IMMUNOLOGY

Natural polymorphism of Ym1 regulates pneumonitis through alternative activation of macrophages

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We have positionally cloned the Ym1 gene, with a duplication and a promoter polymorphism, as a major regulator of inflammation. Mice with the RIIIS/J haplotype, with the absence of Ym1 expression, showed reduced susceptibility to mannan-enhanced collagen antibody–induced arthritis and to chronic arthritis induced by intranasal exposure of mannan. Depletion of lung macrophages alleviated arthritis, whereas intranasal supplement of Ym1 protein to Ym1-deficient mice reversed the disease, suggesting a key role of Ym1 for inflammatory activity by lung macrophages. Ym1-deficient mice with pneumonitis had less eosinophil infiltration, reduced production of type II cytokines and IgG1, and skewing of macrophages toward alternative activation due to enhanced STAT6 activation. Proteomics analysis connected Ym1 polymorphism with changed lipid metabolism. Induced PPAR-γ and lipid metabolism in Ym1-deficient macrophages contributed to cellular polarization. In conclusion, the natural polymorphism of Ym1 regulates alternative activation of macrophages associated with pulmonary inflammation.

INTRODUCTION

Allergic asthma and other complex chronic pulmonary diseases, such as chronic obstructive pulmonary disease and chronic bronchitis, are characterized by airway obstruction and the pathological structure of inflammatory alterations in the lungs. Although many loci have been found to be associated with asthma, few results have been replicated, because of the complex and context-dependent interactions of the genetic variants with environmental factors, as well as with other genes (1). Therefore, it has been difficult to conclusively identify critical genetic and pathogenic factors in pulmonary inflammation. Candidate genes thought to be important based on human studies have been genetically modified in mouse models to understand disease mechanisms. However, most of the genes in question seem to be linked more to regulation of basic physiology than to pathogenesis per se, and humanized genes may not be physiologically regulated in the mouse model as they are in humans. An alternative approach is to use inbred experimental animals, with diseases and biological pathways mimicking humans, for the genetic analysis. The major associated loci can be identified by using genetic segregating crosses of inbred strains and then phenotyping the offspring for quantitative traits followed by genetic mapping (2). The first locus to be positionally identified was an amino acid replacement single-nucleotide variant in the Ncf1 gene (3). A single-nucleotide polymorphism (SNP) in the human Ncf1 gene and a spontaneous mutation of the Ncf1 gene in mice were subsequently shown to be significantly correlated with systemic lupus erythematosus; Ncf1 variants are also likely to play a critical role in many other inflammatory diseases such as rheumatoid arthritis, multiple sclerosis, and psoriasis (4–9). Further studies of this newly identified pathway offer a fruitful direction for future research on human immune diseases.

Here, we have addressed another major locus identified from genetic segregating intercrosses between B10.RIII and RIIIS/J mice, using collagen-induced arthritis (CIA) and experimental autoimmune encephalomyelitis (EAE) as inflammatory diseases traits. Using these crosses, the loci Eae3/Cia5 and Eae2 located on murine chromosomes 3 and 15 were identified (10, 11). Furthermore, in a partial advanced intercross between Eae3/Cia5 and Eae2 bicongenic mice, the original loci were fine-mapped, and a 2-Mb RIIIS/J-derived congenic segment on chromosome 3 located within the Cia22 locus, including a cluster of chitinase-like genes, was identified (12). Subsequent microarray analysis of the selected genes in the chitinase gene cluster indicated Ym1 as the major differentially expressed candidate gene (13).

Ym1 [chitinase-like protein 3 (Chil3)] is a member of chitinase-like proteins (CLPs) but was originally identified as an eosinophil chemotactic factor, which is strongly induced in mice infected with tissue and gastrointestinal nematode parasites (14). It is also considered a signature marker for alternatively activated macrophages (AAMs) (15), although a potential functional role of Ym1 on macrophage polarization has not yet been assessed. A recent study reported that Ym1/2 promoted interleukin-17 (IL-17)–mediated neutrophilia and was involved in nematode killing and host damage in mice (16). It was also suggested that Ym1-induced oligodendrocytogenesis, and silencing Ym1 increased the severity of EAE (17). Furthermore, Ym1/2 promoted T112 (T helper 2) cytokine expression implicated in regulating allergic inflammation by inhibiting 12/15(S)-lipoxgenase in mice (18). Ym1 Ly6Chhi monocytes have been shown to be associated with the resolution of inflammation and tissue healing (19). It seems that Ym1, which lacks chitinase activity, has important but diverse functions; however, a conclusive role of Ym1 in complex diseases such as pulmonary inflammation has so far been lacking.

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In this study, we have positionally identified Ym1 polymorphism and developed a congenic mouse strain with a RIIS/J-derived haplotype that leads to reduced Ym1 expression; these findings provide an opportunity to further investigate the regulation of Ym1 and its role in the immune response. We demonstrate that the lack of expression due to mutations in the promoter of the Ym1 gene alleviates pulmonary inflammation and promotes the alternative activation of macrophages. Thus, the Ym1 gene contains a positionally identified genetic polymorphism, offering new insights into the immune system and the pathogenesis of inflammatory disorders.

RESULTS
Promoter polymorphism of Ym1 controls its gene expression
To address the potential of Ym1 as a candidate gene for a major locus regulating inflammatory disease, Ym1 congenic deficient mouse strain (BR.Ym1<sup>-/-</sup> mice) carrying a 2-Mb RIIS/J fragment was generated by introgressing the congenic fragment into the B10.RIII background (Fig. 1A). The tissue distribution of Ym1 showed remarkable expression of Ym1 mRNA was strongly expressed in lung, spleen, and bone marrow but only weakly expressed in other tissues, including stomach and lymph nodes, while Ym2 mRNA was highly expressed in stomach but undetectable in the other tissues analyzed (Fig. 1B). We found that the expression of Ym1 mRNA in both lung and spleen was remarkably low in RIIS/J-derived congenic mice compared with B10.RIIII, Balb/c, and B6/NJ, as analyzed with real-time quantitative polymerase chain reaction (RT-qPCR) (Fig. 1C). In addition, we validated the serum levels of Ym1 protein of different inbred mouse strains with an enzyme-linked immunosorbent assay (ELISA), and in line with our qPCR results, we could detect Ym1 in the serum of B10.RIIII mice but not in the BR.Ym1<sup>-/-</sup> littermate congenics (Fig. 1D).

Ym1 expression differs considerably between different inbred and wild-derived mouse strains, which might be due to natural polymorphism in their respective promoter regions. Therefore, we decided to take a closer look at their genetic differences using the online database from Ensembl together with our own sequencing data. A copy number variation was found—a 187-kbp (kilo–base pair) fragment covering the Ym1 gene (from 106,123,423 bp to 106,311,121 bp in GRCh38/mm10). C57Black strains, LP/J-, NOD-, and 129-derived strains have this duplication, but other strains including Balb/c and RIIS/J strains do not (Fig. 1E). In general, mouse strains harboring the duplication show increased Ym1 expression (data from ImmGen), suggesting that Ym1 expression is strongly associated with this copy number variant. However, Balb/c mice without the duplication still showed higher Ym1 expression than RIIS/J mice (Fig. 1D), indicating that there are additional polymorphisms affecting Ym1 expression.

The additional effect could be explained by differences in the Ym1 promoter sequences in the different mouse strains. SNPs were found in SPRET, PWK, NZO, and RIIS/J strains, and the RIIS/J strain was found to have a distinct haplotype in the promoter region of Ym1 gene, with four SNPs compared to C57Black strains (Fig. 1E). Subsequently, we cloned Ym1 promoters of both B10.RIIII and RIIS/J mice into luciferase reporter plasmids (Fig. 1F; designated as pGL-B10.RIIII and pGL-RIIS/J), mimicking the four SNPs, respectively, by site mutation (Fig. 1F; pGL-SNP1, pGL-SNP2, pGL-SNP3, and pGL-SNP4). We carried out a dual-luciferase reporter assay to test the effect of each SNP on the regulation of Ym1 expression, and the results showed that the promoter from RIIS/J mice had a lower transcription activity than the one from wild-type B10.RIIII mice (Fig. 1G). The results also showed that it was SNP2, SNP3, and SNP4, but not SNP1, that reduced the transcription activity compared with the wild-type promoter sequence. SNP2 had the largest effect, indicating that it might be the key site controlling the Ym1 expression (Fig. 1G). Together, the natural polymorphism controls Ym1 expression.
In addition, the immune cell populations in spleen and peripheral blood from naïve Ym1 congenic deficient mice and its wild-type control B10.RIII mice, which differed in Ym1 expression, were analyzed by flow cytometry. Except the slightly decreased proportion of eosinophils in BR.Ym1° congenic mice, there were almost no difference between the two strains (fig. S1, A and B).

Ym1-deficient mice are protected from mannan-induced arthritis, dependent on macrophages

As Ym1 was originally identified in segregating partial advanced intercross associated with arthritis, we next determined the effect of Ym1 deficiency on arthritis pathology to confirm its disease phenotype. Collagen antibody–induced arthritis (CAIA) model, which is developed in the absence of an adaptive immune system (αβT and B cells), is induced after intravenous injection of anti-CII antibodies, leading to mild arthritis (20). Usually, an intraperitoneal injection of lipopolysaccharide (LPS) or mannan is given a few days after the initial transfer of the CII-specific antibodies to enhance the arthritis susceptibility (21, 22). We found that the Ym1 alleles had no impact on LPS-enhanced CAIA susceptibility or severity, whereas deficiency of Ym1 led to a reduction in severity of mannan-enhanced CAIA (Fig. 2A). Mannan-CAIA is a different disease than LPS-CAIA, best demonstrated by the contrasting effect in Ncf1-mutated mice, which have a lower capacity to make an oxidative burst by NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) oxidase (Nox2) complex (22). LPS-CAIA is ameliorated, whereas mannan-CAIA is markedly enhanced in Ncf1-mutated mice.

Ym1 is highly expressed in lung macrophages and is likely to have an important role in regulating the immune response in the lung. We crossed our Ym1 congenic mice with Ncf1-mutated mice on B10.RIII background (BR.Ncf1°.Ym1Δ mice) and exposed the mice by treating them intranasally with mannan directly. It is well known that sterile aspergillus induces severe inflammation in the lung (23), and we chose to treat them intranasally with mannan based on our earlier observation that a single intraperitoneal injection of mannan induces an acute form of psoriasis and psoriatic arthritis (PsA) in Ncf1-mutated mouse strains (24). Intranasal exposure of mannan in Ncf1-deficient B10.RIII mice induced psoriasis and chronic development of arthritis (Fig. 2, B and C). Ym1-deficient littermate mice were completely protected against the disease, demonstrating that the presence of Ym1 is critical for the development of chronic arthritis induced by mannan (i.e., PsA) in Ncf1-deficient mice (Fig. 2, B and C). To further confirm the importance of macrophages, we used clodronate-liposome to deplete the lung-resident macrophages of BR.Ncf1° mice (fig. S2) and found that the severity of mannan-induced PsA (MIP) was alleviated significantly (Fig. 2D). In addition, to directly prove the role of Ym1 in disease pathogenesis, we tried to reestablish arthritis severity through recombinant Ym1 protein administration. Mannan was injected intranasally in BR.Ncf1°.Ym1Δ mice, which were subsequently treated with or without Ym1 protein. As expected, BR.Ncf1°.Ym1Δ mice supplemented with Ym1 protein exhibited enhanced disease severity compared to mock [phosphate-buffered saline (PBS)]–treated mice of the same strain, thereby displaying comparable severity levels with BR.Ncf1° mice (Fig. 2E). Because both CAIA and mannan-induced arthritis are independent on adaptive immunity, our results suggested that Ym1 play a role in regulating innate immune response with the involvement of macrophages.

Fig. 2. Ym1 deficiency in congenic mice protects arthritis development induced by mannan through macrophages. (A) B10.RII and BR.Ym1° congenic mice (n = 11 to 18 per group) were injected intravenously with four monoclonal antibodies at day 0 and boosted with LPS (25 μg) or mannan (2 mg) at day 5 to induce CAIA. The arthritis severity was scored. BR.Ncf1° and BR.Ncf1°.Ym1Δ mice (n = 6 per group) were administrated with 20 mg of mannan intranasally (i.n.) to induce psoriasis and chronic arthritis. (B) Arthritic joint phenotype and psoriasis-like skin lesions in the hind paws. Photo credit: Wenhua Zhu, Xi’an Jiaotong University Health Science Center. (C) The arthritis and psoriasis severity was scored. The area under the arthritis scoring curve until D28 between the two strains was analyzed statistically (P < 0.05). (D) Clodronate-liposome (CL-lipo) or PBS-liposome (PBS-lipo) was treated intranasally to BR.Ncf1° mice (n = 6 per group) to deplete macrophages. Two days later, mice were administrated with mannan intranasally to induce arthritis. (E) BR.Ncf1° and BR.Ncf1°.Ym1Δ mice (n = 7 per group) were administrated with mannan intranasally with or without Ym1 protein supplement to induce arthritis. Disease severity was scored using a macroscopic scoring system. All values are expressed as means ± SEM. *P < 0.05.

Ym1-deficient mice with mannan intranasal treatment show less pneumonitis

To further delineate the role of Ym1 for regulation of macrophage functions, we treated mice with mannan intranasally, which primarily induces pneumonitis (Fig. 3A) and is followed by a late secondary immune response leading to arthritis. First, lung tissues from both BR.Ncf1° mice and BR.Ncf1°.Ym1Δ mice at days 0, 2, and 5 after mannan treatment were collected, and lung index (the lung weight normalized by the body weight) was calculated. BR.Ncf1° mice showed higher lung index than congenic mice (Fig. 3B). Histopathological changes of the lungs were observed by evaluating the inflammation around the airways and in interstitia. Results showed...
that the inflammatory cell infiltration was increased significantly at day 5 in BR.Ncf1* mice but not in BR.Ncf1*.Ym11 mice (Fig. 3C). BR.Ncf1*.Ym11 mice had mild inflammation around the airways compared with BR.Ncf1* mice (Fig. 3C).

Then, the immune cells in the bronchoalveolar lavage fluid (BALF) were analyzed by flow cytometry. The number of total BALF cells, eosinophils, and infiltrated interstitial macrophages was increased after mannan treatment in BR.Ncf1* mice and showed significant difference between the two strains at day 2 (Fig. 3, D to F). The major constituent cell type of BALF cells is alveolar macrophages (25), where their proportion was significantly decreased in BR.Ncf1* mice because of immune cell infiltration, but was only slightly changed...
in BR.Ncf1*Ym1Δ mice (Fig. 3G). Meanwhile, the cell population in Ym1 protein supplement experiment was also observed by flow cytometry, and we found an increase in total BALF cells and of eosinophils (Fig. 3, H and I). Thereby, the increased proportion of alveolar macrophages in BR.Ncf1*Ym1Δ mice were reversed when the mice were treated with Ym1 protein (Fig. 3J).

Cytokine expression in the lung tissues of mannan-treated mice were also determined by RT-qPCR. Results showed that both Il4 and Tgfb1 mRNA expression were increased at day 2 in BR.Ncf1* mice (Fig. 3K). In contrast, BR.Ncf1*Ym1Δ mice showed reduced Il4 expression, but not Tgfb1 expression, suggesting a specific regulatory effect by Ym1 on IL-4 (Fig. 3K).

In addition, the Ym1 protein level in BALF was determined by ELISA, and the result showed that the Ym1 concentration in the BALF of BR.Ncf1* mice was increased with a higher degree of inflammation, whereas no change was observed in BR.Ncf1*Ym1Δ mice (Fig. 3L). Besides, the Ym1 expression in immune cells of BALF at day 2 was also detected by flow cytometry. BALF cells from BR.Ncf1* mice showed higher Ym1 expression than from BR.Ncf1*Ym1Δ mice (Fig. 3M). Ym1 was mainly expressed by lung-resident macrophages, and alveolar macrophages from BR.Ncf1* mice showed higher Ym1 expression than those from BR.Ncf1*Ym1Δ mice (Fig. 3N).

Low expression of Ym1 also alleviates antigen-induced pneumonitis

The above results showed that Ym1 deficiency weakened the innate immune response in lung triggered by mannan. Innate immune regulation contributes to the activation and maintenance of adaptive immunity. To further confirm the role of Ym1, the classical antigen-induced pulmonary inflammation (AIPI) model using ovalbumin (OVA) was induced in B10.RIII and BR.Ym1Δ mice (Fig. 4A). In the OVA group of both strains, only the wild-type B10.RIII mice showed increased interstitial infiltration of inflammatory cells, although both Ym1-deficient and wild-type mice showed increased inflammation around the airways (Fig. 4B). Besides, Ym1-deficient mice in the OVA group had decreased mucus hypersecretion compared with wild-type mice (Fig. 4C).

Immunocytes in the BALF were analyzed by flow cytometry, and results showed that Ym1-deficient mice in the OVA group had less cell numbers, especially eosinophils and B cells, as compared with...
Ym1 counteracts alternative activation of alveolar macrophages in pneumonitis

Ym1 is commonly used as a signature marker for alternative activation of macrophages in mice, and deficiency in Ym1 may therefore influence the macrophage polarization state during disease development. To analyze the alternative activation of alveolar macrophages, the polarization markers were determined in both mannann- and OVA-induced pulmonary inflammation. In lungs with an inflammatory response due to mannan intranasal treatment, the proportion of Arg1+ MRC1+ cells in alveolar macrophages of BR.Ncf1* mice were decreased during disease but remained relatively stable in BR.Ncf1*Ym1* mice (Fig. 5A). Alveolar macrophages from congenic mice showed higher Arg1 and MRC1 expression levels than those from BR.Ncf1* mice (Fig. 5A and fig. S3A). In both strains, there was no difference in inducible nitric oxide synthase (iNOS) expression (Fig. 5A). Particularly, the increased proportion of Ym1-deficient mice showed decreased trend compared with wild-type mice, but Ym1 seemed to have no effect on Tgfb1 expression regulation (Fig. 4H). Combined with the above results, it is clear that Ym1 deficiency alleviated pneumonitis induced by OVA associated with a reduced IL-4 response.

Ym1-regulating STAT6 limits the alternative activation of macrophages during polarization

To determine the pathway by which Ym1 down-regulated alternative activation, we investigated macrophage polarization in vitro. Bone marrow cells were collected from B10.RIII, BR.Ym1Δ, BR.Ncf1*, and BR.Ncf1*.Ym1Δ mice and differentiated to macrophages followed by polarization to classically activated macrophages (CAMs) using LPS and interferon-γ (IFN-γ) or AAMs using IL-4 and IL-13. Expression of polarization markers was compared between Ym1-deficient mice and their littermate wild-type controls. The Ym1 levels were high in AAMs from wild-type B10.RIII mice and BR.Ncf1* mice and low in the Ym1-deficient congenic mice (Fig. 5D). Particularly, Ym1 expression could only be induced by IL-4/IL-13, but not IL-10, suggesting a relatively unique effect of Ym1 on IL-4–induced AAMs (fig. S4A).

Subsequently, the AAM markers Arg1 and MRC1 were detected by Western blotting. As expected, AAMs derived from Ym1-deficient mice showed higher Arg1 and MRC1 expression than that from their wild-type control mice (Fig. 5E). The results underscore the importance of Ym1 for the AAM phenotype in vitro. In addition, IL-4/IL-13 could induce the increased Tgfb1 mRNA expression in macrophages derived from Ym1-deficient mice because these mice had lower basal expression of Tgfb1. However, there was no difference in Tgfb1 expression between the two strains, again suggesting the limited effect of Ym1 on transforming growth factor (TGF) regulation (fig. S4B). On the other hand, we assessed IL-6, IL-12, and IL-10 levels by ELISA in CAMs. IL-6 levels did not differ in any of the four strains, while IL-12 and IL-10 levels also showed no difference in Ncf1 wild-type strains (fig. S4, C to E). However, compared with BR.Ncf1* mice, CAMs from BR.Ncf1*.Ym1Δ congenic mice showed low IL-12 concentration but high IL-10 concentration (fig. S4, D and E), which indicated that Ym1 with a basal expression level might interact with Ncf1 to regulate CAMs, although the effect seemed to be very limited.

Therefore, we next observed the changes of AAM markers comprehensively during proliferation. The mRNA expression of Ym1, Arg1, MRC1, and Fizz1 was determined by RT-qPCR at 0, 3, 6, 12, 24, and 48 hours after IL-4 and IL-13 stimulation. Ym1 expression was increased gradually during polarization in macrophages from BR.Ncf1* mice but maintained at a low level in macrophages from BR.Ncf1*.Ym1Δ mice (Fig. 5F). Both Arg1 and MRC1 expression in BR.Ncf1* macrophages were quickly induced at 3 and 6 hours but started to decrease later (Fig. 5F). Macrophages with Ym1 deficiency showed different expression patterns, in which Arg1 expression continued to increase and reached the peak at 24 hours, and MRC1 expression was also higher than that from BR.Ncf1* mice (Fig. 5F). Fizz1 expression in macrophages from both mouse strains was increased gradually, suggesting a regulatory pattern independent from Ym1 (Fig. 5F). We analyzed the protein levels of Ym1, Arg1, and MRC1 by Western blotting, and the expression of Ym1 was found to be consistent with that of mRNA (Fig. 5G). Arg1 and MRC1 protein expression in both strains was gradually induced, however, and macrophages from congenic mice showed higher expression (Fig. 5G and fig. S5A). STAT6 (signal transducer and activator of transcription 6), the most critical transcriptional factor induced by IL-4, controls the process of alternative activation of macrophages (27). Here, the activation of STAT6 signaling pathway was detected by Western blotting. The result showed that macrophages from congenic mice had higher phosphorylation level of STAT6 during the polarization process (Fig. 5H and fig. S5B). Therefore, Ym1 down-regulating STAT6 activation limits alternative activation of macrophages.

Ym1 regulates lipid metabolism and PPAR-γ to orchestrate polarization in macrophages

To comprehensively study the role of Ym1 in macrophages, protein profiles of primary macrophages from B10.RIII mice and BR.Ym1Δ congenic mice were determined by label-free mass spectrum analysis to find out the differentially expressed proteins and relevant pathways. Ninety-four proteins showed differential expression (fold change > 1.2 and P < 0.05) between the two groups in 4053 identified proteins totally. Compared with B10.RIII mice, 49 proteins were up-regulated and 45 proteins were down-regulated in congenic mice (Fig. 6A). Differentially expressed proteins were then hierarchically clustered and analyzed by the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment. Lipid metabolism–associated pathways and peroxisome proliferator–activated receptor
Lipid metabolism pathways in which Ym1 might be involved have earlier been suggested to be involved in macrophage polarization (28). We hypothesized that the regulation of PPAR signaling pathways is involved in the alternative activation of macrophages from BR.Ncf1* and BR.Ncf1*.Ym1 mice. Using Western blotting, we could show that IL-4/IL-13 could induce the expression of PPAR-γ and PPAR-δ, and AAMs from congenic mice showed higher PPAR-γ expression (Fig. 6C and fig. S8A). In addition, AAMs from congenic
Ym1 is predominantly expressed by macrophages and is also commonly used as a signature marker for AAMs (15). However, little is known about the regulatory role of Ym1 in the alternative activation of macrophages contributing to inflammation, as illustrated in Fig. 6F.

**DISCUSSION**

To understand the pathogenic molecular mechanisms underlying inflammatory diseases, it is essential to identify the underlying causative natural polymorphisms. In this study, we have identified disease-associated Ym1 polymorphism, with conserved and widespread variation in Ym1 expression, and thereby function, in mice. An increasing body of literature points toward the role of catalytically active chitinases and chitinase-like proteins (C/CLPs) and their involvement in infection, inflammation, tissue injury, and remodeling (29–35). However, besides the enzymatic activity, we still do not know how to associate C/CLPs with many immunomodulatory effects. Worse still, unlike other C/CLPs, the lack of Ym1 gene knockout mice until now has limited research on Ym1 function. But now with the low-expressing allele isolated in the congenic strain, we can initiate definitive physiological studies of the role of Ym1 in inflammatory diseases.

We identified the Ym1 gene using the CIA model but later found a stronger effect along a pathway that was induced by mannan, which is important for the induction of a range of different inflammatory disease; here, we tested psoriasis, PsA, and autoimmune arthritis in mice (22, 24). During this study, we also found that exposure of lung macrophages lacking the capacity to produce ROS (reactive oxygen species) with mannan leads to chronic relapsing arthritis. This could therefore be a model that could explain why smoking and air pollution could enhance the severity of many chronic inflammatory diseases (36). Ym1 exerted its role in regulating arthritis by modifying function in local tissue macrophages in the lung. Because Ym1 is strongly expressed in lung macrophages, we tried to observe its direct regulatory effect in the lung using both mannan-induced and classic antigen–induced pneumonitis models. Reduced pathological score, as well as decreased eosinophil infiltration, type II cytokine expression, and IgG1 production all suggest that lung inflammation is alleviated at low levels of Ym1 expression.

Previous studies using mice deficient in oxidative burst due to loss-of-function mutations in components of the NOX2 complex–like Ncf1 (p47phox) or Cyp1a (gp91phox) and certain colonies of SWISS mice indicated that these mice develop a severe crystalline macrophage or eosinophilic pneumonia characterized by crystallization of Ym1 protein in the lungs of affected animals (37, 38). This is followed by massive infiltration of the respective granulocytes displaying a hyperinflammatory phenotype, thereby producing a whole range of cytokines. In our study, mannan intranasally induced arthritis and pneumonitis in Ncf1–mutated mice suggests the interactive contribution of Ncf1 and Ym1 to the pathogenesis of disease. Further investigation of their interaction mechanism would be a fruitful direction for future research.

Ym1 is predominantly expressed by macrophages and is also commonly used as a signature marker for AAMs (15). However, little is known about the regulatory role of Ym1 in the alternative activation of macrophages in pulmonary inflammation. All values are expressed as means ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001.
of macrophages. It was found that iNOS-deficient mice displayed enhanced CAM polarization (39), which gives us a new understanding of the role of polarization markers on regulating macrophages. Acidic mammalian chitinase (AMCase) was also found to be of critical importance in the control of type 2 immune responses because knocking in enzymatically inactive AMCase enhanced type 2 immune responses to inhaled house dust mites (40). A recent study also found opposing effects of Ym1 on type 2 immunity during nematode infection (41), which suggests the diverse roles of C/CLPs in regulating the immune response. In the present study, we found that Ym1-deficient macrophages showed an enhanced AAM phenotype. The increased expression of Ym1 during macrophage polarization might act as a brake to control or limit the induction of AAM markers Arg1 and MRC1. Our finding reveals a novel function of Ym1 in controlling the alternative activation of macrophages.

It has been demonstrated that CAMs favor enhanced glycolysis, whereas AAMs favor β-oxidation orchestrated by STAT6 and PPARγ, whereas AAMs favor opposing effects of Ym1 on type 2 immunity during nematode infection. A recent study also found that the natural polymorphism of Ym1 controls its expression and thereby will influence the alternative activation of macrophages in the control of pneumonitis. Our results raise the possibility that Ym1 polymorphism might be correlated with differential susceptibility not only to pneumonitis but also to parasite infections. Our data identify chitinase-like genes as critical regulators of macrophages in immune responses, thus opening new possibilities for understanding and therapeutic intervention of pneumonitis and other inflammatory diseases.

MATERIALS AND METHODS

Mice and ethic statement

B10.RIII H2b MHC (major histocompatibility complex) haplotype-bearing mice were originated in J. Klein’s laboratory (Max Planck Institute for Biology, Tübingen) and maintained in our breeding colony. RIIIS/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The BR.Cia22.Chitinase (henceforth denoted BR.Ym1Δ) congenic founder was obtained by recombination-assisted breeding from a partial advanced intercross previously described and subsequently backcrossed (12). To ensure that BR.Ym1Δ mice were devoid of contaminating RIIIS/J alleles, strain purity of congenic mice was assessed using a custom-made 8k Illumina SNP chip (48). No SNPs were observed between congenic and B10.RIII parental strains. Ym1 congenic mice were crossed with Ncf1m1J point mutation mice on the B10.RIII background (named as BR.Ncf1*Ym1Δ). All above mouse strains, as well as C57BL/6N (B6N) and Balb/c, were kept under specific pathogen–free environment in the animal housing facilities of the Department for Medical Biochemistry and Biophysics, Section for Medical Inflammation Research, Karolinska Institute, Stockholm. They were housed in individually ventilated polystyrene cages containing wood shavings in a climate-controlled environment with a 14-hour light cycle and fed with standard rodent chow and water ad libitum. For all experiments, 10- to 14-week-old age- and sex-matched congenic and wild-type littermates controls have been used. All experimental procedures were approved by local ethical committees (Stockholm, Sweden; permit numbers M107/07, M109/07, N169/10, N66/10, N490/12, and N351/16).

Genomic DNA was isolated from toe tissue samples using the sodium hydroxide extraction method. Microsatellite markers were amplified using standard PCR. The size of the PCR fragments was determined using an ABI AB3730 DNA Analyzer system (Applied Biosystems, Foster City, CA, USA).

RNA quantitation using qPCR

Spleen, thymus, lymph nodes, stomach, lung, liver, heart, kidney, small intestine, colon, skin, brain, and bone marrow cells were obtained from age- and sex-matched congenic mice and wild-type counterparts after carbon dioxide asphyxiation. Total RNA from the above tissues was isolated by using TRIzol Reagent (Invitrogen), and complement- DNA (cDNA) was synthesized using the First Strand cDNA Synthesis Kit (Fermentas). RT-PCR was performed by Agilent Stratagene Mx3005P with FastStart Universal SYBR Green Master (Roche) to assess the gene expression. The relative gene expression normalized by β-actin was calculated with the 2−ΔΔCT method. The primers used were described in table S1. In addition, PCR production of Ym1 and Ym2 in the indicated tissues of B10.RIII wild-type mice was separated and determined by DNA electrophoresis on 1.5% agarose gel.

Transcriptional activity assay of Ym1 promoter

The genomic DNA was extracted from B10.RIII and RIIIS/J mice, and the promoter of Ym1 gene, a 2074-bp DNA fragment (from −1788 to +274, the transcriptional start site was denoted as +1), was isolated and cloned to the pGLO.17 plasmid by Biomatik, which were denoted as pGL-B10.RIII and pGL-RIIIS/J, respectively. The site mutations were performed using pGL-B10.RIII as the template to make the promoter containing one of four SNPs according to a commercial site mutation kit (Q5 Site-Directed Mutagenesis Kit, New England Biolabs), which were named as pGL-SNP1, pGL-SNP2, pGL-SNP3, and pGL-SNP4. The primers used for site mutation were described in table S1. All plasmids, including the pRL-TK vector (Promega, Fitchburg, USA) that served as a control, were prepared with the EZNA Endo-free Plasmid Mini Kit (Omega Bio-tek, Norcross, USA). The constructs were sequenced to prove sequence integrity before use.

Human embryonic kidney (HEK) 293T cells cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, USA) containing 10% fetal bovine serum (FBS) (Gibco, USA) were used for a dual-luciferase
tissues were prepared for hematoxylin and eosin (H&E) staining. The lung index, weight normalized by body weight (lung index) was calculated. Lung tissues were prepared for histological analysis. Control mice were sensitized by the intraperitoneal injection of 0.2 ml of emulsion solution mixed with 10 µg (in 100 µl) of OVA (Sigma-Aldrich, St. Louis, MO, USA) and 100 µl of Imject Alum (Pierce, Thermo Fisher Scientific) at days 0 and 7. During days 14 to 20, mice were challenged with 1% OVA aerosol for 30 min per day. Control mice were sham-sensitized and exposed to the same volume of PBS. Mice were sacrificed at day 21. Lung tissues were prepared for histological analysis and cytokine expression detection. Inflammatory cells in BALF were analyzed by flow cytometry. Sera were collected for determining the concentration of Ym1- and OVA-specific IgG1 and IgE.

**Lung histology**

Lung tissues were stained with H&E to observe pathological changes, with periodic acid–Schiff (PAS) to detect mucus production. The scoring of lung histology was performed in a double-blind fashion. First, a scoring system to estimate the severity of peribronchial infiltration of inflammatory cells is as follows: 0, no cells; 1, a few cells; 2, half ring of cells around airway; 3, a ring of cells and one cell layer deep; 4, a ring of cells and two cell layers deep; 5, a ring of cells and more than two cell layers deep or cell aggregation. Second, to evaluate the inflammatory cell infiltration in lung interstitium, total cell numbers in interstitium were counted by using the Image-Pro Plus software. In addition, to estimate the extent of mucus production, the integral optical density (IOD) of the airways with PAS staining was analyzed by using the Image-Pro Plus software.

**Flow cytometry**

Single-cell suspensions from BALF cells were labeled with the following monoclonal antibodies in the respective experiments: anti-CD11b–Pacific blue, anti-Ly6G–Alexa Fluor 700, anti-CD11c–APC (allophycocyanin), anti-F4/80–BV605, anti–Siglec F–PerCP (peridinin chlorophyll protein)–Cy5.5, anti–Arg1–FITC (fluorescein isothiocyanate), anti–iNOS–PE (phycocerythrin)–Cy7, anti–MRC1–PE, anti-CD11b–APC, anti-CD11c–APC-Cy7, anti-Ly6G–Pacific blue, anti-F4/80–FITC, anti–TGRβ–Alexa Fluor 700, anti-B220–PE–Cy7, and anti–Ym1–PE. All of these antibodies were sourced from either Becton Dickinson, ebioscience, BioLegend, or Abcam. For intracellular staining, cells were fixed and permeated by using BD Cytofix/Cytoperm before intracellular antibody incubation. For Ym1 intracellular staining, cells were incubated with brefeldin A (10 µg/ml; Selleck) for 4 hours before staining. Samples were analyzed using FACS LSR II and FlowJo software (TreeStar Inc.).

**Bone marrow–derived macrophages and polarization**

The primary bone marrow–derived cells were isolated from B10.RIII, BR.Ym1, BR.Ncf1, and BR.Ncf1. Ym1 mice and cultured in DMEM containing 10% FBS and M-CSF (macrophage colony-stimulating factor) (10 ng/ml) for 7 days to differentiate bone marrow–derived macrophages (BMMs). For macrophage polarization, LPS (100 ng/ml) and IFN-γ (10 ng/ml) were treated to BMM to polarize the CAMs, while IL-4 (20 ng/ml) and IL-13 (50 ng/ml) were treated to BMM to polarize the AAMs. BMM was also treated with IL-10 (20 ng/ml) to compare with IL-4/IL-13–treated BMM. The PPAR-γ inhibitor T0709097 (1 µM; Selleck) or the CPT1 inhibitor etomoxir (200 µM; Selleck) was used to block the alternative activation process of macrophages. The siRNA of mouse STAT6 and PPAR-γ was designed and purchased from GenePharma (Shanghai, China) (sequences described in table S2). Macrophages were transfected with 50 nM siRNA using Lipofectamine 2000 for 24 hours, followed by IL-4/IL-13 stimulation. RNA and protein were extracted from cells for gene and protein expression assay, and cultured media were collected for cytokine production assay.

**Enzyme-linked immunosorbent assay**

For quantification of Ym1 levels, a commercially available ELISA kit was used according to the manufacturer’s protocol (R&D Systems,
Western blotting
Total protein lysates from cells were extracted by using the radioimmunoprecipitation assay (RIPA) solution with a cocktail of protease and phosphatase inhibitors (Roche). The final protein concentration of each sample was determined with a Bradford protein assay (Bio-Rad, USA). Briefly, proteins were extracted, and the concentrations were determined using one-way analysis of variance (ANOVA) or Student’s t test. A P value of less than 0.05 was considered significant.

Label-free quantification proteomics
Thioglycollate-elicited macrophages were isolated from B10.RIII mice and BR.Ym1Δ congenic mice (n = 4 per group), and the label-free quantification proteomics analysis was performed (Novogene, China). Briefly, proteins were extracted, and the concentrations were determined by Bradford protein assay (Bio-Rad, USA). After proteins were digested with Trypsin Gold (Promega), shotgun proteomics analyses were performed using an EASY-nLC 1200 UHPLC system (Thermo Fisher Scientific) coupled with an Orbitrap Q Exactive HF-X mass spectrometer (Thermo Fisher Scientific) operating in the data-dependent acquisition mode.

Statistical analysis
Quantitative data were expressed as means ± SEM. The statistical analysis of differences between experimental groups was performed using one-way analysis of variance (ANOVA) or Student’s t test. A P value of less than 0.05 was considered significant.

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