

Localized Multigene Expression Patterns Support an Evolving Th1/Th2-Like Paradigm in Response to Infections with *Toxoplasma gondii* and *Ascaris suum*

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Human infectious diseases have been studied in pigs because the two species have common microbial, parasitic, and zoonotic organisms, but there has been no systematic evaluation of cytokine gene expression in response to infectious agents in porcine species. In this study, pigs were inoculated with two clinically and economically important parasites, *Toxoplasma gondii* and *Ascaris suum*, and gene expression in 11 different tissues for 20 different swine Th1/Th2-related cytokines, cytokine receptors, and markers of immune activation were evaluated by real-time PCR. A generalized Th1-like pattern of gene expression was evident in pigs infected with *T. gondii*, along with an increased anti-inflammatory gene expression pattern during the recovery phase of the infection. In contrast, an elevated Th2-like pattern was expressed during the period of expulsion of *A. suum* fourth-stage larvae from the small intestine of pigs, along with low-level Th1-like and anti-inflammatory cytokine gene expression. Prototypical immune and physiological markers of infection were observed in bronchial alveolar lavage cells, small intestinal smooth muscle, and epithelial cells. This study validated the use of a robust quantitative gene expression assay to detect immune and inflammatory markers at multiple host tissue sites, enhanced the definition of two important swine diseases, and supported the use of swine as an experimental model for the study of immunity to infectious agents relevant to humans.

Although experimental protozoan and helminth infections of rodents have provided critical information on the Th1/Th2 response paradigm, these models lack critical evaluation of the responses in outbred host species and often do not involve natural host-parasite interactions that are relevant to humans. Furthermore, the phylogenetic distance from mice to humans tempers the extrapolation of results between these species. Pigs that are used as a model of infectious disease can address these limitations because of the similarity between their immune and physiological functions and those of humans. To test this assumption, pigs were infected with two important human zoonotic agents, *Toxoplasma gondii* and *Ascaris suum*, and their responses to the infections were evaluated. *T. gondii* is an intracellular protozoan parasite that causes severe, sometimes lethal disease in animals and humans (27). Felids are the definitive host; however, 300 mammalian species, including pigs, and 20 avian species have been identified as intermediate hosts

(16). Recent data indicate that about 25% of the United States population has detectable antibodies to *T. gondii*, whereas estimates of the exposure in other areas of the world range from 15 to 80% (27). Most humans that are exposed are asymptomatic, but immunocompromised and congenitally infected humans are at risk of developing severe disease, and acute infections in susceptible women during pregnancy cause birth defects and recurrent disease in children (27). Pigs, like humans, can acquire a transplacental *T. gondii* infection (19), but they are typically infected by ingestion of tissue-derived cysts or infective oocysts, which makes them a potential source of food-borne infection for humans. Most of what is known about the immune response to *T. gondii* infection has been derived from murine models; however, pigs also develop protective immunity (17). Infection of mice with *T. gondii* elicits a dominant Th1 response (63, 64); gamma interferon (IFN- γ), interleukin-12 (IL-12), and tumor necrosis factor alpha (TNF- α) are protective, whereas IL-18 and TNF- α contribute to pathology. Th2-associated cytokines, such as IL-4 and IL-10, appear relatively late after infection and may limit immune pathology (32). Most strains of mice are classified as sensitive to clinical toxoplasmosis, while humans and pigs are asymptomatic except when the infection levels are high or the host is immune deficient (10).

Ascaris lumbricoides is an extracellular gastrointestinal nem-

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atode parasite that affects more than one-quarter of humans worldwide, including an estimated 4 million people in the United States (12, 59). A closely related species, *Ascaris suum*, infects more than 50% of pigs raised for food globally, and infective eggs can be transmitted to humans (61). Migrating larvae produce focal liver lesions and eosinophilic pneumonitis in both humans and pigs (45). The development and persistence of a bolus of adult worms in the small intestine is associated with reduced growth and cognitive development in children and intestinal obstructions and aberrant worm migration that often require surgical interventions (13). Rodents do not support the development of the late larval and adult stages of *Ascaris* spp. in the intestine; these later stages are important in human morbidity and epidemiological studies. The protective immune response to *Ascaris* in pigs or humans is dominated by a Th2 response based on evidence of *Ascaris*-induced immunoglobulin E (IgE) production, peripheral eosinophilia, and increased ex vivo lymphocyte Th2 cytokine production (2, 9, 40). There is self-curing of fourth-stage larvae from the small intestine of swine between 2 and 3 weeks after inoculation that is associated with a localized mast cell-dependent immediate-type hypersensitivity response to parasite antigens (3, 44). Study of self-curing in humans is not feasible, but the relationship between high levels of *Ascaris* allergen-specific IgE antibodies in children and low adult worm reinfestation levels is supportive of a similar mechanism of clearance (40).

One limitation of pigs as a model of human infectious disease is the relative lack of defined immunological reagents. Competitive PCR assays have been developed for porcine IFN- γ (33), IL-10, IL-12p35, IL-12p40 (39), and IL-12R β 2 (51). These assays are accurate but time- and labor-intensive. The recent development of real-time fluorogenic PCR technology permits rapid, high-throughput analysis of gene expression. Real-time PCR assays have been described for a panel of bovine (8, 34), equine (35), human (24, 53), and murine (24) cytokines. To date, there have been few reports of studies in which real-time PCR was used to analyze the expression of cytokines in pigs (1, 11, 36, 52). The objective of the present study was to characterize the immunobiology of polarized cytokine responses to experimental infection with *A. suum* and *T. gondii* by using molecular, cellular, and physiological markers of immunity and inflammation. Our hypothesis is that *T. gondii* elicits a prototypical Th1-associated response and *A. suum* elicits a prototypical Th2-associated response that contributes to disease resolution and immunity to infection.

MATERIALS AND METHODS

Animal infection models. Eight- to 14-week-old Poland China X Landrace pigs were obtained from the experimental farm at the Beltsville Agricultural Research Center. They were housed two pigs per pen in stalls with a nonabsorbent concrete floor surface and had access to water and feed ad libitum. The diet was a corn-soybean formulation containing 16% crude protein and vitamins and minerals that exceeded National Research Council guidelines (57). Group I pigs ($n = 4$) were noninfected controls. Group II pigs ($n = 4$) were inoculated per os with 4.5×10^5 oocysts of the VEG strain of *T. gondii* (a type III genotype originally obtained from a patient expressing AIDS) (18, 51). The pigs were examined 7 days after inoculation (DAI) after an overdose of pentobarbital. Group III pigs ($n = 3$) were given the same inoculum and were examined 14 DAI. A second set of pigs was segregated into group A noninfected control pigs ($n = 4$) and group B pigs ($n = 4$) and group C pigs ($n = 4$) that were inoculated per os with infective *A. suum* eggs (57) and examined at 14 and 21 DAI, respectively. The inoculation dose of eggs was based on a test infection that

yielded between 1,500 and 2,000 migrating larvae in the lungs and small intestines of test pigs at 7 and 14 DAI, respectively (57). Both infections were replicated twice, and all procedures were conducted in accordance with approvals and recommendations made by the Beltsville Area Animal Care and Use Committee.

Tissue preparation. Whole blood was obtained by venipuncture in EDTA Vacutainers and mixed 1:2 with phosphate-buffered saline (PBS) (52). Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation by using 1.077d Histopaque (Sigma). The isolated cells were washed twice in PBS, resuspended in TriZol (Invitrogen, Gaithersburg, Md.), and vigorously mixed to lyse all of the cells. Solid tissues were excised with scalpels and forceps and cut into 1-mm cubes; all samples were immediately flash frozen and stored at -80°C until they were processed for RNA isolation.

RNA isolation, cDNA synthesis, and real-time PCR assays. All probes and primers were designed by using the Primer Express software (Applied Biosystems, Foster City, Calif.) with sequences obtained from GenBank or The Institute for Genome Research porcine expressed sequence tag database and were produced by Biosource (Camarillo, Calif.) (Table 1). Primers and fluorescent probes were designed across adjacent exons when possible; however, the intron-exon structures for most of the genes assayed have not been described, and it was presumed that the assays amplified genomic DNA. Therefore, extracted RNA was treated with DNase in the presence of an RNase inhibitor (11). RNA integrity and quantity and genomic DNA contamination were assessed by using an Agilent Bioanalyzer 2100 and an RNA 6000 Labchip kit (Agilent Technologies, Palo Alto, Calif.). cDNA was synthesized by using Superscript RT (Invitrogen) and oligo(dT) (11), and 100 ng of this cDNA per well was used for PCR amplification.

PCR was performed with a Brilliant quantitative PCR core reagent kit (Stratagene, La Jolla, Calif.) and an ABI PRISM 7700 sequence detector system (Applied Biosystems, Foster City, Calif.). The amplification conditions were as follows: 50°C for two min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. Fluorescence signals measured during amplification were processed postamplification and were considered positive if the fluorescence intensity was >20 -fold greater than the standard deviation of the baseline fluorescence. This level was defined as the C_t value. Assays were optimized as follows. The forward and reverse primer concentrations (900, 300 and 50 nM) were tested with a fixed concentration of probe to determine the primer set with the highest signal-to-noise intensity and lowest C_t . The probe concentration was then varied from 250 to 50 nM (in increments of 50 nM), and the concentration with the highest signal-to-noise intensity and lowest C_t was chosen. To determine the linearity and slope for each optimized assay, the cDNA template was serially diluted, and the C_t was measured. In order to assess the reproducibility of each assay, a pool of cDNA obtained from *A. suum*- and *T. gondii*-infected mesenteric lymph node (MLN) tissue was assayed on four consecutive days to establish the coefficient of variation. Because variation in the expression of housekeeping genes (L32, HPRT, EF1-a, and RPL13A) was observed during infection with *A. suum* and *T. gondii*, as well as in other parasite infection studies in which *Trichuris suis* and *Oesophagostomum dentatum* were used (data not shown), we normalized gene expression based upon the constant amount of RNA and cDNA amplified. This method has recently been proposed to be the most reliable means of standardization of quantitative measurements of mRNA expression by real-time PCR provided an accurate estimate of the total RNA can be made (e.g., by use of an Agilent Bioanalyzer) (5).

Real-time PCR assay characterization. The coefficient of variation of all assays was $<2\%$, with a linearity of >0.950 to >0.990 (data not shown). For genes such as the IL-12R β 2 gene, the range of the linear response was >4 logs. The slope of the C_t value line was used as the ordinate, and the value for the log of the input of RNA was used as the abscissa. This described the efficiency of the reaction in which a slope of -3.4 represented 100% efficient PCR; the slopes of the great majority of the assays were within ± 0.2 of 3.4 (data not shown).

Immune staining and phenotypic analysis of BAL cells. Bronchial alveolar lavage (BAL) was obtained from the lungs of pigs infected with either *A. suum* or *T. gondii*. Briefly, the large right lobe of a lung was gravity filled with 500 ml of PBS, and this was followed by massaging for 30 s and draining of the cell suspension into 50-ml polypropylene tubes. The cells were washed and resuspended in RPMI 1640 medium with 5% heat-inactivated fetal bovine serum, and cell counts and viability were determined after trypan blue staining. Aliquots of BAL cells were centrifuged onto glass slides, air dried, and stained with Giemsa stain after methanol fixation, and differential counting was performed with more than 100 cells.

The expression of surface antigen on immune cells isolated from BAL was determined by flow cytometry (52). The BAL cells were stained with specific monoclonal antibodies against porcine macrophages (SWC9-IgG1) (clone 18-5

TABLE 1. Primers and probes

Gene	Forward primer		Reverse primer		Probe		Accession no. ^b
	Concn (nM)	Sequence	Concn (nM)	Sequence (5' to 3')	Concn (nM)	Sequence (5' to 3') ^c	
IFN- α	300	TCAGCTGCAATGCCATCTG	300	AGGAGAGATTTCTCTCAITTTGTG	200	6FAM-TGACCTGCTCTAGACCCACAGCC-TAMRA	X57191
IFN- γ	300	TGGTAGCTCTGGGAACTGAAATG	300	GGCTTTGGCTGGATCTG	200	6FAM-CTTCGAAAAAGCTGATTAAAAATCCGGTAGAT AATCTGC-TAMRA	X53085
IL-2	300	ACAGTTGCTTTTGAAGAAAGTTAAGAA	300	CCTGCTTGGGCATGTAAAAATTT	150	6FAM-CGAGAAATGCTGATCTCTCCAGGATGCTC- TAMRA	X56750
IL-12p35	300	GCCCAGGAATGTTCAAATGC	300	GGGTTTGTTTGGCCTTCTGA	200	6FAM-CAACCCTCCAAAAATCTGCTGAAAGGC- TAMRA	L35765
IL-12 p40	300	AAGCTGTTTCAAGGCTCAAGTATGA	300	TCTTTGGGAGGGTCTGGTTTG	200	6FAM-ACCAGCAGCTTCTTTCATCAGGGACATCA- TAMRA	U08317
IL-12R β 2	300	GGCCAGGAAAGGGACAAAAG	300	CCCCAGCACCTTGTACAGATC	100	6FAM-AAAGTCCACCACCTCCAAAGGGCTCTCAC- TAMRA	AF448143
IL-15	300	GATGCCACATTTGTATATACTGAAAAGTGA	300	GCCTAACTCCAGGAGAAAAGCA	150	6FAM-CATCCCAATTTGCAAAAGTAAACAGCGATGA- TAMRA	U58142
IL-18	300	CGTGTTTGAGGATATGCCTGATT	300	TGGTTACTGCCAGACCTCTAGTGA	100	6FAM-TGACTGTTTTCAGATAAATGCACCTCAGACCGT- TAMRA	AF176949
IL-23	300	GCCTGTCTGGGCTCAA	300	GTAGATCCACATGTCCCATTGGT	200	TET-CACGCTGGCTGGACTGCACATC-BHQ	AB030002
iNOS	300	CGTTATGCCACCAACAATGG	300	AGACCCGGAAGTCTGTCTT	200	6FAM-CATCAGGTGCGCCATCCCGTG-TAMRA	U59390
STAT4	300	GAAAAACCTCTGAAGTACCCTCTATCCT	300	TCACATGGCTGGAGCTGTA	200	TET-TGCTGCTCCACTGAACAGGACT-BHQ	AB020984
TNF- α	300	TGGCCCTTGAAGCATCA	300	CGGGCTTATCTGAGTTTGAGA	150	6FAM-CCCTCTGGCCCAAGGACTCAGATCA-TAMRA	X54001
IL-4	300	GCCGGCCTCGACTGT	300	TCCGCTCAGGAGGCTCTTC	200	6FAM-CTTCGGCACATCTACAGACACCCACAG- TAMRA	X68330
IL-5	300	GACTGGTGGCAGAGACCTTGAC	300	CTTCAATGCATAGTTGGTGAATTTGT	200	6FAM-CTGTCTCTCCATTCATCGAACTGTGCTGAT- TAMRA	AJ010088
IL-10	300	TGAGAACAGCTGCATCCAGTTC	300	TCTGTCTCTTCTGTTTGAAGAAGAA	150	6FAM-CAACCAGCTGCCACACATGC-TAMRA	L20001
IL-13	50	CTGACCACCAAGATGCAGTACT	900	GCTGCAGTGGAGATGTTTGA	150	6FAM-TGCCGCTGGAATCCCTCA-TAMRA	AF385626
Mast cell tryptase	300	ACCGCCCTGGCATCTACAC	300	AGGAGGTGACTGGCTTTTGA	200	6FAM-CGCGTACCCCACTACTTGGACTGGAT- TAMRA	AB038652
STAT6	300	CCTGGGTTGGTGAAGACATGT	300	GCCCTCCAAGAGAAAGCTTAG	200	TET-TGCTGCTCCACTGAACAGGACT-BHQ	TC52764
TGF- β 1	300	AGGGTACCATGCCAATTTCT	300	CCGGTTGTGTGGTTGT	200	6FAM-CACTCAGTACAGCAAGGCTCTGGCTCTGTA- TAMRA	M23703
CD3e	300	ACCTGCACAGTCCGGAGAGAAG	300	CTCCACGCAGTTCTCACACACT	150	6FAM-CGAGCCATAGGCTCTACTGAAAAGCCA- TAMRA	U08270

^a 6FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine; TET, 6-carboxy-2',4,7'-tetrachlorofluorescein; BHQ, black hole quencher.

^b The STAT6 sequence was obtained from the Institute for Genome Research porcine EST database; all other sequences were obtained from the GenBank database.

provided by Y. B. Kim), porcine granulocytes (SWC3-IgG2b) (hybridoma 74-22-15), and isotype controls (Serotec, Raleigh, N.C.). An isotype-specific (Fab')₂ antibody labeled with fluorescein isothiocyanate or R-phycoerythrin (Southern Biotechnology, Birmingham, Ala.) was added. After 15 min on ice, the cells were washed and resuspended in 1% formaldehyde (Polysciences, Warrington, Pa.). Independent gates for monocytes/macrophages and granular cells were used for data collection from more than 10,000 events. The percentage of bound secondary antibody was determined with a Becton Dickinson FACSCalibur flow cytometer and was used as a measurement to determine the relative percentage of SWC3⁻/SWC9⁺ cells (macrophages) and SWC3⁺/SWC9⁻ cells (eosinophils); CellQuest software (Becton Dickinson, San Jose, Calif.) was used to analyze data files.

In vitro muscle contractility of intestinal circular smooth muscle. Mucosa-free segments of jejunum (*A. suum* infected) or ileum (*T. gondii* infected) were suspended in organ baths containing Krebs' buffer solution. Concentration-dependent responses to acetylcholine (1 nM to 1 mM) and frequency-dependent responses to electrical field stimulation (1, 2.5, 5, 10, 20 Hz; duration, 1 ms; 80 V) were determined with separate muscle strips, and tension was recorded as the amount of force per cross-sectional area by using a Grass model 79 polygraph (Grass Medical Instruments, Quincy, Mass.) (65).

Ussing chamber measurements of intestinal epithelial cell function. Segments of mucosae from either uninfected or parasite-infected jejunum (*A. suum* infected) or ileum (*T. gondii* infected) were stripped of muscle and mounted in Ussing chambers. The basal net ion flux across the mucosa (I_{sc}) and the tissue resistance, a measure of tissue permeability, were determined for concentration-dependent changes in I_{sc} to histamine added to the serosal side and, in separate tissues, concentration-dependent changes in I_{sc} to the cumulative addition of glucose to the mucosal side (49).

Plasma cytokine and nitrite assays. Matched enzyme-linked immunosorbent assay antibody pairs were used to measure porcine IFN- γ and IL-4 (Biosource); the results were expressed in picograms per milliliter. Plasma nitrite was determined (Promega, Madison, Wis.) after dilution with an equal volume of sterile distilled H₂O and filtration through a 3,000-molecular-weight-cutoff filter (Centricron; Millipore, Bedford, Mass.).

Statistical analysis. All statistical analyses were performed by using Statview 5.0 for Macintosh (Abacus Concepts, Berkeley, Calif.). Data were analyzed for equality of variance by using Fisher's F test. If the variance was heterogeneous, the appropriate transformation of the data was performed. Plasma cytokine or nitrite levels or tissue mRNA expression (C_t values) was evaluated by one-way analysis of variance to examine the effect of infection. A *P* value of <0.05 was considered statistically significant for all analyses.

RESULTS

Characterization of the pig response to *T. gondii*. All pigs fed *T. gondii* oocysts developed an acute clinical toxoplasmosis during the first week after inoculation. The body temperature was elevated 4 to 7 DAI, and the pigs exhibited inappetence, lethargy, and erythematous ears, nose, and extremities. Pigs in group II that were examined at 7 DAI showed mild enteritis characterized by a pseudomembrane over the ileum mucosa, edematous mesenteries, and fluid in the peritoneum. The MLN were edematous, had pale areas representing necrosis, and were hyperplastic, as were the colonic lymph nodes (CLN) and tracheal-bronchial lymph nodes (TBLN). These clinical features were not apparent in pigs in group III at 14 DAI, indicating that there was resolution of overt disease. Mice fed suspensions of pig ileum, liver, and lungs taken 7 DAI died within 3 weeks of inoculation, and the brains of these mice contained microscopically detectable tachyzoites (15, 18).

There was a significant increase ($P < 0.0001$) in the level of IFN- γ in the serum of *T. gondii*-infected pigs at 7 DAI (group II pigs), and the levels fell to control levels at 14 DAI (group III pigs) (Fig. 1A). Plasma nitrite, which is induced by IFN- γ and is known to accompany *T. gondii* infection in mice, was measured as a surrogate marker of tissue inducible nitric oxide synthase (iNOS) activity. All plasma samples contained mea-

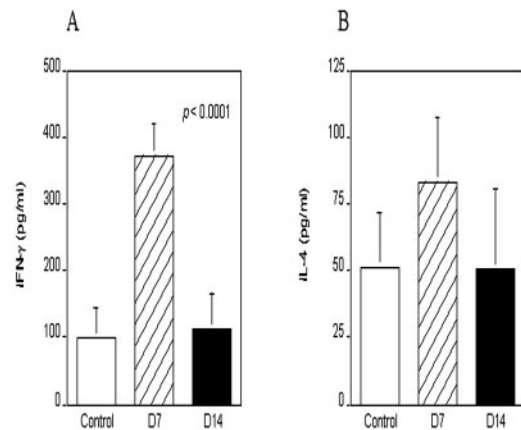


FIG. 1. Plasma IFN- γ and IL-4 levels in *T. gondii*-infected pigs at 7 and 14 DAI. Whole blood was obtained from pigs by venipuncture and mixed with EDTA; the isolated plasma was used to determine IFN- γ levels (A) and IL-4 levels (B) with matched enzyme-linked immunosorbent assay antibody pairs, and the results were expressed in picograms per milliliter. The data are representative of two replicate experiments, and a minimum of three samples were used to obtain each mean value. The differences in IFN- γ were statistically significant ($P < 0.0001$). D7, 7 days after infection; D14, 14 days after infection.

surable amounts of nitrite, but these levels did not vary in response to *T. gondii* infection (data not shown). There was a mean 25% increase in serum IL-4 levels in pigs in group II (7 DAI) compared to uninfected pigs and pigs at 14 DAI that was not statistically significant (Fig. 1B).

Local responses to *T. gondii* in the lung and small intestine. Flow cytometric analysis of BAL cells indicated that there was no change in the relative percentage of macrophages in the experimental groups. The BAL from control uninfected pigs contained 97.4% \pm 0.90% macrophages, while the BAL from pigs at 7 and 14 DAI contained 98.2% \pm 0.58% macrophages (group II) and 94.5% \pm 2.96% macrophages (group III), respectively.

There was a clear time-dependent and selective effect on epithelial cell and smooth muscle function in the ileum of *T. gondii*-infected pigs, indicating that there were periods of acute local response to infection followed by recovery. There was no change in epithelial cell resistance in the ileum of either the uninfected or *T. gondii*-infected pigs, demonstrating that tissue permeability was not impaired by the infection (data not shown). There were, however, statistically significant decreases in Na⁺-linked glucose absorption (Fig. 2C) and chloride secretion in response to histamine (Fig. 2D) in pigs in group II at 7 DAI, which returned to normal at 14 DAI. Likewise, electrical field stimulation of smooth muscle contractility decreased significantly in pigs at 7 DAI but not at 14 DAI (Fig. 3D). There was no generalized difference in smooth muscle contractility between infected and uninfected pigs, however, because there was no response to the cholinergic neurotransmitter acetylcholine (Fig. 3C).

mRNA expression following infection with *T. gondii*. There was a statistically significant increase in Th1-derived type 1 and proinflammatory cytokine gene expression in lymph nodes draining sites of infection during acute disease at 7 DAI (Fig. 4). The levels of IL-15, TNF- α , and iNOS were elevated in the

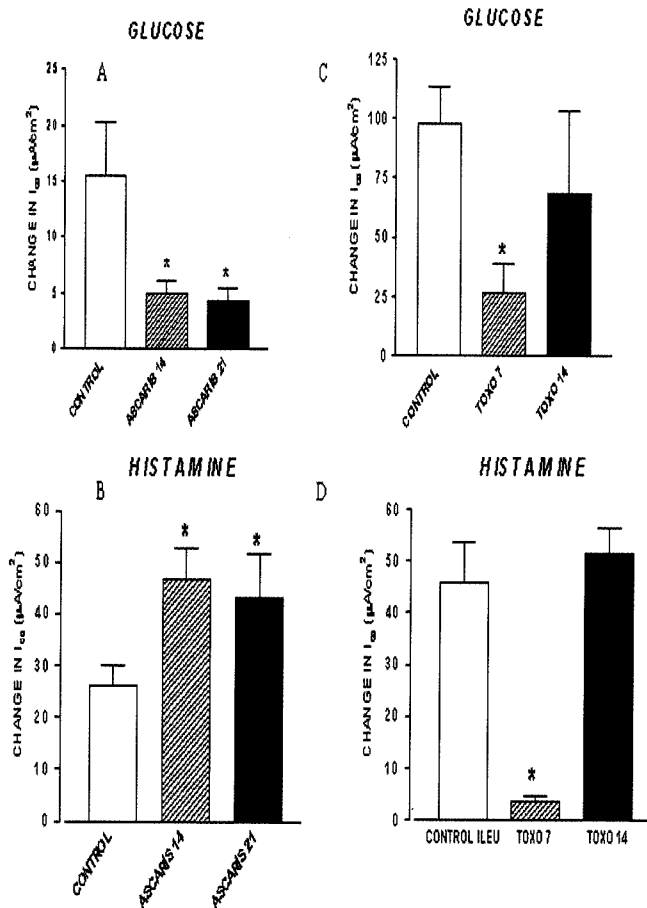


FIG. 2. Ussing chamber measurements of intestinal epithelial cell function in *A. suum*- and *T. gondii*-infected pigs at different times after inoculation. Segments of mucosae from either uninfected or parasite-infected jejunum (*A. suum*-infected pigs at 14 and 21 DAI) (A and B) or ileum (*T. gondii*-infected pigs at 7 and 14 DAI) (C and D) were stripped of muscle and mounted in Ussing chambers. Concentration-dependent changes in I_{sc} with histamine added to the serosal side (B and D) or, in separate tissues, with cumulative addition of glucose to the mucosal side (A and C) were determined. ASCARIS 14, *A. suum*-infected pigs at 14 DAI; ASCARIS 21, *A. suum*-infected pigs at 21 DAI; TOXO 7, *T. gondii*-infected pigs at 7 DAI; TOXO 14, *T. gondii*-infected pigs at 14 DAI; CONTROL ILEU, control ileum.

TBLN, CLN, and MLN, as well as in the liver, and the levels of the signature type 1 cytokine marker IFN- γ were increased in 9 of 11 tissues at 7 DAI compared with control tissues; statistically significant differences in expression in the CLN, jejunal Peyer's patches (PP), liver, MLN, and TBLN were observed. The most dramatic change was an 83.7-fold difference observed in the liver. In contrast, the levels of IFN- γ mRNA were increased in 4 of 11 tissues 14 DAI compared to control tissues, but only the TBLN data were significantly different from the control data. The level of IFN- γ mRNA was also higher at 7 DAI than at 14 DAI in nine tissues, and significant increases were observed for the CLN, ileum, liver, MLN, and TBLN. It is notable that the expression of CD3 ϵ increased ninefold in the liver 7 DAI and was also elevated at 14 DAI, suggesting that there was T-cell involvement in the cytokine patterns observed. There were more selective changes in the expression

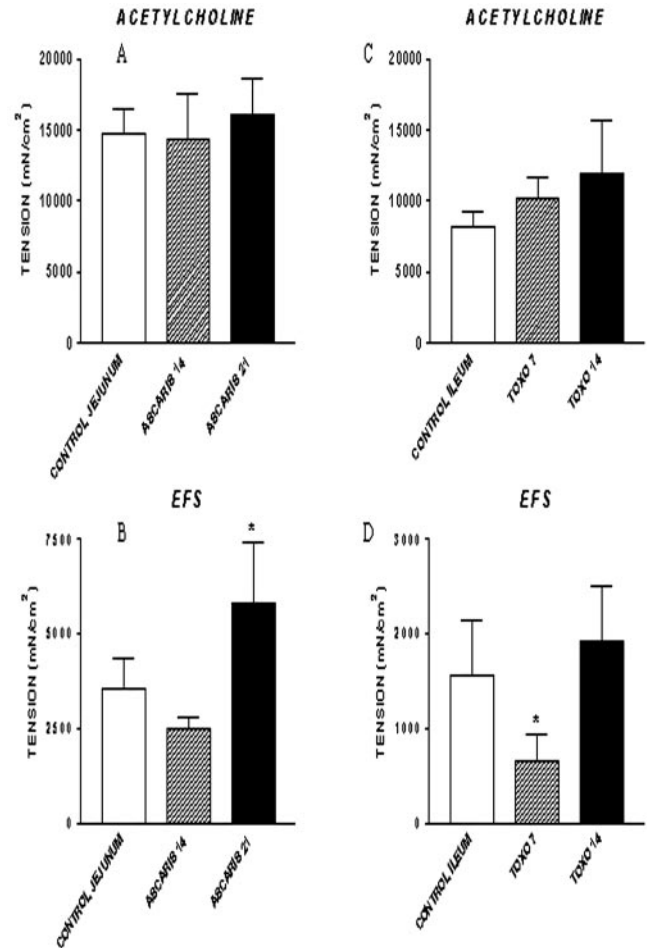


FIG. 3. In vitro muscle contractility of intestinal circular smooth muscle from pigs infected with *A. suum* and *T. gondii*. Mucosa-free segments of jejunum (*A. suum*-infected pigs at 14 and 21 DAI) (A and B) or ileum (*T. gondii*-infected pigs at 7 and 14 DAI) (C and D) were suspended in organ baths containing Krebs' buffer solution; similar tissues were obtained from uninfected control pigs. Concentration-dependent responses to acetylcholine (1 nM to 1 mM) (A and C) and frequency-dependent responses to electrical field stimulation (EFS) (1, 2.5, 5, 10, and 20 Hz; duration, 1 ms; 80 V) (B and D) were determined with separate muscle strips. Tension was recorded on a Grass model 79 polygraph and was expressed as force per square centimeter of cross-sectional area. ASCARIS 14, *A. suum*-infected pigs at 14 DAI; ASCARIS 21, *A. suum*-infected pigs at 21 DAI; TOXO 7, *T. gondii*-infected pigs at 7 DAI; TOXO 14, *T. gondii*-infected pigs at 14 DAI.

of other type 1-associated cytokines, including IL-2, IL-18, IL-23p19, IL-12p35, and IL-12p40; indeed, the level of IL-12p40 mRNA was significantly decreased in 4 or 5 of 11 tissues at 7 DAI. Nevertheless, the prominence of significant increases in the levels of many markers in the liver at 7 DAI followed by reductions in expression at 14 DAI was consistent with a period of rapid response to infection, followed by recovery.

Commensurate with recovery from overt disease was the elevation of anti-inflammatory cytokine gene expression (Fig. 5). The IL-10 mRNA levels, in particular, increased in 10 of 11 tissues at 7 DAI, and there were significant differences in the CLN, MLN, liver, spleen, and TBLN. The IL-10 mRNA levels increased in five tissues at 14 DAI, but only the increase in the

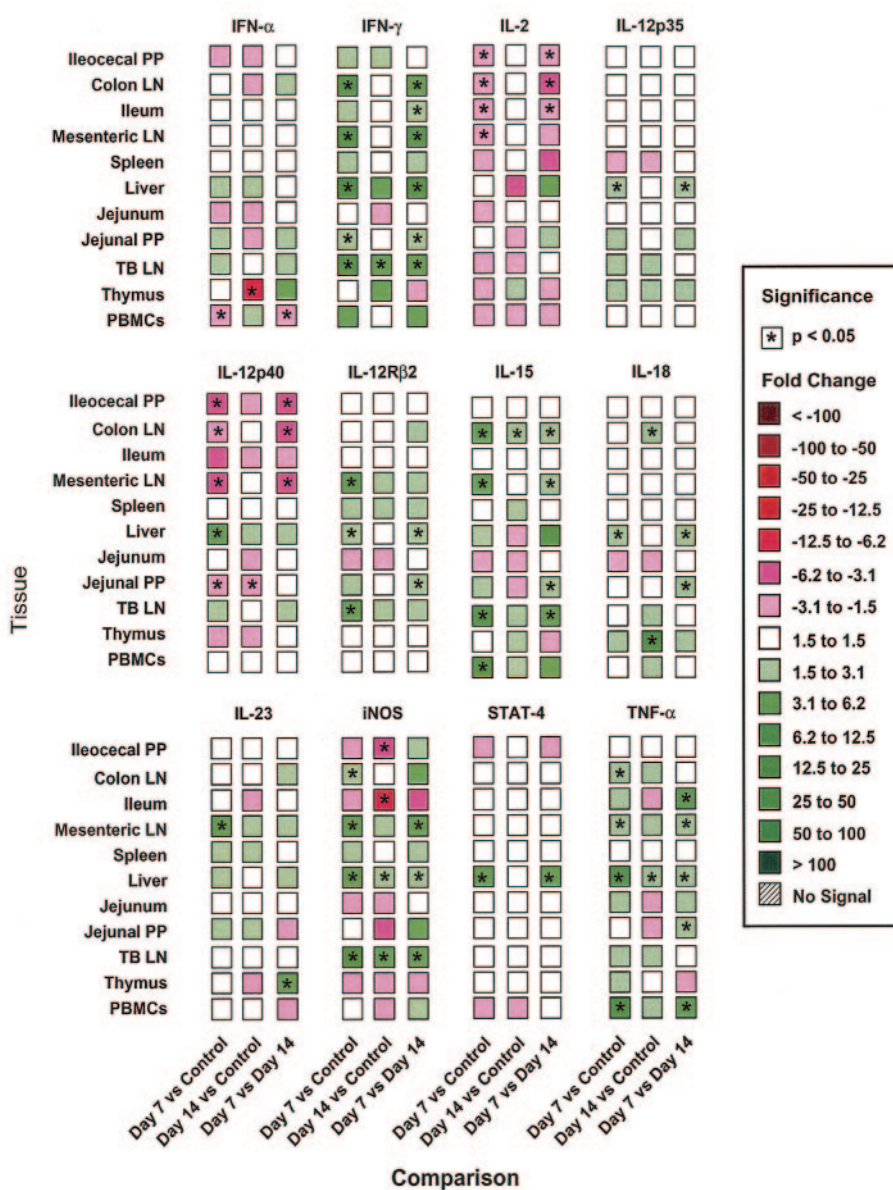


FIG. 4. Real-time PCR assay characterization of 11 different tissues from pigs infected with *T. gondii* and measurement of a cluster of 12 type 1 cytokines and related gene expression products. PCR was performed by using a Brilliant quantitative PCR core reagent kit and an ABI PRISM 7700 sequence detector system. Fluorescence signals measured during amplification were processed after amplification and were considered positive if the fluorescence intensity was >20-fold greater than the standard deviation of the baseline fluorescence. This level was defined as the C_t value. To determine the linearity and slope for each optimized assay, the cDNA template was serially diluted, and the C_t value was determined. Gene expression was normalized based upon the constant amount of RNA and cDNA amplified. A statistical analysis was performed by using Statview 5.0 for Macintosh. Data were analyzed for equality of variance by using Fisher's F test. Tissue mRNA expression (C_t values) was evaluated by one-way analysis of variance to examine the effect of infection. Fold changes are indicated by different colors, and statistical significance is indicated by asterisks ($P < 0.05$). Tissues were obtained from four uninfected control pigs and from pigs infected with *T. gondii* for 7 days (four pigs) and 14 days (three pigs); the data are representative of the data from two separate experiments. LN, lymph nodes.

thymus was statistically significant. The expression of transforming growth factor $\beta 1$ (TGF- $\beta 1$) significantly increased in the CLN and liver at 7 DAI and increased in the MLN at 14 DAI. There was also significantly increased expression of IL-4 mRNA in the liver at 7 DAI that was not observed at 14 DAI, and there were statistically significant increases in the STAT6 levels in the MLN and thymus at 7 DAI and in the MLN at 14 DAI.

Increases in IFN- γ , IL-12p35, iNOS, TNF- α , and IL-12R $\beta 2$ levels should signal a decrease in the expression of type 2 cytokines. This hypothesis was supported by the decrease in IL-5 mRNA expression in 8 of the 10 tissues examined 7 DAI compared with the control; significant differences were observed for the CLN, ileocecal PP, jejunum, and spleen, and decreases were observed in seven tissues at 14 DAI (Fig. 5). However, IL-13 mRNA expression increased in six tissues at 7

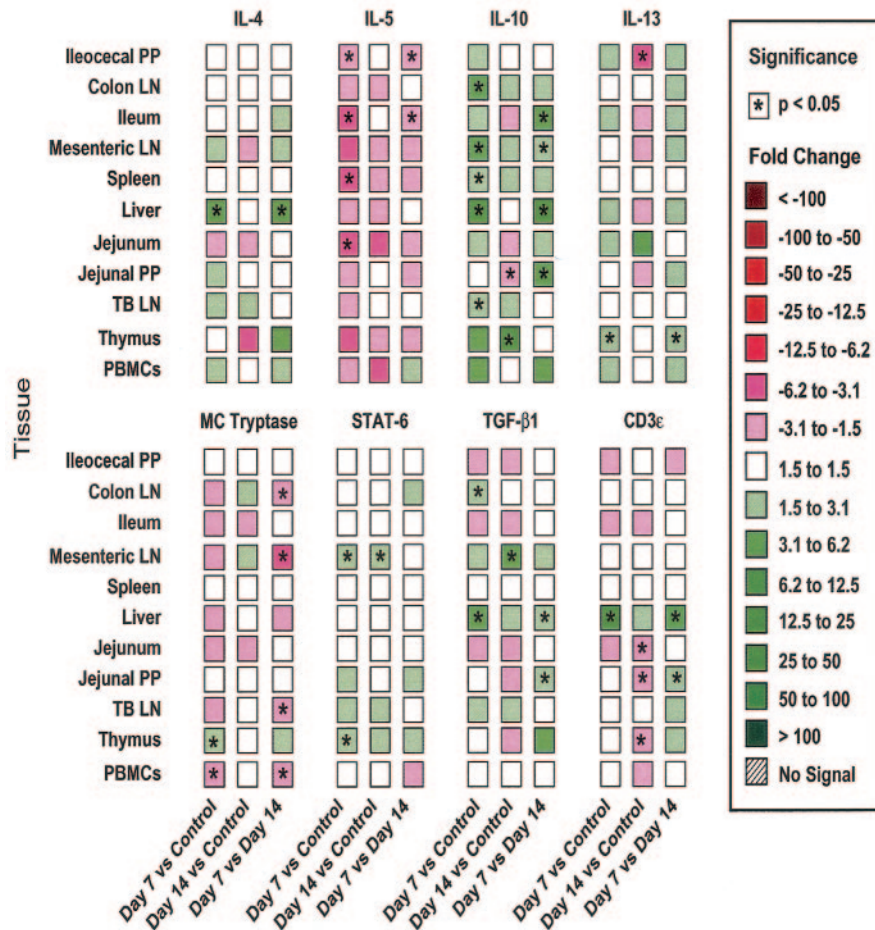


FIG. 5. Real-time PCR assay characterization of 11 different tissues from pigs infected with *T. gondii* and measurement of a cluster of eight type 2 and anti-inflammatory cytokines. PCR was performed as described in the legend to Fig. 4. Tissues were obtained from four uninfected control pigs and from pigs infected with *T. gondii* for 7 days (four pigs) and 14 days (three pigs); the data are representative of the data from two separate experiments. LN, lymph nodes.

DAI, with statistical significance only for the thymus, but decreased in five tissues at 14 DAI. Only the decreased expression in ileocecal PP was statistically significant.

Characterization of host response to *A. suum*. Pigs generally tolerated infection with *A. suum* without overt signs of disease. The only gross indicator of infection was the appearance of fibrotic lesions or white spots that covered the surface and parenchyma of the livers of pigs in groups B and C as a result of migrating *A. suum* larvae. The lesions on the livers of pigs in group C at 21 DAI were less intense than those on the livers of pigs in group B at 14 DAI (data not shown), indicating that there was disease resolution and recovery in the absence of subsequent larval migration. There was no significant increase in the plasma levels of IFN- γ , IL-4, or nitrite in *A. suum*-infected pigs at either 14 or 21 DAI compared to the levels in uninfected pigs (data not shown).

Local responses to *A. suum* in the lung and small intestine. The relative percentages of macrophages and granulocytes in the BAL determined by flow cytometry showed that in control pigs 93.3% \pm 1.1% of the cells were alveolar macrophages (group A). There was a significant reduction in the percentage of macrophages at 14 DAI (average \pm standard deviation,

43.1% \pm 8.5%; $P < 0.01$) compared to the percentage in uninfected pigs. The relative number of macrophages was also reduced at 21 DAI (78.0% \pm 5.0%), but the difference did not reach full statistical significance ($P = 0.05$). In contrast, there were significant increases in the percentages of granulocytes at 14 DAI (50.6% \pm 9.5%) ($P < 0.01$) (group B) and at 21 DAI (18.9% \pm 5.5%) (group C) compared to the percentage in control animals (0%) (group A). Differential histochemical staining of fixed BAL cells (data not shown) confirmed both the relative differences in alveolar macrophages between groups and that the majority of the granulocytes were eosinophils. Staining also showed that for all infected pigs, other cell types, such as lymphocytes and neutrophils, were present at levels of $<5\%$.

There was a clear time-dependent but selective effect on epithelial cell and smooth muscle function in the jejunum of *A. suum*-infected pigs, indicating that there was a period of change in intestinal physiology that was coincidentally related to self-curing of the fourth-stage larvae (44) and increased mucosal mast cell hyperplasia (3). There was no change in epithelial cell resistance in the jejunum in either the uninfected or *A. suum*-infected pigs, indicating that tissue permeability

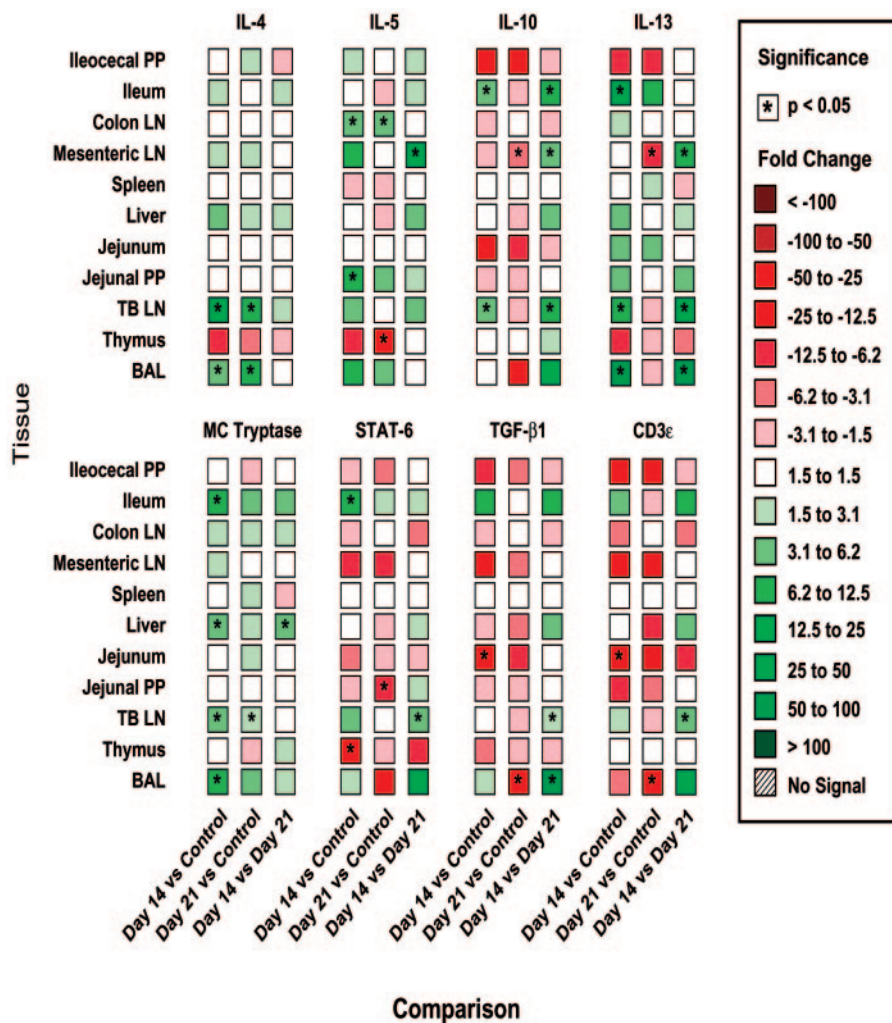


FIG. 6. Real-time PCR assay characterization of 11 different tissues from pigs infected with *A. suum* and measurement of a cluster of eight type 2 and anti-inflammatory cytokines. PCR was performed as described in the legend to Fig. 4. Tissues were obtained from four uninfected control pigs and from pigs infected with *A. suum* for 14 days (four pigs) and 21 days (four pigs); the data are representative of the data from two separate experiments. LN, lymph nodes.

was not generally impaired by the infection (data not shown). There was, however, a significant decrease in Na⁺-linked glucose absorption (Fig. 2A) and a statistically significant (*P* < 0.05) increase in Cl⁻ secretion in response to histamine in pigs in groups B and C at 14 and 21 DAI compared to the values for uninfected pigs (group A) (Fig. 2B), which supported the hypothesis that there was a net increase in fluid in the intestinal lumen during the period of self-curing. In addition, electrical field stimulation of smooth muscle contractility increased significantly in pigs in group C at 21 DAI compared to uninfected pigs (group A) and pigs infected with *A. suum* for 14 days (group B) (Fig. 3B). There was, however, no change in smooth muscle contractility with the cholinergic neurotransmitter acetylcholine at either time after infection with *A. suum* compared to the contractility in uninfected pigs (Fig. 3A).

mRNA expression after *A. suum* infection. The cytokine gene expression pattern induced by *A. suum* was much more polarized toward a Th2-like response than the pattern observed in *T. gondii*-infected pigs, especially in the small intes-

tine, lungs, and draining lymph nodes (Fig. 5 and 6). Gene expression for the Th1-associated molecules (Fig. 7), including IFN-γ, IL-12p35, IL-12Rβ2, IL-23p19, STAT4, and TNF-α, was mostly decreased or only modestly increased in *A. suum*-infected pigs compared to the expression in the controls, but the differences were not statistically significant. IL-2, however, was significantly upregulated in ileocecal PP and jejunal PP at 14 DAI and in jejunal PP at 21 DAI. Gene expression for the type 1 cytokine effector molecule that contributes to control of *T. gondii*, iNOS, was downregulated by *A. suum* infection in 9 of 10 tissues examined at 14 and 21 DAI; however, statistical significance was observed only in ileocecal PP, jejunal PP, and thymus at 14 DAI and in the MLN and TBLN at 21 DAI.

The levels of Th2-associated cytokines (Fig. 6), in contrast, were increased by *A. suum* infection. IL-4 gene expression was significantly increased in BAL and TBLN at 14 and 21 DAI. The level of IL-5 was significantly increased in CLN and jejunal PP at 14 DAI and in the CLN at 21 DAI. General induction of IL-13 mRNA expression was observed in most tissues at 14

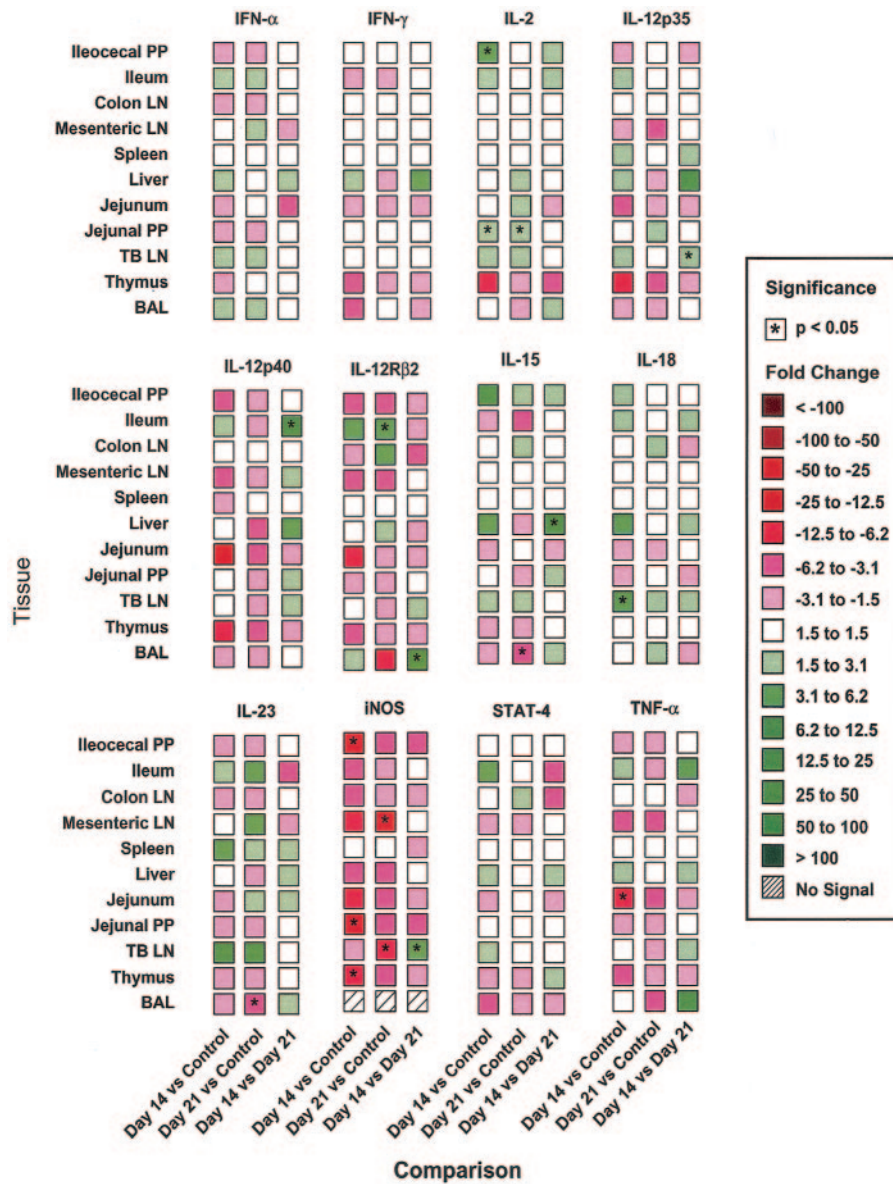


FIG. 7. Real-time PCR assay characterization of 11 different tissues from pigs infected with *A. suum* and measurement of a cluster of 12 type 1 cytokines and related gene expression products. PCR was performed as described in the legend to Fig. 4. Tissues were obtained from four uninfected control pigs and from pigs infected with *A. suum* for 14 days (four pigs) and 21 days (four pigs); the data are representative of the data from two separate experiments. LN, lymph nodes.

DAI along with significant increases in the ileum, BAL, and TBLN, but these changes did not persist at 21 DAI, when significantly decreased IL-13 gene expression was observed in MLN. It is notable that the level of IL-18, which is purported to regulate IL-13 expression, significantly increased in TBLN at 14 DAI. Mast cell tryptase gene expression, a marker of mucosal mast cell activity, was induced in most tissues at 14 and 21 DAI, and there was significant induction in the ileum, TBLN, liver, and BAL. IL-10 expression was increased in the ileum and TBLN at 14 DAI and was decreased in MLN at 21 DAI. STAT6 expression, TGF- β 1 expression, and CD3 ϵ expression were generally decreased by *A. suum* infection at the times examined.

DISCUSSION

Large-animal models can arguably provide a proof of principle for the broad evolutionary theories of immune function. They can be especially useful in the analysis of natural host-pathogen interactions in outbred populations for the design of control strategies (25). There are, however, limitations on the technical tools that are necessary to evaluate the immune responses of diverse large-animal species. The present study demonstrated that the technology for quantitative determination of multiple-target gene expression by real-time PCR analysis can test hypotheses concerning the immune responses of pigs to infectious agents. This study is novel, first because of

the inherent flexibility of the multiwell PCR array format to analyze different sets of genes of interest in various combinations in pigs (in this case Th1- and Th2-associated gene expression) and second because the model uses zoonotic infections that evoke immunological and physiological responses in a large-animal species that cannot be systematically evaluated in humans or inbred mouse strains. Thus, it provides insight into immune and inflammatory protective mechanisms. The results indicate that a prototypical Th1 response to *T. gondii* is expressed in pigs. Furthermore, the remarkable resilience of pigs to infection was evidenced by the rapid resolution of disease and the elaboration of some of the gene expression pathways responsible for host protective and antiparasite mechanisms. Likewise, the expression of a largely Th2-derived response to the parasitic nematode *A. suum* fit the concept of a type 2 cytokine response orchestrating an immediate hypersensitivity response and facilitating intestinal worm expulsion.

During infection of pigs with *T. gondii*, the magnitude of changes in proinflammatory gene expression, IFN- γ , IL-15, iNOS, and TNF- α was greatest at 7 DAI and was reduced at 14 DAI, supporting the observed clinical changes that indicated that there was partial resolution of overt disease at 14 DAI. IFN- γ mRNA was induced in all tissues analyzed except the jejunum and thymus and was reflected in an increase in the level of the circulating plasma IFN- γ protein. Multiple studies have established that IFN- γ production is associated with a protective immune response to *T. gondii* infection in murine models (30). TNF- α is induced early after *T. gondii* infection in murine models and is important for resistance to infection, as well as in host tissue pathology (63). Although there have been reports that IL-2 production is decreased in mice infected with *T. gondii* (7), IL-2-knockout (KO) mice are susceptible to infection (60), and treatment with recombinant murine IL-2 increases survival (48). Thus, the generalized decrease in IL-2 mRNA in the gut-associated lymphoid tissues in our pig model of *T. gondii* infection was unexpected, but it may reflect the enteritis and necrosis in the MLN at 7 DAI and contribute to the immune suppression that is often seen during infection with *T. gondii* (32).

We previously observed a decrease in IL-12p35 and IL-12R β 2 expression in PBMC isolated from pigs infected with *T. gondii* for >10 DAI (52). Here, we observed upregulation of IL-12R β 2 in selected tissues at 7 DAI that was reduced at 14 DAI. IL-12R β 2 is expressed on activated murine T cells (55), is maintained in an IFN- γ -dependent manner during Th1 differentiation, and is lost in an IL-4-dependent fashion during Th2 differentiation (50, 55). In the present study, IL-12p35 and IL-12p40 were significantly induced only in the liver at 7 DAI. In murine models, IL-12 p35 is produced early after acute *T. gondii* infection (46). There was a more generalized downregulation of swine IL-12p40 at multiple tissues sites during the infection that is difficult to reconcile with current knowledge of the biology of murine or human IL-12p40. Solano-Aguilar et al. (52), however, showed that biologically active recombinant swine IL-12 did not induce marked IL-12R β 2 gene expression or IFN- γ production in naïve pig PBMC, suggesting that it may not be a major Th1 activator. Alternately, IL-12p40 is the heterodimeric partner of IL-12p35 and IL-23p19 (42) that also forms homodimers that inhibit IL-12p70 signaling through the

IL-12 receptor when it is produced in excess (26). Thus, the role of IL-15, IL-18, and IL-23p19 in the development of pig Th1 responses, particularly those in mucosal tissues, needs to be evaluated further.

IL-15 was significantly induced in several tissues in response to *T. gondii* infection. It is synthesized by murine macrophages in response to in vitro exposure to *T. gondii* (14), and it is partially responsible for the protective responses induced by memory CD8⁺ T cells (28, 29, 31). IL-18 was significantly induced by *T. gondii* infection only in swine liver at 7 DAI. The role of IL-18 in the murine response to *T. gondii* is complex because it can enhance IL-12-mediated resistance to *T. gondii* in SCID mice, but overproduction can lead to pathology and mortality (41), which may explain the tighter regulation of generalized expression of IL-18 in pigs.

iNOS was upregulated in multiple tissues following *T. gondii* infection of pigs, but the plasma nitrite levels did not change. This suggests that infection in pigs leads to strict local regulation of NO-dependent effector mechanisms which may be necessary to control the multiple downstream effects of NO on many immune and nonimmune cell types. Inappropriately regulated iNOS has consequences in mice infected with *T. gondii* because iNOS KO mice have greater parasite burdens but survive longer than wild-type controls (63). Nitric oxide has not been associated with antiparasitic effects during infection with gastrointestinal nematodes, but it has been implicated in the accompanying tissue pathology (22); it was significantly downregulated in most tissues from *A. suum*-infected pigs.

The overall lack of correlation between STAT4 mRNA expression and a Th1 response in most infected pig tissues was surprising because STAT4 KO mice rapidly succumb to acute *T. gondii* infection (6). The increase in STAT4 mRNA in liver after infection with *T. gondii* may reflect the presence of activated T cells or macrophages (4). The overall lack of correlation between STAT4 and Th1 cytokine mRNA expression may mean that phosphorylation of STAT4, not regulation of mRNA levels, is required for the induction or maintenance of Th1 responses (62).

IL-4 was significantly induced by *T. gondii* only in the livers of pigs at 7 DAI. Previous reports showed that IL-4 is induced in the lung and spleen late after infection of mice with *T. gondii* (20, 32). The role of IL-4 in the resolution of *T. gondii* infection in mice is confounding because infection of IL-4 KO mice with *T. gondii* leads to increased mortality but a lower number of cysts in the brains of the surviving mice (43). IL-5 was broadly downregulated in most tissues examined at 7 DAI, while the levels of IL-4, IL-10, and IL-13 were reduced at 14 DAI, suggesting that there was a generalized reduction in Th2-derived cytokines as a consequence of increased levels of IFN- γ , IL-12p35, and TNF- α . IL-10 levels were significantly increased at 7 DAI in most pig tissues by *T. gondii* infection. This interleukin is produced early after murine *T. gondii* infection (41), and IL-10R KO mice rapidly succumb to doses of *T. gondii* that are not lethal to the wild-type strain (54); death is associated with increased tissue destruction due to uncontrolled effects of proinflammatory cytokines. Studies in mice suggest that the liver is a major organ affected by *T. gondii* infection (23, 41). Our study confirmed and extended these data. The magnitude of the changes in IFN- γ and iNOS gene expression was greatest in the liver, and expression of CD3 ϵ was elevated 10-fold,

suggesting that infiltrating or expanding T cells play a role. In fact, recent studies have shown that the positive regulators of Th1 responses, IRF1, MYD88, and STAT1, were found to be expressed in the livers of *T. gondii*-infected pigs prior to the simultaneous upregulation of IFN- γ , HLX1, and TBX21 gene expression (11). The concomitant and significant increases in the levels of the anti-inflammatory cytokines IL-4, IL-10, and TGF- β in the liver at 7 DAI were predictive of the decreases in the levels of proinflammatory cytokines at 14 DAI and improved clinical features.

The resolution of overt disease expression during infection with *T. gondii* in pigs is supported by the physiological data for intestinal function in the ileum at 7 and 14 DAI. The epithelial cell resistance, a measure of mucosal permeability, was unchanged during the infection, but Na⁺-coupled glucose absorption and responses to histamine were significantly reduced at 7 DAI and returned to normal levels at 14 DAI. This suggests that the barrier function is maintained at the expense of lost responsiveness to selected secretagogues and glucose metabolism but that the deficiency is quickly normalized. This was also reflected in a reduced response to electrical field stimulation of intestinal smooth muscle at 7 DAI that returned to normal at 14 DAI, suggesting that there was significant, but transient, suppression of intestinal function.

The cellular changes in the livers and lungs of *A. suum*-infected pigs and the physiological changes in the small intestine are characteristic of the effects of enhanced type 2 cytokine expression. Intestinal mucosal resistance in the jejunum was not altered during the infection, while Na⁺-coupled glucose absorption and Cl⁻ secretion in response to histamine were increased at 14 and 21 DAI compared to the data for uninfected control pigs. Smooth muscle contractility increased significantly from 14 to 21 DAI, indicating that there was a local response to fourth-stage *A. suum* larvae in the jejunum, and the increase was correlated with increases in IL-4, IL-5, and IL-13 gene expression. This mimics the IL-4-, IL-13-, and STAT6-dependent weep and sweep response that supports clearance or self-curing of murine gastrointestinal nematode parasites like *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus* (38, 49, 65). The net effect of increased fluid in the intestinal lumen and enhanced smooth muscle contractility between 14 and 21 DAI coincides with the expulsion of fourth-stage larvae from their predilection site in the jejunum to the ileum during expulsion from the intestine (44). A histamine-dependent increase in epithelial cell secretion during this period is also consistent with the mucosal mast cell hyperplasia in vivo, the increase in mast cell tryptase gene expression shown here, and the parasite antigen-specific induction of histamine release from isolated porcine mucosal mast cells in vitro between 14 and 21 DAI (3).

We observed significant increases in gene expression for IL-4, IL-5, IL-13, and mast cell tryptase in specific tissues and draining lymph nodes at 14 and 21 DAI with *A. suum*, which were commensurate with the development of type 2 effector pathways that coordinate protective responses to nematode parasites (21). The lack of a more generalized and sustained type 2 cytokine response may have been due to the rapid migration of *A. suum* larvae in the host or to the fact that regulation of these cytokines by T cells is dependent on different activation stimuli, kinetics, transcription factors, and cell

types (37). Increased STAT6 gene expression in the pig ileum at 7 DAI is consistent with its role in murine intestinal responses to nematode infection (2, 56, 58), but STAT6 activity, like STAT4 activity in *T. gondii*-infected pigs, may be more a function of the state of phosphorylation than a function of gene expression (62).

The notable absence of gene expression for type 1 cytokines and the anti-inflammatory cytokine TGF- β 1 in *A. suum*-infected pigs compared to pigs infected with *T. gondii* suggests that there is a definitive skewing toward a Th2-like response. There were also significant increases in IL-10 gene expression in the ileum and TBLN at 14 DAI that were indicative of the activity of IL-10 as a type 2-related cytokine that regulates resistance and local immune pathology during intestinal nematode infections in mice (47). There apparently is not a need, however, for a more generalized increase in the levels of IL-10 and other anti-inflammatory cytokines to control tissue responses like those observed during infection with *T. gondii*.

In summary, we established that *T. gondii* infection elicits a prototypical Th1-associated response, including generalized increases in IFN- γ , iNOS, and TNF- α levels, as well as localized increases in the liver that include increases in IL-12 levels. In contrast, infection with *A. suum* induces a prototypical Th2-associated response that includes increased IL-4, IL-5, IL-13, and mast cell tryptase mRNA expression and is supported by physiological and cellular responses that are characteristic of immunity to gastrointestinal nematode parasites. The pig can model specific molecular and physiological aspects of these infections that cannot be easily studied in rodents, such as the rapid emergence of anti-inflammatory pathways to control overt infection with *T. gondii* and the role of intestinal stages of *A. suum* in mucosal immune activation and modulation.

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