

Transcriptomic Analysis of the Host Response to *Giardia duodenalis* Infection Reveals Redundant Mechanisms for Parasite Control

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ABSTRACT The immune system has numerous mechanisms that it can use to combat pathogens and eliminate infections. Nevertheless, studies of immune responses often focus on single pathways required for protective responses. We applied microarray analysis of RNA in order to investigate the types of immune responses produced against infection with the intestinal pathogen *Giardia duodenalis*. Infection with *G. duodenalis* is one of the most common causes of diarrheal disease in the world. While several potential antiparasitic effector mechanisms, including complement lysis, nitric oxide (NO), and α -defensin peptides, have been shown to inhibit parasite growth or kill *Giardia in vitro*, studies *in vivo* have thus far shown clear roles only for antibody and mast cell responses in parasite control. A total of 96 transcripts were identified as being upregulated or repressed more than 2-fold in the small intestine 10 days following infection. Microarray data were validated using quantitative PCR. The most abundant category of transcripts was antibody genes, while the most highly induced transcripts were all mast cell proteases. Among the other induced transcripts was matrix metalloprotease 7 (Mmp7), the protease responsible for production of mature α -defensins in mice. While infections in Mmp7-deficient mice showed only a small increase in parasite numbers, combined genetic deletion of Mmp7 and inducible nitric oxide synthase (iNOS, Nos2) or pharmacological blockade of iNOS in Mmp7-deficient mice resulted in significant increases in parasite loads following infection. Thus, α -defensins and NO are redundant mechanisms for control of *Giardia* infections *in vivo*.

IMPORTANCE The immune system has multiple weapons which it uses to help control infections. Many infections result in activation of several of these response mechanisms, but it is not always clear which responses actually contribute to control of the pathogen and which are bystander effects. This study used the intestinal parasite *Giardia duodenalis* to examine the redundancy in immune responses during infections in mice. Our results showed that at least four distinct mechanisms are activated following infections. Furthermore, by blocking two pathways at the same time, we showed that both mechanisms contribute to control of the infection, whereas blocking single responses showed no or minimal effect in these cases.

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Giardia duodenalis is one of the most common protozoan infections of humans, as well as other mammals throughout the world, and is a leading cause of diarrheal disease in these species (1–3). Symptomatic infections occur in about 20 to 80% of humans with positive stool samples and are characterized by nausea, vomiting, epigastric pains, and diarrhea (1, 2, 4, 5). These symptoms are associated with nutrient malabsorption and can lead to weight loss and malnutrition in children, exposing this vulnerable group to failure to thrive and developmental problems (6, 7). Disease resolves spontaneously in most cases, although the acute phase of the disease can develop into chronic disease in spite of a healthy immune system (8). In these cases, symptoms of the disease will reappear for short and recurrent periods (2, 3, 9). The mechanisms explaining interactions between the host and the parasite leading to parasite clearance and disease pathogenesis are poorly understood.

Data from humans suggest that antibody responses are important in preventing chronic infections, although roles for cellular responses have not been excluded (1, 7). Available data from mouse models of *G. duodenalis* infection describe cell-mediated mechanisms of parasite control, especially early during infections, though the precise nature of these is still unknown (7). Secretory IgA responses are also important in mice, especially late during the infections (10–13). It is now also known that mast cells play a significant role in clearing this infection and that these cells also contribute to abnormal motility in infected animals (7). Nevertheless, mice lacking antibodies can rapidly eliminate *G. duodenalis* infections (14), and the additional effector mechanisms leading to parasite elimination remain to be defined.

In an effort to better characterize the initial host-parasite interaction, Roxström-Lindquist et al. successfully used transcriptomics in an *in vitro* setting to show induction of several mediators,

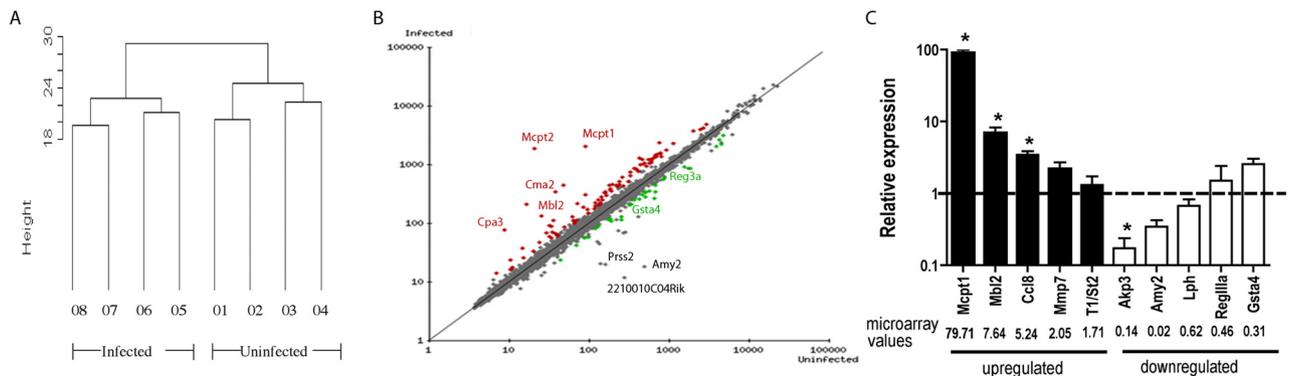


FIG 1 Microarray analysis of *Giardia* infection in C57BL/6 mice. Total RNA was isolated from four uninfected C57BL/6J mice and from four mice 10 days after infection with *G. lamblia*. Probes generated from each mouse were hybridized to Affymetrix gene chips. A cluster dendrogram (A) was produced using Affymetrix software, and samples were grouped together as coming from either uninfected or infected C57BL/6J mice. The scale on the left vertical bar indicates the Manhattan distance. Data were then processed using the RMA algorithm with the GeneSpring software program and displayed as a scatterplot (B). Red points correspond to genes significantly upregulated following infection, while the green dots correspond to significantly downregulated genes. Spots with the greatest changes in expression level are identified (mast cell protease 1 [Mcpt1], mast cell protease 2 [Mcpt2], mast cell carboxypeptidase A [Cpa3], mast cell chymase 2 [Cma2], mannose binding lectin C [Mbl2], trypsin 2 [Prss2], trypsin 1 [2210010C04Rik], glutathione S-transferase [Gsta4], islet regenerating protein III alpha [Reg3a], and pancreatic amylase [Amy2]). A threshold value ≥ 1.5 -fold change in expression following infection with *G. lamblia* and an adjusted *P* value cutoff of 0.05 by analysis of variance (ANOVA) were used to control the false discovery rate (4). Note that while changes in Amy2, Prss2, and 2210010C04Rik are not statistically significant using RMA preprocessing as shown, they were identified as significantly regulated following CHP and/or GC-RMA preprocessing (see Table S1 in the supplemental material). Real-time quantitative PCR was used to validate microarray results for expression of Mcpt1, Amy2, Mbl2, Gsta4, Reg3a, chemokine (C-C motif) ligand 8 (Ccl8), interleukin 1 receptor like 1 (T1/ST2; the IL-33 receptor), matrix metalloproteinase 7 (Mmp7), lactase phlorizin hydrolase (Lph), and intestinal alkaline phosphatase 3 (Akp3) (C). Transcripts whose expression was induced by infection based on microarray analysis (filled bars) or repressed (open bars) were examined in an additional four infected and four uninfected mice. The fold change as measured on the microarray using GC-RMA preprocessing is indicated for each gene. The fold differences in gene expression (\pm SD) relative to those for uninfected mice (dashed line) are shown. *, *P* < 0.05.

such as the chemokine CCL20, following *Giardia* interaction with a human colon carcinoma cell line (15). We also recently described the ability of *Giardia* extracts to induce a limited dendritic cell response *in vitro* using bone marrow-derived dendritic cells (16). While valuable, these studies were both performed *in vitro* and utilized a single cell type at a time.

To further understand host defense mechanisms and to identify effector mechanisms responsible for the control of *G. duodenalis*, we chose to examine the transcriptional response of whole small intestinal tissue following *Giardia* infection using oligonucleotide arrays. Transcripts identified as being induced or repressed more than 2-fold in infected mice were then analyzed using quantitative PCR (QPCR). While we identified numerous transcripts from B cells and mast cells, as would be expected based on previous reports, we also identified matrix metalloproteinase 7 (Mmp7) as being induced following infection. The role of Mmp7 was then confirmed *in vivo* in conjunction with Nos2 using pharmacological inhibitors of Nos2 in Mmp7-deficient mice and by breeding mice deficient in both Mmp7 and Nos2.

RESULTS

In this study, we analyzed gene expression in the duodenum of C57BL/6J mice infected with *G. duodenalis* [strain GS(M)H7] and compared it to that for uninfected mice. Samples were collected 10 days postinfection, when we expected mechanisms contributing to the elimination of the parasite to be most apparent. RNA from 1-cm segments of intestine from each mouse (*n* = 4/group) was isolated, labeled, and hybridized onto microarrays each containing 12,488 probe sets. The eight samples were correctly segregated into infected and uninfected samples by the image capture

software based solely on the signal intensity and distribution of transcripts (Fig. 1A).

Transcriptome data analysis following infection. Quantitative data from the microarray were analyzed using two different preprocessing algorithms in an effort to focus efforts on those transcripts whose expression differed consistently from baseline levels following infection. A total of 7,296 (out of 12,488) genes had a present or marginal signal from which we established a quality control (QC) gene list with which further analyses were carried out. The QC gene list data were normalized, and a *t* test was performed that identified 96 transcripts as being either induced or repressed by more than 50% (Fig. 1B; see also Fig. S1 in the supplemental material). We manually assigned each of these transcripts to functional categories (Table 1; see also Table S1). We also subjected all 96 genes to Gene Ontology (GO) term analysis (see Table S2).

Of the 96 genes detected, 71 (74%) of the genes were upregulated while 25 (26%) genes were notably downregulated. Gene Ontology (GO) term analysis identified pathways associated with immune function and protease activity (see Table S2 and Fig. S2 in the supplemental material). However, we found it more informative to manually assign transcripts to functional categories (Table 1). For example, the most common category of upregulated probe sets was B cell transcripts. Indeed, 42% of all identified transcripts were B cell transcripts. Most of these transcripts corresponded to various immunoglobulin heavy- and light-chain variable gene segments which are not assigned to GO terms. The Igh-6 (IgM) and Igh-4 (IgG1) constant region genes and CD79b, the Ig β signaling component of membrane immunoglobulin, were also identified (see Table S1). The most strongly induced transcripts

TABLE 1 Summary of regulated transcript families identified by microarray analysis

Gene family ^a	No. (%) of transcripts identified by algorithm(s)					
	CHP and GC-RMA ^b	GC-RMA ^c	CHP ^c	GC-RMA only ^d	CHP only ^d	All detected genes
Mast cell	5 (9)	5 (8)	5 (6)	0 (0)	0 (0)	5 (5)
B cell	29 (54)	37 (56)	32 (50)	8 (67)	3 (13)	40 (42)
Immune	6 (11)	6 (9)	7 (8)	0 (0)	1 (3)	7 (8)
Paneth cell	4 (7)	4 (6)	4 (5)	0 (0)	0 (0)	4 (3)
Glutathione	1 (2)	1 (2)	3 (4)	0 (0)	2 (7)	3 (4)
Enterocyte	2 (4)	4 (6)	2 (2)	2 (17)	0 (0)	4 (4)
Digestive	2 (4)	2 (3)	6 (7)	0 (0)	4 (10)	6 (4)
Other	5 (9)	7 (11)	25 (30)	2 (17)	20 (63)	27 (28)
Total	54	66	84	12	30	96

^a Gene family identification was assigned manually.

^b CHP and GC-RMA refers to genes identified using both algorithms.

^c Refers to genes identified with this algorithm, whether or not they were also identified using the other algorithm.

^d "Only" refers to genes identified only with this algorithm.

were all mast cell genes. Mast cell protease 1 (Mcpt1), Mcpt2, mast cell carboxypeptidase A3 (Cpa3), mast cell chymase 2 (Cma2), and Fcε receptor transcripts showed an average fold induction of 54 (range, 14 to 114). Nine transcripts which we classified as other immune functions were also upregulated, as were three Paneth cell genes. Four enterocyte transcripts showed downregulation, as did five transcripts identified as encoding digestive enzymes. Twenty-seven transcripts were classified as "other" and represented a heterogeneous group, including transcripts with no clearly defined function and several transcription factors. Given that *Giardia* infection is known to induce strong antibody and mast cell responses, this pattern of gene induction suggested that the microarray approach was a reliable way to identify changes in gene expression following infection.

Analysis of the microarray data using the preprocessing algorithm GC-content Robust Multi-Array (GC-RMA) yielded 66 genes, while the CHP preprocessing algorithm identified 84. Fifty-four transcripts were identified as differentially expressed by both the CHP and GC-RMA algorithms (see Fig. S1 and Table S1 in the supplemental material). Interestingly, focusing on this overlapping set of 54 transcripts eliminated almost all of the transcripts assigned as "other" (Table 1; see also Table S1).

To validate the microarray results, we performed quantitative real-time PCR (QPCR) with 10 transcripts (Fig. 1C). Five were identified as induced on the microarray, and four were identified as repressed. We also analyzed expression of lactase-phlorizin hydrolase (Lph) since reduced lactase activity is a hallmark of *Giardia* pathogenesis in humans and in animal models. Lph expression was not downregulated more than 50% according to microarray results, and we wanted to determine if genes whose expression was affected by infection were missed in our analysis. Other genes selected for analysis included both strongly and weakly affected transcripts. For example, Mcpt1 was among the most strongly induced genes based on the microarray (80-fold), while T1/ST2 was only weakly induced (1.7-fold). As seen in Fig. 1C, QPCR confirmed microarray results for most but not all of the chosen transcripts. Mcpt1 transcripts increased 90-fold by QPCR ($P < 0.0003$), compared to an 80-fold upregulation on the microarray. Mannose-binding lectin (Mbl2) transcripts were 6.95-fold increased by QPCR ($P < 0.013$), compared to a 7.64-fold increase on the array. Matrix metalloproteinase 7 (Mmp7) showed a 2-fold upregulation by QPCR ($P < 0.06$), with a very similar upregulation of transcripts on the array (2-fold). Alkaline phosphatase 3

(Akp3) was downregulated 7.1-fold on the microarray and was 6-fold downregulated by QPCR ($P < 0.0001$). Though downregulated on the array, islet regenerating protein 3α (Reg3a) and glutathione *S*-transferase α4 (Gsta4) were upregulated according to the QPCR (1.5-fold and 2.5-fold, respectively), although in a statistically insignificant manner. The microarray data indicated a 38% reduction in Lph transcripts. Because we demanded a 2-fold change in gene expression, this did not appear in our list of affected genes. QPCR confirmed a 34% reduction in Lph mRNA, although this was not statistically significant. Interestingly, the reduction in disaccharidase activity reported in infected humans and mice is also typically in the 30% range (17).

We have previously reported that tumor necrosis factor (TNF)-deficient mice have a defect in clearing parasites following *Giardia* infection (18) (Fig. 2A). Parasite clearance did not seem to be delayed because of defects in previously described mechanisms, such as mast cells, IgA, or interleukin 6 (IL-6) (18). Since TNF did not appear to be required for the mechanisms previously implicated in controlling *Giardia* infections, we analyzed in TNF-deficient mice expression levels of several genes identified by microarray analysis of infections in wild-type mice (Fig. 2). Like their wild-type counterparts, TNF-deficient mice had a very robust induction of Mcpt1, consistent with the mast cells observed histologically (18). Interestingly, while wild-type mice showed significant induction of two genes involved in innate immune responses, Mbl2 and Mmp7, the TNF-deficient mice failed to induce Mbl2 transcripts (although they were initially higher in TNF-deficient mice than in wild-type mice) and also had lower levels of Mmp7 transcripts than wild-type mice at all times examined (Fig. 2C and 2D). Gsta4 and Reg3a also showed higher transcript levels in wild-type mice than in TNF-deficient mice at day 12 postinfection. Interestingly, Akp3 and Lph were both repressed after infection in wild-type mice but actually showed higher levels of expression in TNF-deficient mice. T1/ST2 mRNA levels were greater in uninfected TNF-deficient mice than in wild-type mice, although no differences were seen postinfection.

Numerous mechanisms have been proposed to contribute to elimination of *Giardia duodenalis* infection. For example, α-defensins are able to kill trophozoites *in vitro*, although their role *in vivo* has not been determined (19). *Mmp7* encodes the protease responsible for conversion of α-defensin propeptides into active antimicrobial peptides (20). Since Mmp7 was induced following infection, we decided to determine if Mmp7-deficient

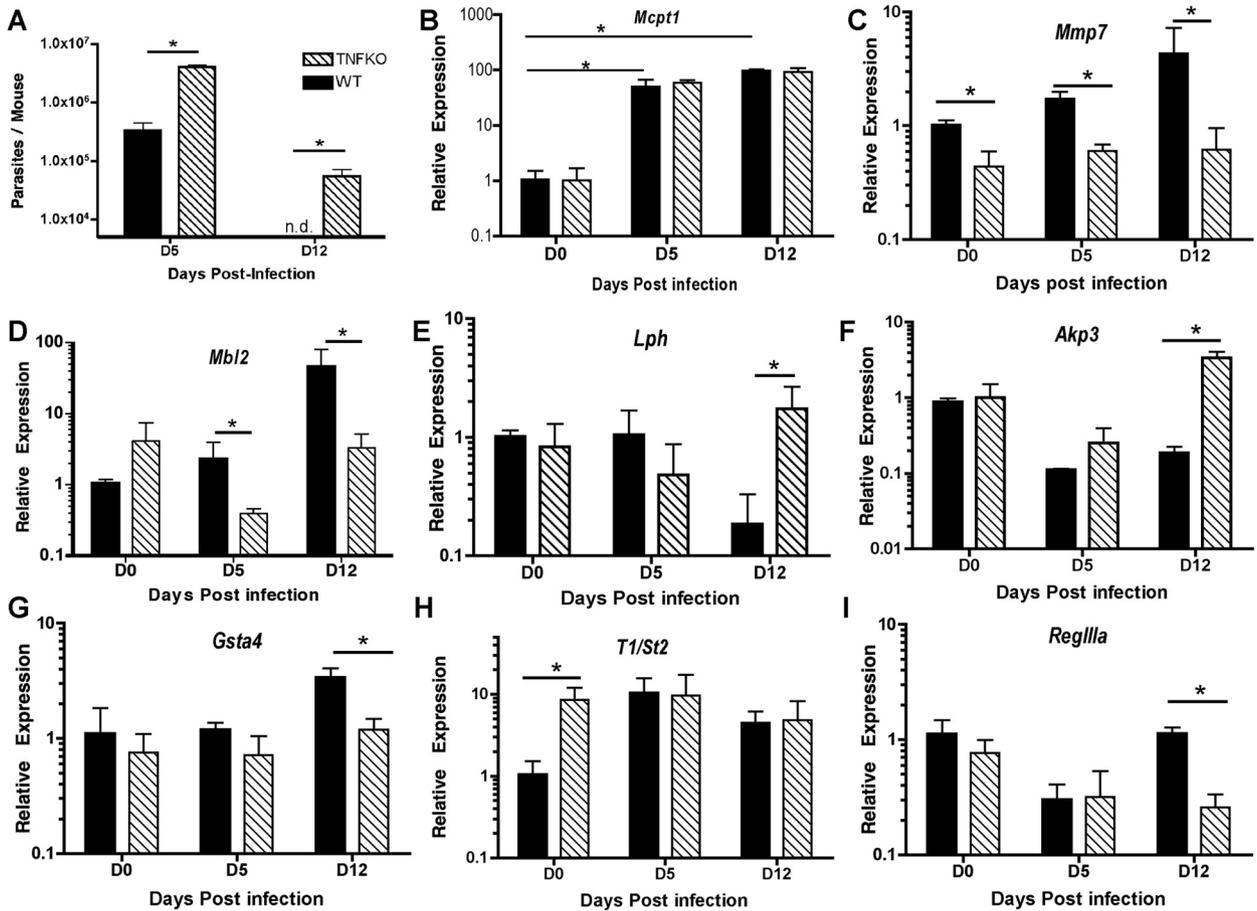


FIG 2 Relative mRNA expression levels following infection in TNF-deficient mice. Wild-type B6X129 F2 mice (solid bars) and TNF gene-targeted mice on a mixed C57BL/6 and 129 background (hatched bars) were infected with *G. lamblia* as described in Materials and Methods. (A) Parasite numbers in the small intestine were determined 5 and 12 days postinfection ($n = 4/\text{group}$). n.d., not detected. (B to I) Intestinal mRNA levels for mice analyzed in panel A and uninfected controls were analyzed by real-time RT-PCR. PCR was repeated between two and four times per mouse per gene. Error bars represent the range of fold difference in gene expression relative to that for uninfected wild-type mice. *, $P < 0.05$.

mice had a defect in control of *G. duodenalis* infections. Eckmann had previously published that *Mmp7*-deficient mice controlled *G. muris* infections at least as well as wild-type mice (4), and pre-

liminary experiments found small differences in the *G. duodenalis* infection model (Fig. 3 and data not shown). We have previously reported that nitric oxide (NO) produced by inducible nitric ox-

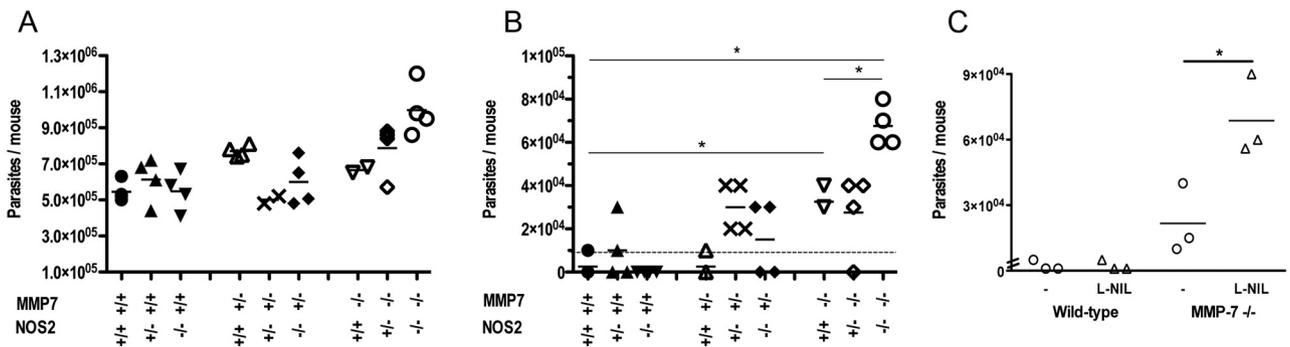


FIG 3 Infections in mice lacking *Mmp7* and *Nos2*. F2 offspring were genotyped for *Mmp7* and *Nos2* and infected on day 0 with *G. duodenalis*. Mice were euthanized on day 5 (A) or day 13 (B), and trophozoites in the small intestine were quantified. Each symbol represents an individual mouse. Medians were compared using a two-tailed Mann-Whitney test, since most groups included mice with no detectable parasites. (C) Wild-type and *Mmp7*-deficient mice were infected on day 0 and treated on days 2, 4, 6, and 8 with 10 mg/kg L-NIL to inhibit NO production by *Nos2*. All mice were euthanized on day 10, and trophozoites in the small intestine were enumerated. Each symbol represents an individual mouse. Means were compared using a two-tailed t test. *, $P < 0.05$.

ide synthase (iNOS), another antimicrobial mechanism able to inhibit parasite replication *in vitro*, was not necessary for parasite control *in vivo* (21). We therefore wondered if perhaps these mechanisms acted in a redundant fashion. To test this hypothesis, we intercrossed Nos2- and Mmp7-deficient mice and infected and genotyped animals from the F2 generation. Figure 3 shows that offspring which were homozygous for deletion of the Mmp7 gene had a defect in elimination of the parasite. Regardless of genotype, all mice examined on day 5 were infected with *Giardia* (Fig. 3A). At day 13, in contrast, wild-type mice had mostly reduced parasite loads below the limit of detection, whereas almost all Mmp7-deficient mice still had parasites in the small intestine (Fig. 3B). Mice lacking both Mmp7 and Nos2 had more parasites than mice lacking Mmp7 alone (Fig. 3B). To confirm these data, we also infected Mmp7 single-knockout mice and treated half the mice with the Nos2-specific inhibitor N-iminoethyl-L-Lysine (L-NIL; ref. 21). Again, more parasites were seen in Mmp7 knockout mice than in wild-type mice treated with saline controls (Fig. 3C). Inhibition of Nos2 in wild-type mice had no effect on parasite loads, as previously reported. Treatment of Mmp7 knockout mice with L-NIL, however, increased parasite loads from an average of 30,000/mouse to more than 70,000. Together these data suggest that both α -defensins and nitric oxide produced by Nos2 have a role in eliminating *Giardia duodenalis* infections but that α -defensins can compensate for the absence of NO.

DISCUSSION

The immune mechanisms responsible for elimination of *Giardia* from the infected host and those that may contribute to pathology during infection have not been clearly defined. By applying microarray technology to *Giardia* infection, we have identified 96 transcripts whose expression was either increased or decreased by more than 2-fold. These transcripts could be grouped into several functional categories and confirmed the importance of previously identified pathways known to be important in responses to *Giardia*, including B cells and mast cells. Several new pathways which have not yet been explored for this disease were also identified. For example, the mannose binding lectin (Mbl2) gene was induced following infection. MBL has recently been shown to bind trophozoites and induce parasite lysis *in vitro* (22). Our analyses suggest that MBL also plays a role in controlling infections *in vivo* (E. Tako, E. Li, and S. M. Singer, unpublished data). In this study, we further analyzed the role of matrix metalloprotease 7 (Mmp7) during *G. duodenalis* infections in mice and showed that this protease acts redundantly with inducible nitric oxide synthase to contribute to control of infections.

IgA and mast cells. The most obvious result of microarray analysis was that numerous genes from B cells and mast cells were induced following infection. B cell transcripts were the largest category and included both variable gene segments and the constant region genes for IgM (*Igh-6*) and IgG1 (*Igh-4*). Strong antibody responses against *Giardia* are well documented in humans and animal infections, although their exact role in parasite control has been debated (23). In contrast, while only five transcripts clearly associated with mast cells were identified, these represented the five transcripts with the largest induction. For example, mast cell protease 1 (Mcp1) transcripts were 86-fold more abundant at 10 days postinfection, consistent with our previous report that serum levels of this protease are significantly higher in infected mice (24). Mice genetically deficient in mast cells or treated with

anti-c-kit antibodies to block mast cell responses exhibit delayed parasite elimination, defining an important role for these cells in parasite immunity (24). Together these results indicate that this microarray screen successfully identified pathways important for control of giardiasis.

Malabsorption. A number of transcripts whose expression was significantly reduced following infection were identified. Many of these were from genes important for enterocyte function and nutrient absorption. These included genes for pancreatic amylase (*Amy2*), whose transcripts were found at only 1 to 2% of normal levels following infection, along with intestinal alkaline phosphatase (*Akp3*), trypsin (*Try4*), and chymotrypsinogen B (*Ctrb1*). The diarrhea in giardiasis is known to be malabsorptive, and reduced nutrient uptake can lead to defects in physical and cognitive development in children (7, 25). However, many of the enzymes whose expression is reduced are thought to be expressed at more significant levels in the liver or pancreas. It is therefore unclear what role reduced intestinal expression of these genes might have in this process. In contrast, while decreased lactase activity is a hallmark of giardiasis, lactase phlorizin hydrolase (*Lph*) gene expression was reduced only ~30% at day 10 postinfection. The magnitude of this reduction is consistent with activity levels in patients. Interestingly, when comparing the effects of infection in wild-type and TNF-deficient mice, we noted that reduced expression of *Akp3* and *Lph* was not seen in the absence of TNF, suggesting an immune etiology for nutrient malabsorption, consistent with adoptive transfer studies performed by Scott et al. (26).

Other transcripts. This microarray screen identified many transcripts that did not fit easily into these major categories and that could be of significant interest for further study. For example, three transcripts associated with glutathione and cellular redox regulation were identified, suggesting that infection and/or the immune response against it may produce oxidative stress for the epithelial cells. One potential pathway is the induction of nitric oxide production through Nos2. We have previously shown that Nos2 levels increase *in vivo* following infection, although Nos2-deficient mice had no defect in eliminating the parasites (21, 27). Glutathione can help protect cells from damage by NO produced in this manner. Interestingly, Nos2 was not detected as being increased in this analysis. It is likely that the small sample size ($n = 4$ per group) resulted in our failing to identify additional transcripts that are regulated following *Giardia* infection.

Matrix metalloprotease 7. We also identified the transcript Mmp7 on our microarray and confirmed by PCR that it was induced ~2-fold following infection. This gene encodes a protease required for conversion of α -defensin precursors expressed in the crypts to active antimicrobial peptides (20). While some α -defensin peptides have been shown to lyse *Giardia duodenalis* trophozoites *in vitro* (19), mice lacking Mmp7 were shown to control *G. muris* infections as well as wild-type mice (4). In contrast, Mmp7-deficient mice infected with *G. duodenalis* exhibited slightly elevated parasite numbers in the small intestine compared with control mice. These differences were statistically significant in our experiments. Interestingly, concurrent elimination of Nos2, either by genetic deletion or by pharmacological inhibition, resulted in significant increases in the numbers of trophozoites recovered from infected mice compared to results with Mmp7 deletion alone. This suggests that nitric oxide (from Nos2) and α -defensins (from Mmp7) may act redundantly to help reduce parasite numbers following infection. Elimination of either anti-

microbial has a minimal effect, while elimination of both at the same time produces a more clear result. In addition to roles for IgA and mast cells, this indicates at least four mechanisms contributing to elimination of intestinal infections with *Giardia*.

Giardia trophozoites attach to the surface of intestinal epithelial cells, and it is thought that attachment helps prevent their elimination due to the normal forces moving intestinal contents downstream. We have previously shown that infection leads to an increase in the force of intestinal contractions, resulting in an increased rate of transit of intestinal contents (28). Antimicrobials like IgA, NO, and α -defensins likely act in and beneath the mucus layers of the intestinal tract, while mast cell-dependent hypermotility will be more effective at removing trophozoites detached from the epithelial surface and in the intestinal lumen. Our data support significant redundancy in the mechanisms used by the immune system to clear this intestinal parasite.

Recent data from the Global Enteric Multicenter Study suggest that infection with *G. duodenalis* in children is actually associated with a small reduced risk of developing severe and life-threatening diarrhea (29, 30). *G. duodenalis* infection is known to produce nutrient malabsorption and intestinal cramping but is not typically associated with bloody stools or the severe dehydration used to define severe diarrhea in this study. The ability of *Giardia* to enhance the production of antimicrobial mechanisms, such as NO and α -defensins, may reduce the ability of other pathogens to colonize and/or cause disease. For example, both NO and α -defensins have been shown to help reduce colonization by *Salmonella* (31, 32).

MATERIALS AND METHODS

Mice, parasites, and infections. C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME). In addition, we also purchased a B6.129S4-*Mbl1^{tm1Kata} Mbl2^{tm1Kata}/J* breeding pair from Jackson Laboratories (Bar Harbor, ME) for breeding at the Georgetown University Division of Comparative Medicine. All animals were kept under specific-pathogen-free conditions. All experiments were performed with the approval of the Georgetown University Animal Care and Use Committee. *G. duodenalis* strain GS(M)H7 was cultured to confluence and used for infections as previously described (33). Briefly, 5- to 6-week-old female mice ($n = 4$ /group) were infected by gavage with 1×10^6 parasites, each in 0.1 ml phosphate-buffered saline (PBS). Parasites were counted by collecting intestinal segments from 10 cm of the distal duodenum and proximal jejunum just below the ligament of Trietz. This is where maximal parasite growth occurs due to the presence of bile from the common bile duct. Collected fragments were minced in 4 ml of cold PBS and then kept on ice for 30 min for parasite release before counting on a hemocytometer. Differences between the median number of parasites per group were compared using Mann-Whitney tests.

RNA preparation and gene expression profiling by using Affymetrix GeneChip microarrays. Total RNA was prepared from a 1- to 2-cm segment of the small intestine from each mouse. RNA was purified by using the RNA Stat-60 reagent (Tel-test, Inc., Midland, TX). RNA was further cleaned using the Qiagen Rneasy kit (Qiagen, Hilden, Germany). RNA quality was assessed using spectrophotometric analysis (NanoDrop Technologies, Wilmington, DE) and electrophoresis prior to cDNA synthesis. Five to ten micrograms of total RNA from each sample was used to generate double-stranded cDNA using a T7 oligo(dT) primer; 1 μ g of this product was then used in an *in vitro* transcription reaction in the presence of biotinylated deoxynucleoside triphosphates (dNTPs) using a transcript labeling kit to yield 50 to 100 μ g of biotin-labeled cRNA target. The resulting 20 μ g of full-length cRNA was subjected to metal-catalyzed acid hydrolysis to a length of 20 to 200 bp, and the fragmented cRNA was hybridized to the Affymetrix array and subjected to gene expression anal-

ysis on an Affymetrix MG_UV74Av2 high-density oligonucleotide array representing more than 12,000 mouse genes. Hybridization, staining, and washing of all arrays were performed at the microarray core facility of the Lombardi Comprehensive Cancer Center, Georgetown University. A streptavidin-phycoerythrin stain (Molecular Probes, Eugene, OR) was used to detect hybridized target sequences. The detection and quantitation of target hybridization were performed with a GeneArray scanner 3000 set to the manufacturer's defaults (Affymetrix, Santa Clara, CA).

Microarray data handling and analysis. Affymetrix MicroArray Suite software, version 5.0 (Affymetrix, Santa Clara, CA), was used to preprocess the data files. The resulting CHP files were imported into the software program GeneSpring GX, version 7.3.1 (Agilent Technologies, Santa Clara, CA). In addition, Affymetrix CEL files were imported into GeneSpring and preprocessed using the GC-RMA algorithm. Two separate experiments were created (CHP and GC-RMA). For each experiment, each gene was normalized to its median expression in the control samples. Gene-level quality control was applied to the normalized CHP experiment. The original gene list was filtered to a QC gene list in which each gene had a "present" or "marginal" flag value in at least 2 of the 8 chips. Gene Ontology analysis was performed using the software program GOS-tat (34).

QPCR. Quantitative real-time reverse transcription (RT)-PCR (QPCR) was carried out using Taqman chemistry with probes and primers (see Table S2 in the supplemental material) designed using the Beacon Designer 4.0 software program (Premier Biosoft International, Palo Alto, CA) for mast cell protease 1 (*Mcpt1*), lactase phlorizin hydrolase (*Lph*), pancreatic amylase isoenzyme 2 (*Amy2*), alkaline phosphatase 3 (*AKP3*), matrix metalloproteinase 7 (*Mmp7*), mannose-binding lectin C (*Mbl2*), chemokine (C-C motif) ligand 8 (*Ccl8*), glutathione S-transferase, alpha 4 (*Gsta4*), and islet regenerating protein III alpha (*RegIII α*). β -Actin primer and probe sequences were obtained from Overbergh et al. (35). Briefly, total RNA was isolated from 1- to 2-cm fragments of mouse intestine using RNA Stat-60 (Tel-test, Inc., Midland, TX). RNA isolation was followed by DNase treatment (Ambion, CA) to remove genomic DNA contamination. The RNA was then used for first-strand cDNA synthesis. Two micrograms of total RNA was primed with oligo(dT)₁₂₋₁₈ and reverse transcribed into cDNA (Superscript II; Invitrogen Corp., Carlsbad, CA) in a total volume of 25 μ l. Aliquots of cDNA target template were diluted serially and mixed with 200 nM of primers and 200 to 400 nM of Taqman probes (see Table S2 in the supplemental material). The reactions were carried out in a PCR master mix containing 2.5 mM MgCl₂, 10 mM dNTP, and 2.5 U Surestart *Taq* DNA polymerase (Stratagene, La Jolla, CA) in a total volume of 25 μ l. The PCR reactions were carried out in duplicate on an MX3000P cycler (Stratagene, La Jolla, CA). The two-step cycling program was set as follows: an initial step of denaturation at 95°C for 10 min, followed by 40 thermal cycles of denaturation (15 s at 95°C) and elongation (1 min at 60°C). The relative quantification of the target mRNAs was carried out using the comparative method according to the instruction manual. The mRNA expression levels for all samples were normalized to the levels for the beta-actin housekeeping gene.

Data filtering and statistical analysis For each microarray experiment, a Student *t* test with a false discovery rate (FDR) of 0.05 was applied to test the hypothesis that a gene's expression does not differ between infected and control mice. The resulting statistically significant gene list was further reduced to identify those genes that were differentially expressed at least 1.5-fold. Adjusted *P* values were also calculated using the Benjamini and Hochberg method (36). Statistical probability at *P* values of <0.05 was considered significant. Comparisons of parasite numbers between mouse groups were performed using the GraphPad Prism software program (GraphPad Software, San Diego, CA).

Microarray data accession number. Microarray data have been deposited in the Gene Expression Omnibus at NCBI with series accession no. GSE23372.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00660-13/-DCSupplemental>.

- Figure S1, TIF file, 0.7 MB.
- Figure S2, TIF file, 18.6 MB.
- Table S1, XLSX file, 0.1 MB.
- Table S2, XLSX file, 0.1 MB.

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