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Research Article

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

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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/6J (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 sedimentation plus residual worms from the intestinal mesenteric vessels. Weighed liver samples were digested in 5% KOH at 37°C overnight to count the released eggs under a microscope. The released eggs were microscopically counted. Parasite burden was measured by the total number of worms recovered, released eggs in the liver, eggs per pair counted, and eggs per gram liver sample counted. Two independent experiments were carried out.

2.3. Detection of Schistosome-Specific IgG Antibodies in Serum by Enzyme-Linked Immunosorbent Assay (ELISA). Serum was prepared from the peripheral blood sample at day 0, 3 weeks, and 6 weeks after *S. japonicum* infection. The levels of *S. japonicum* soluble adult worm preparation-(SWAP-) and egg antigen-(SEA-) specific IgG antibodies in sera were measured using an indirect ELISA. The concentrations of coated SWAP and SEA were 6 μg/mL and 5 μg/mL, respectively. All serum samples were diluted to 1 : 100 in phosphate buffered saline (PBS). Secondary goat anti-mouse IgG labeled with horseradish peroxidase (HRP) (ABD, USA) was diluted to 1 : 1500 and used to detect the primary antibody. Each sample was assayed in triplicate. On each plate, positive and negative tests were provided as quality controls. OD (optical density) values were read at 450 nm (Clinibio 128C, ASYS Hitch GmbH, Austria).

2.4. Isolation of Splenocytes, Calculation of the Percentage of CD8$^+$ and NK Cell by Flow Cytometry, and Purification of CD8$^+$ and NK Cell Subsets by Magnetic-Activated Cell Sorting (MACS). Spleens were aseptically removed when 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 1×10^8 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 ManagerTM 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; 60°C for 20 sec; 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.

2.8. Immunization of Mice with SEA and Cytotoxicity Assay of Purified CD8$^+$ and NK Cells. A volume of 100 μL PBS containing 50 μg of soluble egg antigen (SEA) was, respectively, injected at 3 different subcutaneous sites on the back of IFNg 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.

Table 1: qRT-PCR primers and products.

Gene symbol	Primer sequence (5′ → 3′)	Product (bp)
β-actin	F: 5′CCTCTATGCCAACACAGTGC3′ R: 5′GTACTCCTGCTTGCTGATCC3′	211
gzma	F: 5′TGTGAAACCAGGAACCAGATG3′ R: 5′GGTGATGCCTCGCAAAATA3′	256
gzmb	F: 5′TGCTCTGATTACCCATCGTCC3′ R: 5′GCCAGTCTTTGCAGTCCTTTATT3′	89
gzmk	F: 5′CCCACTGCTACTCTTGGTTTC3′ R: 5′GGCATTTGGTCCCATCTCTA3′	252
prf1	F: 5′CCCACTCCAAGGTAGCCAAT R: 5′GCTGTAAGGACCGAGATGCG	265
fasl	F: 5′GGTTCTGGTGGCTCTGGTT3′ R: 5′ACTTTAAGGCTTTGGTTGGTG3′	105
ccl5	F: 5′ACCACTCCCTGCTGCTTTG3′ R: 5′CACACTTGGCGGTTCCTTC3′	131
klrk1	F: 5′GCAGTTCTTGCCTCACTTCC3′ R: 5′AGCTCCTCCTCGTCTTCTTC3′	97
tnfsf9	F: 5′TGTTCCTATCTTCACCCGC3′ R: 5′GACTGTCTACCACCAACTCCTT3′	290

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 ^{3}H-TdR 10 μCi were cultured in an incubator (37°C, 5% CO_2) 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 1×10^7/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% CO_2, 37°C for 4 hours. A Liquid Scintillation Counting System collected the data. Cytotoxicity of NK cells is calculated by the following formula:

$$\frac{^3\text{H-TdR release value of Experiment} - {}^3\text{H-TdR release value of Blank well}}{^3\text{H-TdR release value of Maximum well} - {}^3\text{H-TdR release value of Blank well}} \times 100\%. \quad (1)$$

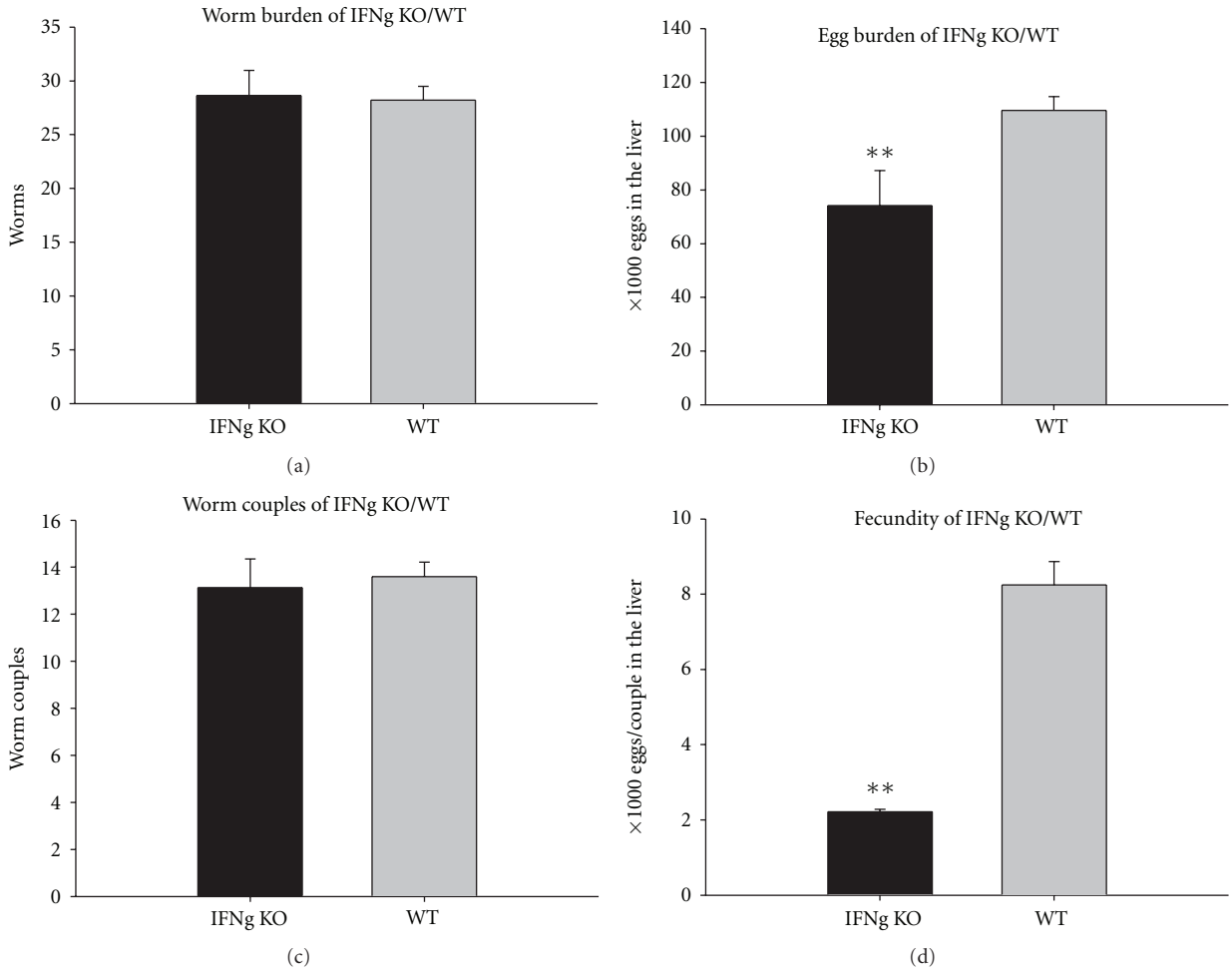

Figure 1: Parasite burden of IFNg KO mice and WT mice ($n = 10$, resp.) at 6 weeks after-infection with *Schistosoma japonicum* (compared with WT mice, $^{**}P < 0.01$). (a) Total worms were recovered by portal perfusion at 6 weeks after-infection. (b) Eggs deposited in the liver were counted after digestion 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 (χ^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

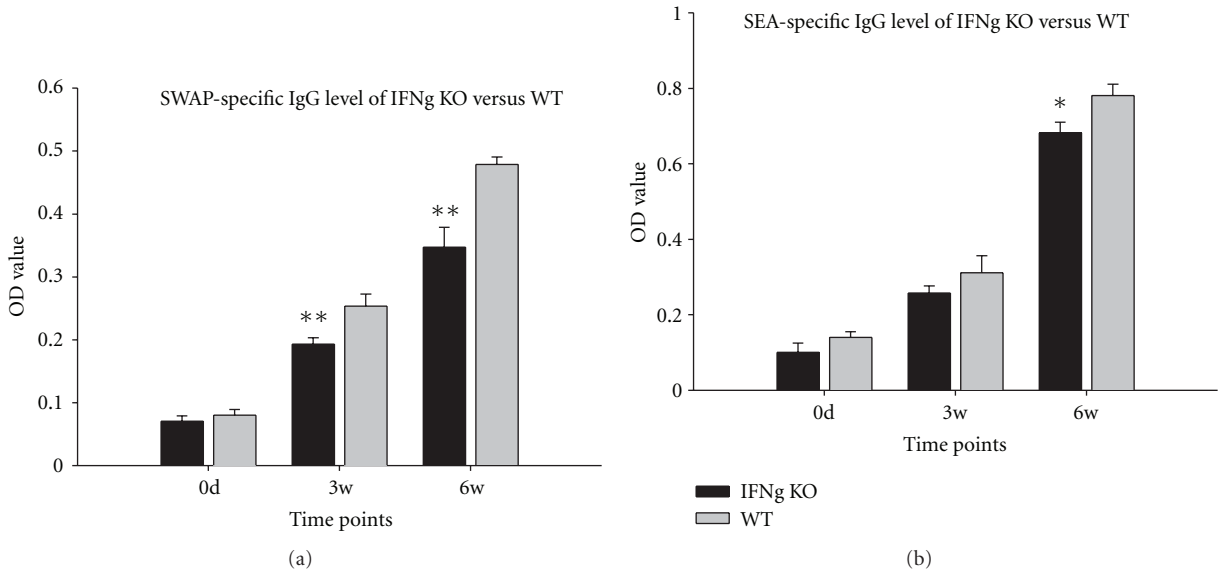

Figure 2: Dynamics of SWAP- and SEA-specific IgG antibody levels in IFNg KO and WT mice ($n = 10$, resp.) according to ELISA of sera harvested at day 0, 3 weeks, and 6 weeks after-infection (compared with WT mice, $^*P < 0.05$, $^{**}P < 0.01$). Data are representative of two independent experiments with the similar results.

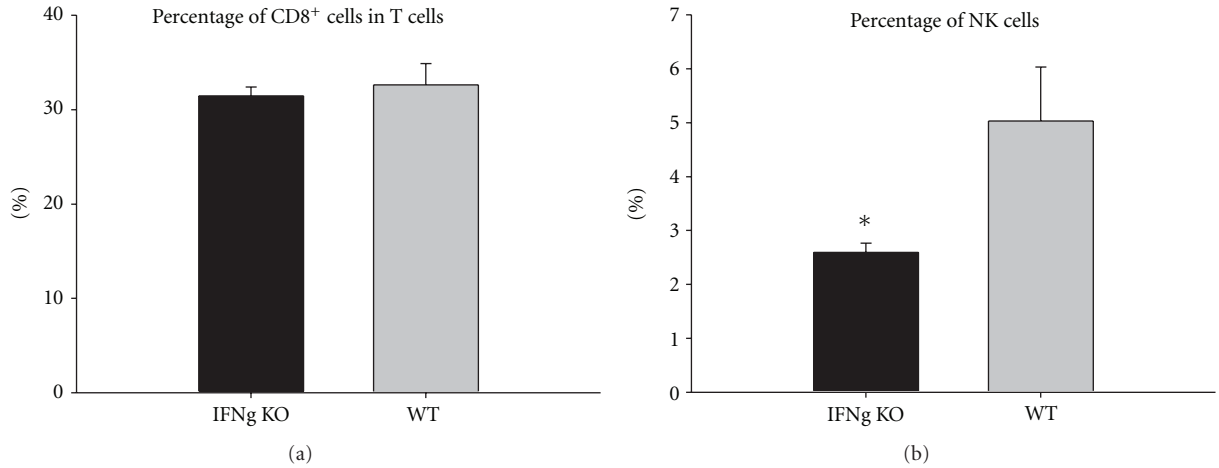

Figure 3: Percentage of $CD8^+$ cells among T cells and NK cells among spleen cells as determined by FACS at 6 weeks after-infection with *Schistosoma japonicum* (compared with WT mice, $^*P < 0.05$).

to lay eggs at approximately 3 weeks after infection. Thus, SEA-specific IgG antibody level in sera was significantly elevated at 6 weeks after-infection. Comparably, SEA-specific IgG antibody level in sera from IFNg KO mice at 6 weeks after-infection was also lower than that from WT mice (Figure 2(b)).

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-γ Enhanced the Activation of T Cells during Acute Infection with S. japonicum. To assess the effects of IFN-γ 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 IFNg 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 IFNg 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 IFNg KO mice were higher than those of WT mice ($P < 0.05$). More importantly, levels of IL-10 in sera from IFNg KO mice were much lower than those in WT mice, which might contribute to immune activation in IFNg KO mice.

3.5. Microarray and qRT-PCR Analyses of Splenocytes Showed That Some Genes Related to Cytotoxicity Were Significantly Upregulated in IFNg KO Mice. Based on the above-described parasitological and immunological differences between *S. japonicum*-infected IFNg 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 IFNg 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 IFNg 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, cxcl2, ccr5,* and *cxcr6*) and cytotoxicity-related molecules (such as *gzma, gzmb, gzmk, prf1, fasl*, *klrc1*, *klrd1*, *klrg1,* and *klrk1*) were significantly upregulated in IFNg 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 *klrk1* were significantly higher in IFNg 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 genes expression associated with CD8$^+$ cells and/or NK cells in IFNg KO mice infected with *S. japonicum*, we purified CD8$^+$ cells and NK cells from splenocytes of IFNg 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 IFNg 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 IFNg 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 IFNg KO mice.

3.7. Schistosoma japonicum-Specific Egg Antigen Could Activate CD8$^+$ T Cells in IFNg 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, IFNg 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 *tnfsf9* in purified CD8$^+$cells was measured by qRT-PCR. Although only gzmb and tnfsf9 were significantly higher in IFNg KO mice relative to WT mice, other genes showed a trend of enhanced expression in IFNg KO mice. Thus, *Schistosoma japonicum* SEA might activate the cytotoxic ability of CD8$^+$ cells in IFNg 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 IFNg 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

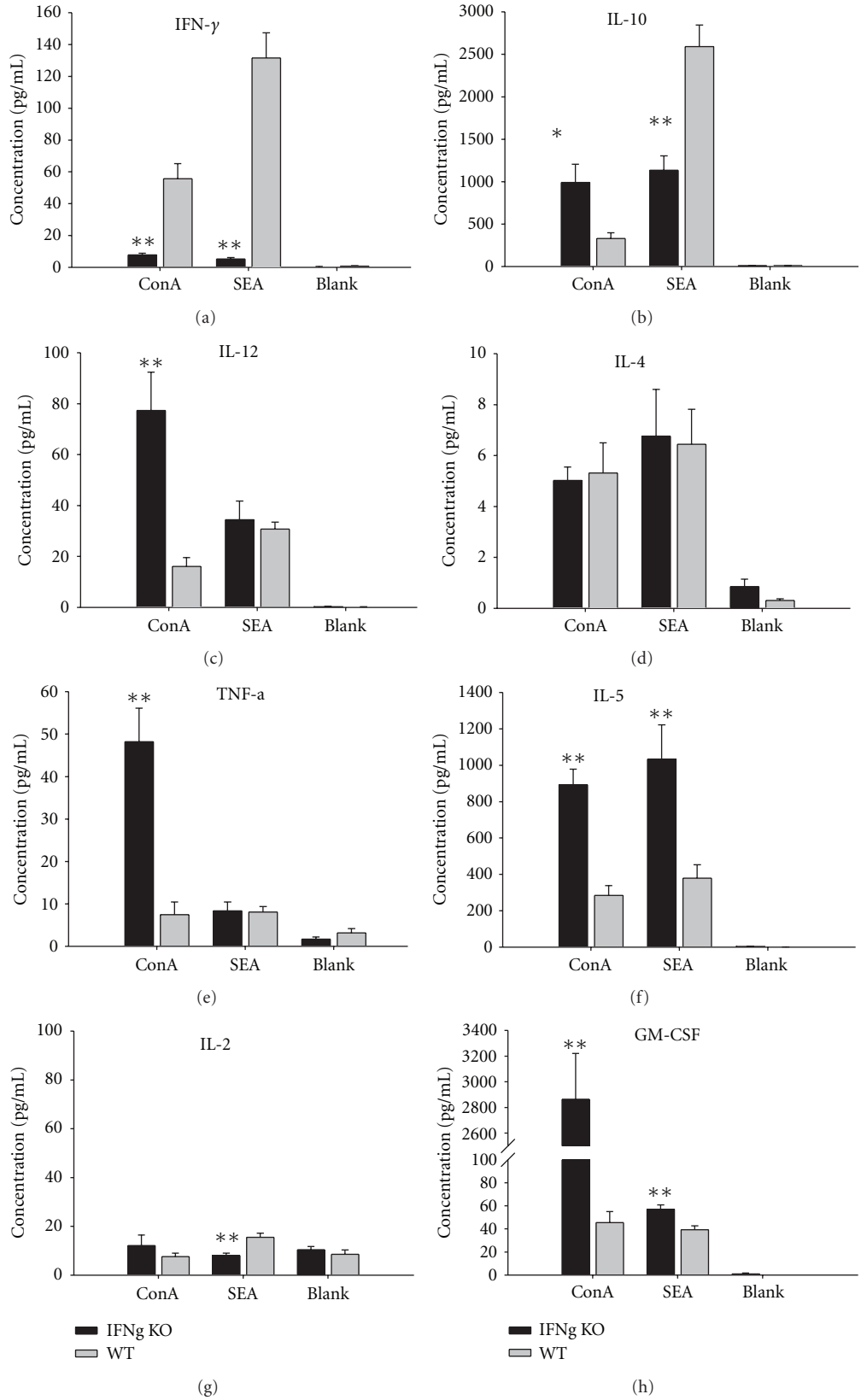

Figure 4: Type 1/Type 2 cytokine levels in the supernatant of splenocyte cultures of IFNg KO and WT mice ($n = 10$, resp.) at 6 weeks after *Schistosoma japonicum* infection by Bio-Plex detection (compared with WT mice, $^{*}P < 0.05$, $^{**}P < 0.01$). Data are representative of two independent experiments with the similar results.

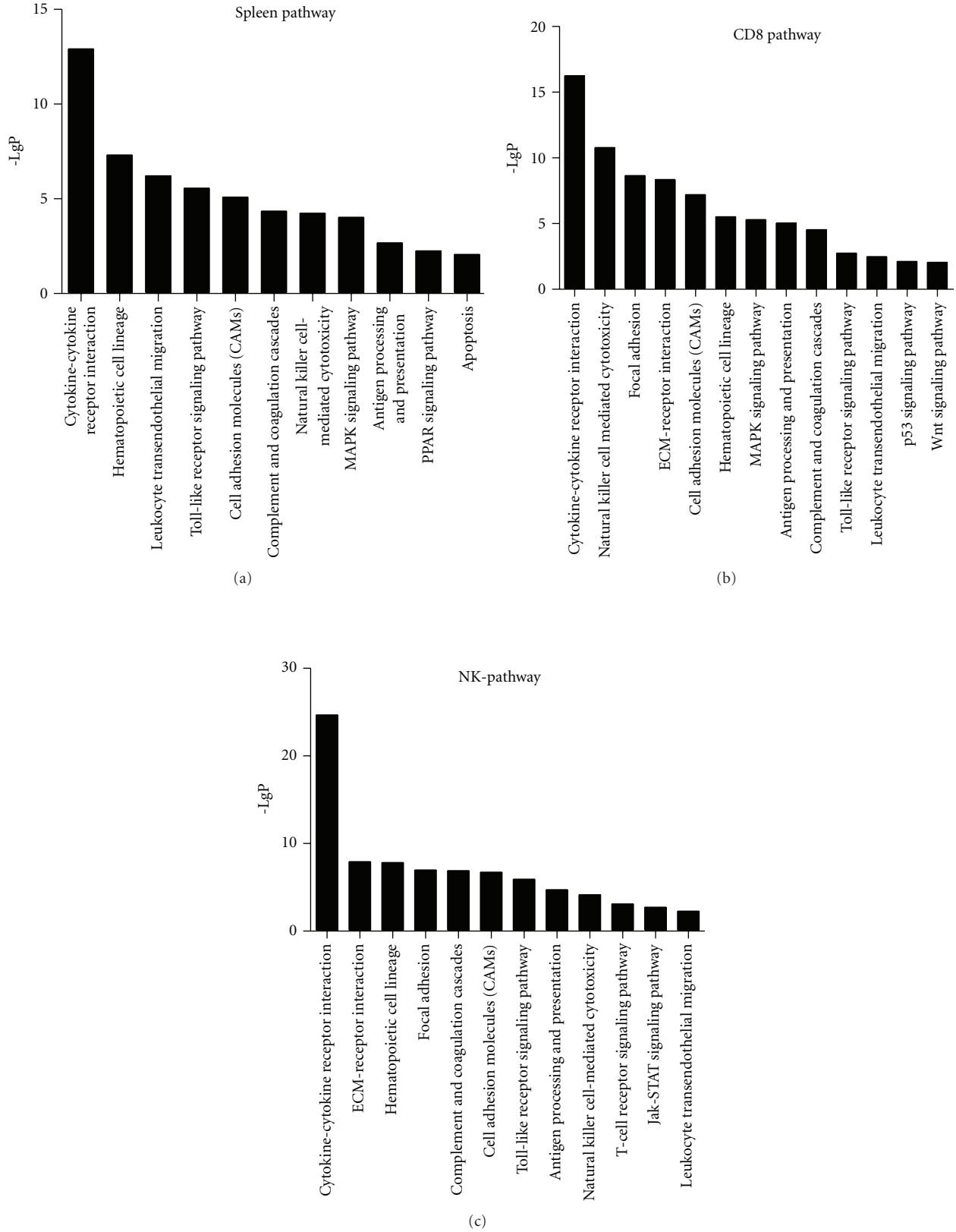

Figure 5: Significantly upregulated pathways with differentially expressed genes in splenocytes, purified $CD8^+$ cells, and NK cells in 6-week *Schistosoma japonicum*-infected IFNg KO mice compared with WT mice based on KEGG and GENEMAP databases. The value of "-LgP" stands for the significance of a particular pathway category.

Table 2: Signal intensities of some characteristic genes in spleen cells, purified $CD8^+$ and NK cells from 6-week *Schistosoma japonicum*-infected mice.

Gene Symbol	Probe Set ID	Spleen cells			Purified $CD8^+$ cells			Purified NK cells		
		SI* of IFNg KO	SI of WT	Change**	SI of IFNg KO	SI of WT	Change**	SI of IFNg KO	SI of WT	Change**
ccl2	1420380_at	241.3	48.3	Increase	80.5	54.1	No change	219.8	43	Increase
ccl4	1421578_at	674.3	293.7	No change	2028.4	874.2	Increase	6176.4	2540.6	Increase
ccl5	1418126_at	19335.4	6000.8	Increase	32722	27917.8	No change	25966.1	29725.3	No change
ccl9	1417936_at	3048.6	2043.1	Increase	939.3	493.4	Increase	14887.6	10318.9	Increase
ccr2	1421188_at	414.5	141.6	No change	586.5	211.5	Increase	4102.4	1825.7	Increase
ccr3	1422957_at	141.5	97	No change	247	29.4	Increase	575.3	130.2	Increase
ccr5	1424727_at	594.2	257.3	Increase	1422.7	1105	Increase	1757.1	1424.8	Increase
cxcl2	1449984_at	3436.5	1584.8	Increase	2948.7	690.5	Increase	5645.8	1727.6	Increase
cxcr6	1422812_at	2547.3	438.4	Increase	6390	1782.7	Increase	8213.3	2695.1	Increase
csf1r	1419873_s_at	3946.4	2641.7	Increase	4913.4	1210.7	Increase	4166.8	2346.6	Increase
il1b	1449399_a_at	2322.7	1027.5	Increase	1919.8	1355.5	Increase	1587.8	1663	No change
il18	1417932_at	1280	941	No change	1722.4	658.9	Increase	1498.4	840.9	Increase
il18r1	1421628_at	2336.8	279.4	Increase	5965.7	2332.4	Increase	10221.2	4810	Increase
il18rap	1456545_at	1326.6	392.7	Increase	3042	1722	Increase	5229.3	4758.1	No change
Il6	1450297_at	115	44.9	No change	20.7	33.6	No change	5888.1	2484.2	Increase
tnf	1419607_at	291.9	195	No change	472.2	253.8	Increase	906.7	551.2	Increase
gzma	1417898_a_at	13097.3	2514.9	Increase	22373.6	11803.6	Increase	32005.9	35300.9	No Change
gzmb	1419060_at	6568.9	1127.1	Increase	17907	6863.3	Increase	14257.1	14818.8	No Change
gzmk	1422280_at	2049.2	400.9	Increase	10278.1	2464.3	Increase	3299.7	1849.7	Increase
prf1	1451862_a_at	677.4	262.8	Increase	3098.3	2575.4	Increase	2369.2	2338.4	No Change
fasl	1449235_at	514.6	232.2	Increase	1198.3	497	Increase	2026	1903.7	No Change
klrc1	1425005_at	1237.6	272.5	Increase	2809.8	1154.7	Increase	9477.8	6187.2	Increase
klrd1	1460245_at	2968.6	1158.5	Increase	12620.7	11113.5	Increase	14710.6	10420.8	Increase
klrg1	1420788_at	2706.3	315.4	Increase	4224.1	1961.4	Increase	6138.2	4830.9	Increase
klrk1	1450495_a_at	1109.5	459.2	Increase	6708.1	2665	Increase	10344.4	8147.4	Increase
cd14	1417268_at	2277.3	1158.6	Increase	2004.7	515	Increase	2112.1	870.8	Increase
cd28	1417597_at	1154.5	742.5	No change	5199.3	3430.5	Increase	2448.6	1127.7	Increase
cd3e	1422105_at	2558.9	1382.3	Increase	5797.7	5959.3	No change	3458.3	3198.7	No change
cd8a	1444078_at	1131	634.3	Increase	12587.6	13751.8	No change	1425.6	2022	Decrease
cd8b1	1426170_a_at	3568.2	1429.2	Increase	27357.8	25575	No change	4259.4	3833.2	No change

* SI stands for "signal intensity." ** Indicates statistically significant difference between IFNg KO and WT groups.

(cells/molecules), which might participate in eliminating larvae in the early stages of infection. Contrary to expectations, our studies showed that IFN-γ-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-γ concomitant with an impaired antibody response had no significant impact on the schistosomula. It is possible that the disruption of IFN-γ 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-γ 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-γ 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-γ and respond to IFN-γ. 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

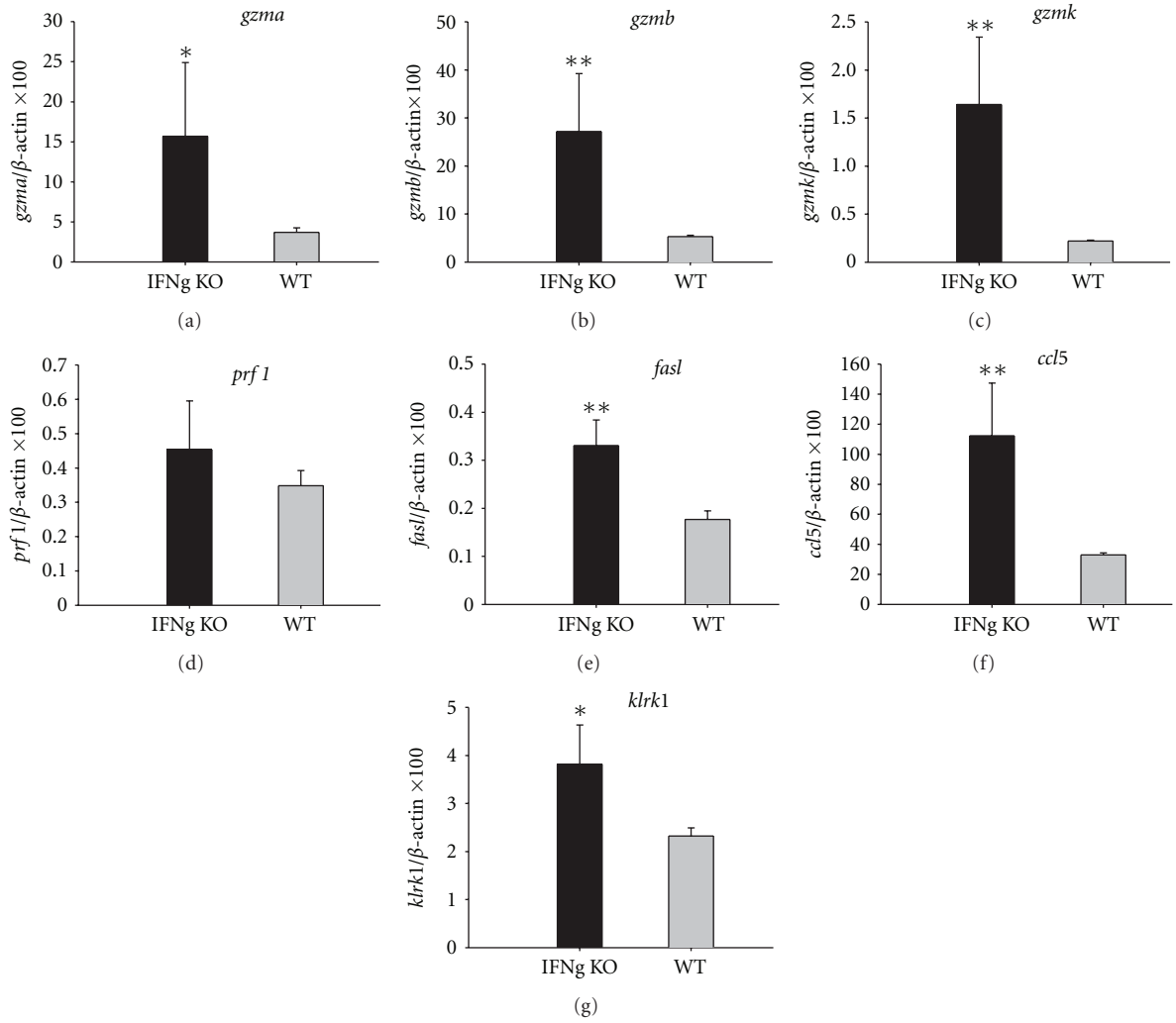

Figure 6: Relative transcription levels of *gzma*, *gzmb*, *gzmk*, *prf1*, *fasl*, *ccl5*, and *klrk1* in splenocytes of *Schistosoma japonicum*-infected IFNg KO and WT mice by real-time PCR. Data were analyzed using the Mann-Whitney test for statistical support (compared with WT mice, $^{*}P < 0.05$, $^{**}P < 0.01$).

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 cytotoxin 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.

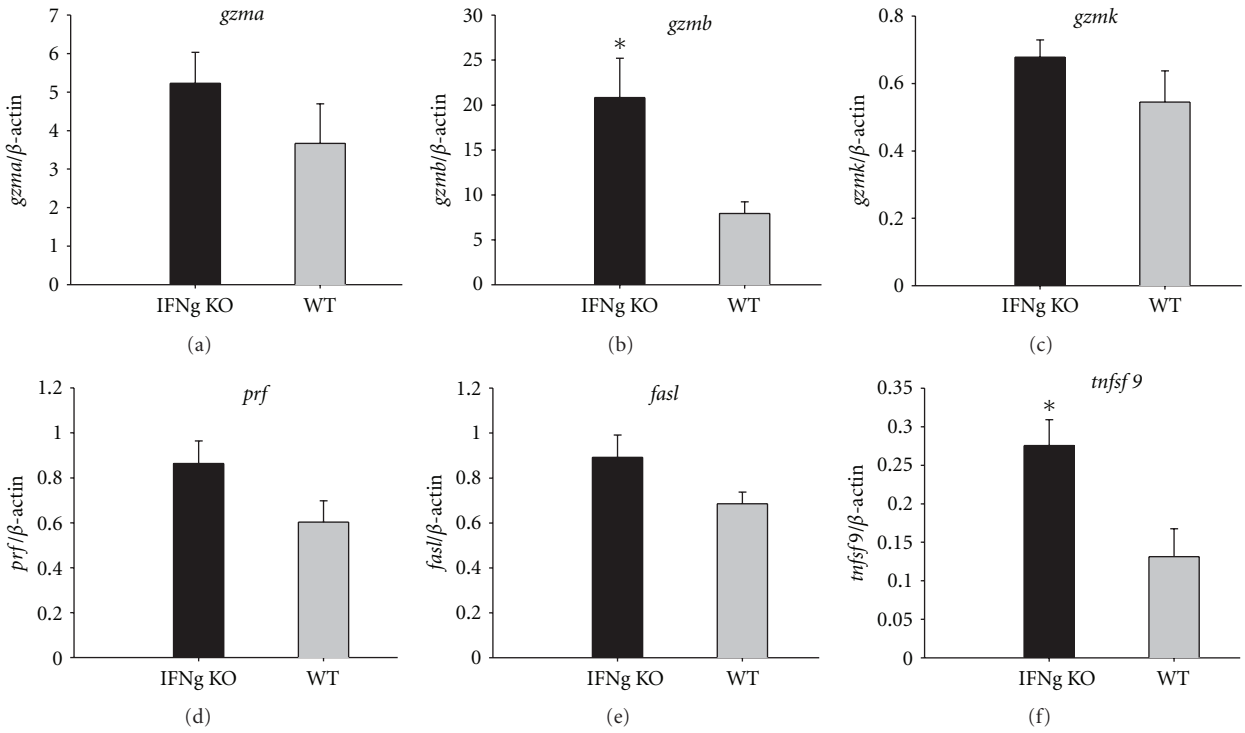

Figure 7: Expression of *gzma, gzmb, gzmk, prf1, fasl* and *tnfsf9* in purified $CD8^+$ cells from SEA-immunized mice, as measured by qRT-PCR (compared with WT mice, $^*P < 0.05$, $^{**}P < 0.01$).

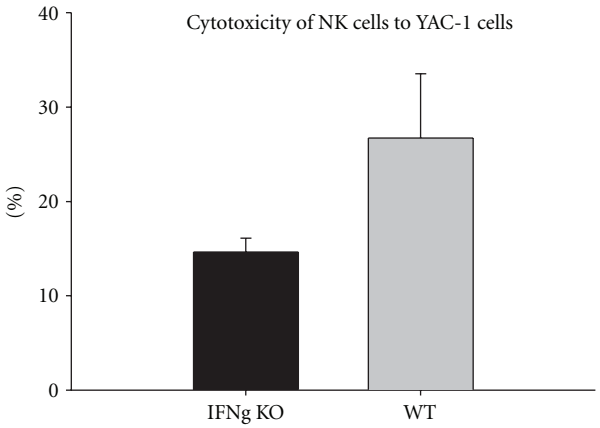

Figure 8: Cytotoxicity of NK cells exposed to YAC-1 cells from IFNg KO and WT mice immunized with SEA.

In our model of *Schistosoma japonium* infection, it was implied that IFN-γ 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-γ 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-γ might play a negative role depending on the concentrations of IFN-γ, microenvironments, different infectious agents, or different phases of the immune response. Besides well-known protective effects, the negative modulation of IFN-γ has been gaining increasing attention. IFN-γ negatively regulates activation and migration of dendritic cells and NK cells [22–24]. Also, IFN-γ 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 cytotoxity. Further studies of the mechanism through which IFN-γ 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.

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