Research Article

Upregulated Expression of Cytotoxicity-Related Genes in IFN-γ Knockout Mice with Schistosoma japonicum Infection

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Received 9 June 2011; Accepted 28 July 2011

Academic Editor: Jorge Morales-Montor

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It is well accepted that IFN-γ is important to the development of acquired resistance against murine schistosomiasis. However, the in vivo role of this immunoregulatory cytokine in helminth infection needs to be further investigated. In this study, parasite burden and host immune response were observed in IFN-γ knockout mice (IFNg KO) infected with Schistosoma japonicum for 6 weeks. The results suggested that deficiency in IFN-γ led to decreased egg burden in mice, with low schistosome-specific IgG antibody response and enhanced activation of T cells during acute infection. Microarray and qRT-PCR data analyses showed significant upregulation of some cytotoxicity-related genes, including those from the granzyme family, tumor necrosis factor, Fas Ligand, and chemokines, in the spleen cells of IFNg KO mice. Furthermore, CD8+ cells instead of NK cells of IFNg KO mice exhibited increased transcription of cytotoxic genes compared with WT mice. Additionally, Schistosoma japonicum-specific egg antigen immunization also could activate CD8+ T cells to upregulate the expression of cytotoxic genes in IFNg KO mice. Our data suggest that IFN-γ is not always a positive regulator of immune responses. In certain situations, the disruption of IFN-γ signaling may up-regulate the cytotoxic T-cell-mediated immune responses to the parasite.

1. Introduction

Interferon gamma (IFN-γ) is a cytokine with multiple immunoregulatory functions that mediates the host defense against various pathogen infections. The broad effects of IFN-γ include activation of macrophages and antiviral immunity, enhancement of antigen presentation, induction of MHC-peptide complexes, orchestration of lymphocyte-endothelial interactions, regulation of T cell polarization toward Th1, cellular proliferation, and stimulation of apoptosis [1]. The importance of these diverse IFN-γ-mediated functions is also highlighted by the examination of schistosome infections, which are classically a type of multicellular parasitic infections.

It is well accepted that T-cell-mediated immunity, mainly that mediated by CD4+ T cells, is important to the development of acquired resistance against schistosomes. Following infection by normal cercariae, a predominant Th1 immune reaction is observed in the early phase, which then shifts to an egg-induced Th2-biased profile. Many immunization studies, especially using a variety of animal models vaccinated with attenuated cercariae [2–6] suggest that Th1 cytokines, including IFN-γ and IL-2, and the activated macrophages may be beneficial in preventing schistosomiasis. Also, some immuno-epidemiological studies on reinfection following drug treatment have shown that people living in endemic areas acquire some form of protective immunity after years of exposure to Schistosoma mansoni, Schistosoma haematobium, or Schistosoma japonicum [7–9]. Th1 response (particularly IFN-γ production) to schistosomulum antigen is hypothesized to be the key to schistosomiasis resistance in these subjects [10, 11]. Thus, an important strategy for vaccine design and development of an immune response against schistosomes involves induction of inherent IFN-γ production, which will facilitate the mounting of a Th1 response, especially at the early stage of infection [12].

It has been theoretically speculated that increased worm burdens and/or higher worm fecundity would be present in Schistosoma japonicum-infected IFN-γ knockout mice (IFNg KO mice). However, in our studies, a very interesting
phenomenon showed that the absence of IFN-γ made little difference in the worm burdens, while lower egg burdens were observed in IFNg KO mice. To explore some other possible immunological events in the absence of IFN-γ signaling in *Schistosoma japonicum* infection, the characteristics of the host immune responses were investigated in infected IFNg KO mice with lower egg burdens.

2. Materials and Methods

2.1. Experimental Mice and Parasites. Six- to eight-week-old female IFN-γ knockout (IFNg KO) mice and the wild-type (WT) control C57BL/6 (B6) mice were purchased from Model Animal Research Center of Nanjing University (Nanjing, China). All mice were maintained and bred under specific pathogen-free conditions at Nanjing Medical University. All experiments were undertaken with the approval of Nanjing Medical University Animal Ethics Committee. *Schistosoma japonicum* (*S. japonicum*, a Chinese mainland strain) cercariae were maintained in Oncomelania hupensis snails as the intermediate host, which were purchased from Jiangsu Institute of Parasitic Disease (Wuxi, China).

2.2. Infection with *S. japonicum* and Assessment of Parasite Burden. IFNg KO mice and WT mice were percutaneously infected with 40 ± 2 *S. japonicum* cercariae through their shaved abdomen. There were ten mice in each group. At 6 weeks after-infection, all mice were sacrificed to measure the parasitological parameters. After perfusion of the thoracic aorta, the recovery of worms was calculated by perfusate shaved abdomen. There were ten mice in each group. At 6 weeks after-infection, all mice were sacrificed. Spleen cells were prepared from infected and uninfected mice and 6-week *S. japonicum*-infected IFNg KO and WT mice were sacrificed. Spleen cells were prepared by gently forcing spleen tissue through a fine nylon net into incomplete RPMI 1640 containing 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. After removal of erythrocytes, the cells were resuspended and diluted to a final concentration of 1.0 × 10^7 cells/mL. The viability of splenocytes was >95%, as assessed by trypan blue dye exclusion.

Each one of the 1.0 × 10^6 splenocytes was, respectively, put into a tube and labeled with FITC anti-mouse CD19 to stand for “B cells,” labeled with APC anti-mouse CD3 and FITC anti-mouse CD4 to mark “CD3+CD4+ cells”, and labeled with APC anti-mouse CD3, PE anti-mouse NK1.1 and FITC anti-mouse CD8a to count the percentage of CD3−NK1.1+ or CD3+CD8+ cells in the splenocytes by flow cytometry.

For isolation of mouse CD8+ and NK cells from a mouse spleen cell suspension, the splenocytes were readjusted to a concentration of 1 × 10^6 cells into 400 μL buffer, incubated with 100 μL mouse anti-CD8a cell microbeads or mouse anti-NK cell (CD49b, DX5) microbeads (Miltenyi Biotec GmbH, Germany) for 15 min at 4–8°C, and washed with buffer one time. NK+ and CD8a+ cells were separated using magnetic activated cell sorting (MACS; Mini Macs, Miltenyi Biotec), by applying the cell suspension to a plastic column equipped with an external magnet. The sorted NK and CD8+ cell suspensions were, respectively, incubated with PE-conjugated rat anti-mouse CD49b (DX5) monoclonal antibody or FITC-conjugated rat anti-mouse CD8a monoclonal antibody to confirm the purity by flow cytometry (Miltenyi Biotec GmbH, Germany). Purified NK and CD8+ cells were used for the microarray analysis.

2.5. Measurement of Type 1/Type 2 Cytokine Levels in the Splenocyte Culture Supernatants. Isolated splenocytes harvested from uninfected mice and 6-week *S. japonicum*-infected IFNg KO and WT mice were cultivated without or with 10 μg/mL SEA and 1 μg/mL ConA for 72 hours. Next, the supernatants were collected for the Th1/Th2 cytokine assay. Cytokine levels were examined using the Bio-Plex mouse Th1/Th2 cytokine assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for interleukin (IL)-12p70, IFN-γ, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-2, IL-4, IL-5, and IL-10 according to the recommended procedure and protocols of the manufacturer. Parameters were read on the Bio-Plex suspension array system, and the data were analyzed using Bio-Plex Manager™ software with 5 PL curvetting.

2.6. Microarray Analyses of Splenocytes, Purified CD8+, and NK Cells from *S. japonicum*-Infected Mice. The isolated splenocytes, CD8+, and NK cells harvested from IFNg KO and WT mice at 6 weeks after-infection were subjected to gene expression profile analyses. First, total RNA was extracted using the TRIzol reagent (Invitrogen Life Technologies) and purified using the RNeasy kit (QIAGEN).
An equal amount of total RNA from five to six mice per group was mixed and cDNA was generated using the One- Cycle Target Labeling and Control Reagents (Affymetrix). The cRNA was made with the GeneChip IVT Labeling Kit (Affymetrix). Biotin-labeled, fragmented (200 nt or less) cRNA was hybridized for 16 hours at 45°C to Affymetrix Mouse 430 2.0 arrays (Affymetrix) by the Microarray Facility. The arrays were washed and stained and were subsequently read using a GeneChip Scanner 3000. The fluorescence signal was excited at 570 nm, and data were collected on a confocal scanner at 3 μm resolution. Data sorting and analysis were acquired by GeneSpring GX7.0 software (Agilent). After the normalization and filtering procedure, the system identified the differentially expressed genes that had differences of 2-fold or greater. These genes were placed into pathways based on the KEGG and GENEMAP databases. Significant pathways with differentially expressed genes were identified (P < 0.05) by use of the Fisher’s Exact Test and Chi-square (χ^2) test. The -LgP is given to assess the significance of a particular pathway category.

2.7. Quantitative Real-Time PCR (qRT-PCR). Total RNA was extracted from the isolated splenocytes harvested from IFNg KO and WT mice at 6 weeks after-infection using TRIzol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer’s instructions. The total RNA was transcribed to cDNA using a commercially available reverse transcription kit (Epicentre, USA). The cDNA was employed as a template in the following real-time PCR. Primers specific for β-actin, granzyme A (gzma), granzyme B (gzmb), granzyme K (gzmk), perforin 1 (prf1), Fas Ligand (fasl), chemokine (C-C motif) ligand 5 (ccl5), killer cell lectin-like receptor, subfamily K, member 1 (klrk1), and tumor necrosis factor (ligand) superfamily, member 9 (tnfsf9) are shown in Table 1. The PCR reaction was carried out in a 10 μL reaction mixture containing 2 μL of cDNA, 2× Master Mix (eENZYME, USA), and 0.625 μL of forward primer and reverse primer, respectively (Invitrogen, CA). The qRT-PCR was performed using an ABI 7900 Real-time PCR system with the following program: 95°C for 10 min, 40 cycles at 95°C for 15 sec, and at 68°C for 1 min. To create the PCR melting curve, the amplified product was submitted to incubation at 95°C for 2 min; 99°C for 10 sec. β-actin was used as an internal control. The relative transcription levels of individual target genes were normalized using the internal control. The identity and purity of the PCR product were confirmed by melting curve analysis. All data were analyzed using PE Applied Systems Sequence Detector 1.3 software. The threshold cycle number was used to quantify the target gene transcription level for each sample using the comparative threshold cycle method. The results represent the expression level of the target gene relative to the expression level of β-actin.

KO and WT mice. Booster immunizations were conducted at 1 and 2 weeks after the initial vaccination. The animals were sacrificed at 7 days after the last immunization. NK cells and CD8+ cells separated from the splenocytes were, respectively, submitted to cytotoxicity and qRT-PCR assays.

The gene transcription levels for some cytotoxic molecules, including gzma, gzmb, gzmk, prf1, fasl, and tnfsf9, were detected in purified CD8+ cells by qRT-PCR, as described above. For the cytotoxicity assay of NK cells, 1 × 10^6/mL of YAC-1 cells labeled with 3H-TdR 10 μCi were cultured in an incubator (37°C, 5% CO2) for 2 hours, shaking every 30 min. Next, YAC-1 cells were washed with the RPMI-1640 in triplicate and adjusted to 1 × 10^5/mL. A volume of 100 μL of YAC-1 cells and 100 μL of purified NK cells of 1 × 10^5/mL were added to 96-well plates (effector-target ratio = 100 : 1). Additionally, 100 μL of YAC-1 cells and 100 μL of RPMI-1640 were added to wells as the blank control. A total of 100 μL of YAC-1 cells and 100 μL of 1% Triton X-100 were added to wells as the maximum release control. All plates were cultured in 5% CO2, 37°C for 4 hours. A Liquid Scintillation Counting System collected the data. Cytotoxicity of NK cells is calculated by the following formula:

\[
\frac{3H-TdR \text{ release value of Experiment} - 3H-TdR \text{ release value of Blank well}}{3H-TdR \text{ release value of Maximum well} - 3H-TdR \text{ release value of Blank well}} \times 100\%.
\]
Figure 1: Parasite burden of IFNg KO mice and WT mice (n = 10, resp.) at 6 weeks after-infection with Schistosoma japonicum (compared with WT mice, **P < 0.01). (a) Total worms were recovered by portal perfusion at 6 weeks after-infection. (b) Eggs deposited in the liver were counted after digestion of the liver with 5% KOH. (c) Worm pairs were recovered by portal perfusion at 6 weeks after-infection. (d) Eggs deposited per worm couple in the liver. Data are representative of two independent experiments with the similar results.

2.9. Statistical Analysis. The data are presented as mean ± SEM. Significance was tested using unpaired t-test, ANOVA, or the Mann-Whitney test where appropriate, or in the case of microarray data, Fisher’s Exact Test, Chi-square ($\chi^2$) test. All statistics were analyzed with SPSS 16.0 software. Significant values were indicated as follows: *P < 0.05, **P < 0.01.

3. Results

3.1. Deficiency of IFN-γ Signaling Led to Decreased Egg Burden. To investigate the outcome of infection with S. japonicum in the absence of IFN-γ, parasite burden was evaluated at six weeks after the 40 cercariae challenge. Two independent animal experiments showed that the total egg number in the liver of IFNg KO mice was significantly lower than that in WT mice (P < 0.01), although there was little difference in worm recovery between these two groups, as in one of these experiments shown in Figures 1(a)–1(c).

The number of eggs per pair of worms is a significant index of the fecundity of Schistosoma japonicum, which can exclude the difference of pairs and be objective to assess the pathological damage of liver by the deposit of eggs. As shown in Figure 1(d), the number of eggs per pair in IFNg KO mice was much lower than that in WT mice, indicating that the absence of IFN-γ might have a deleterious effect on the fecundity of worms.

3.2. IFN-γ-Deficient Mice Displayed a Low Schistosome-Specific IgG Antibody Response. To study the humoral response in the acute infection, schistosome-specific IgG levels in sera were determined by ELISA. With the progress of S. japonicum infection, SWAP-specific IgG antibody levels in mice sera continued to rise. Although there was no difference in worm numbers between IFNg KO and WT mice, SWAP-specific IgG antibodies of IFNg KO mice at 3 and 6 weeks after-infection were significantly lower than those of WT mice (Figure 2(a)). S. japonicum worms begin
3.3. The Percentage of T-Cell Subsets of IFNg KO Mice Were Comparable to WT Mice, While There Were Fewer NK Cells in the Splenocytes of IFNg KO Mice. Before infection, no significant difference was observed in the total number of cells in the spleens of the IFNg KO mice compared to WT mice, nor were there any alterations of splenic cell populations with respect to CD3, CD4, CD8, CD19, and NK1.1 surface markers. At 6 weeks after S. japonicum infection, there were no significant differences in the percentage of CD3\(^+\), CD4\(^+\), CD8\(^+\), and B cells among the spleen cells between IFNg KO and WT mice. Also, little differences were observed in the percentage of CD4\(^+\) and CD8\(^+\) cells among T cells between these two mice groups. However, the percentage of NK cells in IFNg KO mice was significantly lower than that in WT mice (\(P < 0.05\)). Figure 3 showed the percentages of CD8\(^+\) cells among CD3\(^+\) T cells and NK cells among the spleen cells at 6 weeks after S. japonicum infection.

3.4. Deficiency of IFN-\(\gamma\) Enhanced the Activation of T Cells during Acute Infection with S. japonicum. To assess the effects of IFN-\(\gamma\) deficiency on the cellular immune response,
Th1/Th2 cytokines, IFN-γ, IL-12, TNF-α, IL-2, IL-10, IL-4, IL-5, and GM-CSF in the splenocyte culture supernatant were measured. All cytokine levels before infection were very low and close to baseline (data not shown). At 6 weeks after *S. japonicum* infection, cytokine expression of both IFNγ KO and WT mice without any stimulation also stayed at low levels (Figure 4). With ConA stimulation, IL-12, TNF-α, IL-5, IL-10, and GM-CSF of IFNγ KO mice were significantly higher than those of WT mice (*P < 0.05*). Furthermore, with specific stimulation of SEA, IL-5 and GM-CSF levels in IFNγ KO mice were higher than those of WT mice (*P < 0.05*). More importantly, levels of IL-10 in sera from IFNγ KO mice were much lower than those in WT mice, which might contribute to immune activation in IFNγ KO mice.

3.5. Microarray and qRT-PCR Analyses of Splenocytes Showed That Some Genes Related to Cytotoxicity Were Significantly Upregulated in IFNγ KO Mice. Based on the above-described parasitological and immunological differences between *S. japonicum*-infected IFNγ KO and WT mice, a gene expression profiling approach was used to compare the functional gene expression changes in the spleen cells. All differentially expressed genes with 2-fold or greater changes were placed into pathways based on the KEGG and GENEMAP databases. The value of “LGp” stands for the significance of a specific pathway in IFNγ KO mice compared with that in WT mice (Figure 5). Pathway analysis of splenocytes (Figure 5(a)) showed that several immune-related pathways, including cytokine-cytokine receptor interaction, hematopoietic cell lineage, leukocyte transendothelial migration, Toll-like receptor signaling pathway, cell adhesion molecules (CAMs), complement and coagulation cascades, natural killer cell-mediated cytotoxicity, MAPK signaling pathway, antigen processing and presentation, PPAR signaling pathway, and apoptosis, were significantly enhanced in IFNγ KO mice. The differentially expressed genes in the pathways of cytokine-cytokine receptor interaction and natural killer cell mediated cytotoxicity were listed in Table 2. For some genes, transcription levels of proinflammatory factors chemokines and their receptors (such as *ccl2*, *ccl5*, *ccl5*, *ccr5*, and *cxc6*) and cytotoxicity-related molecules (such as *gzma*, *gzmb*, *gzmk*, *prf1*, *fasl*, *klate1*, *klate1*, and *klate1*) were significantly upregulated in IFNγ KO mice.

Notably, some genes belonging to the signaling pathway of natural killer cell-mediated cytotoxicity should be mentioned. Several genes related to cytotoxic effects, including *gzma*, *gzmb*, *gzmk*, *prf1*, and *fasl*, and some genes related to activating and recruiting killer cells were also examined by qRT-PCR detection. Relative transcription levels of *gzma*, *gzmb*, *gzmk*, *fasl*, *ccl5*, and *klate1* were significantly higher in IFNγ KO mice than those in WT mice, which were consistent with the microarray data (Figure 6).

3.6. Microarray Data for Purified CD8+ Cells and NK Cells Revealed That the CD8+ Cell Subset Might Play More Important Role in the Cytotoxic Effect in *S. japonicum* Infection. To investigate the cytotoxic gene expression associated with CD8+ cells and/or NK cells in IFNγ KO mice infected with *S. japonicum*, we purified CD8+ cells and NK cells from splenocytes of IFNγ KO and WT mice by MACS for further microarray analysis. The purity of CD8+ cells and NK cells are about 99% and 80%, respectively. The differentially expressed genes between IFNγ KO and WT mice with 2-fold or greater changes were also placed into pathways based on the KEGG and GENEMAP databases. Most of the increased immune-related pathways in purified CD8+ and NK cells (Figures 5(b) and 5(c)) were seen in spleen cells, such as cytokine-cytokine receptor interaction, hematopoietic cell lineage, leukocyte transendothelial migration, Toll-like receptor signaling pathway, cell adhesion molecules (CAMs), complement and coagulation cascades, natural killer cell-mediated cytotoxicity, antigen processing, and presentation. Furthermore, it was found that natural killer cell-mediated cytotoxicity exhibited more significance of enhancement in purified CD8+ cells than in NK cells. As listed in Table 2, those genes associated with cytotoxicity, including the granzyme family members *gzma*, *gzmb*, *gzmk*, and *prf1*, *fasl*, and *tnf*, strongly enhanced the transcriptional levels in CD8+ cells of IFNγ KO mice compared with those of the WT mice. Unlike CD8+ cells, there was little difference in those transcripts in NK cells. In addition, NK cells might not be excluded from function as regulators of immune response to *S. japonicum* infection through upregulated transcription of some cytokines, chemokines and CD molecules, such as *ccl2*, *ccl4*, *il18*, *il18r1*, *il6*, *cd14*, and *cd28*, in IFNγ KO mice.

3.7. *Schistosoma japonicum*-Specific Egg Antigen Could Activate CD8+ T Cells in IFNγ KO Mice. To ascertain whether the specific antigen may directly induce the cytotoxic activity of CD8+ cells or NK cells, rather than complicated factors in the infectious course, IFNγ KO and WT mice were immunized with *Schistosoma japonicum*-specific egg antigen (SEA). Next, CD8+ cells and NK cells were sorted from the splenocytes. As Figure 7 illustrates, expression of *gzma*, *gzmb*, *gzmk*, *prf1*, *fasl*, and *tnf* in purified CD8+ cells was measured by qRT-PCR. Although only *gzmb* and *tnf* were significantly higher in IFNγ KO mice relative to WT mice, other genes showed a trend of enhanced expression in IFNγ KO mice. Thus, *Schistosoma japonicum* SEA might activate the cytotoxic ability of CD8+ cells in IFNγ KO mice. Meanwhile, to assess the cytotoxicity of NK cells, purified NK cells stimulated by *Schistosoma japonicum* SEA were cocultured with YAC-1 cells, which are specific target cells for activated NK cells. As Figure 8 illustrates, cytotoxicity of NK cells from IFNγ KO mice was decreased, although there was no significant difference between these two groups.

4. Discussion

It has been well documented that IFN-γ plays significant protective roles in the host response to *Leishmania*, *Toxoplasma gondii* [13], *Plasmodium* [14], *Candida albicans* [15], and other intracellular pathogens. As for many extracellular metazoan parasites, such as schistosomes, most studies support the hypothesis that the Th1 response, especially IFN-γ secretion, can activate macrophages and/or other effectors...
Figure 4: Type 1/Type 2 cytokine levels in the supernatant of splenocyte cultures of IFNγ KO and WT mice (n = 10, resp.) at 6 weeks after Schistosoma japonicum infection by Bio-Plex detection (compared with WT mice, *P < 0.05, **P < 0.01). Data are representative of two independent experiments with the similar results.
Figure 5: Significantly upregulated pathways with differentially expressed genes in splenocytes, purified CD8+ cells, and NK cells in 6-week Schistosoma japonicum-infected IFNγ KO mice compared with WT mice based on KEGG and GENEMAP databases. The value of “-LgP” stands for the significance of a particular pathway category.
(cells/molecules), which might participate in eliminating larvae in the early stages of infection. Contrary to expectations, our studies showed that IFN-\(\gamma\)-deficient mice infected with *Schistosoma japonicum* were found to have a significantly decreased egg burden in the liver compared to WT mice, while no obvious difference in worm burdens between these two groups. In the early stage of *Schistosoma japonicum* infection, the deficiency of IFN-\(\gamma\) concomitant with an impaired antibody response had no significant impact on the schistosomula. It is possible that the disruption of IFN-\(\gamma\) signaling altered some immunological or physiological internal environmental of the host, either as direct effect or as compensatory consequence, so the worm fecundity might be affected or some eggs might be destroyed.

Killer cell-mediated cytotoxicity was addressed in IFN-\(\gamma\) knockout mice infected with *Schistosoma japonicum*. Microarray data of splenocytes showed that some transcripts of granzymes, perforin, Fasl, and TNF family members that are normally involved in cytotoxicity were significantly upregulated in the absence of IFN-\(\gamma\) during the acute infection. These molecules are mainly induced by two major cytotoxic lymphocyte subsets, natural killer (NK) cells and CD8\(^+\) T cells. Although the effector functions of NK cells and CD8\(^+\) T cells are carried out in similar way, their activation modes and action stages are different [16]. NK cells both produce IFN-\(\gamma\) and respond to IFN-\(\gamma\). In our studies of *Schistosoma japonicum* infection, the number of NK cells from IFNg KO mice was significantly lower than that in wild-type mice, and the transcripts of some cytotoxicity-related genes in splenic NK cells from IFNg KO mice could not be increased. In contrast, purified CD8\(^+\) T cells from *Schistosoma japonicum*-infected IFNg KO mice or

| Gene Symbol | Probe Set ID | Spleen cells | Purified CD8\(^+\) cells | Purified NK cells |
|-------------|--------------|--------------|--------------------------|------------------|
| *cc2*      | 1420380_at   | 241.3        | 48.3                     | Increase         |
| *cc4*      | 1421578_at   | 674.3        | 293.7                    | No change        |
| *cc5*      | 1418126_at   | 19335.4      | 6000.8                   | Increase         |
| *cc9*      | 1417936_at   | 3048.6       | 2043.1                   | Increase         |
| *cc2r*     | 1421188_at   | 414.5        | 141.6                    | No change        |
| *cc3*      | 1422957_at   | 141.5        | 97                       | No change        |
| *cc5*      | 1424727_at   | 594.2        | 257.3                    | Increase         |
| *cxl2*     | 1449984_at   | 3436.5       | 1584.8                   | Increase         |
| *cxt6*     | 1422812_at   | 2547.3       | 438.4                    | Increase         |
| *csf1r*    | 1419873_s_at | 3946.4       | 2641.7                   | Increase         |
| *il1b*     | 1443939_a_at | 2322.7       | 1027.5                   | Increase         |
| *il18*     | 1417932_at   | 1280         | 941                      | No change        |
| *il18r1*   | 1421628_at   | 2336.8       | 279.4                    | Increase         |
| *il18rap*  | 1456545_at   | 1326.6       | 392.7                    | Increase         |
| *il6*      | 1450297_at   | 115          | 44.9                     | No change        |
| *tnf*      | 1419607_at   | 291.9        | 195                      | No change        |
| *gzma*     | 1417989_a_at | 13097.3      | 2514.9                   | Increase         |
| *gzmb*     | 1419060_at   | 6568.9       | 1127.1                   | Increase         |
| *gzmk*     | 1422280_at   | 2049.2       | 400.9                    | Increase         |
| *prf1*     | 1451862_a_at | 677.4        | 262.8                    | Increase         |
| *fas*      | 1449235_at   | 514.6        | 232.2                    | Increase         |
| *krlc1*    | 1425005_at   | 1237.6       | 272.5                    | Increase         |
| *krlk1*    | 1460245_at   | 2968.6       | 1158.5                   | Increase         |
| *krlg1*    | 1420788_at   | 2706.3       | 315.4                    | Increase         |
| *krlk1*    | 1450495_a_at | 1109.5       | 459.2                    | Increase         |
| *cd14*     | 1417268_at   | 2277.3       | 1158.6                   | Increase         |
| *cd28*     | 1417597_at   | 1154.5       | 742.5                    | No change        |
| *cd3e*     | 1422105_at   | 2558.9       | 1382.3                   | Increase         |
| *cda8*     | 1444078_at   | 1131         | 634.3                    | Increase         |
| *cd8b1*    | 1426170_at   | 3568.2       | 1429.2                   | Increase         |

\(^*\) SI stands for "signal intensity." \(^{**}\) Indicates statistically significant difference between IFNg KO and WT groups.
SEA-immunized IFNg KO mice showed higher transcription of these cytotoxic molecules in IFNg KO mice compared to WT mice, which was consistent with upregulated expression of cytotoxic genes in infected spleen cells. Potentially, activation of CD8+ T cells might play more important role in the cytotoxic effect during *Schistosoma japonicum* infection. Induction of CD8+ T cell activation and expression of cytotoxic transcripts requires at least two independent stimuli, activation of the TCR and costimulation via a cytokine milieu [17]. Firstly, most CD8+ T cells express TCR that can recognize a specific MHC I-bound antigenic peptide, which is commonly derived from an intracellular pathogen via antigen processing. However, peptides may also be derived from exogenous antigens that intersect class I presentation pathway after endocytosis by APCs. It is generally accepted that dendritic cell and its derived cytokines are the most efficient at cross-presenting exogenous antigens [18]. Cross-priming of CD8+ T cells could not be excluded in *Schistosoma japonicum* infection, which needs to be clearly elucidated in the future. Secondly, the cytokine milieu, including IL-6/IL-12 and some γc-dependent cytokines, could regulate the expression of granzymes and perforin [19]. We found that deficiency of IFN-γ could influence a wide range of cytokines and other inflammatory molecules, which might activate the immune response in *Schistosoma japonicum* infection. The levels of some of the Th1 and Th2 type cytokines, especially IL-12, in spleen cell culture supernatant from IFNg KO mice were significantly higher than those from WT mice with ConA stimulation. Microarray data of splenocytes from mice infected with *Schistosoma japonicum* showed upregulated gene expression of some proinflammatory factors, chemokines, cytokines, and cell adhesion molecules in IFNg KO mice. These factors might contribute to the recruitment of activated lymphocytes and other immune cells and lead to intensive inflammatory environment. It is suggested that deficiency of IFN-γ signaling may enhance the cellular immune capacity of some certain lymphocytes in response to schistosome antigens.
**Figure 7:** Expression of **gzma**, **gzmb**, **gzmk**, **prf1**, **fasl** and **tnfsf9** in purified CD8\(^+\) cells from SEA-immunized mice, as measured by qRT-PCR (compared with WT mice, *P* < 0.05, **P** < 0.01).

**Figure 8:** Cytotoxicity of NK cells to YAC-1 cells.

In our model of *Schistosoma japonium* infection, it was implied that IFN-\(\gamma\) negatively regulated the CD8\(^+\)T cell response. Dalton et al. reported that T-cell cytolytic activity was enhanced in IFNg KO mice with *Mycobacterium bovis* infection [20]. Another study suggested that CD8\(^+\) T cells from IFN-\(\gamma\) gene knockout donors induce more severe lethal graft-versus-host disease (GVHD) compared to CD8\(^+\) T cells from wild-type (WT) donors in fully MHC-mismatched strain combinations [21]. Thus, it is important to note that, in some specific circumstances, IFN-\(\gamma\) might play a negative role depending on the concentrations of IFN-\(\gamma\), microenvironments, different infectious agents, or different phases of the immune response. Besides well-known protective effects, the negative modulation of IFN-\(\gamma\) has been gaining increasing attention. IFN-\(\gamma\) negatively regulates activation and migration of dendritic cells and NK cells [22–24]. Also, IFN-\(\gamma\) promotes the development and differentiation of regulatory T cells and apoptosis of activated CD4\(^+\)/CD8\(^+\) cells [25]. These studies using IFNg KO mouse model help to define the *in vivo* role of this immunoregulatory cytokine.

Finally, some literature supports the hypothesis that CD8\(^+\) T cells may participate in the protective immunity against schistosomes. In a study of a *Schistosoma mansoni* vaccine candidate molecule, Sm28GST, it was reported that immunization with Sm28GST could induce antigen-specific CTL effects, leading to a decreased parasite burden and alleviated liver pathology. Transfer of Sm28GST-specific CD8\(^+\) cells also conferred protection, and this protective effect of Sm28GST was significantly decreased after treatment with anti-CD8\(^+\) cell antibody [26, 27]. Nevertheless, we do recognize there is no direct evidence regarding how extracellular parasites activated CD8\(^+\) T cells or whether some cytotoxic granules could destroy schistosome eggs. In our present work, the fact that there was no difference in worm numbers suggests that these CD8\(^+\) T cells might not have any deleterious effect on primary infections in IFNg KO mice despite the up-regulation of a range of enzymes involved in expression of cytotoxicity. Further studies of the mechanism through which IFN-\(\gamma\) interacts with CD8\(^+\) T cells may contribute to a better understanding of the immunity during *Schistosoma japonicum* infection.
5. Conclusion

This study shows that IFN-γ knockout has no obvious effect on worm burden and results in reduced egg burden in S. japonicum infection of mice. In IFN-γ knockout mice, many cytotoxicity-associated genes are upregulated during the infection. These results indicate that IFN-γ is not always a positive regulator of immune responses and it might play multiple roles in S. japonicum infection.

Authors’ Contributions

X. Du, D. Zhang, M. Ji, and G. Wu participated in the design of the study, drafted the paper. X. Du, J. Wu, M. Zhang, Y. Gao and M. Hou performed majority of the experiments. All authors read and approved the final paper.

Acknowledgments

This work was supported by National Basic Research Program of China (973 Program 2007CB513106) and the National Science Foundation of China (NSFC) (Project no. 30872368). The authors thank Professor Mike J. Doenhoff from the University of Nottingham for critically reading the paper and providing constructive suggestions.

References

[1] U. Boehm, T. Klamp, M. Groot, and J. C. Howard, “Cellular responses to interferon-γ,” Annual Review of Immunology, vol. 15, pp. 749–795, 1997.
[2] J. P. Hewitson, P. A. Hamblin, and A. P. Mountford, “Immunity induced by the radiation-attenuated schistosome vaccine,” Parasite Immunology, vol. 27, no. 7-8, pp. 271–280, 2005.
[3] M. Eberl, J. A. Langermans, P. A. Frost et al., “Cellular and humoral immune responses and protection against schistosomes induced by a radiation-attenuated vaccine in chimpanzees,” Infection and Immunity, vol. 69, no. 9, pp. 3532–5362, 2001.
[4] T. M. Kariuki and I. O. Farah, “Resistance to re-infection after exposure to normal and attenuated schistosome parasites in the baboon model,” Parasite Immunology, vol. 27, no. 7-8, pp. 281–288, 2005.
[5] Q. D. Bickle, H. O. Bogh, M. V. Johansen, and Y. Zhang, “Comparison of the vaccine efficacy of γ-irradiated Schistosoma japonicum cercariae with the defined antigen Sj62(IrV-5) in pigs,” Veterinary Parasitology, vol. 100, no. 1-2, pp. 51–62, 2001.
[6] F. Tian, D. Lin, J. Wu et al., “Immune events associated with high level protection against Schistosoma japonicum infection in pigs immunized with UV-attenuated cercariae,” Plos One, vol. 5, no. 10, Article ID e13408, 2010.
[7] B. Gryseels, K. Polman, J. Clerinx, and L. Kestens, “Human schistosomiasis,” The Lancet, vol. 368, no. 9541, pp. 1106–1118, 2006.
[8] A. P. Mountford, “Immunological aspects of schistosomiasis,” Parasite Immunology, vol. 27, no. 7-8, pp. 243–246, 2005.
[9] A. G. Ross, A. C. Sleigh, Y. Li et al., “Schistosomiasis in the people’s republic of China: prospects and challenges for the 21st century,” Clinical Microbiology Reviews, vol. 14, no. 2, pp. 270–295, 2001.
[10] R. Corre-Oliveira, I. R. Caldas, and G. Gazzinelli, “Natural versus drug-induced resistance in Schistosoma mansoni infection,” Parasitology Today, vol. 16, no. 9, pp. 397–399, 2000.
[11] I. R. Caldas, R. Correa-Oliveira, E. Colosimo et al., “Susceptibility and resistance to Schistosoma mansoni reinfection: parallel cellular and isotypic immunologic assessment,” American Journal of Tropical Medicine and Hygiene, vol. 62, no. 1, pp. 57–64, 2000.
[12] D. P. McManus and A. Loukas, “Current status of vaccines for schistosomiasis,” Clinical Microbiology Reviews, vol. 21, no. 1, pp. 225–242, 2008.
[13] H. Dlugonska, “Immunity in Toxoplasma gondii infections,” Postepy Higieny i Medycyny Doswiadczalnej, vol. 54, no. 1, pp. 53–65, 2000.
[14] D. S. Korbel, O. C. Finney, and E. M. Riley, “Natural killer cells and innate immunity to protozoan pathogens,” International Journal for Parasitology, vol. 34, no. 13-14, pp. 1517–1528, 2004.
[15] L. M. Lavigne, L. R. Schopf, C. L. Chung, R. Maylor, and J. P. Sypek, “The role of recombinant murine IL-12 and IFN-γ in the pathogenesis of a murine systemic Candida albicans infection,” Journal of Immunology, vol. 160, no. 1, pp. 284–292, 1998.
[16] L. Chavez-Galan, A. M. C. Arenas-Del, E. Zenteno, R. Chavez, and R. Lascurain, “Cell death mechanisms induced by cytotoxic lymphocytes,” Chinese Journal of Cellular and Molecular Immunology, vol. 6, no. 1, pp. 15–25, 2009.
[17] S. Ramanathan, J. Gagnon, and S. Ilangumaran, “Antigen-nonspecific activation of CD8+ T lymphocytes by cytokines: relevance to immunity, autoimmunity, and cancer,” Archivum Immunologiae et Therapiae Experimentalis, vol. 56, no. 5, pp. 311–323, 2008.
[18] A. S. Beignon, M. Skoberne, and N. Bhardwaj, “Type I interferons promote cross-priming: more functions for old cytokines,” Nature Immunology, vol. 4, no. 10, pp. 939–941, 2003.
[19] D. Chowdhury and J. Lieberman, “Death by a thousand cuts: granzyme pathways of programmed cell death,” Annual Review of Immunology, vol. 26, pp. 389–420, 2008.
[20] D. K. Dalton, S. Pitts-Meek, S. Keshav, I. S. Figari, A. Bradley, and T. A. Stewart, “Multiple defects of immune cell function in mice with disrupted interferon-γ genes,” Science, vol. 259, no. 5102, pp. 1739–1742, 1993.
[21] W. Asavaroengchai, H. Wang, S. Wang et al., “An essential role for IFN-γ in regulation of alloreactive CD8 T cells following allogeneic hematopoietic cell transplantation,” Biology of Blood and Marrow Transplantation, vol. 13, no. 1, pp. 46–55, 2007.
IFNγ on NK cells,” *International Immunopharmacology*, vol. 5, no. 6, pp. 1057–1067, 2005.

[25] Z. Wang, J. Hong, W. Sun et al., “Role of IFN-γ in induction of Foxp3 and conversion of CD4+ CD25− T cells to CD4+ Tregs,” *Journal of Clinical Investigation*, vol. 116, no. 9, pp. 2434–2441, 2006.

[26] V. Pancre, H. Gras-Masse, A. Delanoye, J. Herno, A. Capron, and C. Auriault, “Induction of cytotoxic T-cell activity by the protective antigen of Schistosoma mansoni Sm28GST or its derived C-terminal lipopeptide,” *Scandinavian Journal of Immunology*, vol. 44, no. 5, pp. 485–492, 1996.

[27] V. Pancre, I. Wolowczuk, S. Guerret et al., “Protective effect of rSm28GST-specific T cells in schistosomiasis: role of γ interferon,” *Infection and Immunity*, vol. 62, no. 9, pp. 3723–3730, 1994.