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Chitinase and Fizz Family Members Are a Generalized Feature of Nematode Infection with Selective Upregulation of Ym1 and Fizz1 by Antigen-Presenting Cells

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Ym1 and Fizz1 are secreted proteins that have been identified in a variety of Th2-mediated inflammatory settings. We originally found Ym1 and Fizz1 as highly expressed macrophage genes in a *Brugia malayi* infection model. Here, we show that their expression is a generalized feature of nematode infection and that they are induced at the site of infection with both the tissue nematode *Litomosoides sigmodontis* and the gastrointestinal nematode *Nippostrongylus brasiliensis*. At the sites of infection with *N. brasiliensis*, we also observed induction of other chitinase and Fizz family members (ChaFFs): acidic mammalian chitinase (AMCase) and Fizz2. The high expression of both Ym1 and AMCase in the lungs of infected mice suggests that abundant chitinase production is an important feature of Th2 immune responses in the lung. In addition to expression of ChaFFs in the tissues, Ym1 and Fizz1 expression was observed in the lymph nodes. Expression both in vitro and in vivo was restricted to antigen-presenting cells, with the highest expression in B cells and macrophages. ChaFFs may therefore be important effector or wound-repair molecules at the site of nematode infection, with potential regulatory roles for Ym1 and Fizz1 in the draining lymph nodes.

Macrophages are a fundamental feature of chronically inflamed tissue. In the course of long-term inflammation, the macrophage phenotype often shifts away from a highly microbicidal state towards an "alternative activation" pathway as the T-cell cytokine profile shifts from type 1 to type 2 (16). In the case of helminth infection or allergy, the type 2 response can dominate from the outset. Although our understanding of macrophage activation under these type 2 conditions is increasing, whether macrophages promote the disease state or protect against it remains essentially unknown. We and others have recently discovered that macrophages activated by type 2 cytokines in vivo produce high levels of two secreted proteins, Ym1 (9, 12, 51) and Fizz1 (31, 36, 40). In a nematode infection model, we found that Ym1 represents over 10% of the total nematode-elicited macrophage (NeMϕ) mRNA, while Fizz1 is the second most abundant transcript at 2% (31).

Ym1 is a member of a family of mammalian proteins that share homology to chitinases of lower organisms (25). Although Ym1 was originally described as an eosinophil chemotactic factor (38, 39), the dramatic level of production by macrophages and its ability to bind chitin and related glycan structures (9, 46) suggest that eosinophil chemotaxis, a property that remains controversial (9), is not its primary function. Ym1 may have a defensive role by binding fungal or other pathogens containing chitin, but having no apparent chitinase activity, its effector mechanisms remain unclear. These mechanisms may include the sequestration of the pathogens and/or the recruitment of effector immune cells. By its ability to bind heparin, a glycan abundant on the cell surface and the extracellular matrix, Ym1 may also mediate cell-to-cell and cell-to-matrix interactions in a manner similar to that of selectins (46). Finally, a role in the deposition of extracellular matrix during the wound-healing process has been proposed (9). The high level of Ym1 in NeMϕ would be consistent with the role of macrophages activated by type 2 cytokines in tissue repair (20, 41).

The function of Fizz1 is even less well defined. Holcomb et al. first reported Fizz1 as an abundantly secreted protein in the bronchoalveolar lavage fluid of a murine asthmatic model (22). They observed the secretion of Fizz1 from the inflamed pulmonary epithelium by pneumocytes and demonstrated that Fizz1 could inhibit the action of nerve growth factor (NGF) in vitro (22). Fizz1 (also known as Resistin-like moleculeα) is also expressed in adipocytes (43), where it inhibits adipocyte differentiation (4). In agreement with the findings of Raes et al. (40), we have found Fizz1 to be specifically produced by macrophages in response to interleukin-4 (IL-4) both in vivo and in vitro (31, 36). More recently, Fizz1 has been implicated in mediating the deposition of extracellular matrix in an animal model of lung fibrosis (29). Thus, Fizz1 and Ym1 could both participate in the wound-healing function of type 2 cytokine-activated macrophages. This array of biological properties and expression patterns suggests that Fizz1 is highly pleiotropic in function, but its role or relative importance in vivo remains the subject of considerable speculation.

The highly abundant expression of these two proteins in many different contexts as well as the high level expression of human homologues with unknown function (6, 7, 22) suggest that these molecules will be of significant interest to our understanding of responses to infectious agents as well as inflam-

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matory responses in general. We thus chose to examine these genes in more depth by investigating their pattern of expression during the course of two very different nematode infections.

We show here not only that Fizz1 and Ym1 are highly upregulated at the sites of parasite migration and residence during both chronic infection with filarial nematode *Litomosoides sigmodontis* and acute infection with gastrointestinal nematode *Nippostrongylus brasiliensis* but also that additional chitinase and Fizz family members (ChaFFs) are also produced. Notably, Fizz2 and acidic mammalian chitinase (AMCase), a functional chitinase, were induced at the sites of nematode infection but with expression patterns distinct from those of Fizz1 and Ym1. Furthermore, Fizz1 and Ym1 expression was also induced in the draining lymph nodes (LN), where expression was limited to the antigen-presenting cell (APC) population, with the highest expression by macrophages and B cells. These studies suggest that ChaFFs have a wide range of functions during Th2-polarized immune responses that may include both effector and regulatory roles.

MATERIALS AND METHODS

Mice. All experiments used C57BL/6 or BALB/c mice bred in-house or purchased from Harlan UK. Mice were 6 to 8 weeks old at the start of the experiment.

Antibodies. By using the protocol of Holcomb et al. (22), a polyclonal antibody against Fizz1 was similarly raised by immunizing rabbits with the N terminus of Fizz1 (ENKVKELLANPANYP) conjugated with keyhole limpet hemocyanin (Genosphere). A polyclonal antibody against Ym1 was obtained by immunizing rabbits with the Ym1 peptide (IPRLLLTSTGAGIID) conjugated with ovalbumin.

Nematode infection. (i) *B. malayi*. Adult parasites were removed from the peritoneal cavity of infected jirds purchased from TRS Laboratories (Athens, Ga.). C57BL/6 males were surgically implanted intraperitoneally with six live adult female *B. malayi* parasites. At selected intervals that ranged from 1 to 21 days later, the mice were euthanized, and peritoneal exudate cells (PEC) were harvested by thorough washing of the peritoneal cavity with 15 ml of ice-cold medium (RPMI). Control mice were subjected to sham surgery and euthanized at time points that matched those of the implanted mice. The first milliliter of the wash was recovered for Western blot analysis. The mediastinal and parathymic LN draining the peritoneal cavity were recovered, and cell suspensions were prepared. NeMϕ were purified from the PEC by adherence as described previously (36). Mice were injected intraperitoneally with 0.8 ml of 4% thioglycolate medium (Becton Dickinson) brewer modified as a control for non-Th2-polarized inflammation (28, 32). Four days later, the PEC and draining LN were harvested as described above.

(ii) *L. sigmodontis*. Female BALB/c mice were infected subcutaneously with 25 L3 larvae, as described previously (27). 60 days following infection, the mice were euthanized, and the thoracic cavity was thoroughly washed with 5 ml of ice-cold medium. The thoracic lavage cells were recovered for RNA extraction. The mediastinal and parathymic LN draining the thoracic cavity were also removed, and a cell suspension was prepared.

(iii) *N. brasiliensis*. The parasite life cycle was maintained as described previously (26). C57BL/6 male mice were injected subcutaneously with 400 L3 larvae. After 6 days, the mice were sacrificed, and the lung tissue and small intestine were recovered.

Western blot analysis. Twenty microliters or 10 μg of peritoneal exudates was mixed with sample buffer (Invitrogen) supplemented with β-mercaptoethanol (100 μM), heat denatured, and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 4 to 12% gradient bis-Tris Nupage gel (Invitrogen) followed by transfer onto nitrocellulose membrane (Bio-Rad). Cell lysates were prepared according to established protocols (35). In brief, the cell pellets were resuspended in 40 mM Tris with protease inhibitors and sonicated twice for 20 s followed by centrifugation to remove the insoluble debris. Protein (5 μg) was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described above. The membranes were blocked overnight with 0.05% Tween 20 in StartingBlock buffer (Pierce) and then incubated for 2 h at room temperature with a 1:5,000 dilution of anti-Fizz1, a 1:10,000 dilution of anti-Ym1, or a 1:5,000 dilution of control preimmune serum. Incubation for 1 h with a 1:2,000 dilution of peroxidase-conjugated goat anti-rabbit immunoglobulin G (heavy plus light chains; Bio-Rad) was followed by detection by the enhanced chemiluminescence method according to manufacturer's instructions (Amersham Pharmacia Biotech).

Cell preparations for in vitro analysis. The clone D10.G4 (23) was activated as described previously (1) with specific antigen (conalbumin) in the presence of irradiated splenocytes for 3 days before recovery of live cells using a Ficoll gradient. Myelin oligodendrocyte glycoprotein (35–55)-specific Th1-polarized cells were a kind gift from Steve Anderton (2, 14). B cells were purified from spleens of C57BL/6 mice through negative depletion of $CD43^+$ cells by using a MACS column (Miltenyi Biotec) according to previously published protocols (42). The purified B cells (>90% $B220^+$) were cultured in 24-well plates at 1.5×10^6 cells/well. Bone marrow (BM)-derived dendritic cells (DC) and macrophages (Mϕ) were prepared by harvesting the BM from the femurs and tibia of C57BL/6 mice. Differentiation into macrophages was performed according to previously published protocols (11). In brief, the cells were plated at 2.5×10^5 cells/well in Dulbecco's modified Eagle's medium supplemented with 25% fetal calf serum (FCS) (Gibco) and 25% L929 supernatant (ATCC CCL1). The medium was replaced at days 4 and 6, resulting in a pure population of macrophages by day 7 (>90% $F4/80^+$). For differentiation into DC, the cells were plated at 3.75×10^5 cells/well in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 10% granulocyte-macrophage colony-stimulating factor (44), and L-glutamine and penicillin-streptomycin as described above. The medium was replaced on days 3 and 6. On day 7, the loosely adherent immature DC ($CD11c^+$) were removed by gentle pipetting, resulting in >85% $CD11c^+$ purity, and plated at 1.5×10^6 cells/well. B cells, Mϕ, and DC were cultured in medium alone or treated overnight with IL-4 (20 ng/ml; Pharmingen). For cell preparations other than macrophages, F4/80 staining demonstrated minimal macrophage contamination.

Purification of cell populations from the LN. In order to obtain single-cell suspensions, the collected LN were first cut into pieces and digested with type IV collagenase (2.6 mg/ml; Lorne Labs Ltd.) and DNAseI (1 mg/ml; SIGMA) in RPMI for 30 min at 37°C, and the reaction was stopped with 25% FCS. The cells were washed and incubated for 15 min with a solution containing 5% mouse serum, 0.01 mg of anti-CD16/32 per ml, and 5% purified rat immunoglobulin G (Pharmingen) in phosphate-buffered saline supplemented with 2 mM EDTA–0.5% bovine serum albumin–2 mM NaN_3 to block nonspecific binding. Purification was performed by magnetic cell sorting using MiniMacs columns (Miltenyi Biotec) according to the manufacturer's instructions. The antibodies used for purification were as follows: anti-CD4 microbeads (Miltenyi Biotec), anti-CD8α-biotin (Pharmingen), anti-Igκ-biotin (cell clone ATCC HB38), anti-F4/80-biotin (Pharmingen), anti-CD11c microbeads (Miltenyi Biotec), and streptavidin microbeads (Miltenyi Biotec). The purity of the sorting was verified by flow cytometry analysis (BD FACStation and FlowJo software).

RNA extraction and real-time reverse transcription (RT)-PCR. RNA was recovered from cells by resuspension in Trizol (Invitrogen). For RNA extraction of tissue, the tissue was first incubated overnight at 4°C in RNAlater (Ambion) and subsequently homogenized in Trizol. Total RNA was extracted according to the manufacturer's instructions. Following DNAseI treatment (Ambion), 1 μg of RNA was used for the synthesis of cDNA by using Moloney murine leukemia virus reverse transcriptase (Stratagene). Relative quantification of the genes of interest was measured by real-time PCR using the Roche LightCycler. For each gene, five serial 1:4 dilutions of a positive control sample of cDNA were used in each reaction. For cell analysis, macrophage cDNA was used, whereas for whole-tissue analysis, infected tissue cDNA was used as the positive control. Expression levels were then estimated from the standard curve of the positive-control cDNA. Real-time PCR quantification of the housekeeping gene β-actin allowed normalization of the amount of cDNA in each sample after which expression of the genes of interest was assessed. PCR amplifications were performed in 10 μl containing 1 μl of cDNA, 4 mM $MgCl_2$, 0.3 μM primers, and the LightCycler-DNA SYBR Green I mix. The amplification of β-actin, Fizz1, Fizz2, AMCase, and arginase I was performed under the following conditions: a denaturation step for 30 s at 95°C, annealing of primers for 5 s at 55°C, and an elongation step for 12 s at 72°C, for 40 to 60 cycles. SYBR Green fluorescence was monitored after each cycle at 86°C. For Ym1 amplification, the annealing temperature was increased to 63°C and the monitoring of SYBR Green fluorescence was performed at 85°C. Primers for LightCycler PCR analysis were TGGAATCCTGT GGCATCCATGAAAC and TAAAACGCAGCTCAGTAACAGTCCG for β-actin, CAGAAGAATGGAAGAGTCAG and CAGATATGCAGGGAGT CACC for arginase I, GGTCCCAGTGCATATGGATGAGACCATAGA and CACCTCTTCACTCGAGGGACAGTTGGCAGC for Fizz1, TCACAGGTCT GGCAATTCTTCTG and TTTGTCCTTAGGAGGGCTTCCTCG for Ym1,

GTGTTTCCTTTTCATCCTCGTCTC and CAGTGGCAAGTATTTCCAT TCCG for Fizz2, and GTCTGGCTCTTCTGCTGAATGC and TCCATCAAA CCCATACTGACGC for AMCase.

Distinction between Ym1 and Ym2. Ym1 and Ym2 are highly homologous genes that cannot be distinguished with the primers used for real-time PCR. Restriction digestion of the full Ym PCR product with ScaI (Sigma) allowed differentiation between Ym1 and Ym2, as only the Ym1 PCR products are digested (50). cDNA (1 μl) was amplified by using *Taq* polymerase (QIAGEN) for 30 cycles. PCR conditions were as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, which resulted in a 1,156-bp amplicon. The PCR products were purified and digested with ScaI for 2 h. The results of the restriction digest were assessed by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining. Primers for PCR were Ym1-For (TGGGGGATCCGTACCA GCTGATGTGCTACT) and Ym1-Rev (GTAAAGGATCCTCAATAAGGGC CCTTGCA). For comparison, a plasmid containing Ym1 was similarly amplified, purified, and digested.

Data analysis and statistics. Graphs were prepared by using PRISM software (version 3.0; GraphPad Software, Berkeley, Calif.). The two-tailed Mann-Whitney nonparametric *t* test was used to assess the statistical difference between the groups studied, with a P of <0.05 designated as significant.

RESULTS

Fizz1 and Ym1 are secreted in the peritoneal lavage fluid following the implant of *B. malayi* in an IL-4-dependent manner. Localized induction of Fizz1 and Ym1 is readily apparent in peritoneal exudate macrophages following the implant of the human filarial parasite *B. malayi* into the peritoneal cavity of mice (12, 31). We have shown previously by real-time PCR that the induction of both Ym1 and Fizz1 in NeMϕ is IL-4 dependent (31, 36). Fizz1 and Ym1 proteins both have leader peptide sequences and have been shown to be secreted in other disease models (9, 22). We wanted to see whether the very high level of transcription of these two genes was reflected in protein expression. Western blot analysis of the peritoneal supernatants 3 weeks following implant with adult *B. malayi* parasites showed secretion of both proteins in implanted wild-type C57BL/6 mice but no secretion or only basal levels in IL-$4^{-/-}$ mice and in control mice injected with thioglycolate (Fig. 1A). The upregulation of Fizz1 appeared to be more strictly regulated by IL-4, as we did not detect any signal in the groups other than the implanted C57 mice, in contrast to Ym1, where a basal level was detected in the naïve and IL-$4^{-/-}$ mice. Control of expression by type 2 cytokines is consistent with evidence that the Ym1 promoter has STAT-6-responsive elements (51) while the Fizz1 promoter contains functional binding sites for STAT-6 and C/EBP (45).

In order to further verify that real-time RT-PCR measurements reflected protein secretion, we performed a time course of Ym1 and Fizz1 expression, measuring RNA in the peritoneal exudate cells and protein in the peritoneal lavage fluid by Western blot (Fig. 1B). Our data show a close correlation between mRNA levels and protein expression, suggesting that Ym1 and Fizz1 protein secretion is controlled at the RNA transcription level. Thus, measurement of mRNA levels provides a reliable indicator of protein production. Interestingly, in control animals that underwent the surgical procedures without parasite implant, Fizz1 and Ym1 message was upregulated in the first 72 h but returned to baseline by 5 days postsurgery.

Fizz1 and Ym1 are induced at the sites of parasite migration and residence during infection with *L. sigmodontis*. Our analysis of peritoneal exudate cells from mice implanted with *B.*

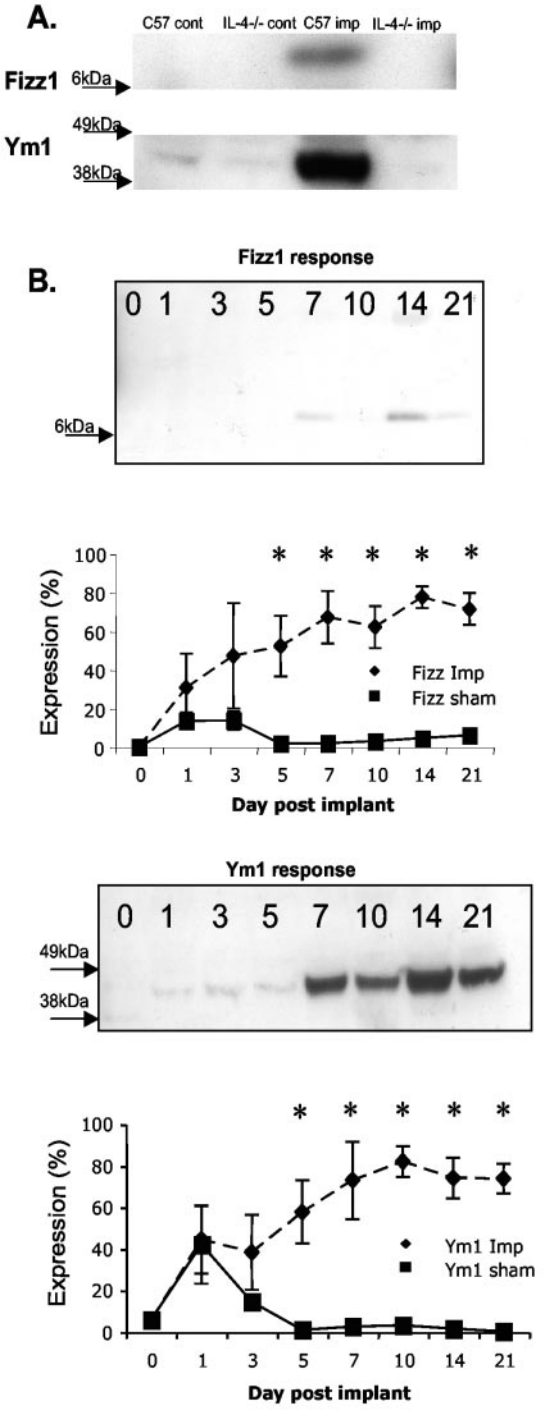

FIG. 1. Fizz1 and Ym1 gene expression reflects protein levels. A. Western blot analysis of the peritoneal lavage fluid from individual mice. C57 or IL-$4^{-/-}$ mice were infected with *B. malayi* (imp) or injected with thioglycolate (cont). B. Time course of Fizz1 and Ym1 expression following sham surgery or *B. malayi* implant (Imp) of C57 mice by Western blot analysis of peritoneal lavage fluid and real-time RT-PCR of the peritoneal exudate cells. Expression is shown as a percentage of pooled *B. malayi* NeMϕ cDNA (± standard deviations [SD] from groups of five mice). An asterisk indicates a significant difference ($P < 0.05$) between the implanted and sham surgery groups at the same time point.

malayi provided valuable insight into Fizz1 and Ym1 expression patterns, but we wanted to extend these studies to a more systemic setting in which the full life cycle of the parasite takes place. We thus examined expression during infection with the rodent filarial nematode *L. sigmodontis*. Larvae injected subcutaneously into BALB/c mice migrate via the lymphatics to the thoracic cavity where they develop into adults and by 2 months postinfection release microfilariae, which circulate in the bloodstream (21).

At 60 days, by which time a patent infection is established, we obtained thoracic lavage cells as well as the parathymic and mediastinal LN, through which the larvae migrate to arrive in the thoracic cavity (3). Using real-time RT-PCR, we measured the induction of Fizz1 and Ym1 and found that both these genes were highly upregulated in the thoracic lavage cells and also significantly elevated in the LN (Fig. 2A and B).

Ym2 is highly homologous to the Ym1 gene but shows expression patterns distinct from those of Ym1: Ym1 expression is predominant in the lung and spleen, and Ym2 expression is found mainly in the stomach (25). As thoracic lavage cells and LN cells had not been previously investigated for Ym1 expression, we performed an ScaI restriction analysis of the Ym PCR products to differentiate between Ym1 and Ym2 transcripts and found that Ym1 was the only Ym transcript expressed in response to *L. sigmodontis* infection (Fig. 2C), consistent with Ym1 being the only transcript in *B. malayi* NeMϕ (31).

The expression levels of both Fizz1 and Ym1 in the thoracic lavage cells were comparable to expression in *B. malayi* NeMϕ. This was not surprising since infection with *L. sigmodontis* results in a type 2 chronic inflammatory environment similar to that induced in response to *B. malayi* implant. Notably, in both settings, macrophages represent a major proportion of the cells recruited to the site of infection (12, 33, 48). The high Fizz1 and Ym1 expression in these settings supports the studies of Raes et al. (40), which argue for the expression of these genes during the chronic stages of an immune response. However, we have also observed Fizz1 and Ym1 induction in the thoracic cavity as early as 10 days post-*L. sigmodontis* infection in both C57BL/6 and BALB/c mice (our unpublished observation) and by 24 h in the *B. malayi* implant model (Fig. 1B), suggesting that the establishment of a chronic infection is not essential for gene expression.

Induction of ChaFFs at the sites of infection with *N. brasiliensis*. Having established that Fizz1 and Ym1 are highly responsive to filarial nematode infection, we chose to investigate whether induction of these genes was broadly characteristic of nematode parasitism by looking at a gastrointestinal infection model using *N. brasiliensis*. This model allowed us to examine the expression of Fizz1 and Ym1 in two different tissues exposed to the same parasite and also provided an acute nematode infection scenario in contrast to chronic infestation with *B. malayi* and *L. sigmodontis*. We measured gene expression in both relevant sites, the lung and small intestine, at 6 days postinfection, by which time the parasite had completed its full life cycle (26, 47).

Fizz1 expression had not previously been reported in the gastrointestinal region, where preferential expression of the homologous gene Fizz2 was observed (22, 43). Thus, we also measured Fizz2 expression in the infected tissue. Both Fizz1 and Fizz2 were induced in the lungs and small intestine of infected mice. Interestingly, the relative levels of Fizz1 and Fizz2 in the different infection sites showed a reciprocal pattern: Fizz1 expression was highest in the lung, whereas Fizz2 was preferentially expressed in the small intestine (Fig. 3A). It would be of interest to investigate this response kinetically to see whether the relative levels of Fizz1 and Fizz2 change over the course of infection with migration of the parasite through the different tissues or whether the Fizz1-to-Fizz2 ratio we observed is a fixed feature of lung biology compared to gut biology.

We also observed high levels of Ym in both the lung and

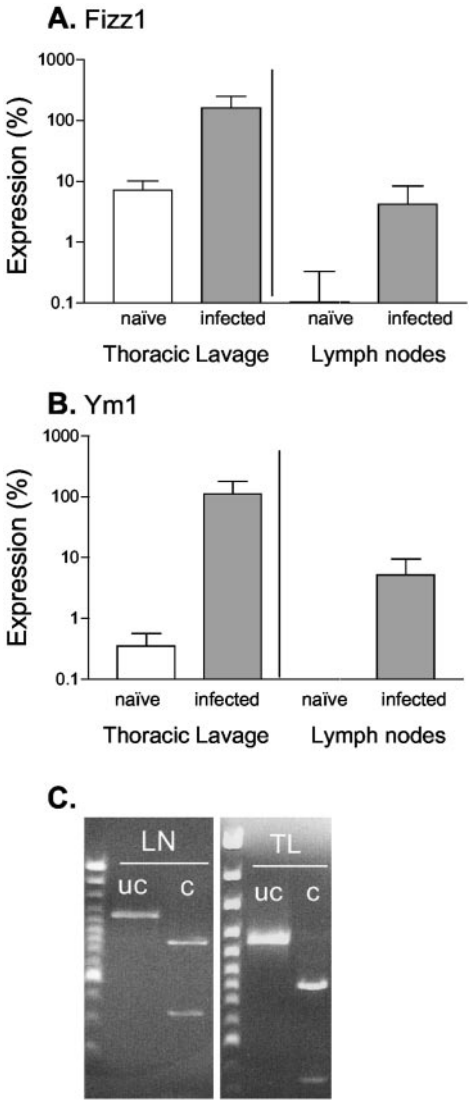

FIG. 2. Fizz1 and Ym1 induction during chronic infection with the filarial nematode *L. sigmodontis* at both the site of infection and draining LN. A, B. Real-time RT-PCR quantification of Fizz1 and Ym1 expression in thoracic lavage and draining LN cells 60 days postinfection with *L. sigmodontis*. Expression is shown as a percentage of pooled *B. malayi* NeMϕ cDNA (± SD from groups of five mice). (C) ScaI restriction digest performed on the Ym PCR products from thoracic lavage (TL) cells and LN cells from infected mice (uc, uncut control; c, cut with ScaI). These data are representative of two separate experiments.

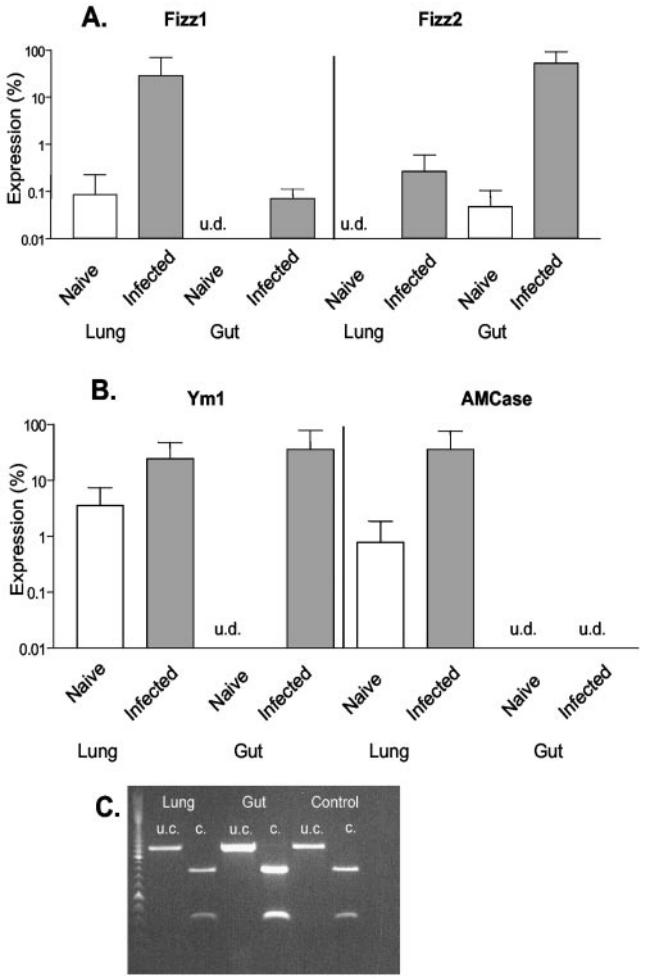

FIG. 3. Infection with *N. brasiliensis* upregulates expression of Fizz and chitinases in multiple tissues. Real-time RT-PCR quantification of Fizz1 and Fizz2 (A) and Ym1 and AMCase (B) in the lung and gut tissue of naïve and BALB/c mice infected with *N. brasiliensis* for 6 days is shown. Expression was measured as the percentage of the highest-expressing infected tissue sample (± SD from groups of five mice). C. Sca1 restriction digest performed on the Ym PCR products of cDNA of both infected tissues. u.d., undetected by 50 amplification cycles; u.c., uncut; c., cut.

small intestines of *N. brasiliensis*-infected mice (Fig. 3B) and confirmed that the gene product was Ym1 by restriction analysis (Fig. 3C). Consistent with previously published observations (24), we observed high background levels of Ym1 in the lungs of naïve mice, but *N. brasiliensis* infection induced a greater than 10-fold increase in expression ($P < 0.05$) over these background levels. As Ym1 expression had not previously been reported in the small intestine, we were surprised to find that induction in the small intestine was comparable to that in the lungs. However, most studies on the expression pattern of Ym1 have investigated gene expression in uninfected tissue. The potent Th2 environment induced by *N. brasiliensis* may cause the recruitment of Ym1-expressing immune cells to the inflamed tissue. This is consistent with recent studies of the gut-dwelling nematode *Trichuris muris* which demonstrated large numbers of F4/80$^+$ macrophages recruited to the site of infection (10).

Webb et al. reported preferential Th2 cytokine-dependent expression of Ym2 in the lungs of mice with allergic pulmonary inflammation (50). In contrast, we report here that Ym1 is preferentially expressed in nematode infection as well as in vitro in response to IL-4 (36). Differences between our studies may indicate that preferential expression of Ym1 or Ym2 varies according to the polarization, intensity, and/or chronicity of the immune response.

By sequence identity, the closest human homologue to Ym1 is the recently described AMCase (6). A murine AMCase has also been identified; thus, the relationship between Ym1 and AMCase in mice is unclear. To help define this relationship, we analyzed the expression of the murine AMCase in this infection model. AMCase followed a stricter expression pattern and was detected uniquely in the lungs (Fig. 3B). As AMCase was upregulated in response to infection, this result implied a broader function for this protein than the suggested housekeeping role of digestion (6). The induction of two distinct chitinase family members following the rapid migration of a nematode parasite through the lungs suggests that this family of molecules must have important but as-yet-unidentified roles to play in lung physiology.

Having observed two additional ChaFF members (Fizz2 and AMCase) induced by nematode infection, we also looked for induction of these genes in NeMϕ and the draining lymph nodes of *L. sigmodontis*-infected mice but could not detect any expression by real-time RT-PCR.

Fizz1 and Ym1 are induced in Mϕ, DC, and B cells but not in helper T cells in response to IL-4. We have shown that Fizz1 and Ym1 induction is common to three different nematode infection models. Induction of Fizz1 and Ym1 is caused by the highly Th2-polarized immune response driven by these nematode infections, as these genes are not upregulated in mice when the Th2 response is impaired (31) (Fig. 1A). Although we initially identified Fizz1 and Ym1 as Mϕ genes, our finding that they were also induced in the draining LN, where macrophages are a small proportion of the total cell population, suggested that other cell types may also express these genes.

Since Fizz1 and Ym1 were expressed in the LN during filarial infection, we focused on cells of the immune system and examined expression of these genes in BM-derived DC (Fig. 4A), Mϕ (Fig. 4B), and B and T lymphocytes (Fig. 4C) activated in a Th2 cytokine environment. In the resting or naïve state, all cell types showed no expression or basal expression of the genes examined. Activation with IL-4 induced expression of Fizz1 and Ym1 in B cells, BM-derived DC, and BM-derived Mϕ. ScaI restriction analysis confirmed that Ym1 was the main Ym gene induced in response to IL-4 (Fig. 4D). We did not observe induction of Fizz1 and Ym1 in Th1-polarized T cells or in the resting or activated Th2 T-cell clone D10.G4 despite the high production of IL-4 from this cell line (34). Therefore, Fizz1 and Ym1 appear to be expressed specifically by the APC population activated under Th2 conditions.

Fizz1 and Ym1 are induced in vivo in the draining LN of mice implanted with *B. malayi*. Since we observed that immune cells other than macrophages expressed Fizz1 and Ym1 when activated by IL-4 in vitro, we asked if this was physiologically relevant in vivo. We chose to look at gene expression in the

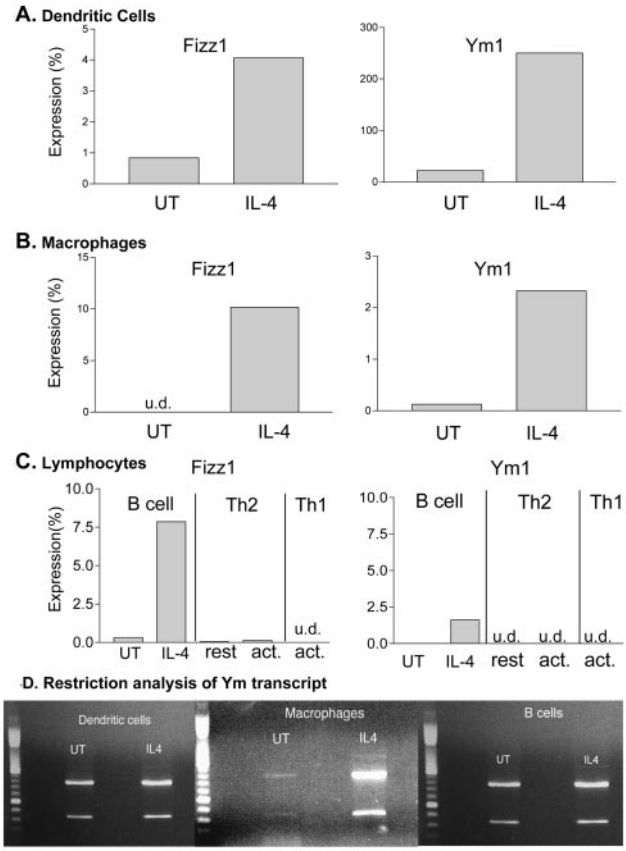

FIG. 4. Fizz1 and Ym1 expression is induced in Th2-activated dendritic cells (A), macrophages (B), and B cells but not in T-helper cells (C). Bone marrow-derived Mϕ, DC, and purified splenic B cells were left untreated (UT) or were treated with IL-4 overnight. Resting Th2 cells (rest) and Th2 cells activated with specific antigen for 3 days (act.) were obtained for expression analysis. Th1-polarized T cells were obtained by activation with immunogenic peptide over 3 weeks. Expression (mean of replicate samples) was measured by real-time RT-PCR as a percentage of pooled *B. malayi* NeMϕ cDNA. In antigen-presenting cells, Ym1 was the sole Ym transcript observed (D). u.d., undetected by 50 amplification cycles. These data are representative of two separate experiments.

draining LN of our original, well-established *B. malayi* implant model, where the adult parasite is inoculated directly into the peritoneal cavity. Thus, unlike the *L. sigmodontis* model, the lymphatics do not represent a site of parasite migration.

Both Fizz1 and Ym1 showed basal or no expression in LN from control, thioglycolate-injected mice; by real-time RT-PCR, the Fizz1 PCR product was not detected after 40 cycles (Fig. 5A), and the Ym1 product was detected by only 30 cycles (Fig. 5B). In response to *B. malayi* implant, however, Fizz1 and Ym1 expression was upregulated, and product was observed by 35 and 20 amplification cycles, respectively (Fig. 5A and B). However, Fizz1 and Ym1 were not as highly expressed as in NeMϕ, where PCR products were detected at 20 and 10 amplification cycles, respectively (Fig. 5A and B), and expression levels were measured as less than 1% of the NeMϕ cDNA (Fig. 5C). This result was consistent with our findings in the *L. sigmodontis* infection model (Fig. 2) and in the mesenteric lymph nodes of *N. brasiliensis*-infected mice (data not shown).

In order to verify that RNA data reflected protein expression, we compared Fizz1 and Ym1 protein levels in the draining LN cells, in NeMϕ, and in the peritoneal exudate fluid of mice implanted with *B. malayi* by Western blot (Fig. 5D). When equivalent amounts of protein (5 μg) were loaded, it was clear that NeMϕ expressed the highest levels of Fizz1 and Ym1. In particular, Ym1 levels were strikingly higher than Fizz1 levels, consistent with previous work in our laboratory showing that Ym1 represented 10% of the total NeMϕ RNA and that Fizz1 represented 2% of the transcript (31). Consistent with the real-time PCR results, Ym1 protein was also detected in the draining LN cells, although at a far lower level than in NeMϕ. We could not detect Fizz1 in the LN cells, probably due to the lower sensitivity of Western blot analysis in comparison to RT-PCR.

Fizz1 and Ym1 expression in the draining LN is limited to the APC population. Having observed Fizz1 and Ym1 expression in the draining LN of mice implanted with *B. malayi*, we chose to investigate which cell types in the LN were responsible for the expression of these genes. Analysis by flow cytometry showed that the proportion of cell types present in the draining LN of mice implanted with *B. malayi* was as follows: B cells, 60.6%; $CD4^+$ T cells, 18%; $CD8^+$ T cells, 17%; DC, 4%; and Mϕ, 0.4%.

Using positive magnetic bead selection, we purified the different cell populations from the draining LN cells of mice implanted with *B. malayi* and looked for Fizz1 and Ym1 expression by real-time RT-PCR. For the B cells and $CD4^+$ and $CD8^+$ T cells, the purification yielded over 90% purity. In the case of the Mϕ and DC, which are the least-represented cell types in the lymph nodes, we obtained 65% Mϕ and 86.1% DC from starting populations of under 5% starting material. In all cell preparations except Mϕ, we ensured that there was minimal Mϕ contamination by staining for F4/80 (data not shown).

In support of the in vitro data, Fizz1 and Ym1 were expressed in B cells, Mϕ, and DC. Macrophages were the highest-expressing cell type, followed by B cells and finally DC, which expressed low levels of Fizz1 and Ym1 (Fig. 6). Although a very low level of Ym1 expression was seen in $CD4^+$ and $CD8^+$ T cells, this level was no greater than the basal level we typically observe in unstimulated cells. This was a potentially surprising finding, as Ym1 (or ECF-L) was first described as a product of $CD8^+$ T cells during a nematode infection (39). However, this study described only an eosinophil chemotactic activity in the supernatant of spleen cells that is inhibited on depletion of CD8 cells. The assays did not directly measure Ym1 production and did not specifically show Ym1 expression by CD8 cells. These assays were performed in vitro with extracts from whole *Toxocara canis* parasites which may contribute to the eosinophil chemotaxis. Furthermore, eosinophil chemotaxis may not be the appropriate assay for Ym1, especially since this function is still controversial (9, 50). Production of Ym1 (mRNA or protein) by T lymphocytes has thus never been shown, and our data suggest that T cells are not a significant source of Ym1.

Thus, both in vitro and in vivo investigations of ChaFF expression profiles have shown that while Fizz2 and AMCase have tissue-specific expression patterns, Fizz1 and Ym1 are additionally expressed in immune cells with specific expression in antigen-presenting cells but not T cells.

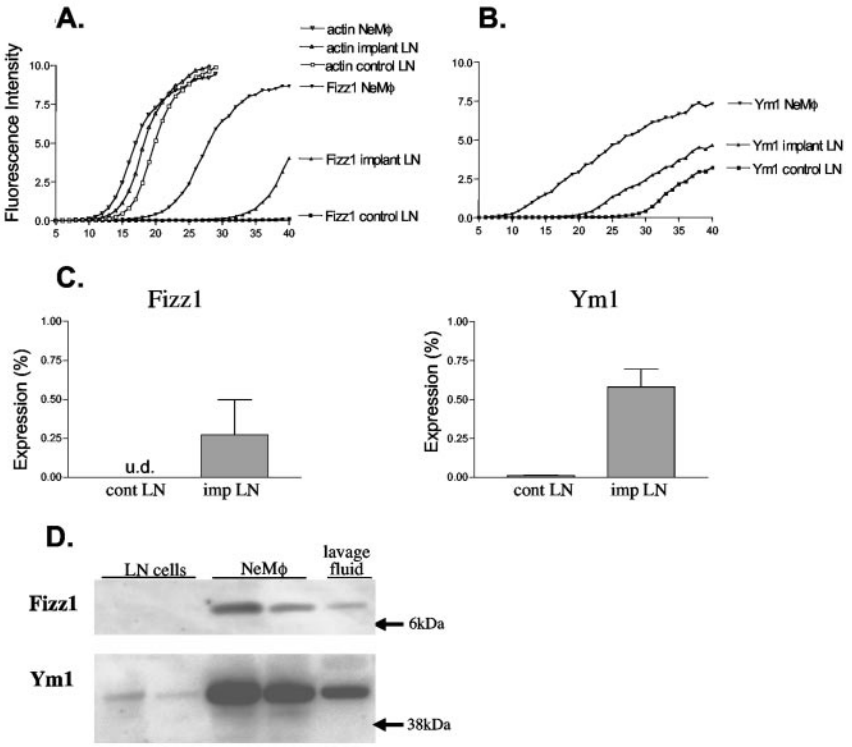

FIG. 5. Fizz1 and Ym1 are induced in vivo in the draining lymph nodes of *B. malayi*-implanted mice. The draining lymph nodes from control mice injected with thioglycolate (cont LN) and mice implanted with *B. malayi* (imp LN) were recovered and prepared for gene expression and Western blot analysis. A, B. Real-time RT-PCR shows the increase in fluorescence intensity during amplification of β-actin, Fizz1, and Ym1. C. Gene expression as the percentage of pooled NeMϕ cDNA (± SD from replicate samples). D. Western blot analysis for Fizz1 and Ym1 in 5 μg of protein of lymph node cell lysate, NeMϕ lysate, and lavage fluid from *B. malayi*-implanted mice. Duplicate lanes represent individual mice. These data are representative of two separate experiments.

DISCUSSION

The potential importance of Fizz1 and Ym1 in Th2-mediated immune responses was first highlighted by their extraordinarily abundant expression in macrophages recruited to the site of chronic *B. malayi* infection (12, 31, 33, 36) and during chronic trypanosomiasis (40), both profound Th2 environments. Despite their abundance in Th2 settings (9, 22, 45, 51), their function is still unknown. Our findings that these genes and other ChaFFs are induced in diverse nematode infection models and at varying sites of infection imply a broader range of function for these novel protein families than has previously been appreciated. First, they are produced not only during chronic infection but also in an acute setting. Second, they are produced in a wide range of tissues, appearing to be a necessary response to nematodes during migration or residence. Third, they are produced in the draining lymph nodes, dramatically expanding their potential functions.

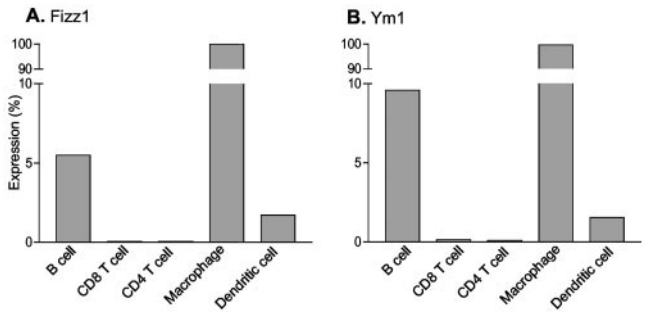

FIG. 6. Fizz1 and Ym1 are expressed in vivo in antigen-presenting cells but not T lymphocytes. Purified cell populations from the pooled draining lymph nodes of six *B. malayi*-implanted mice were measured for Fizz1 (A) and Ym1 (B) by real-time PCR. Expression levels of each sample are shown as a percentage of the lymph node macrophages (mean of replicate samples). These data are representative of two separate experiments.

Ym1 has been strongly implicated in tissue repair (25, 50), and more recently, Fizz1 has been shown to have angiogenic properties, stimulating actin and collagen expression (29, 49). Data from control animals in this study provide some of the most striking evidence that ChaFFs have related roles in tissue repair. Mice that underwent surgery but that did not receive any parasite material rapidly upregulated both Fizz1 and Ym1 following surgery. This expression was transient and returned to nondetectable levels by 5 days postsurgery, by which time the surgical wound had completely healed. Neutrophils are the first cell type recruited to the site of implant or surgery but are replaced by NeMϕ in infected animals (12). Neutrophils have been shown to express Ym1 (18), but expression of Ym1 and Fizz1 by NeMϕ is dependent on IL-4 (Fig. 1A) and requires $CD4^+$ T cells (P. Loke et al., unpublished observation). This finding suggests that ChaFFs can be produced rapidly at the

site of injury but that sustained production by macrophages requires Th2 cells. We have yet to determine the precise source of early ChaFF production or the requirement for IL-4 or IL-13.

Our finding that both Ym1 and AMCase are produced in the lung during *N. brasiliensis* infection suggests that the production of chitinase family members is a characteristic feature of Th2-mediated responses in the lung. The constant exposure of the lung to chitin-bearing organisms such as fungi highlights the potential importance of chitinases as a first line of innate defense. AMCase has functional chitinase activity, and the fact that it is most highly expressed in the stomach of naïve mice has led to its proposed role in the breakdown of ingested chitins (6). The upregulation of AMCase in response to infection argues for an additional role in the defense against lung-invading pathogens. Although Ym1 lacks functional chitinase activity, it may have an effector role in direct pathogen binding and promoting extracellular matrix deposition. Furthermore, the putative ability to recruit eosinophils could contribute to effector functions (38).

During *N. brasiliensis* infection, Ym1 was significantly upregulated in both the lungs and small intestine, but AMCase was uniquely expressed in the lungs. In contrast to the lungs, the small intestine has a measure of protection against external organisms by the upper gastrointestinal tract. There may be no requirement for AMCase expression in the small intestine since AMCase is highly expressed in the stomach and salivary glands. Alternatively, the restriction of AMCase expression to the lung during *N. brasiliensis* infection may be explained by the different exposure to the parasite in each tissue. In the lungs, more trauma results from the migration of the parasite through the tissue than in the small intestine, where surface colonization is more common (15, 37). Differences between Ym1 and AMCase may also reflect the cell types that express AMCase, which have not yet been determined. We did not observe AMCase expression in NeMϕ or in lymph nodes, suggesting that the expression profile of this gene may be significantly different from that of Ym1, which seems to be expressed by cells of the immune system (12, 18, 24, 51). AMCase may be expressed by resident cells in the lung for an immediate defense response to injury, while Ym1 may be expressed by cells recruited to the inflammatory site.

During infection with *N. brasiliensis*, we also observed Ym1 induction as early as 24 h in the lungs and as late as 15 days in both the lungs and small intestine, by which time the parasite had been expelled (our unpublished observation). The postulated function of Ym1 in mediating extracellular matrix deposition and tissue repair (25, 50) may explain Ym1 expression several days after the expulsion of the parasite, where it may be required for wound healing. However, in pulmonary diseases, these same properties may cause pathology. In a model of Th2-polarized pulmonary inflammation, the induction of Ym1 and AMCase was correlated with increased granuloma formation and fibrosis (41). Ym1 has also been reported to crystallize spontaneously in pulmonary disease models (17, 18). Interestingly, we did not observe crystal structures in the macrophages recruited, the peritoneal exudates, or thoracic lavage fluid in the *B. malayi* and *L. sigmodontis* infection models. The crystallization of Ym1 may be site specific and may occur only in dysregulated Th2 pulmonary inflammation as is seen in mice which overexpress IL-4 or IL-13 in the lungs (13; our unpublished observation) and in the lungs of a schistosome vaccination model (our unpublished observation) and in various immunodeficient mouse models (17, 18).

In a strikingly similar scenario, the proteins Fizz1 and Fizz2 were also induced in response to nematode infection. One reported function of Fizz1 is the inhibition of NGF (22). NGF and its receptor are expressed by cells associated with Th2-mediated inflammation including eosinophils, mast cells, and Th2 cells themselves (5). In allergic lung inflammation, NGF appears to both augment the Th2 response and enhance airway smooth muscle contractility leading to airway hyperresponsiveness (8). Fizz1 may thus be a critical regulator of pulmonary inflammation through effects on Th2 responsiveness as well as by modulating sensory nerve stimulation. In an analogous manner, the induction of Fizz2 in the small intestine may influence the tissue response to nematode infection. Recently, Zhao et al. reported that IL-4/IL-13 enhanced smooth muscle responsiveness to enteric nerve stimulation, which likely contributes to the IL-4/IL-13-dependent expulsion of gastrointestinal nematode parasites (52). Together with our observation that Fizz2 is induced in the small intestine of *N. brasiliensis*-infected mice and the known regulation of this gene by IL-4/IL-13, these properties may implicate Fizz2 in the expulsion of the parasite through influences on enteric nerve stimulation. Fizz2 induction in response to bacterial colonization of the gastrointestinal tract has also been reported previously (19), suggesting a broader function in the defense against gut pathogens.

In contrast to AMCase and Fizz2, which were induced uniquely at the sites of infection and were not expressed by NeMϕ or the lymph node cells, Ym1 and Fizz1 were expressed in the draining lymph nodes, suggesting that they may have additional immunomodulatory functions. Macrophages were the cell type with the highest expression of Ym1/Fizz1 by far. As macrophages are effector cells that are recruited to the site of infection, high levels of these ChaFFs may be necessary in this setting. The lymph node, on the other hand, has a highly organized structure, where the interactions between B cells and DC with T cells are favored by their close proximity; dendritic cells will present antigen and activate naïve T cells, whereas cross talk between B and T cells will occur in the germinal centers. In this case, lower levels of protein may be needed. Although the expression profiles of Ym1 and Fizz1 are remarkably similar, their functions are likely to be distinct. Based on the ability of Ym1 to bind glycosaminoglycans and other extracellular matrix molecules, its function in the lymph nodes may be to mediate APC-T-cell interactions. Fizz1, on the other hand, may have a more regulatory role. Previous studies on this protein have focused on its expression at the sites of infection or inflammation, where it may control local tissue responses (22). In the lymph nodes, Fizz1 could have an autocrine function on the APCs and/or could act on T cells. This may occur through inhibitory effects on NGF, which is known to be involved in the development and differentiation of immune cells, particularly B cells (5). We have previously shown that NeMϕ can effectively drive Th2 differentiation (30), and it is possible that Fizz1 is involved in this T-cell polarization. As dendritic cells were the lowest-expressing APC, Fizz1 may not have an essential role in influencing the

initial activation of naïve T cells but may function at a later stage to influence fully activated effector T cells.

The abundant production of ChaFFs appears to be a programmed response to nematode infection and as such is likely to have significant consequences for the host. Although AMCase, Ym1, Fizz1, and Fizz2 are all expressed at the site of infection, Ym1 and Fizz1 are additionally induced in the APCs of the draining LN. This implies a broader function for these two proteins and suggests that they function at multiple levels during a Th2-mediated immune response.

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