no. 7183, pp. 103–107, 2008.
- [22] V. Hornung, A. Ablasser, M. Charrel-Dennis, et al., "AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC," *Nature*, vol. 458, no. 7237, pp. 514–518, 2009.
- [23] T. Fernandes-Alnemri, J.-W. Yu, P. Datta, J. Wu, and E. S. Alnemri, "AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA," *Nature*, vol. 458, no. 7237, pp. 509–513, 2009.
- [24] T. Bürckstümmer, C. Baumann, S. Blüml, et al., "An orthogonal proteomic-genomic screen identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome," *Nature Immunology*, vol. 10, no. 3, pp. 266–272, 2009.
- [25] T. L. Roberts, A. Idris, J. A. Dunn, et al., "HIN-200 proteins regulate caspase activation in response to foreign cytoplasmic DNA," *Science*, vol. 323, no. 5917, pp. 1057–1060, 2009.
- [26] F. Martinon, A. Mayor, and J. Tschopp, "The inflammasomes: guardians of the body," *Annual Review of Immunology*, vol. 27, pp. 229–265, 2009.
- [27] J. Ferluga and A. C. Allison, "Role of mononuclear infiltrating cells in pathogenesis of hepatitis," *The Lancet*, vol. 1, no. 8090, pp. 610–611, 1978.
- [28] Y. Mizoguchi, H. Tsutsui, K. Miyajima, et al., "The protective effects of prostaglandin E1 in an experimental massive hepatic cell necrosis model," *Hepatology*, vol. 7, no. 6, pp. 1184–1188, 1987.
- [29] H. Tsutsui, Y. Mizoguchi, and S. Morisawa, "Importance of direct hepatocytolysis by liver macrophages in experimental fulminant hepatitis," *Hepato-Gastroenterology*, vol. 39, no. 6, pp. 553–559, 1992.
- [30] T. Yoshimoto, K. Nakanishi, S. Hirose, et al., "High serum IL-6 level reflects susceptible status of the host to endotoxin and IL-1/tumor necrosis factor," *Journal of Immunology*, vol. 148, no. 11, pp. 3596–3603, 1992.
- [31] J. A. Russell, "Management of sepsis," *The New England Journal of Medicine*, vol. 355, no. 16, pp. 1699–1713, 2006.
- [32] K. Kawa, H. Tsutsui, R. Uchiyama, et al., "IFN- γ is a master regulator of endotoxin shock syndrome in mice primed with heat-killed *Propionibacterium acnes*," *International Immunology*, vol. 22, no. 3, pp. 157–166, 2010.
- [33] T. P. Clemmer, C. J. Fisher Jr., R. C. Bone, et al., "Hypothermia in the sepsis syndrome and clinical outcome. The Methylprednisolone Severe Sepsis Study Group," *Critical Care Medicine*, vol. 20, no. 10, pp. 1395–1401, 1992.
- [34] D. G. Remick and H. Xia, "Hypothermia and sepsis," *Frontiers in Bioscience*, vol. 11, no. 1, pp. 1006–1013, 2006.
- [35] M. Levi and H. Ten Cate, "Disseminated intravascular coagulation," *The New England Journal of Medicine*, vol. 341, no. 8, pp. 586–592, 1999.
- [36] M. S. Sawdey and D. J. Loskutoff, "Regulation of murine type 1 plasminogen activator inhibitor gene expression in vivo. Tissue specificity and induction by lipopolysaccharide, tumor necrosis factor- α , and transforming growth factor- β ," *Journal of Clinical Investigation*, vol. 88, no. 4, pp. 1346–1353, 1991.
- [37] L. Romics Jr., A. Dolganiuc, K. Kodys, et al., "Selective priming to toll-like receptor 4 (TLR4), not TLR2, ligands by *P. acnes* involves up-regulation of MD-2 in mice," *Hepatology*, vol. 40, no. 3, pp. 555–564, 2004.
- [38] M. Imamura, H. Tsutsui, K. Yasuda, et al., "Contribution of TIR domain-containing adapter inducing IFN- β -mediated IL-18 release to LPS-induced liver injury in mice," *Journal of Hepatology*, vol. 51, no. 2, pp. 333–341, 2009.
- [39] H. Yoneyama, K. Matsuno, Y. Zhang, et al., "Regulation by chemokines of circulating dendritic cell precursors, and the formation of portal tract-associated lymphoid tissue, in a granulomatous liver disease," *Journal of Experimental Medicine*, vol. 193, no. 1, pp. 35–49, 2001.
- [40] H. Yoneyama, S. Narumi, Y. Zhang, et al., "Pivotal role of dendritic cell-derived CXCL10 in the retention of T helper cell 1 lymphocytes in secondary lymph nodes," *Journal of Experimental Medicine*, vol. 195, no. 10, pp. 1257–1266, 2002.
- [41] N. Van Rooijen and A. Sanders, "Elimination, blocking, and activation of macrophages: three of a kind?" *Journal of Leukocyte Biology*, vol. 62, no. 6, pp. 702–709, 1997.
- [42] M. A. Collart, D. Belin, J.-D. Vassalli, S. de Kossodo, and P. Vassalli, " γ interferon enhances macrophages transcription of the tumor necrosis factor/cachectin, interleukin 1, and urokinase genes, which are controlled by short-lived repressors," *Journal of Experimental Medicine*, vol. 164, no. 6, pp. 2113–2118, 1986.
- [43] K. Takeda, H. Tsutsui, T. Yoshimoto, et al., "Defective NK cell activity and Th1 response in IL-18-deficient mice," *Immunity*, vol. 8, no. 3, pp. 383–390, 1998.

- [44] A. Kumanogoh, T. Shikina, K. Suzuki, et al., "Nonredundant roles of Sema4A in the immune system: defective T cell priming and Th1/Th2 regulation in Sema4A-deficient mice," *Immunity*, vol. 22, no. 3, pp. 305–316, 2005.
- [45] K. Matsui, T. Yoshimoto, H. Tsutsui, et al., "Propionibacterium acnes treatment diminishes CD4⁺NK1.1⁺ T cells but induces type I T cells in the liver by induction of IL-12 and IL-18 production from Kupffer cells," *Journal of Immunology*, vol. 159, no. 1, pp. 97–106, 1997.
- [46] G. Trinchieri, "Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity," *Annual Review of Immunology*, vol. 13, pp. 251–276, 1995.
- [47] Y. Hyodo, K. Matsui, N. Hayashi, et al., "IL-18 up-regulates perforin-mediated NK activity without increasing perforin messenger RNA expression by binding to constitutively expressed IL-18 receptor," *Journal of Immunology*, vol. 162, no. 3, pp. 1662–1668, 1999.
- [48] J. Sawaki, H. Tsutsui, N. Hayashi, et al., "Type 1 cytokine/chemokine production by mouse NK cells following activation of their TLR/MyD88-mediated pathways," *International Immunology*, vol. 19, no. 3, pp. 311–320, 2007.
- [49] T. Merlin, A. Sing, P. J. Nielsen, C. Galanos, and M. A. Freudenberg, "Inherited IL-12 unresponsiveness contributes to the high LPS resistance of the *Lps*^d C57BL/10ScCr mouse," *Journal of Immunology*, vol. 166, no. 1, pp. 566–573, 2001.
- [50] H. Tsuji, N. Mukaida, A. Harada, et al., "Alleviation of lipopolysaccharide-induced acute liver injury in Propionibacterium aches-primed IFN- γ -deficient mice by a concomitant reduction of TNF- α , IL-12, and IL-18 production," *Journal of Immunology*, vol. 162, no. 2, pp. 1049–1055, 1999.
- [51] I. Hritz, A. Velayudham, A. Dolganiuc, et al., "Bone Marrow-derived immune cells mediate sensitization to liver injury in a myeloid differentiation factor 88-dependent fashion," *Hepatology*, vol. 48, no. 4, pp. 1342–1347, 2008.
- [52] L. Romics Jr., A. Dolganiuc, A. Velayudham, et al., "Toll-like receptor 2 mediates inflammatory cytokine induction but not sensitization for liver injury by Propionibacterium acnes," *Journal of Leukocyte Biology*, vol. 78, no. 6, pp. 1255–1264, 2005.
- [53] C. Kalis, M. Gumenscheimer, N. Freudenberg, et al., "Requirement for TLR9 in the immunomodulatory activity of Propionibacterium acnes," *Journal of Immunology*, vol. 174, no. 7, pp. 4295–4300, 2005.
- [54] H. Tsutsui, K. Matsui, N. Kawada, et al., "IL-18 accounts for both TNF- α - and Fas ligand-mediated hepatotoxic pathways in endotoxin-induced liver injury in mice," *Journal of Immunology*, vol. 159, no. 8, pp. 3961–3967, 1997.
- [55] H. Tsutsui, N. Kayagaki, K. Kuida, et al., "Caspase-1-independent, Fas/Fas ligand-mediated IL-18 secretion from macrophages causes acute liver injury in mice," *Immunity*, vol. 11, no. 3, pp. 359–367, 1999.
- [56] T. Yoshimoto, K. Takeda, T. Tanaka, et al., "IL-12 up-regulates IL-18 receptor expression on T cells, Th1 cells, and B cells: synergism with IL-18 for IFN- γ production," *Journal of Immunology*, vol. 161, no. 7, pp. 3400–3407, 1998.
- [57] S. Mariathasan and D. M. Monack, "Inflammasome adaptors and sensors: intracellular regulators of infection and inflammation," *Nature Reviews Immunology*, vol. 7, no. 1, pp. 31–40, 2007.
- [58] F. Martinon and J. Tschoop, "Inflammatory caspases and inflammasomes: master switches of inflammation," *Cell Death and Differentiation*, vol. 14, no. 1, pp. 10–22, 2007.
- [59] K. Matsui, H. Tsutsui, and K. Nakanishi, "Pathophysiological roles for IL-18 in inflammatory arthritis," *Expert Opinion on Therapeutic Targets*, vol. 7, no. 6, pp. 701–724, 2003.
- [60] M. Yamamoto, K. Yaginuma, H. Tsutsui, et al., "ASC is essential for LPS-induced activation of procaspase-1 independently of TLR-associated signal adaptor molecules," *Genes to Cells*, vol. 9, no. 11, pp. 1055–1067, 2004.
- [61] M. Terada, H. Tsutsui, Y. Imai, et al., "Contribution of IL-18 to atopic-dermatitis-like skin inflammation induced by Staphylococcus aureus product in mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 23, pp. 8816–8821, 2006.
- [62] E. Seki, Y. Kondo, Y. Iimuro, et al., "Demonstration of cooperative contribution of MET- and EGFR-mediated STAT3 phosphorylation to liver regeneration by exogenous suppressor of cytokine signaling," *Journal of Hepatology*, vol. 48, no. 2, pp. 237–245, 2008.
- [63] P. Matzinger, "The danger model: a renewed sense of self," *Science*, vol. 296, no. 5566, pp. 301–305, 2002.
- [64] A. Rubartelli and M. T. Lotze, "Inside, outside, upside down: damage-associated molecular-pattern molecules (DAMPs) and redox," *Trends in Immunology*, vol. 28, no. 10, pp. 429–436, 2007.
- [65] M. T. Lotze, H. J. Zeh, A. Rubartelli, et al., "The grateful dead: damage-associated molecular pattern molecules and reduction/oxidation regulate immunity," *Immunological Reviews*, vol. 220, no. 1, pp. 60–81, 2007.
- [66] M. T. Lotze and K. J. Tracey, "High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal," *Nature Reviews Immunology*, vol. 5, no. 4, pp. 331–342, 2005.
- [67] Q. Zhang, M. Raouf, Y. Chen, et al., "Circulating mitochondrial DAMPs cause inflammatory responses to injury," *Nature*, vol. 464, no. 1, pp. 104–107, 2010.
- [68] C.-J. Chen, H. Kono, D. Golenbock, G. Reed, S. Akira, and K. L. Rock, "Identification of a key pathway required for the sterile inflammatory response triggered by dying cells," *Nature Medicine*, vol. 13, no. 7, pp. 851–856, 2007.
- [69] G. Haraldsen, J. Balogh, J. Pollheimer, J. Sponheim, and A. M. Küchler, "Interleukin-33-cytokine of dual function or novel alarmin?" *Trends in Immunology*, vol. 30, no. 5, pp. 227–233, 2009.
- [70] J. Xu, X. Zhang, R. Pelayo, et al., "Extracellular histones are major mediators of death in sepsis," *Nature Medicine*, vol. 15, no. 11, pp. 1318–1321, 2009.

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 infections⁶. 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- κ B, AP-1, C/EBP β , 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. I κ B ζ , an I κ B family member, positively regulates certain genes such as *Il6* by interacting with NF- κ Bp50 or inducing histone modification^{17,18}, whereas ATF3 negatively regulates expression¹⁹. In contrast, transcription factors such as STAT6 and peroxisome proliferator-activated receptor- γ (PPAR γ) are involved in polarization of M2 macrophages^{5,20}.

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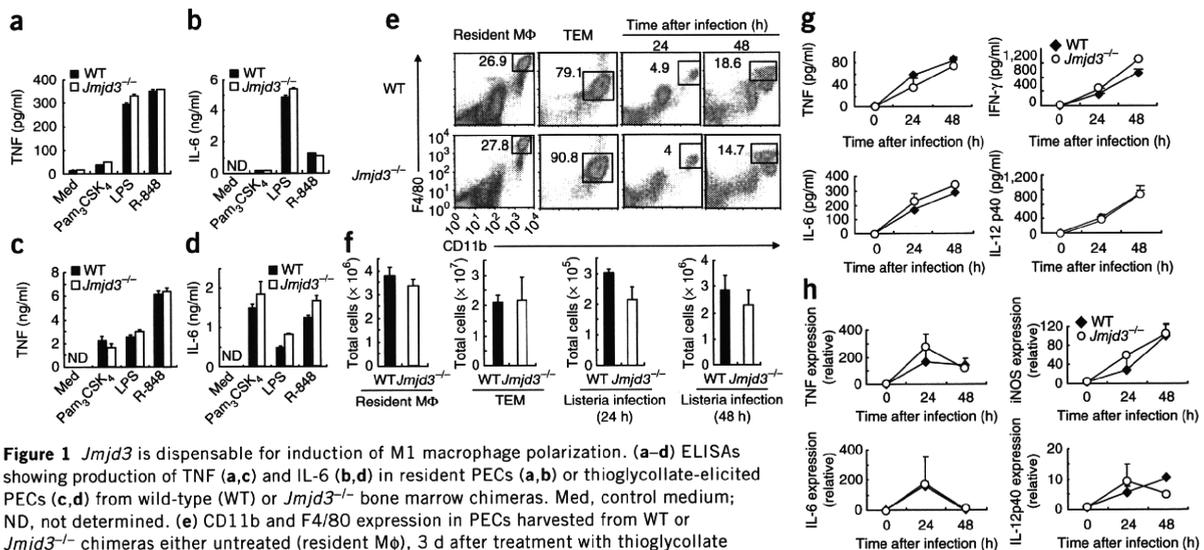


Figure 1 *Jmjd3* is dispensable for induction of M1 macrophage polarization. (a–d) ELISAs showing production of TNF (a,c) and IL-6 (b,d) in resident PECs (a,b) or thioglycollate-elicited PECs (c,d) from wild-type (WT) or *Jmjd3*^{-/-} bone marrow chimeras. Med, control medium; ND, not determined. (e) 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 expression¹⁶. 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 fashion²⁶. *Jmjd3* has also been identified as an early TLR-inducible gene in mouse macrophages by microarray analysis²⁷. 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* expression²⁶. 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 Ly6Ch⁺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 (Pam₃CSK₄, 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

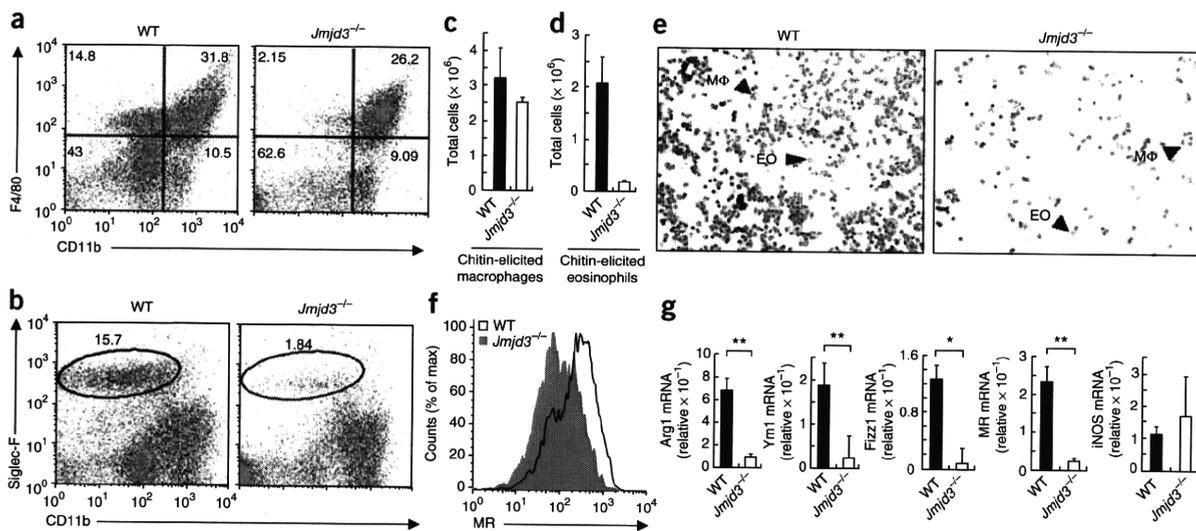


Figure 2 Crucial role of *Jmjd3* in M2 macrophage polarization in response to chitin administration. (a,b) Expression of CD11b and either F4/80 (a) or Siglec-F (b) in PECs harvested from wild-type (WT) or *Jmjd3*^{-/-} chimeric mice 2 d after peritoneal injection with chitin. Quadrants and numbers in 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. M ϕ , 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 fungi³¹. 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).

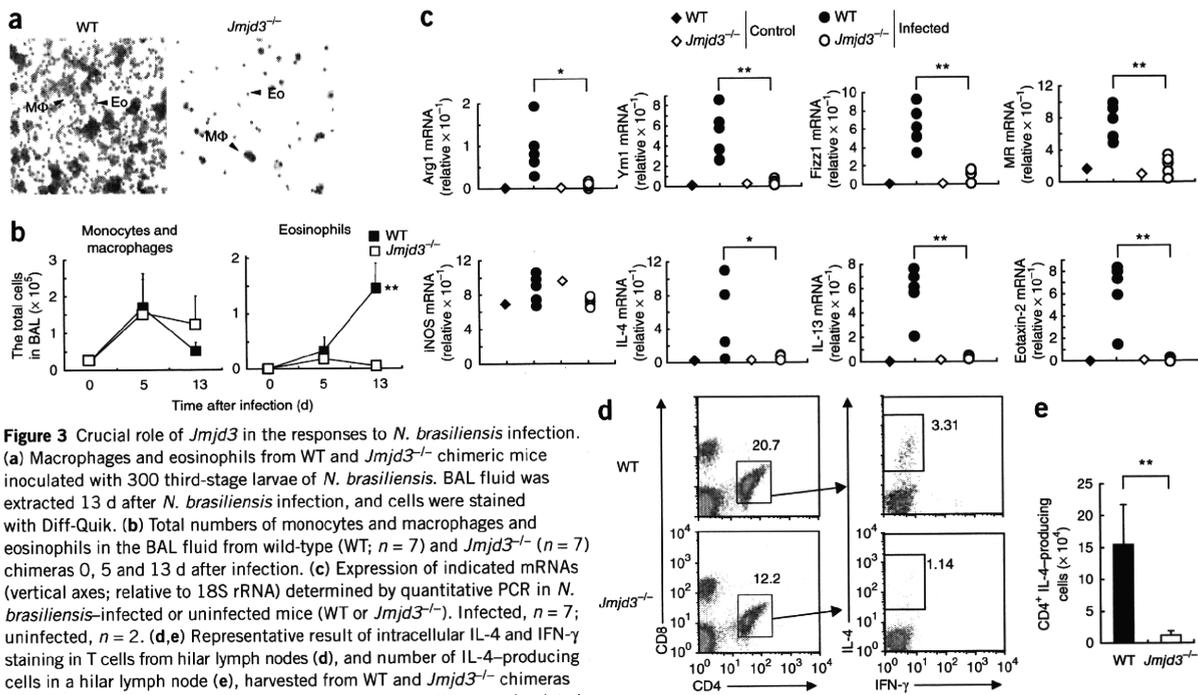


Figure 3 Crucial role of *Jmjd3* in the responses to *N. brasiliensis* infection.

(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

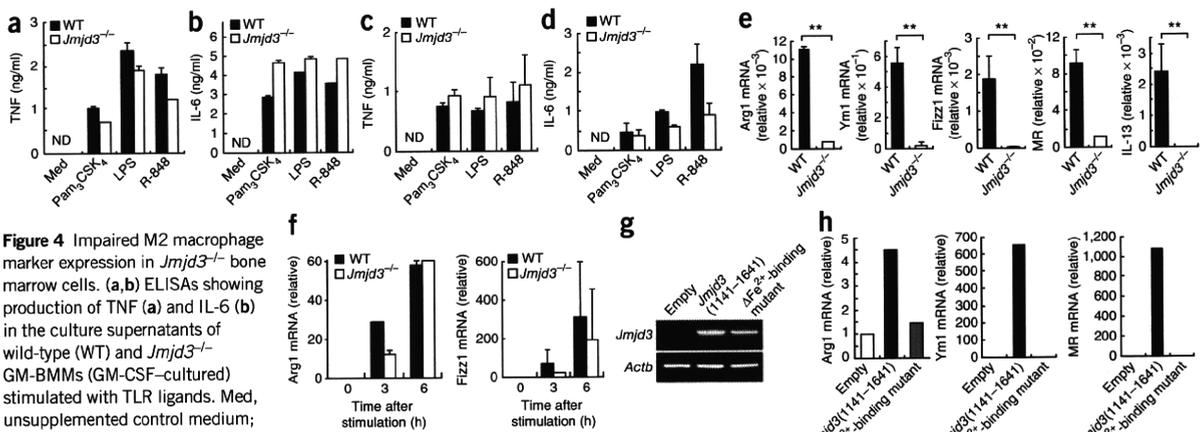


Figure 4 Impaired M2 macrophage marker expression in *Jmjd3*^{-/-} bone marrow cells.

(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.).

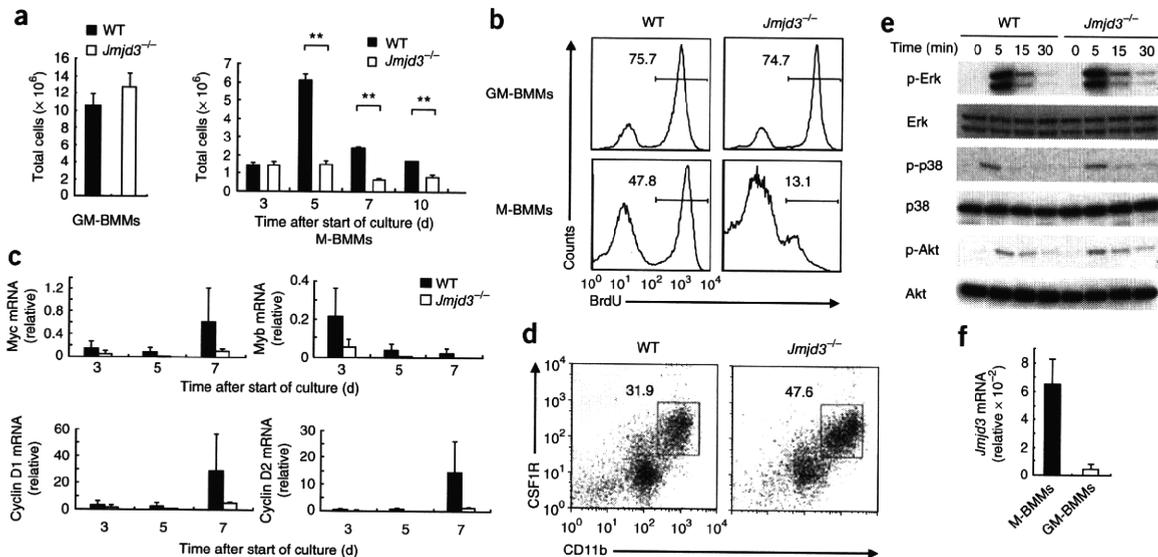


Figure 5 *Jmjd3* is required for the cell-cycle progression of M-BMMs. (a) Numbers of GM-BMMs and M-BMMs generated from wild-type (WT) and *Jmjd3*^{-/-} bone marrow cells. Bone marrow cells from WT and *Jmjd3*^{-/-} chimeras were cultured in the presence of GM-CSF for 5 d or M-CSF for time indicated (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 stimulation³⁴; 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).

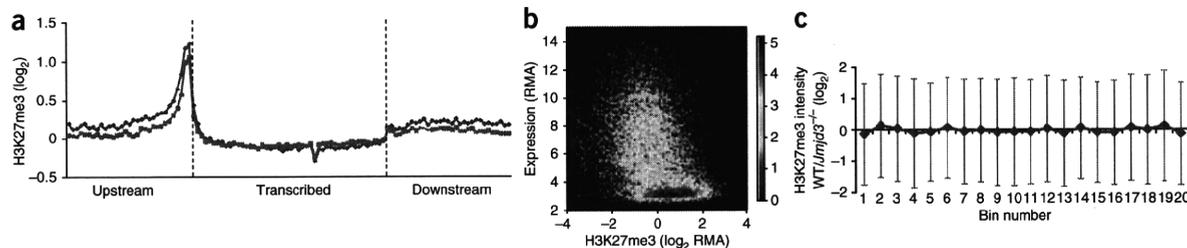


Figure 6 Genome-wide H3K27me3 modifications in M-BMMs. (a) Genome-wide distribution of H3K27me3 in wild-type (red) and *Jmjd3*^{-/-} M-BMMs (blue), determined with ChIP-Seq. H3K27me3 tags that mapped to transcribed regions (based on the genome-wide RefSeq mouse gene annotations in the UCSC database) 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*.

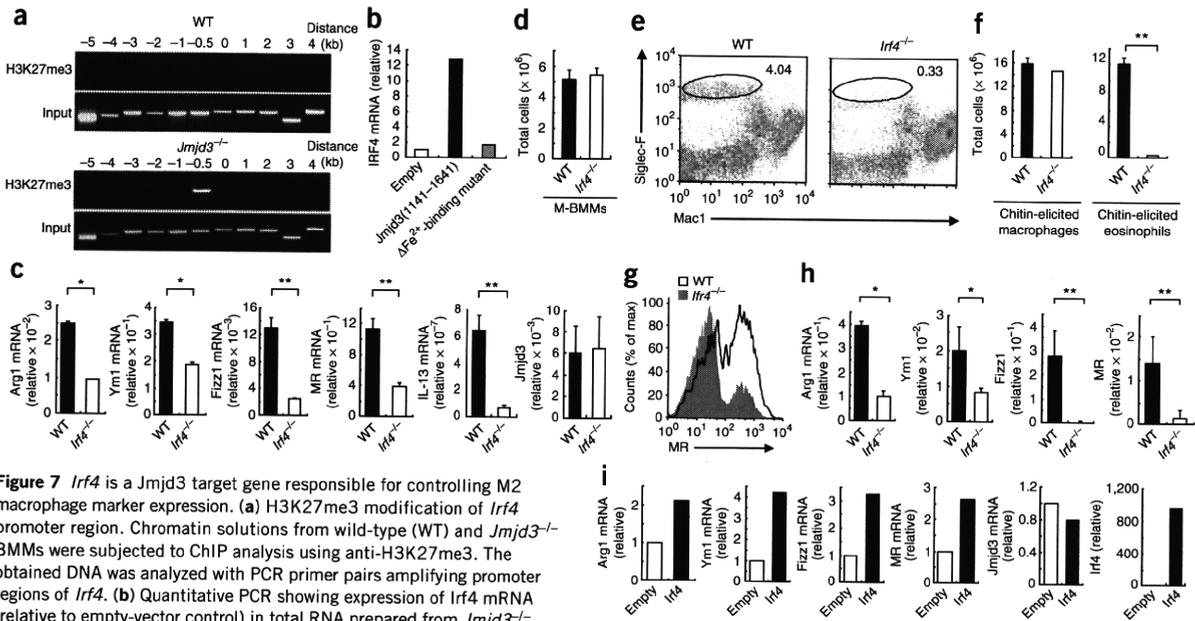


Figure 7 *Irf4* is a *Jmjd3* target gene responsible for controlling M2 macrophage marker expression. (a) H3K27me3 modification of *Irf4* promoter region. Chromatin solutions from wild-type (WT) and *Jmjd3*^{-/-} M-BMMs were subjected to ChIP analysis using anti-H3K27me3. The obtained DNA was analyzed 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 anti-bacterial 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 output²⁸. 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 infection³⁶. 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 stimulation³⁴. 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 report³⁴ 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 responses³⁹. Indeed, *Irf4*^{-/-} mice have been found to show defective T_H2 responses to *N. brasiliensis* infection⁴⁰. 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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- Takeuchi, O. & Akira, S. Pattern recognition receptors and inflammation. *Cell* **140**, 805–820 (2010).
- Medzhitov, R. Origin and physiological roles of inflammation. *Nature* **454**, 428–435 (2008).
- Beutler, B. Microbe sensing, positive feedback loops, and the pathogenesis of inflammatory diseases. *Immunol. Rev.* **227**, 248–263 (2009).
- Mantovani, A., Sozzani, S., Locati, M., Allavena, P. & Sica, A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol.* **23**, 549–555 (2002).
- Gordon, S. Alternative activation of macrophages. *Nat. Rev. Immunol.* **3**, 23–35 (2003).
- Benoit, M., Desnues, B. & Mege, J.L. Macrophage polarization in bacterial infections. *J. Immunol.* **181**, 3733–3739 (2008).
- Bronte, V. & Zanovello, P. Regulation of immune responses by L-arginine metabolism. *Nat. Rev. Immunol.* **5**, 641–654 (2005).
- Nair, M.G., Guild, K.J. & Artis, D. Novel effector molecules in type 2 inflammation: lessons drawn from helminth infection and allergy. *J. Immunol.* **177**, 1393–1399 (2006).
- Nair, M.G. *et al.* Chitinase and Fizz family members are a generalized feature of nematode infection with selective upregulation of Ym1 and Fizz1 by antigen-presenting cells. *Infect. Immun.* **73**, 385–394 (2005).
- Stein, M., Keshav, S., Harris, N. & Gordon, S. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J. Exp. Med.* **176**, 287–292 (1992).
- Mantovani, A. *et al.* The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol.* **25**, 677–686 (2004).
- Verreck, F.A. *et al.* Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco) bacteria. *Proc. Natl. Acad. Sci. USA* **101**, 4560–4565 (2004).
- Martinez, F.O., Gordon, S., Locati, M. & Mantovani, A. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *J. Immunol.* **177**, 7303–7311 (2006).
- Fleetwood, A.J., Lawrence, T., Hamilton, J.A. & Cook, A.D. Granulocyte-macrophage colony-stimulating factor (CSF) and macrophage CSF-dependent macrophage phenotypes display differences in cytokine profiles and transcription factor activities: implications for CSF blockade in inflammation. *J. Immunol.* **178**, 5245–5252 (2007).
- Fleetwood, A.J., Dinh, H., Cook, A.D., Hertzog, P.J. & Hamilton, J.A. GM-CSF- and M-CSF-dependent macrophage phenotypes display differential dependence on type I interferon signaling. *J. Leukoc. Biol.* **86**, 411–421 (2009).
- Medzhitov, R. & Horng, T. Transcriptional control of the inflammatory response. *Nat. Rev. Immunol.* **9**, 692–703 (2009).
- Yamamoto, M. *et al.* Regulation of Toll/IL-1-receptor-mediated gene expression by the inducible nuclear protein I κ B ζ . *Nature* **430**, 218–222 (2004).
- Kayama, H. *et al.* Class-specific regulation of pro-inflammatory genes by MyD88 pathways and I κ B ζ . *J. Biol. Chem.* **283**, 12468–12477 (2008).
- Gilchrist, M. *et al.* Systems biology approaches identify ATF3 as a negative regulator of Toll-like receptor 4. *Nature* **441**, 173–178 (2006).
- Charo, I.F. Macrophage polarization and insulin resistance: PPAR γ in control. *Cell Metab.* **6**, 96–98 (2007).
- Wei, G. *et al.* Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4⁺ T cells. *Immunity* **30**, 155–167 (2009).
- Barski, A. *et al.* High-resolution profiling of histone methylations in the human genome. *Cell* **129**, 823–837 (2007).
- Schuettengruber, B., Chourout, D., Vervoort, M., Leblanc, B. & Cavalli, G. Genome regulation by polycomb and trithorax proteins. *Cell* **128**, 735–745 (2007).
- Hong, S. *et al.* Identification of JmjC domain-containing UTX and JMJD3 as histone H3 lysine 27 demethylases. *Proc. Natl. Acad. Sci. USA* **104**, 18439–18444 (2007).
- Lan, F. *et al.* A histone H3 lysine 27 demethylase regulates animal posterior development. *Nature* **449**, 689–694 (2007).
- De Santa, F. *et al.* The histone H3 lysine-27 demethylase Jmjd3 links inflammation to inhibition of polycomb-mediated gene silencing. *Cell* **130**, 1083–1094 (2007).
- Matsushita, K. *et al.* Zc3h12a is an RNase essential for controlling immune responses by regulating mRNA decay. *Nature* **458**, 1185–1190 (2009).
- De Santa, F. *et al.* Jmjd3 contributes to the control of gene expression in LPS-activated macrophages. *EMBO J.* **28**, 3341–3352 (2009).
- Barradas, M. *et al.* Histone demethylase JMJD3 contributes to epigenetic control of INK4a/ARF by oncogenic RAS. *Genes Dev.* **23**, 1177–1182 (2009).
- Agger, K. *et al.* The H3K27me3 demethylase JMJD3 contributes to the activation of the INK4A-ARF locus in response to oncogene- and stress-induced senescence. *Genes Dev.* **23**, 1171–1176 (2009).
- Bowman, S.M. & Free, S.J. The structure and synthesis of the fungal cell wall. *Bioessays* **28**, 799–808 (2006).
- Reese, T.A. *et al.* Chitin induces accumulation in tissue of innate immune cells associated with allergy. *Nature* **447**, 92–96 (2007).
- Kreider, T., Anthony, R.M., Urban, J.F. Jr. & Gause, W.C. Alternatively activated macrophages in helminth infections. *Curr. Opin. Immunol.* **19**, 448–453 (2007).
- Ishii, M. *et al.* Epigenetic regulation of the alternatively activated macrophage phenotype. *Blood* **114**, 3244–3254 (2009).
- Da Silva, C.A., Hartl, D., Liu, W., Lee, C.G. & Elias, J.A. TLR-2 and IL-17A in chitin-induced macrophage activation and acute inflammation. *J. Immunol.* **181**, 4279–4286 (2008).
- Mantovani, A. & Sica, A. Macrophages, innate immunity and cancer: balance, tolerance, and diversity. *Curr. Opin. Immunol.* **22**, 231–237 (2010).
- Ahyi, A.N., Chang, H.C., Dent, A.L., Nutt, S.L. & Kaplan, M.H. IFN regulatory factor 4 regulates the expression of a subset of T $\text{H}2$ cytokines. *J. Immunol.* **183**, 1598–1606 (2009).
- Klein, U. *et al.* Transcription factor IRF4 controls plasma cell differentiation and class-switch recombination. *Nat. Immunol.* **7**, 773–782 (2006).
- Zheng, Y. *et al.* Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T $\text{H}2$ responses. *Nature* **458**, 351–356 (2009).
- Honma, K. *et al.* Interferon regulatory factor 4 differentially regulates the production of T $\text{H}2$ cytokines in naive vs. effector/memory CD4⁺ T cells. *Proc. Natl. Acad. Sci. USA* **105**, 15890–15895 (2008).
- Honma, K. *et al.* Interferon regulatory factor 4 negatively regulates the production of proinflammatory cytokines by macrophages in response to LPS. *Proc. Natl. Acad. Sci. USA* **102**, 16001–16006 (2005).
- Negishi, H. *et al.* Negative regulation of Toll-like-receptor signaling by IRF-4. *Proc. Natl. Acad. Sci. USA* **102**, 15989–15994 (2005).

ONLINE METHODS

Generation of *Jmjd3*^{-/-} mice. The *Jmjd3* gene was isolated from genomic DNA extracted from embryonic stem cells (GSI-1) 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 described⁴¹. 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). Pam₃CSK₄ and R-848 were prepared as described²⁷. 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 production⁴³.

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.

43. Saitoh, T. *et al.* TWEAK induces NF-κB p100 processing and long lasting NF-κB activation. *J. Biol. Chem.* **278**, 36005–36012 (2003).

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 (MMC), 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/EBP α , 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 Fc ϵ R1 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 *Mcp8Cre* 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 Fc ϵ R1 α chain still develop systemic anaphylaxis,

indicating involvement of an alternative pathway. Fc γ R-deficient mice that lack the expression of Fc ϵ R1 and stimulatory Fc γ R 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 factor (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 *Mcp8Cre* 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 TGF β and IL-6 in the mouse and by TGF β and IL-6/IL-21 in the human. Treg cells are induced by TGF β 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 appropriate 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 lymphopoietin (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

endogenous basophils are crucially involved in the development of Th2 cells *in vivo* by injecting DNP-OVA/anti-DNP IgE complexes into naïve mice. We found that a single iv administration of low dose DNP-OVA/anti-DNP IgE complexes into naïve mice rapidly and preferentially induced OVA-specific Th2 cells. This induction of OVA-specific Th2 by injection of Ag/IgE complex is strongly inhibited by depletion of basophils through injection of anti-FcεR1 antibody [34]. Furthermore, IL-3 treatment renders mice highly susceptible to Th2 cell-inducing action of IgE complex by increasing the number of basophils [34]. Taken together, these results strongly indicate that basophils are also potent Th2 cell-inducing APC *in vivo*.

Atopic individuals are characterized by increased numbers of basophils at sites of allergic inflammation [36–38]. Human mature basophils lack, or only weakly express, HLA-DR but have the potential to display increased HLA-DR expression when stimulated with IL-3 [34]. As levels of IL-3 and other factors may be increased at sites of allergic inflammation, we speculated that accumulated basophils might increase their HLA-DR expression and, thus, play a role as APC in peripheral tissues and augment Th2/IgE response.

To test our hypothesis, we injected Ag/IgE complexes into naïve mice, and, indeed, showed that this treatment efficiently induced Ag-specific Th2 cells *in vivo* [34]. Since naïve mice do not possess Ag-specific IgE, we speculate that this Ag/IgE complex-dependent induction of Ag-specific Th2 cell via basophils mainly account for the mechanism how repetitive exposure to allergen augment allergic inflammation. Once atopic individuals start to produce antigen-specific IgE, their concentration of Ag/Ag-specific IgE complexes may steadily increase. Subsequently, these complexes might be efficiently taken up by basophils via the FcεR1, resulting in these basophils becoming potent Th2 cell-inducing APC, which induce progressive allergic inflammation by enhancing Th2/IgE responses in these individuals. Furthermore, Ag/IgE stimulation upregulates the expression of FcεR1 on basophils [39,40]. Indeed, there is tight correlation between FcεR1 expression on basophils, and IgE levels in human peripheral blood [41], suggesting a positive feedback mechanism for IgE-mediated immediate type hypersensitivity. Furthermore, Denzel *et al.* reported that basophils could bind large amounts of antigens via FcεR1-bound IgE and produce IL-4 and IL-6, cytokines that are known to be required for antibody production in the spleen [42]. These basophils also activate Th2 type T cells, which help B cell proliferation and IgG1 production. Depletion of basophils decreased production of IgG1 [42]. Thus, Ag/IgE complexes and basophils play a crucial role in progression of allergic disease, and, therefore, antibody therapy against IgE or basophils might be

an effective therapeutic means of treating persistent allergic disease.

Basophils as Th2 cell-inducing APC in allergy and helminth-infected hosts

Two groups revealed that basophils induce Th2 cells in the absence of DCs *in vivo* [31,32]. These studies suggest that basophils are involved in allergen-induced or helminth-induced Th2 responses by functioning as Th2 cell-inducing APC [31,32]. Artis and colleagues, by using MHC II^{CD11c} transgenic mice, where MHC class II expression is restricted to CD11c⁺ DCs, demonstrated that these mice, when inoculated with *T. muris*, fail to develop Th2 responses or expel helminths [32]. They simultaneously demonstrated that this infection induced the development of Th1 cells, suggesting that CD11c⁺ cells are required for the generation of Th1 cells. Then, they demonstrated that basophils are dominant Th2 cell-inducing APCs that express IL-4 and MHC class II. They proved this by the evidence that depletion of basophils *in vivo* reduced protective Th2 cell response to *T. muris* in wild type mice [32]. However, Min and colleagues have demonstrated that basophil depletion in *N. brasiliensis*-infected mice does not affect the development of Th2 cells, suggesting that basophils may not be essential for Th2 differentiation in mice infected with the murine hookworm, *N. brasiliensis* [43]. Nevertheless, this infection induces recruitment of basophils into the draining lymph nodes with similar kinetics as those in other models, suggesting that basophils are not required for protection against primary *N. brasiliensis* infection. However, interestingly, basophils are essential for protection against secondary *N. brasiliensis* infection, even in the absence of mast cells and CD4⁺ T cells, which are both required for efficient worm expulsion during primary infection with *N. brasiliensis* [44]. A recent study by Voehringer and colleagues has demonstrated that *Mcpt8Cre* mice with constitutive and selective depletion of basophils, normally develop Th2 cells, a IgG1/IgE response, and eosinophilia upon infection with *N. brasiliensis* [14^{**}]. However, they further demonstrated that basophils are essential for protection against secondary *N. brasiliensis* infection [14^{**},44]. These observations suggest a complex role for basophils in host defense against parasites, and also underline the complexity of regulation of acquired Th2 immunity by innate cells.

Medzhitov and colleagues also demonstrated that skin DCs are not required for mounting Th2 responses to papain. They previously reported that, like injection of the soluble antigens of *S. mansoni* eggs, papain injections rapidly induce recruitment of basophils to the lymph node [30^{**},31]. As this treatment simultaneously induces recruitment of DC, they initially considered that basophils function as accessory cells, and DC present Ag in the presence of basophil-derived IL-4. However, they

have demonstrated that skin DC are not required for the development of Th2 cells in draining lymph nodes [31]. They subcutaneously injected papain into the ear, where skin DCs capture antigens and present Ag-derived peptides to naïve T cells at draining lymph nodes. If skin DCs capture antigens and present this in the lymph node, rapid removal of these Ag-pulsed DCs by prompt excision of the injection site should inhibit Th2 response. However, this treatment failed to inhibit development of Th2 cells, suggesting that Ag captured by skin DC is not required for induction of papain-specific Th2 development. They demonstrated that soluble papain can directly enter lymph nodes from the injection site. Furthermore, selective depletion of CD11c⁺ DCs did not inhibit Th2 development to papain, suggesting the presence of Th2-inducing APC other than DCs in the lymph nodes. Medzhitov and colleagues, as well as Artis and colleagues used CD11c-restricted diphtheria toxin receptor mice, in which CD11c-expressing DC are efficiently depleted upon delivery of diphtheria toxin [31,32]. They demonstrated that DC depletion only inhibited the development of Th1 cells without affecting the development of Th2 response. These results strongly indicate that other types of APC might be required for Th2 cytokine-dependent immune responses. Subsequently, they identified basophils as another type of Th2 cell-inducing APC, as this papain-induced Th2 cell differentiation could be blocked by the depletion of basophils by means of anti-FcεR1 antibody MAR-1 [31]. They have clearly shown that basophils can take up, process, and present soluble antigens. Furthermore, they demonstrated that basophils form immune synapses with T cells within 60 min of co-culture [31]. Finally, they demonstrated that adoptive transfer of OVA-pulsed basophils induced Th2 response in MHC class II-deficient mice.

The above-mentioned studies showed that basophils are potent APCs that preferentially induce Th2 cells both *in vitro* and *in vivo*. Mice deficient in the Src family tyrosine kinase, Lyn, have increased numbers of basophils, exhibit spontaneous Th2 differentiation, and produce IgE autoantibodies against various autoantigens, including dsDNA, that cause lupus-like nephritis in Lyn^{-/-} mice [29]. Thus, basophils regulate not only allergic diseases and anti-helminth immunity but also autoimmune diseases.

Following studies

However, recent studies questioned the role of basophils as Th2 cell-inducing APCs. Tang *et al.* suggested that DCs and basophils cooperatively induce a Th2 cell response against papain [45]. Phythian-Adams *et al.* reported that depletion of CD11c⁺ DCs during the priming stage of Th2 response against the helminth *Schistosoma mansoni* severely impaired Th2 induction, while basophil depletion using MAR-1 antibody had no mea-

surable effect, underlining the important role of DCs in the development of Th2 response against helminth infection [46]. Furthermore, Hammad *et al.* demonstrated that Th2 responses against house dust mite are initiated by FcεR1-expressing inflammatory DCs, which is amplified by basophils [47]. Voehringer and colleagues examined basophil function using newly developed basophil-ablated mice. In this model they could not find the evidence that basophils are required for Th2 cell differentiation *in vivo* [14**]. They speculated that antibody-mediated depletion of basophils with MAR-1 (anti-FcεR1) may simultaneously deplete FcεR1 expressing inflammatory DCs [47]. They also suggested bystander effects of antibody-treatment, including activation of mast cells and macrophages, as well as the formation of immune complexes.

Conclusion

The data on the role of basophils as APCs are confusing but the issue is still interesting. As we reported previously [34], basophils pulsed with Ag/IgE complexes have potent Th2 cell-inducing APC activity both *in vitro* and *in vivo*. Basophils take-up intact OVA and present OVA-peptide in conjunction with MHC class II to OVA-peptide specific naïve CD4⁺ T cells. Furthermore, when injected into naïve animal, these Ag-pulsed basophils also exhibited Th2 cell-inducing APC activity *in vivo*. However, their role as initiating cells of primary Th2 response might be minor. I suspect their important role for secondary Th2 response. Nevertheless, since basophils often show strong protective effect against helminth infection, they are still very interesting cells. To fully understand the role of basophils in Th2-mediated anti-helminth immunity, we definitively need further studies of these cells *in vivo*.

References and recommended reading

Papers of particular interest published within the period of review have been highlighted as:

- of special interest
- of outstanding interest

1. Galli SJ, Tsai M, Piliponsky AM: **The development of allergic inflammation.** *Nature* 2008, **454**:445-454.
2. Galli SJ, Kalesnikoff J, Grimbaldeston MA, Piliponsky AM, Williams CM, Tsai M: **Mast cells as "tunable" effector and immunoregulatory cells: recent advances.** *Annu Rev Immunol* 2005, **23**:749-786.
3. Min B: **Basophils: what they 'can do' versus what they 'actually do'.** *Nat Immunol* 2008, **9**:1333-1339.
- This is the comprehensive review of basophils and highlight the importance of basophils in immune response, parasite infection, and allergic inflammation. Potential involvement of basophils in clinical setting is also discussed.
4. Karasuyama H, Mukai K, Tsujimura Y, Obata K: **Newly discovered roles for basophils: a neglected minority gains new respect.** *Nat Rev Immunol* 2008, **9**:9-13.

This review summarized newly discovered novel roles for basophils in allergic response and clearly showed that the basophils are functionally distinct from mast cells.

5. Sullivan BM, Locksley RM: **Basophils: a nonredundant contributor to host immunity.** *Immunity* 2009, **30**:12-20.
6. Malbec O, Daéron M: **The mast cell IgG receptors and their roles in tissue inflammation.** *Immunol Rev* 2007, **217**:206-221.
7. Hida S, Yamasaki S, Sakamoto Y, Takamoto M, Obata K, Takai T, Karasuyama H, Sugane K, Saito T, Taki S: **Fc receptor gamma-chain, a constitutive component of the IL-3 receptor, is required for IL-3-induced IL-4 production in basophils.** *Nat Immunol* 2009, **10**:214-222.
- This paper showed the first time the requirement of cross-talk between FcR γ and IL-3R for basophil IL-4 production. FcR γ constitutively associates with the common β chain of IL-3R and transduce IL-3 activation signal by recruiting Syk.
8. Yoshimoto T, Tsutsui H, Tominaga K, Hoshino K, Okamura H, Akira S, Paul WE, Nakanishi K: **IL-18, although antiallergic when administered with IL-12, stimulates IL-4 and histamine release by basophils.** *Proc Natl Acad Sci USA* 1999, **96**:13962-13966.
9. Kondo Y, Yoshimoto T, Yasuda K, Futatsugi-Yumikura S, Morimoto M, Hayashi N, Hoshino T, Fujimoto J, Nakanishi K: **Administration of IL-33 induces airway hyperresponsiveness and goblet cell hyperplasia in the lungs in the absence of adaptive immune system.** *Int Immunol* 2008, **20**:791-800.
- This paper showed the first time that basophils simultaneously express IL-18R and IL-33R and strongly produce IL-4 and IL-13 when stimulated with IL-18 and/or IL-33 together with IL-3. IL-33 administration induces IL-13-dependent airway hyperresponsiveness and goblet cell hyperplasia in SCID mice, suggesting the presence and involvement of IL-33-responsive innate cells in allergic inflammation.
10. Yoshimoto T, Nakanishi K: **Roles of IL-18 in basophils and mast cells.** *Allergol Int* 2006, **55**:105-113.
11. Arinobu Y, Iwasaki H, Akashi K: **Origin of basophils and mast cells.** *Allergol Int* 2009, **58**:21-28.
12. Mukai K, Matsuoka K, Taya C, Suzuki H, Yokozeki H, Nishioka K, Hirokawa K, Etori M, Yamashita M, Kubota T *et al.*: **Basophils play a critical role in the development of IgE-mediated chronic allergic inflammation independently of T cells and mast cells.** *Immunity* 2005, **23**:191-202.
13. Tsujimura Y, Obata K, Mukai K, Shindou H, Yoshida M, Nishikado H, Kawano Y, Minegishi Y, Shimizu T, Karasuyama H: **Basophils play a pivotal role in immunoglobulin-G-mediated but not immunoglobulin-E-mediated systemic anaphylaxis.** *Immunity* 2008, **28**:581-589.
- Basophils are dispensable for IgE-mediated anaphylactic shock but are indispensable for IgG1-mediated systemic anaphylaxis. Thus, basophils play a crucial role in IgG1-mediated anaphylaxis.
14. Ohnmacht C, Schwartz C, Panzer M, Schiedewitz I, Naumann R, Voehringer D: **Basophils orchestrate chronic allergic dermatitis and protective immunity against helminths.** *Immunity* 2010, **33**:364-374.
- Newly discovered functions of basophils *in vivo* are mainly obtained from mice depleted of basophils by using monoclonal antibody specific for Fc ϵ R1 (MAR-1) or CD200R3(Ba103). The possibility that basophils and/or nonbasophils are activated by the Ab treatment has been concerned. Thus, establishment of basophil-deficient mice has been eagerly needed. These authors performed analysis of the functions of basophils *in vivo* and questioned the importance of basophils-induced systemic anaphylaxis and Th2 cell-inducing APC function of basophils *in vivo*.
15. Strait RT, Morris SC, Yang M, Qu XW, Finkelman FD: **Pathways of anaphylaxis in the mouse.** *J Allergy Clin Immunol* 2002, **109**:658-668.
16. Zhu J, Paul WE: **CD4 T cells: fates, functions, and faults.** *Blood* 2008, **112**:1557-1569.
17. Trinchieri G: **Interleukin-12 and the regulation of innate resistance and adaptive immunity.** *Nat Rev Immunol* 2003, **3**:133-146.
18. Seder RA, Paul WE: **Acquisition of lymphokine-producing phenotype by CD4⁺ T cells.** *Annu Rev Immunol* 1994, **12**:635-673.
19. Korn T, Bettelli E, Oukka M, Kuchroo K: **IL-17 and Th17 cells.** *Annu Rev Immunol* 2009, **27**:485-518.
20. Sakaguchi S, Yamaguchi T, Nomura T, Ono M: **Regulatory T cells and immune tolerance.** *Cell* 2008, **133**:775-787.
21. Takeda K, Kaisho T, Akira S: **Toll-like receptors.** *Annu Rev Immunol* 2003, **21**:335-376.
22. Amsen D, Antov A, Flavell RA: **The different faces of Notch in T-helper-cell differentiation.** *Nat Rev Immunol* 2009, **9**:116-124.
23. Ito T, Yang M, Wang YH, Lande R, Gregorio J, Perng OA, Qin XF, Liu YJ, Gilliet M: **TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand.** *J Exp Med* 2005, **202**:1213-1223.
24. Airmaniana V, Haensler J, Lacroix-Desmazes S, Kaveri SV, Bayry J: **Novel cellular and molecular mechanisms of induction of immune responses by aluminum adjuvants.** *Trends Pharmacol Sci* 2009, **30**:287-295.
25. Anderson CF, Mosser DM: **A novel phenotype for an activated macrophage: the type 2 activated macrophage.** *J Leukoc Biol* 2002, **72**:101-106.
26. Padigel UM, Hess JA, Lee JJ, Lok JB, Nolan TJ, Schad GA, Abraham D: **Eosinophils act as antigen-presenting cells to induce immunity to *Strongyloides stercoratis* in mice.** *J Infect Dis* 2007, **196**:1844-1851.
27. Oh K, Shen T, Le Gros G, Min B: **Induction of Th2 type immunity in a mouse system reveals a novel immunoregulatory role of basophils.** *Blood* 2007, **109**:2921-2927.
- In general, naive CD4⁺ T cells stimulated with Ag-pulsed DCs develop into Th1 cells. However, in this report, authors showed that basophils have the capacity to induce the development of naive CD4⁺ T cells stimulated with Ag-pulsed DCs into Th2 cells by production of IL-4, which simultaneously inhibits development of Th1 cells.
28. Hida S, Tadachi M, Saito T, Taki S: **Negative control of basophil expansion by IRF-2 critical for the regulation of Th1/Th2 balance.** *Blood* 2005, **106**:2011-2017.
29. Charles N, Hardwick D, Daugas E, Illei GG, Rivera J: **Basophils and the T helper 2 environment can promote the development of lupus nephritis.** *Nat Med* 2010, **16**:701-707.
- This paper showed that activation of basophils by complexes of auto-antigen/autoantigen-reactive IgE causes their homing to lymph nodes, where they promote Th2 cell differentiation and enhance the production of autoantibodies, causing lupus-like nephritis in Lyn^{-/-} mice.
30. Sokol CL, Barton GM, Farr AG, Medzhitov R: **A mechanism for the initiation of allergen-induced T helper type 2 responses.** *Nat Immunol* 2008, **9**:310-318.
- This paper showed the mechanisms how innate immune cells senses protein allergens and induce the development of Th2 cells *in vivo*. Basophils were recruited to lymph node in response to subcutaneous injection of protease allergens (e.g. papain) or schistosoma soluble egg antigens. Then, basophils were directly activated to produce IL-4 and TSLP, which in combination induce Th2 cell differentiation *in vivo*.
31. Sokol CL, Chu NQ, Yu S, Nish SA, Laufer TM, Medzhitov R: **Basophils function as antigen-presenting cells for an allergen-induced T helper type 2 response.** *Nat Immunol* 2009, **10**:713-720.
32. Perrigoue JG, Saenz SA, Siracusa MC, Allenspach EJ, Taylor BC, Giacomin PR, Nair MG, Du Y, Zaph C, van Rooijen N *et al.*: **MHC class II-dependent basophil-CD4⁺ T cell interactions promote Th2 cytokine-dependent immunity.** *Nat Immunol* 2009, **10**:697-705.
33. Kim S, Shen T, Min B: **Basophils can directly present or cross-present antigen to CD8 lymphocytes and alter CD8 T cell differentiation into IL-10-producing phenotypes.** *J Immunol* 2009, **183**:3033-3039.
34. Yoshimoto T, Yasuda K, Tanaka H, Nakahira M, Imai Y, Fujimori Y, Nakanishi K: **Basophils contribute to Th2-IgE responses in vivo via IL-4 production and presentation of peptide-MHC class II complexes to CD4⁺ T cells.** *Nat Immunol* 2009, **10**:706-712.
35. Min B, Prout M, Hu-Li J, Zhu J, Jankovic D, Morgan ES, Urban JF Jr, Dvorak AM, Finkelman FD, LeGros G *et al.*: **Basophils produce IL-4 and accumulate in tissues after infection with a Th2-inducing parasite.** *J Exp Med* 2004, **200**:507-517.

36. Irani AM, Huang C, Xia HZ, Kepley C, Nafie A, Fouda ED, Craig S, Zweiman B, Schwartz LB: **Immunohistochemical detection of human basophils in late-phase skin reactions.** *J Allergy Clin Immunol* 1998, **101**:354-362.
37. Koshino T, Arai Y, Miyamoto Y, Sano Y, Itami M, Teshima S, Hirai K, Takaishi T, Ito K, Morita Y: **Airway basophil and mast cell density in patients with bronchial asthma: relationship to bronchial hyperresponsiveness.** *J Asthma* 1996, **33**:89-95.
38. Macfarlane AJ, Kon OM, Smith SJ, Zeibecoglou K, Khan LN, Barata LT, McEuen AR, Buckley MG, Walls AF, Meng Q *et al.*: **Basophils, eosinophils, and mast cells in atopic and nonatopic asthma and in late-phase allergic reactions in the lung and skin.** *J Allergy Clin Immunol* 2000, **105**:99-107.
39. Lantz CS, Yamaguchi M, Oettgen HC, Katona IM, Miyajima I, Kinet JP, Galli SJ: **IgE regulates mouse basophil Fc epsilon RI expression in vivo.** *J Immunol* 1997, **158**:2517-2521.
40. Yamaguchi M, Lantz CS, Oettgen HC, Katona IM, Fleming T, Miyajima I, Kinet JP, Galli SJ: **IgE enhances mouse mast cell Fc(epsilon)RI expression in vitro and in vivo: evidence for a novel amplification mechanism in IgE-dependent reactions.** *J Exp Med* 1997, **185**:663-672.
41. Saini SS, Klion AD, Holland SM, Hamilton RG, Bochner BS, Macglashan DW Jr: **The relationship between serum IgE and surface levels of FcepsilonR on human leukocytes in various diseases: correlation of expression with FcepsilonRI on basophils but not on monocytes or eosinophils.** *J Allergy Clin Immunol* 2000, **106**:514-520.
42. Denzel A, Maus UA, Rodriguez Gomez M, Moll C, Niedermeier M, Winter C, Maus R, Hollingshead S, Briles DE, Kunz-Schughart LA *et al.*: **Basophils enhance immunological memory responses.** *Nat Immunol* 2008, **9**:733-742.
43. Kim S, Prout M, Ramshaw H, Lopez AF, LeGros G, Min B: **Cutting edge: basophils are transiently recruited into the draining lymph nodes during helminth infection via IL-3, but infection-induced Th2 immunity can develop without basophil lymph node recruitment or IL-3.** *J Immunol* 2010, **184**:1143-1147.
44. Ohnmacht C, Voehringer D: **Basophils protect against reinfection with hookworms independently of mast cells and memory Th2 cells.** *J Immunol* 2010, **184**:344-350.
45. Tang H, Cao W, Kasturi SP, Ravindran R, Nakaya HI, Kundu K, Murthy N, Kepler TB, Malissen B, Pulendran B: **The T helper type 2 response to cysteine proteases requires dendritic cell-basophil cooperation via ROS-mediated signaling.** *Nat Immunol* 2010, **11**:608-617.
46. Phythian-Adams AT, Cook PC, Lundie RJ, Jones LH, Smith KA, Barr TA, Hochweller K, Anderton SM, Hammerling GJ, Maizels RM *et al.*: **CD11c depletion severely disrupts Th2 induction and development in vivo.** *J Exp Med* 2010, **207**:2089-2096.
47. Hammad H, Plantinga M, Deswarte K, Pouliot P, Willart MA, Kool M, Muskens F, Lambrecht BN: **Inflammatory dendritic cells – not basophils – are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen.** *J Exp Med* 2010, **207**:2097-2111.

Production of a Transgenic Mosquito Expressing Circumsporozoite Protein, a Malarial Protein, in the Salivary Gland of *Anopheles stephensi* (Diptera: Culicidae)

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We are producing a transgenic mosquito, a flying syringe, to deliver a vaccine protein to human beings via the saliva the mosquito deposits in the skin while biting. The mosquito produces a vaccine protein in the salivary gland (SG) and deposits the protein into the host's skin when it takes the host's blood. We chose circumsporozoite protein (CSP), currently the most promising malaria vaccine candidate, to be expressed in the SG of *Anopheles stephensi*. To transform the mosquitoes, plasmid containing the CSP gene under the promoter of female SG-specific gene, as well as the green fluorescent protein (GFP) gene under the promoter of 3xP3 as a selection marker in the eyes, was injected into more than 400 eggs. As a result, five strains of GFP-expressing mosquitoes were established, and successful CSP expression in the SG was confirmed in one strain. The estimated amount of CSP in the SG of the strain was 40 ng per mosquito. We allowed the CSP-expressing mosquitoes to feed on mice to induce the production of anti-CSP antibody. However, the mice did not develop anti-CSP antibody even after transgenic mosquitoes had bitten them several times. We consider that CSP in the SG was not secreted properly into the saliva. Further techniques and trials are required in order to realize vaccine-delivering mosquitoes.

Key words: malaria, salivary gland, flying syringe, transgenic mosquito, vaccine

Mosquitoes are nuisance to human beings because they puncture the skin to obtain blood, which they use for their eggs. They deposit saliva in the skin when they take blood, and this causes itching [1]. They also transmit pathogens of diseases such as malaria, filariasis, yellow fever, and dengue fever. People have long tried to prevent mosquitoes from biting using mosquito nets [2] and repellents [3]. Moreover, people have attacked mosquitoes with insecticides to eliminate them [4, 5]. These struggles have continued not only in developing countries but

also in advanced countries.

In the last 30 years, developments in genetic engineering have allowed us to add new genes to wild-type cells or to remove protein genes from them [6]. These technologies were used at first in bacteria or yeast to obtain recombinant proteins for experiments and to understand the mechanisms of protein molecules. More recently, many transgenic experiments have been applied to plants and animals, including vertebrates and insects. Recombinant-DNA-modified soybean, cotton, and corn are widely adopted and harvested around the world [7]. Transgenic mice, sheep, dogs, zebra fish, flies, silkworms, and so on have appeared in laboratories as well [8-12]. The objectives of genetic engineering in animals are

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