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Original Article

Switched phenotypes of macrophages during the different stages of *Schistosoma japonicum* infection influenced the subsequent trends of immune responses

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Immunomodulation

Abstract *Background:* Macrophages play crucial roles in immune responses during the course of schistosomal infections.
Methods: We currently investigated influence of immunocompetent changes in macrophages via microarray-based analysis, mRNA expression analysis, detection of serum cytokines, and subsequent evaluation of the immune phenotypes following the differentiation of infection-induced lymphocytes in a unique T1/T2 double-transgenic mouse model.
Results: The gradual upregulation of genes encoding YM1, YM2, and interleukin (IL)-4/IL-13 receptors in infected mice indicated the role of type 2 alternatively activated macrophages (M2, AAMφs) in immune responses after *Schistosoma japonicum* egg production. FACS analysis showed that surface markers MHC class II (IA/IE) and CD8α$^+$ of the macrophages also exhibited

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a dramatic change at the various time points before and after egg-production. The transgenic mouse experiments further demonstrated that the shifting of macrophage phenotypes influenced the percentage of helper T (Th)-2 cells, which was observed to be higher than that of Th1 cells, which increased only at 3 and 5 weeks post-infection. The differentiation of effector B cells showed a similar but more significant trend toward type-2 immunity.

Conclusion: These results suggest that the infection of mice with *S. japonicum* resulted in a final Th2- and Be2-skewed immune response. This may be due to phenotypic changes in the macrophages. The influence of alternatively activated macrophages was also activated by *S. japonicum* egg production. This study elucidated the existence of variations in immune mechanisms at the schistosome infection stages.

Introduction

Schistosome is an important human intestinal helminth.[1] The eggs laid by females may be captured by hepatic capillaries, or penetrate the intestinal wall to finish their life cycle.[1–3] The main immunopathology includes the acute inflammatory response, which is initiated by the infective larvae infilter into the host's skin in the early stage, and the granulomatous inflammation and fibrotic reaction that are resulted from the parasitic eggs being captured by tissues in the later stage[4–7]

Previous studies have revealed that parasites could secrete several derived complexes, which partially or entirely regulate the gene expressions or cell functions of hosts, thereby modulating the trend of immune responses, and limit the scale of damage with host's histologic reactions and then promote the parasites survival.[8,9] Other studies have also shown that the development of the immunopathology seems to be controlled by a widely modulated immune trend when the mouse was infected by *Schistosoma japonicum*.[10,11] Importantly, the interactions of immune responses between the schistosomes and the infected host are not a one-way reaction, but a series of complicated immune network systems. Immune responses of different pathways are initiated in each schistosome infection stage, and several cell modulatory responses are involved.[12–16] Both the larvae and eggs secrete diversified antigens into the host tissues, combining the chemokines of immunocytes or restraining their receptors function, thereby modulating subsequent immune responses to a suitable development mode for the schistosomes.[17–19] Using three different susceptible and non-susceptible hosts to study the immune response mechanisms for *S. japonicum* infection revealed the great differences in the pathology, T cell subsets differentiation and gene expression among each other.[20] The scale and traits of these responses are controlled by the activated cell types of many innate immune responses, especially the macrophages and their secreted cytokines.[14,21,22]

The macrophage (Mφ) is an indispensable element for innate immune defenses initiating by exotic pathogenic infections. Studies have shown that the macrophage would adjust the functional mode and further modulate subsequent development of acquired immune responses due to variations in the cytokine microenvironment of peripheral tissues.[23–25] Macrophage typing has been proven to have many subtypes, with obviously different functions and molecular characters.[26] However, the most important two kinds are the type-1, classically activated macrophages (M1, CAMφ) with characteristics of anti-infection, pro-inflammation and cell killing, differentiated from LPS or Type1 cytokines; and the alternatively activated macrophages (AAMφ), differentiated from Type2 cytokines (M2), which is related to the responses of anti-inflammation, tissue repair, and humoral immunity.[27,28] The macrophages with the two expression profiles would affect the subsequent trend of immune responses, and modulate the occurrence of disparate types of inflammatory responses, respectively.[29–31] The recent studies showed that the differentiation of the macrophages mostly inclines to induce M2 to participate in modulating the host's immunity during the parasite's chronic infection in order to develop into an advantageous environmental pattern for the existence of parasites.[32–34] Array studies of *S. japonicum* also showed that many transcription factors were activated stepwise in systemic and localized liver immune responses, and recruited a lot of macrophages into the liver during the granulomatous inflammation and hepatic fibrosis.[35,36] We previously found that many gene expressions related to TLR, IFN, MHC and TNFrsf were down-regulated to dampen the inflammation and cytotoxicity in hepatic granuloma pathology.[37] However, further proof is necessary that the significant switch of immune transformation associated with entire type-1 and type-2 responses during infection is initiated by the gene phenotype changes of macrophages. Therefore, establishing an accurate detection for Th1/Th2 immune trends in the various infection stages when studying the immunomodulation of the schistosomes is important.[13,38] A T1/T2 doubly transgenic mice which has been used to monitor the activation of different phenotypes of lymphocyte subsets in the *Schistosoma mansoni* infection were also employed here.[39–41]

In this study, we discussed the roles of the gene expression profile and functional changes from M1 to M2 during infection, and the subsequent impacts on the immunomodulation of the host against the *S. japonicum*. For discussing the combined modulation between macrophages and lymphocytes, the comparison of the phenotype

changes of the macrophages in mice infected were analyzed, and then further effects to differentiations of T and B cells were observed through T1/T2 double transgenic mice. In together, the representative clusters of gene modulation of the host in different stages of infection could be screened and analyzed in the cellular molecular aspect, including the host defending against infections, controlling the host's pathological changes, and restraining the growth of schistosomes, which are helpful to future studies on schistosome immunology and genomics.

Methods

Laboratory animals and parasites

Eight-week-old male C57BL/6j mice, from the National Laboratory Animal Center, Taipei, Taiwan were obtained and used in this study. The interferon gamma (IFN-γ): hThy1, IL-4:mThy1.1 double transgenic mice (T1/T2 doubly transgenic mice, BALB/c genetic background) were generated and maintained as previously described.[39] In brief, primers Tg1 and Tg2 were used to amplify the *mIFN-γ*:*hThy1* transgene (T1), and primers Tg3 and Tg4 were used to amplify the *mIL-4*:*mThy1.1* transgene (T2). The DNA sequences of the PCR primers were as follows: Tg1, '-GCTGTCTCATCGTCAGAGAGC-3'; Tg2, '-TCAAGGACAGGAGATCTTAGGG-3'; Tg3, '-CCAAGATATCAGAGTTTCCAAGG-3'; and Tg4, '-AGAGGCTACTTCCCGGGATG-3'. A 600 bp DNA fragment was amplified by primers Tg1/Tg2, and another 600 bp DNA fragment was also amplified by primers Tg3/Tg4 (see supplemental 1). Animals were provided with water and mouse chow (Labdiet 5001, PMI Nutrition International LLC, MO) ad libitum. Animals were housed in a rodent facility at 22 $\pm$ 1 °C with a 12-h light—dark cycle for acclimatization.

The schistosomes used in this experiment were the Hubei strain of *S. japonicum* (SjCH) from Tongji Medical University of Hubei Province, China. The parasite was maintained in laboratory passage in *Oncomelania hupensis chiui* snails and C57BL/6j mice. Each mouse was infected with 30 cercariae in precutaneous infection mode to maintain life cycle.

Ethics statement

Animal experiments were carried out under humane conditions with approval with license number: LAC-101-0304 from the Institutional Animal Care and Use Committee (IACUC) of Taipei Medical University, and conducted in accordance with NIH Guide for the Care and Use of Laboratory Animals (DHHS publication No. NIH 85—23, revised 1996).

Animal experiments

Three groups of six to eight weeks ages old C57BL/6j male mice were used in this experiments. In the first group, C57BL/6j male mice were divided into five subgroups. Each mouse here was infected with 35 cercariae through skin infection. Mouse spleens were removed for subsequent microarray tests at 1, 4.5, 8, 12, and 18 weeks post-infection (p.i.) (n = 5 for each subgroup). A control group was used which consisted of the same number of normal mice. The second group of C57BL/6j male mice was divided into seven subgroups (n = 7 for each subgroup), and were all infected with *S. japonicum* in the same mode. The mice infected with the cercariae were sacrificed, and adult worms were isolated from the portal and mesenteric veins and counted. Sera from the group infected with *S. japonicum* were collected at 1, 3, 5, 7, 9, 12, and 18 weeks p.i. These mice were then sacrificed, and their spleens and livers were then harvested for real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis and histopathological experiments, respectively. The other group of C57BL/6j male mice were infected in the same mode and divided into seven subgroups, too (n = 5 for each subgroup). These mice were sacrificed and the splenocytes were removed for FACS at 1, 3, 5, 7, 9, 12, and 18 weeks p.i. Non-infected mice were used as both control groups. The screened T1/T2 double transgenic mice with infection were also divided into seven groups (n = 5 for each group), and then treated as described above at the same time point p.i. for FACS tests. Non-infected transgenic mice were used as the control groups.

Histopathological treatment

Mice sacrificed and the entire liver of each mouse was then removed and treated immediately. Liver tissue specimens (0.1 cm^3) were collected and fixed immediately in 10% neutral formalin. The fixed specimens were embedded in paraffin and cut into 5 μm-thick sections. Tissue specimens from similar locations in the liver of uninfected mice served as controls. These sections were stained with hematoxylin-eosin (H&E) and observed under a microscope for inflammatory responses and granuloma formation.

Preparation of spleen cells

After the spleen of a mouse was taken out and put in a dish, a 2.5 ml syringe with 23 G needle containing 10%FBS-RPMI culture fluid (containing 100 U/ml penicillin (Sigma, St. Louis, MO, USA), 0.1 mg/ml streptomycin (Sigma)) was used for washing out the spleen cells. Then the cell suspension was added into 5 ml Ficoll-Paque™ PLUS (GE Healthcare, Sweden), centrifuged at 2,000 rpm and 25 °C for 35 min. Afterwards, the lymphocytes were removed and avoided to aspirate granulocyte layer; then the same amount of FBS-RPMI culture fluid was added, and then centrifuged at 1,800 rpm and 4 °C for 5 min. After the supernatant was removed, 10 ml RBC lysis buffer (0.15M NH4Cl, 1 mM KHCO3, 0.1 mM Na2EDTA, pH = 7.2—7.4) were added, mixed gently, centrifuged at 1,800 rpm and 4 °C for 5 min to break the erythrocytes. Then the supernatant was removed, cleaned with FBS-RMPI culture fluid once. The cell density was adjusted for subsequent experiments. In addition, the spleen from another group of mice for FACS test was taken out and the cells were washed as above and directly put in a 15 ml centrifuge tube, centrifuged at 1,800 rpm and 4 °C for 5 min. When the supernatant was removed, 10 ml RBC lysis buffer were added and mixed uniformly to break the erythrocytes, centrifuged at

1,800 rpm and 4 °C for 5 min. Then the supernatant was removed, cleaned with FBS-RMPI culture fluid once, and the cell density was adjusted for subsequent experiments.

Use of the magnetic-activated cell sorting (MACS) system for purifying macrophages

Spleen cells of the mice treated as described above were collected. The $CD11b^+$ cells were purified from total spleen cells, and lymphocytes were removed using the Ficoll—Paque™ Plus (BD Biosciences), while carefully avoiding the aspiration of the granulocyte layer. The remainder were then further purified using MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) to obtain $CD11b^+$ cells, by using magnetic microbeads with a CD11b marker. Cell purities were determined to be >95% by flow cytometry using anti-CD11b antibodies (BD Biosciences). All purified cells were also stained with anti-F4/80 antibodies (BD Biosciences) to determine whether they had the characteristics of macrophages (Supplemental Fig. 2A).

Preparation of splenic macrophage RNA

The preparation of macrophage RNA for microarray experiments was commissioned to Welgene Biotech Co., Ltd. for the purification of the nucleic acid. Total RNA was extracted from the purified macrophages using TRIzol ® Reagent (Invitrogen, USA), and then using an RNeasy Mini Kit (Qiagen, Germany). Purified RNA was quantified at OD260 nm using an ND-1000 spectrophotometer (Nanodrop Technology, USA) and quantified using a Bioanalyzer 2100 (Agilent Technology, USA) with an RNA 6000 Nano LabChip® Kit (Agilent Technologies, USA). The purity of all measured RNA specimens was above a ratio of 2.0. There were two independent peak values of 28S and 18S in each specimen, and all RNA integrity number (RIN) values were above 7 (Supplemental Fig. 2B and 2C).

Microarray experiment

The microarray experiment was also commissioned to Welgene Biotech, Co., Ltd. to make the experimental analysis of gene arrays. This experiment adopted Agilent Technologies, Inc. (CA) and Agilent custom mouse immune arrays. The custom array collected 15,051 probes representing 9704 genes related to the function categories of immune, stress, cytotoxicity, osteoclast, myeloid cell, signal transduction, cytokine and inflammatory. The design format was based on Agilent custom 8 pack design, in which each pack contains identical 15,744 features with 82 columns and 192 rows. 0.5 μg of total RNA was amplified by a low RNA input fluor linear amp kit (Agilent Technologies, USA) and labeled with Cy3 or Cy5 (CyDye, PerkinElmer, USA) during the *in vitro* transcription process. The infected test group RNA was labeled by Cy5 and RNA from the normal control group was labeled by Cy3. 2 μg of Cy-labled cRNA was fragmented to an average size of about 50—100 nucleotides by incubation with a fragmentation buffer at 60 °C for 30 min. Correspondingly fragmented labeled cRNA is then pooled and hybridized to Agilent custom mouse oligo microarray (Agilent Technologies, USA) at 60 °C for 17 h. After washing and drying by nitrogen gun blowing, microarrays are scanned with an Agilent microarray scanner (Agilent Technologies, USA) at 535 nm for Cy3 and 625 nm for Cy5. Scanned images are analyzed by Feature extraction 9.1 software (Agilent Technologies, USA), an image analysis and normalization software is used to quantify signal and background intensity for each feature, substantially normalizing the data by rank-consistency-filtering LOWESS method (See supplemental 2D). Related microarray data has been submitted to the Gene Expression Omnibus (GEO), and the accession number is GSE46719. Those that performed abnormally or with excessive background values were deleted. Statistical significance was set at a fold change cutoff of 4.0. Genes with inconsistent reactions in two chips were also removed.

Isolation of RNA, cDNA synthesis, and real-time reverse transcriptase-polymerase chain reaction (real-time RT-PCR)

Real-time polymerase chain reaction (PCR) was used for mRNA quantification assessment. Total RNA was reverse transcribed using the Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Promega, Madison, WI, USA) to generate cDNA. Each cDNA pool was stored at −20 °C until real-time PCR analysis was completed. Primer pair specificity was validated by performing RT-PCR using common reference RNA (Stratagene, La Jolla, CA, USA) as a DNA template. The primers used were shown in Table 1, and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18s were used as endogenous reference genes. Real-time PCR was performed using the lightcycler® nano real-time PCR system (Roche Diagnostics, Mannheim, Germany) using LightCycler® 480 SYBR Green I Master (Roche Diagnostics). Briefly, 10 μl reactions were utilized, containing 2 μl of Master Mix, 2 μl of 0.75 μM forward primer and reverse primer, and 6 μl of the cDNA sample. Each sample was run in triplicate. The real time PCR program included 3 min at 95 °C; 45 cycles of 10 s durations at 95 °C, and 30 s at 60 °C. Data analysis was performed using the LightCycler Nano software version 1.0 (Roche). The gene was considered differentially expressed only when there was an upregulation of >2-fold or a downregulation of <0.5-fold.

FACS tests of cell surface markers

5% CD16/CD32 antibodies (2.4G2, Pharmingen, San Diego, Calif.) 100 μl were added in the prepared spleen cells then put on ice and reacted for 30 min to block the binding site of non-antigenic specificity. Afterwards washed with PBS, the cells were reacted with 10 μl biotin-binding monoclonal antibodies on ice for 30 min, and centrifuged after PBS washed. Then fluorochrome isothiocyanate (FITC), phycoerythrin (PE) and Cy-chrome (Cy) labeling monoclonal antibodies were added and stained at 4 °C for 30 min, and the cells were transferred to the flow cytometer tubes (FALCON® 352052) after washed with 1xPBS twice, and analyzed by FACS flow cytometer (Becton Dickenson Biosciences, San Jose, CA), finally analyzed by CellQuest™ software (Becton Dickenson

Table 1 Primer pairs for candidate genes used in real time PCR.

Gene name	Primer sequences
Interleukin (IL)-12α	forward 5′-ACA TGG TGA AGA CGG CCA G-3′ reverse 5′-GAA GTC TCT CTA GTA GCC AG-3′
IL-12β	forward 5′-TGC TCA TGG CTG GTG CAA AGA AAC-3′ reverse 5′-AGA CGC CAT TCC ACA TGT CAC TG-3′
Interferon-γ (INF-γ)	forward: 5′-CTT CCT CAT GGC TGT TTC TG-3′ reverse: 5′-TGT CAC CAT CCT TTT GCC AG-3′
IL-1α	forward: 5′-ACG GCT GAG TTT CAG TGA GAC C-3′ reverse: 5′-CAC TCT GGT AGG TGT AAG GTG C-3′
T-bet	forward: 5′-CAC TAA GCA AGG ACG GCG AA-3′ reverse: 5′-CCA CCA AGA CCA CAT CCA CA-3′
CCL5	forward: 5′-CCT GCT GCT TTG CCT ACC TCT C-3′ reverse: 5′-ACA CAC TTG GCG GTT CCT TCG A-3′
IL-4	forward: 5′-CAG AGA GTG AGC TCG TCT G-3′ reverse: 5′-GGT GCA GCT TAT CGA TGA ATC-3′
IL-4Rα	forward: 5′-ATC TGC GTG CTT GCT GGT TCT-3′ reverse: 5′-CTG GTA TCT GTC TGA TTG GAC CG-3′
IL-13	forward: 5′- GAC CCA GAG GAT ATT GCA TG -3′ reverse: 5′- CCA GCA AAG TCT GAT GTG AG -3′
IL-13Rα1	forward: 5′-CAT CTT CTC CTC AAA AAT GGT GCC-3′ reverse: 5′-GGA TTA TGA CTG CCA CTG CGA C-3′
Arg-1	forward: 5′-GGG TGG AGA CCA CAG TCT G-3′ reverse: 5′-AGT GTT GAT GTC AGT GTG AGC-3′
YM-1	forward: 5′-TTA TCC TGA GTG ACC CTT CTA AG-3′ reverse: 5′-TCA TTA CCC TGA TAG GCA TAG G-3′
IL-27Rα1	forward: 5′- CCA ACC TGT CTC TGG TGT GCT T -3′ reverse: 5′- TAC TCC AAC GGT TTC CTG GTC C -3′
IL-21R	forward: 5′- CAC TGA CTA CCT CTG GAC CAT C -3′ reverse: 5′- GCA GAA GGT CTC TTG GTC CTG A -3′
IL-1β	forward: 5′- TTG AAG AAG AGC CCA TCC TC -3′ reverse: 5′- CAG CTC ATA TGG GTC CGA C -3′
IL-6	forward: 5′- GCC TTC CCT ACT TCA CAA GT -3′ reverse: 5′- GAA TTG CCA TTG CAC AAC TCT -3′
CCL2	forward: 5′-ACT CAC CTG CTG CTA CTC ATT CAC-3′ reverse: 5′-ATG TAT GTC TGG ACC CAT TCC TTC-3′
Tumor Necrosis Factor -alpha (TNF-α)	forward: 5′- CCT CAC ACT CAG ATC ATC TTC -3′ reverse: 5′- CGG CTG GCA CCA CTA GTT G -3′
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	forward: 5′- GCA TCC ACT GGT GCT GCC -3′ reverse: 5′- TCA TCA TAC TTG GCA GGT TTC -3′
18s	forward: 5′- ACA ATA CAG GAC TCT TTC GAG -3′ reverse: 5′- GAG CTT TTT AAC TGC AGC AAC -3′

Biosciences). Required antibodies for flow cytometry analysis were listed in Table 2.

Cytokine ELISA

Concentrations of IFN-γ, IL-2, IL-4, IL-10, IL-5, and tumor necrosis factor alpha (TNF-α) in the sera of normal control mice and mice infected at different time points were measured using standard sandwich ELISA kits (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Briefly, 96-well ELISA plates were coated overnight at 4 °C with 100 μl capture antibody. The next day, the capture antibody was discarded, wells were washed, and 200 μl ELISA/ELISASPOT diluent was added. After 1 h incubation, the wells were washed and incubated with 100 μl of serum samples or standards for 2 h. Plates were then washed and 100 μl of detection antibodies was added and incubated for 1 h. The wells were washed and incubated with 100 μl of Avidin-horseradish peroxidase enzyme for 30 min. After washing, 100 μl of 3,3′,5,5′-tetramethylbenzidine substrate was added and incubated for 15 min. In order to stop the reaction, 50 μl 10% sulfuric acid was applied, and the optical density was determined at 450 nm.

Statistical analysis

The experimental data were used to calculate the average mean and standard deviation. Analysis of variance was used to analyze the differences in cytokine or cell marker expression between various infected groups and controls. Statistical differences between groups

Table 2 Required fluorescent antibodies for flow cytometry analysis. The antibodies used in the experiment were from Serotec (Pharmingen, San Diego, CA), the types are listed as below.

Cell type	fluorescent antibody	Cell clone and company brand
$CD4^+$	FITC conjugated anti-mouse CD4	L3T4, clone RM4-5, Pharmingen
$CD8^+$	Cy-chrome-conjugated anti-mouse CD8α	Ly2, clone 53—6.7, Pharmingen
B	FITC-conjugated anti-mouse CD45R/B220	clone RA3-6B2, Pharmingen
Macrophage	FITC-conjugated anti-mouse CD11b/Mac-1	clone M1/70, Pharmingen
	PE-conjugated anti-MHC class II (I-A/I-E)	clone M5/114, Pharmingen
	APC-conjugated anti-mouse CD8α	Ly2, clone 53—6.7, Pharmingen
Type1	PE-conjugated anti-human CD90	L3T4, clone RM4-5, Pharmingen
Type2	Cy-chrome-conjugated streptavidin and biotin-conjugated anti-mouse thy1.1	Pharmingen clone OX-7, Pharmingen

were determined using Student's *t*-test. Statistical significance was set at $p < 0.05$.

Results

The worm burden and liver immunohistological changes of infected mice were examined at different time-points

To confirm the pathological changes at different time points after infection, mice infected with 35 cercariae were sacrificed at 1, 3, 5, 7, 9, 12, and 18 weeks p.i., and adult worms and liver tissues were collected to analyze worm burden and schistosome egg-induced pathogenesis. As shown in Fig. 1A, approximately 10—20 *S. japonicum* adult worms were observed from 5 to 9 weeks p.i., and decreased to less than 10 worms at 12—18 weeks p.i. Moreover, eggs were observed in the liver at 5 weeks p.i. and pathological injury of the liver was initiated significantly from 7 weeks p.i. onwards, which gradually increased with granuloma formation (12 weeks) and fibrosis (18 weeks) in the liver (Fig. 1B).

Analysis of specific gene expression profiles of spleen $CD11b^+$ cell RNA of infected mice at different time points

The mouse cDNA custom microarray used in this experiment was identical as our previous report.[37] We here focused on investigate the role of the macrophages in immune responses after infected with *S. japonicum* and was conducted based on seven functional classifications: inflammation, cytokine, cytotoxicity, myeloid cell, superoxide, osteoclast, and other immune responses. Two independent experiments were normalized and 2473 unique probes were sorted out by the gene expression rates of more than 2 or less than 0.5 were selected as significant differences. 303 unigenes were analyzed to determine the profile changes at each time point of different stages. The difference in the gene expression profile of specific gene clusters from mice splenic macrophages by hierarchical gene tree analysis was significant as the infection time grew.

According to the aforesaid data, the splenic macrophages experienced a large gene expression profile change between 4.5 weeks and 8 weeks after being infected with *S. japonicum*. Therefore, GeneSpring was used to screen the genes with obvious changes in macrophage gene expression during this transformation period (switch point, 4.5—8wk pi), and sorted them according to their functions in order to discuss the trend of the expression profiles of gene clusters related to Inflammation, Cytokine, Cytotoxicity, and Immune response (Fig. 2, **for detailed data presented as** Tables 3—6).

1. Gene clusters with obvious changes in the gene expression of splenic macrophages, occurring at the switch point after the mice were infected with *S. japonicum*, and which is related to inflammation (Table 3). This cluster mainly includes two groups of genes with functions related to inflammation that have similar expression trends from repression to inducement or from inducement to repression, and between 4.5 weeks and 8 weeks. Those genes upregulated after 4.5 weeks are analyzed, Attractin *(Atrn)* and P-selectin *(Selp)* would promote the aggregation and adhesion of immunocytes,[42,43] and the chemokine monocyte chemotactic protein-3 *(CCL7)* would also promote the chemotaxis of monocytes, and the enhancement of chemokine receptor *CCR1* expression shows the ability of macrophages to accept the stimulation of chemokines, which is obviously promoted when the eggs appear.[44,45] Toll-interleukin 1 receptor domain-containing adapter protein (*Tirap*) is related to the information transfer of TLR4.[46] As for the genes repressed by eggs after 4.5 weeks, including chemokines *Cxcl9* and *Cxcl10* subjected to interferon-inducibility, the functions promote the chemotaxis and maturity of Th1 cell.[47,48] Lymphotoxin α (*Lta*) modulates inflammatory, immunostimulatory, and antiviral reactivities, it also influences inflammation index factor macrophage inflammatory protein -3β (*CCL19*) chemotactic Type 1 immune response.[49] *CD180* and Lymphocyte antigen (*Ly86*) jointly modulate the information transfer of TLR4,[50] and *TLR3* and Protein kinase C, α (*Prkca*) influence antiviral cellular poisoning response.[51] According to the above data, after 4.5 weeks, the inflammatory active genes of macrophages, especially those related to cell-mediated effector responses were repressed, but the genes related to cell proliferation, chemotaxis, and adhesion functions are upregulated.

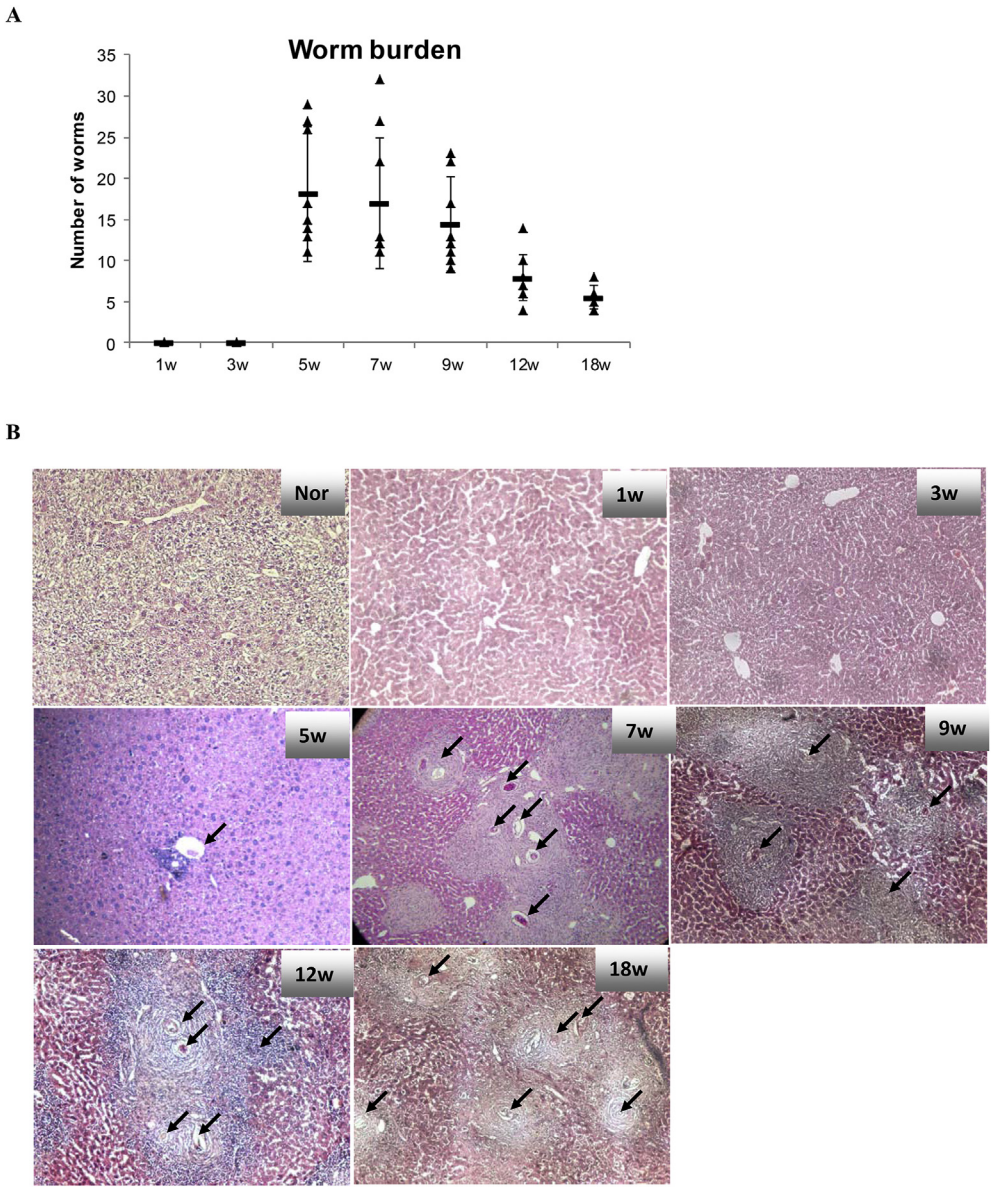

Fig. 1. Worm burden and liver pathology induced by schsitosome infection at different time points. (A). Mice after the different times of infection were sacrificed respectively and the adult worm burdens in each group were determined. Data from 3 separate experiments are expressed as mean ± SD. (B) Histopathology of liver tissue sections of normal (Nor) or *S. japonicum*-infected mice at different time points (as indicated week of post-infection). Paraffin-embedded liver tissues from schistosoma-infected mice were stained using H&E. Tissue images were captured with a microscope (Leica) under 100 × magnifications. Sites of egg deposition are shown with arrows.

2. Gene clusters with obvious changes in the gene expressions of splenic macrophages, at the switch point after *S. japonicum* post-infection, and which is related to cytokine (Table 4). This cluster also includes two groups of genes related to cytokine that have similar expression trends from repression to inducement or from inducement to repression, which is between 4.5 weeks and 8 weeks. The upregulated clusters include a Chemokine-like factor (*Cklf*), chemotactic immunocyte aggregation.[52] *IL-11* cytokine gene promoting the generation of bone cell and acute-phase proteins, Colony stimulating factor 2 receptorβ(*Csf2rb1, 2*) is the receptor of IL-3/IL-5/GM-CSF cytokines; *IL-1r2*, *IL-1rap*, as expressed in Type2 macrophages and *IL-18r1* are IL-1 receptor family receptor, they can repress or modulate the function of IL-18, IL-1 cytokines in activating macrophages.[44,53] *IL-28rα*, *IL-8rα/β* receptors also increase the specificity of IL-28, IL-8 chemotaxis and effect[54]; Irak3 differentiates monocytes into macrophages. The repressed clusters, such as Bone morphogenetic proteins (*Bmp3/7*), induce the formation of bone and cartilage. However, it restores the expression in 12 weeks p. i.; macrophage inflammatory protein-1γ (*CCL9*) activates osteoclasts through a Ccr1 receptor. Macrophage chemokine receptors *Ccr6* and *Ccr9* influence subsequent maturity and differentiation of B-cells and T-cells, respectively; whereas, *Cxcr3*

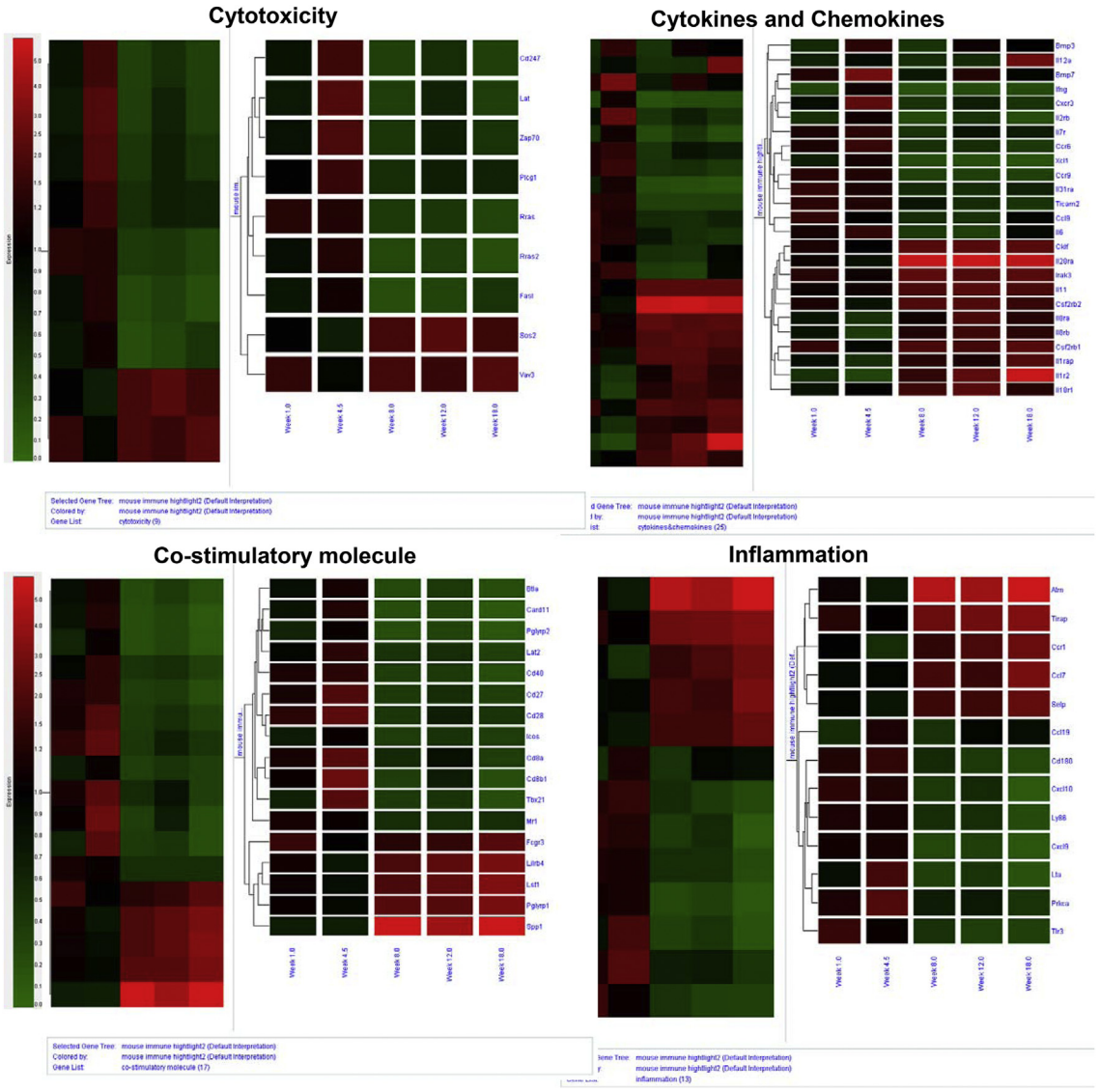

Fig. 2. Analyzing the microarray expression of specific genes of splenic CD11b $^{+}$ cell from the mice infected with *S. japonicum* at different time points. Non-infected mice are classified as the control group, compared with the test group of 1 week, 4.5 weeks, 8 weeks, 12 weeks and 18 weeks post-infection, different expression change genes are classified by cluster analysis. The hierarchical clustering result shows four clusters of different expression profiles, including Cytotoxicity, Cytokines and Chemokines, Co-stimulatory molecule, and Inflammation-related gene clusters are divided. Red indicates induced genes, green repressed genes, and black indicates genes without significant difference. See the information of Tables 3—6 for gene designations and expression magnification values and images.

promotes a Th1 reaction as affected by Cxcl9 and Cxcl10 [48]. Two typical genes of Type1 immune response, Interferon γ (*IFN*γ) and *IL- 12*α are downregulated when eggs appear, the inflammation related cytokine *IL-6* also shows a similar type. However, *IL-12*α reappears in 18 weeks p. i.. The decrease in *IL-7r*, *IL-2r*β and *IL- 31ra* cytokines receptors means a decrease in the effects of *IL-7*, *IL-2* and *IL-31*.[54] Toll-like receptor adaptor molecule 2 (*Ticam2*) is Toll/interleukin-1 receptor, concerned with the signal transduction of Toll receptor.[55] *Xcl1* is related to the activation of CD8 T-cell.[56] This cluster shows the regulations of the whole macrophage on Type1 immunity are changed from an upward expression in 4.5 weeks to repression after 8 weeks, and the upregulated genes are mostly cytokines modulating the cell growth. Another important point is that many changes related to cytokine genes are receptors rather than cytokines themselves, whether the receptor accepts the specificity of cytokine factors in immunoregulation is more important than the number of factors.

3. Gene clusters with obvious changes in the gene expressions of splenic macrophages, at the switch point after *S. japonicum* post-infection, and which are related to cytotoxicity (Table 5). This cluster includes Cytotoxicity related genes that have similar expression trends from repression to inducement or from inducement to repression, which are between 4.5 weeks and 8 weeks. There are only two upregulated genes after 8 weeks, as

Table 3 Inflammation related genes with significant change in mRNA expression of macrophage in spleen cells of the mice infected with *S. japonicum* at the switch point (4.5—8 weeks) as compared with normal mice.

Change of gene expression	Category and gene symbol	Common Name	Description and GeneBank accession no.
Induced	Atrn	mg; Mgca; AW558010; mahogany; mKIAA0548	*Mus musculus* attractin, mRNA [NM_009730]
	Ccl7	fic; marc; mcp3; MCP-3	*Mus musculus* chemokine (C—C motif) ligand 7, mRNA [NM_013654]
	Ccr1	Cmkbr1; Mip-1a-R	*Mus musculus* chemokine (C—C motif) receptor 1, mRNA [NM_009912]
	Selp	Grmp; CD62P; P-selectin	*Mus musculus* selectin, platelet, mRNA [NM_011347]
	Tirap	Mal; wyatt; Tlr4ap; AA407980; C130027E04Rik	*Mus musculus* 16 days embryo head cDNA, RIKEN full-length enriched library, clone:C130027E04 product:toll-interleukin 1 receptor (TIR) domain-containing adaptor protein, full insert sequence [AK081514.1]
Inhibitive	Ccl19	ELC; CKb11; MIP3B	*Mus musculus* chemokine (C—C motif) ligand 19, mRNA [NM_011888]
	CD180	Ly78; RP105; F630107B15	*Mus musculus* CD180 antigen, mRNA [NM_008533]
	Cxcl10	C7; IP10; CRG-2; INP10; IP-10; Ifi10; mob-1; gIP-10	*Mus musculus* chemokine (C-X-C motif) ligand 10, mRNA [NM_021274]
	Cxcl9	CMK; Mig; Scyb9; crg-10; BB139920	*Mus musculus* chemokine (C-X-C motif) ligand 9, mRNA [NM_008599]
	Lta	LT; Ltx; Tnfb; TNFSF1; LTalpha; Tnfsf1b; LT-alpha	*Mus musculus* lymphotoxin A, mRNA [NM_010735]
	Ly86	MD1; MD-1	*Mus musculus* lymphocyte antigen 86, mRNA [NM_010745]
	Prkca	Pkca	*Mus musculus* protein kinase C, alpha, mRNA [NM_011101]
	Tlr3	AI957183	*Mus musculus* toll-like receptor 3, mRNA [NM_126166]

based on analysis, Son of Sevenless 2 (*Sos2*) activates Ras/MAP kinase pathway,[57] and *Vav3* influences *FcγR* mediated cell activation initiating oxidative burst.[58] On the contrary, 7 genes are repressed by the eggs after 8 weeks, T-cell receptor zeta (*CD247*) is combined with Zeta-chain - associated protein kinase 70 (*Zap70*), in order to phosphorylate the Linker of Activated T cells (Lat) protein and Phospholipase C,γ1(*Plcg1*) protein functions to activate Ras, and play a leading role in initiating the cytotoxicity of T-cells and NK cells.[59,60] The function of the Fas ligand (*Fasl*) is the activation of cytotoxic T-cells and the cell apoptosis[61]; whereas, related RAS viral (*Rras*) and *Rras2* modulate ras viral oncogene to initiate apoptosis and NK antivirus functions.[62] This cluster shows that the infection obviously represses the ability of the host's macrophages to modulate cytotoxicity.

4. Gene clusters with obvious changes in the gene expressions of splenic macrophages, at the switch point after *S. japonicum* post-infection, and which is related to the immune response (Table 6). This cluster includes immune response related genes changed from repression to inducement, or from inducement to repression, which is between 4.5 weeks and 8 weeks. The trend is that there are 5 upregulated genes, such as Fc fragment of IgG, low affinity III, and the receptors (*Fcgr3*) are antibody receptors that influence the ability of phagocytosis or anti-microbes.[63] Leukocyte immunoglobulin-like receptors, subfamily B, member 4 (*Lilrb4*) would combine MHC class I to repress the immune response from being stimulated.[64] Leukocyte specific transcript 1 (*Lst1*) is related to the repression of lymphopoiesis and the maturity of dendritic cells.[65] Peptidoglycan recognition protein 1 (*Pglyrp1*) is a carbohydrate recognition antigen, concerned with microbes surface antigen recognition.[66] Osteopontin (*Spp1*) is induced by Bmp protein, the functions include regulation and control in the initial stages of immunity, and the formation of granuloma and tissue fibrosis.[67] In comparison to the induced genes, the repressed immune genes are the majority. B and T lymphocyte attenuator (*Btla*) are presented in Th1 cell, and combine with TNFSF14 to negatively modulate the immune response of lymphocytes.[68] Caspase recruitment domain family, member 11 (*Card11*) modulates cell apoptosis and NF-κB activation.[69] This cluster also includes many common cell surface markers, such as *CD27, CD28, CD40, CD8α/β 1*, and Inducible T-cell co-stimulators (*Icos*), which modulate and control the immunologic functions of B, T-cell hyperplasia differentiation and formation of germinal

Table 4 Cytokine related genes with significant change in mRNA expression of Macrophage in spleen cells of the mice infected with *S. japonicum* at the switch point (4.5—8 weeks) as compared with normal mice.

Change of gene expression	Category and gene symbol	Common Name	Description and GeneBank accession no.
Induced	Cklf	C32; CKLF1; CKLF3; CKLF4; CKLF5; Cklf2	*Mus musculus* chemokine-like factor, transcript variant 1, mRNA [NM_029295]
	Csf2rb1	Bc; Il3r; AIC2B; Il3rb; Il5rb; CDw131; Il3rb1	*Mus musculus* NOD-derived CD11c + ve dendritic cells cDNA, RIKEN full-length enriched library, clone:F630014N20 product:colony stimulating factor 2 receptor, beta 1, low-affinity (granulocyte-macrophage), full insert sequence. [AK154286]
	Csf2rb2	Bil3; Il3r; AIC2A; Il3rb; Il3rb2; BetaIl3; Csfgmrb	*Mus musculus* colony stimulating factor 2 receptor, beta 2, low-affinity (granulocyte-macrophage), mRNA [NM_007781]
	Il11	IL-11	*Mus musculus* interleukin 11, mRNA [NM_008350]
	Il18r1	Il1rrp; Il18ralpha	*Mus musculus* interleukin 18 receptor 1, mRNA [NM_008365]
	Il1r2	CD121b; Il1r-2	*Mus musculus* interleukin 1 receptor, type II, mRNA [NM_010555]
	Il1rap	AI255955; AV239853; IL-1RAcP; IL-1R AcP	*Mus musculus* interleukin 1 receptor accessory protein, transcript variant 1, mRNA [NM_008364]
	Il28ra	IFNLR1; CRF2-12	*Mus musculus* interleukin 28 receptor alpha, mRNA [NM_174851]
	Il8ra	CXCR1	*Mus musculus* interleukin 8 receptor, alpha, mRNA [NM_178241]
	Il8rb	CD128; CXCR2; IL8RA; CDw128; IL-8Rh; IL-8rb	*Mus musculus* interleukin 8 receptor, beta, mRNA [NM_009909]
	Irak3	IRAK-M; AI563835; 4833428C18Rik	*Mus musculus* interleukin-1 receptor-associated kinase 3, mRNA [NM_028679]
Inhibitive	Bmp3	9130206H07; 9530029I04Rik	*Mus musculus* bone morphogenetic protein 3, mRNA [NM_173404]
	Bmp7	OP1	*Mus musculus* bone morphogenetic protein 7, mRNA [NM_007557]
	Ccl9	CCF18; MRP-2; Scya9	*Mus musculus* chemokine (C—C motif) ligand 9, mRNA [NM_011338]
	Ccr6	Cmkbr6	*Mus musculus* chemokine (C—C motif) receptor 6, mRNA [NM_009835]
	Ccr9	Cmkbr10; GPR-9-6	*Mus musculus* chemokine (C—C motif) receptor 9, mRNA [NM_009913]
	Cxcr3	CD183; Cmkar3	*Mus musculus* chemokine (C-X-C motif) receptor 3, mRNA [NM_009910]
	Ifng	Ifg; IFN-g; IFN-gamma	*Mus musculus* interferon gamma, mRNA [NM_008337]
	Il12a	p35; Ll12a; Il-12a; IL-12p35	*Mus musculus* interleukin 12a, mRNA [NM_008351]
	Il2rb	p70; CD122; Il-2/15Rbeta	*Mus musculus* interleukin 2 receptor, beta chain, mRNA [NM_008368]
	Il31ra	Il31ra	*Mus musculus* interleukin 31 receptor A, mRNA [NM_139299]
	Il6	Il-6	*Mus musculus* interleukin 6, mRNA [NM_031168]
	Il7r	CD127; IL-7Ralpha	*Mus musculus* interleukin 7 receptor, mRNA [NM_008372]
	Ticam2	TRAM; Tirp; Trif; TICAM-2	*Mus musculus* toll-like receptor adaptor molecule 2, mRNA [NM_173394]
	Xcl1	LTN; ATAC; Lptn; SCM-1	*Mus musculus* chemokine (C motif) ligand 1, mRNA [NM_008510]

Table 5 Cytotoxicity related genes with significant change in mRNA expression of Macrophage in spleen cells of the mice infected with *S. japonicum* at the switch point (4.5—8 weeks) as compared with normal mice.

Change of gene expression	Category and gene symbol	Common Name	Description and GeneBank accession no.
Induced	Sos2	Sos2	PREDICTED: *Mus musculus* Son of sevenless homolog 2 (Drosophila), mRNA [XM_127051]
	Vav3	AA986410; MGC27838; A530094I06Rik	*Mus musculus* vav 3 oncogene, transcript variant 2, mRNA [NM_013654.3]
Inhibitive	CD247	CD3; T3z; CD3h; CD3z; TCRk; Tcrz	*Mus musculus* CD247 antigen, mRNA (cDNA clone MGC: 60425 IMAGE: 30074812), complete cds. [BC052824.1]
	Fasl	gld; CD178; CD95L; Fas-L; Faslg; Tnfsf6; Fas-Ligand	*Mus musculus* Fas ligand (TNF superfamily, member 6), mRNA [NM_010177]
	Lat	Lat	*Mus musculus* linker for activation of T cells, mRNA [NM_010689]
	Plcg1	CDed; Plc-1; Plcg-1; AI894140; Plc-gamma1	*Mus musculus* 2 days neonate thymus thymic cells cDNA, RIKEN full-length enriched library, clone: E430007P16 product:hypothetical protein, full insert sequence. [AK169695]
	Rras	AI573426; MGC129444; MGC129445	*Mus musculus* Harvey rat sarcoma oncogene, subgroup R, mRNA [NM_009101]
	Rras2	TC21; C86394; 2610016H24Rik	*Mus musculus* related RAS viral (r-ras) oncogene homolog 2, mRNA [NM_025846]
	Zap70	Srk; TZK; mur; 70 kDa; mrtle; ZAP-70; AI327364	*Mus musculus* zeta-chain (TCR) associated protein kinase, mRNA [NM_009539]

centers.[45] Linkers for activation of T cells family, member 2 (*Lat2*) is related to the activation of B-cells.[70] Major histocompatibility complex, class I-related (*Mr1*) is concerned with MHC class1 regulated cellular poisoning reaction.[71] Unlike the rising trend of *Pglyrp1*, Peptidoglycan recognition protein 2 (*Pglyrp2*) shows a descending trend.[72] T-box 21 (*Tbx21*) is the specific transcription factor of Th1 cell, inducing IFN-γ to regulate the activation of Th1 reaction. This cluster shows the regulation of schistosome infection on macrophages, there is an apparent inhibitory action when the eggs appear, and the upregulated gene clusters contain negatively modulated factors, which are otherwise tissue repair related. An obvious down-regulation in immune response is found in the repressed immune gene clusters, especially Type1 immunity.

The expression trend of spleen CD11b$^+$ cell typing labeled genes of the infected mice at different time points

In order to prove the changes in macrophage typing from M1(CAMϕ) to M2 (AAMϕ) in the course of *S. japonicum* infection resulted in different switches of the gene expression profile, these two subtypes of indexed representative genes were screened, then to discuss the trend of these genes and the influences on macrophage phenotype changes. Fig. 3A showed the histograms of expression trends of AAMϕ index gene Chitinase 3-like protein 3 and 4 (*YM1, YM2*)[73,74] and AAMϕ differentiation induced associated factors *IL-4Rα, IL-13Rα 1, IL-21R, and IL-27Rα* at different time points.[27,75—77] The result showed that *YM1* and *YM2* were repressed before 4.5 week, in the early stage of infection. However, they obviously upregulated at 8 week, when a large number of eggs appeared, and then continued rising until reaching the maximum at 18 week. On the other hand, the receptors of *IL-4* and *IL-13*, the major factors of AAMϕ phenotype differentiation, *IL-4Rα* and *IL-13Rα1* reached the maximum at 12 week. In addition, the expression of *IL-13Rα1* was repressed before 8 week, but *IL- 4Rα* was not. The other two receptors *IL-21R* and *IL-27Rα* regulated AAMϕ phenotype differentiation, and were presented on the AAMϕ. However, the result was unexpectedly contrary to the AAMϕ expression trend, which reached the maximum at 4.5 week, and was repressed after 8 weeks. On the contrary, Fig. 3B showed the expression trend of factors chemokine (C—C motif) ligand 5 (*Ccl5*), *IFN-γ*, *IL-1α*, and *IL-12α/β* as closely related to inducement of activated CAMϕ at various time points.[44] *Ccl5* and *IFN-γ*, which were related to inflammatory and Type1 responses, respectively, reached the maximum at 4.5 week, and declined after 8 week. Another pro-inflammatory *IL-1α* becomes repressed instantly at 1 week p. i.. The expression of two subunits *IL-12α/β* of IL-12, was similar to IFN-γ, which declined obviously after 8 weeks. But *IL-12α* rather had a strong inducement of expression at 18 weeks in the chronic stage. Therefore, it is proven that the macrophages in the spleen of mouse changed from to between 4.5 week and 8 week p. i., and this transformation may be accomplished by the microenvironment jointly effects that regulated synergistically the complicated immunocyte factors from the schistosome and the host interaction.

Table 6 Immune responses related genes with significant change in mRNA expression of Macrophage in spleen cells of the mice infected with *S. japonicum* at the switch point (4.5—8 weeks) as compared with normal mice.

Change of gene expression	Category and gene symbol	Common Name	Description and GeneBank accession no.
Induced	Fcgr3	CD16	*Mus musculus* Fc receptor, IgG, low affinity III, mRNA [NM_010188]
	Lilrb4	HM18; ILT3; gp49; CD85K; Gp49b; LIR-5	*Mus musculus* leukocyte immunoglobulin-like receptor, subfamily B, member 4, mRNA [NM_013532]
	Lst1	B144	*Mus musculus* leukocyte specific transcript 1, mRNA [NM_010734]
	Pglyrp1	PGRP; Tag7; Tasg7; PGRP-S; Pglyrp; Tnfsf3l	*Mus musculus* peptidoglycan recognition protein 1, mRNA [NM_009402]
	Spp1	OP; Bsp; Eta; Opn; Ric; BNSP; BSPI; Opnl; Spp-1	*Mus musculus* secreted phosphoprotein 1, mRNA [NM_009263]
Inhibitive	Btla	MGC124217; MGC124218; A630002H24	*Mus musculus* B and T lymphocyte associated, transcript variant 2, mRNA [NM_177584]
	Card11	BIMP3; CARMA1	*Mus musculus* caspase recruitment domain family, member 11, mRNA [NM_175362]
	CD27	S152; Tp55; Tnfrsf7	*Mus musculus* tumor necrosis factor receptor superfamily, member 7, transcript variant 1, mRNA [NM_001033126]
	CD28	CD28	*Mus musculus* CD28 antigen, mRNA [NM_007642]
	CD40	IGM; Bp50; RAP; Tnfrsf5	*Mus musculus* CD40 antigen, transcript variant 3, mRNA [NM_170701]
	CD8a	Ly-2; Ly-B; Ly-35; Lyt-2; BB154331	*Mus musculus* 2 days neonate thymus thymic cells cDNA, RIKEN full-length enriched library, clone: E430016B14 product: CD8 antigen, alpha chain, full insert sequence. [AK088414]
	CD8b1	CD8b; Ly-3; Ly-C; Lyt-3	*Mus musculus* CD8 antigen, beta chain 1, mRNA [NM_009858]
	Icos	H4; AILIM; Ly115	*Mus musculus* inducible T-cell co-stimulator, mRNA [NM_017480]
	Lat2	LAB; NTAL; WSCR5; Wbscr5; Wbscr15	*Mus musculus* linker for activation of T cells family, member 2, transcript variant 2, mRNA [NM_022964]
	Mr1	H2ls	*Mus musculus* major histocompatibility complex, class I-related, mRNA [NM_008209]
	Pglyrp2	tagL; PGRP-L; Pglyrpl; tagl-beta; tagL-alpha	*Mus musculus* peptidoglycan recognition protein 2, mRNA [NM_021319]
	Tap1	Y3; TAP; APT1; Ham1; MTP1; PSF1; RING4; Tap-1	*Mus musculus* transporter 1, ATP-binding cassette, sub-family B (MDR/TAP), mRNA [NM_013683]
	Tbx21	TBT1; T-bet; Tblym	*Mus musculus* T-box 21, mRNA [NM_019507]

Analysis of the related cytokine mRNA expressions in spleen CD11b$^+$ cell during schistosome infection

To confirm the effects of schistosomal infection on macrophage-induced cytokine and chemokine expression, mRNA levels of spleen macrophages from mice after infection for 1, 3, 5, 7, 9, 12, and 18 weeks p.i. were investigated. Subsequently, the specific mRNA expression levels of classically activated macrophages (CAMφ)-related, alternatively activated macrophages (AAMφ)-related, and inflammatory genes were detected and evaluated. The levels of mRNA expression were normalized to the housekeeping genes *GADPH* and *18S*, which have internal RNA control properties. As shown in Fig. 4A, the expression of all CAMφ-related genes such as *IFN-γ*, *IL-12α*, *β*, *T-bet*, *IL-1α*, and *Ccl5* were downregulated from 9 to 18 weeks p.i., except for the upregulation of *IFN-γ* at 3 and 5 weeks, *IL-12α* at 3 and 18 weeks, *IL-1α* at 1 week, and *T-bet* at 9 weeks ($p < 0.05$). Among these genes, the downregulation of *IL-12β* was especially obvious from the beginning to the end of infection. *IL-1α* showed similar results, except for the first week.

In contrast, the expression of AAMφ-related genes, including *IL-4*, *IL-4Rα*, *IL-13*, *IL-13Rα1*, *IL-21R*, *IL-27Rα*, *Arg-1*, and *YM-1* showed different stages of up- or down-regulated patterns according to different characteristics

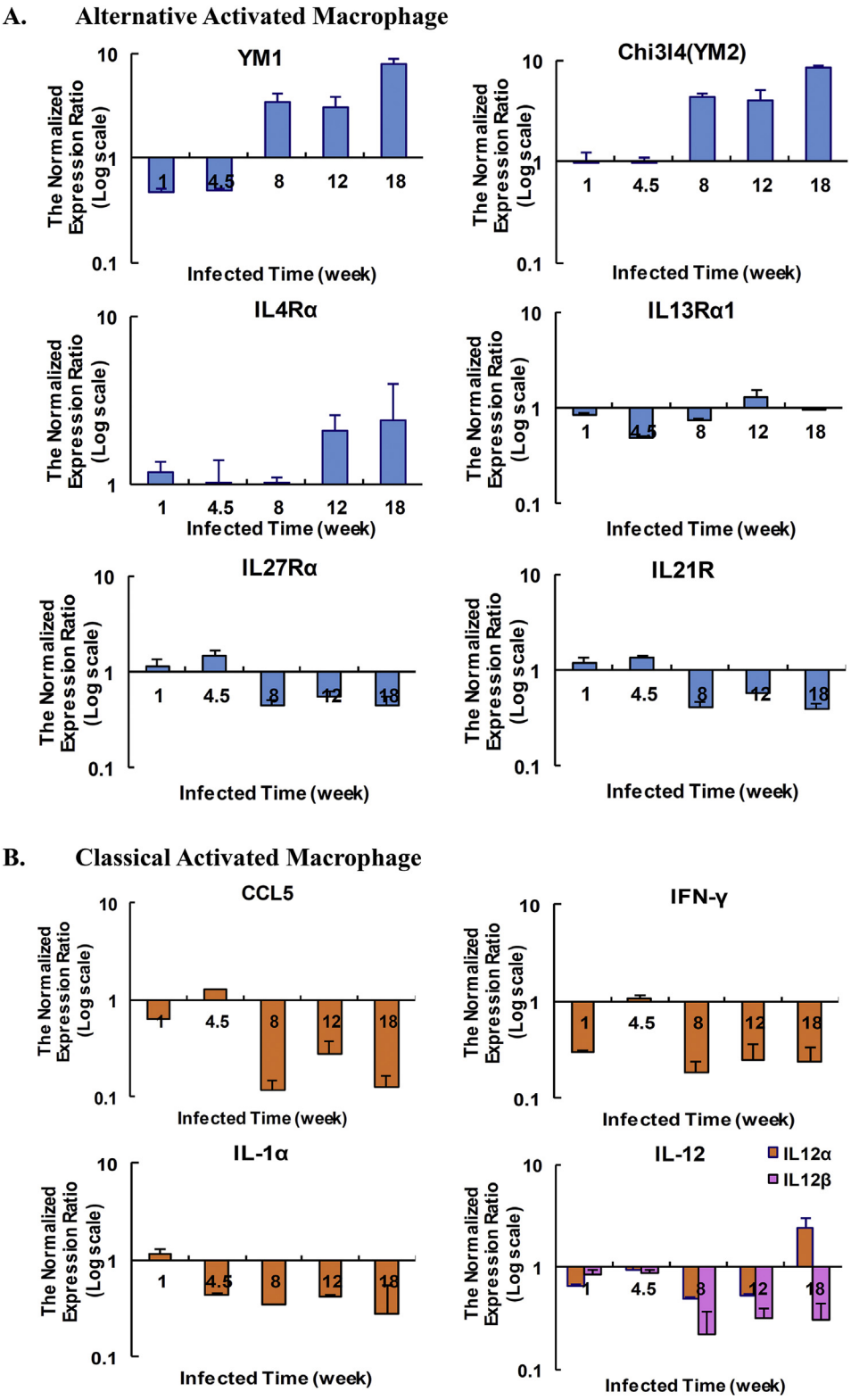

Fig. 3. mRNA expression of Alternative Activated Macrophage- and Classical Activated Macrophage-related genes in spleen CD11b $^{+}$ cells at different time points when the mice are infected with *S. japonicum* by array analysis. The histograms of (A) AAMφ-related genes (1.*YM1*, 2.*YM2*, 3.*IL-4Rα*, 4.*IL-13Rα*, 5.*IL-27Rα*, 6.*IL-21R*) and (B) CAMφ-related genes (1.*CCL5*, 2.*INF-γ*, 3.*IL-1α*, 4.*IL-12α/β*) were shown, respectively. The X-axis shows different infection times; the Y-axis shows corrected expression magnification compared with the control group; the numerical values are presented in logarithm. The respective values are expressed as mean + SD. The designations of genes for expression are indicated above each figure. In addition, IL-12 represents the gene expression trend of two subunits.

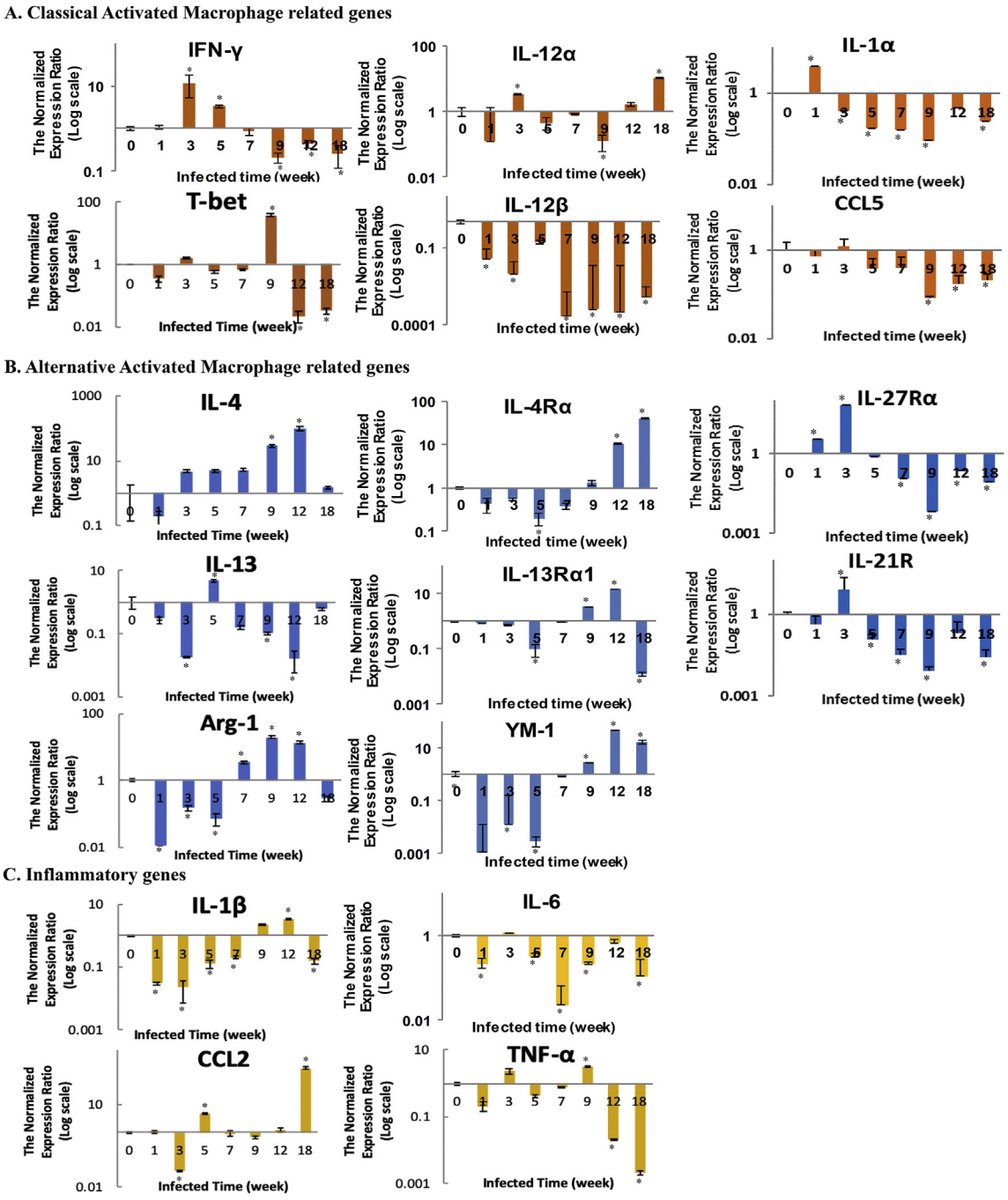

Fig. 4. Changes of mRNA expression in spleen CD11b $^{+}$ cells at different time points when the mice are infected with *S. japonicum* by qRT-PCR analysis. cDNA derived from CD11b^{+} splenocytes of mice with schistosome infection at different weeks were analyzed for (A) CAMφ-related genes (*IFN-γ, IL-12α, β, IL-1α, T-bet and CCL5*), (B) AAMφ-related genes (*IL-4, IL-4Rα, IL-13, IL-13Rα1, IL-27Rα, IL-21R, Arg-1 and YM-1*), and (C) Inflammatory genes (*IL-1β, IL-6, CCL2 and TNF-α*) by real-time RT-PCR. All data were normalized to the internal reference Rn18s analyzed by software and depicted as bar charts. The traces represent the mean from three independent experiments, which are expressed as mean ± SD. The X-axis shows different infection times, including normal control group and the test group of 1 week, 3 weeks, 5 weeks, 7 weeks, 9 weeks, 12 weeks and 18 weeks post-infection; The ratios of normal mRNA expression to the internal control are set at one and the numerical values of the Y-axis are presented in logarithm. (*$p < 0.05$, p value is the comparison between each time point and the control group of Week 0).

(Fig. 4B). *IL-4* gradually increased from 3 weeks p.i. and reached its maximum at 12 weeks p.i., whereas *IL-4Rα* was only significantly upregulated at 12 and 18 weeks p.i. ($p < 0.05$). In contrast, *IL-13* was upregulated only at 5 weeks p.i. and downregulated significantly at 3 and 12 weeks p.i., whereas *IL-13Rα1* was downregulated at 5 weeks p.i. but upregulated at weeks 9–12 p.i., and then downregulated at Week 18 p.i. ($p < 0.05$). Th2 precursor cytokines, *IL-21R* and *IL-27Rα*, both upregulated at 3 weeks and then switched at 5 weeks, and significantly downregulated from to 7–18 weeks ($p < 0.05$). Th2 protein markers, *Arg-1* and *YM-1*, both showed significant downregulation from 1 to 5 weeks p.i., and then reversed to upregulation at 7–12 weeks p.i. ($p < 0.05$).

We also detected the expression of inflammatory genes, such as *IL-1β*, *IL-6*, *CCL2*, and *TNF-α*, and found that most of these genes, especially *IL-6*, were downregulated during infection, except for *IL-1β* at 9 and 12 weeks, *CCL2* at 5 and 18 weeks, and *TNF-α* at 3 and 9 weeks p.i. ($p < 0.05$). These results demonstrate that schistosomal infection induces early CAMφ activation and causes the switch to the AAMφ phenotype after egg production. More importantly, cytokines and chemokines expressed by different phenotypes of macrophages are involved in many complex and detailed immunoregulatory processes, especially those related to AAMφ, which are modulated through the divergent expression patterns of cytokines and their receptors at different time points. The trends of qRT-PCR results were confirmed to be very similar to the microarray data.

Analysis of related cytokines in serum during schistosome infection

To further confirm the effect of macrophage-induced chemotaxis after *Schistosoma japonicus* infection on subsequent host immune phenotypes, cytokines in mouse sera were tested at 1, 3, 5, 7, 9, 12 and 18 weeks p.i. Our results indicated a different characteristic pattern between type-1 and type-2 cytokine expression over the course compared to uninfected mice. As shown in Fig. 5, INF-γ was strongly expressed from 1 week and peaked at 3 weeks p.i., ($p < 0.001$) then consistently upregulated until 9 weeks p.i. ($p < 0.01$) and declined at 12 weeks p.i. of the chronic phase. IL-2 expression only showed two raised peaks at 3- and 7-weeks p.i. during infection ($p < 0.01$) (Fig. 5A).

In contrast, IL-4 expression increased gradually from 3 to 7 weeks p.i. ($p < 0.01$), peaked at 9 weeks p.i., ($p < 0.001$), and then decreased at 18 weeks p.i. Unlike the IL-4 expression levels, IL-10 and IL-5 expression levels increased only at 7–9 ($p < 0.05$) and 9–12 weeks p.i. ($p < 0.001$), respectively (Fig. 5B). In addition, the expression of TNF-α in serum from mice infected with *S. japonicum* was similar to that of IL-5, which increased only at 9–12 weeks p.i. ($p < 0.01$) (Fig. 5C). It was revealed that type-1 cytokine expressions in the serum were mainly increased until 7 weeks p.i., while type-2 cytokines and TNF-α were enhanced from 9 to 12 weeks p.i., which was gradually induced and sustained by egg appearance and granuloma pathogenesis. The expressed trends of cytokines in sera were also similar to the results of both qRT-PCR and microarrays for macrophage gene expressions.

Analysis of the expression trend of spleen CD11b$^+$ cell labeled proteins of the infected mice at different time points

In order to identify the trend of macrophage typing change in the course of *S. japonicum* infection, we used the flow cytometer to detect the expression of splenic macrophage surface markers of the infected mice at different time points. According to Fig. 6A, all the ratio of CD11b$^+$ macrophages was higher than the control group after infection with *S. japonicum* ($p < 0.01$). Except for the expressions at 3 week and 5 week, which slightly declined, the other time points were significantly higher. In addition, on the expression of macrophage related surface markers, this study analyzed MHC class II IA/IE protein and CD8α protein, which inducing acquired immune response related to antigen presenting and helping tend to Type1 immune response, respectively,[78–81] hereby to discuss the activity and the function typing change of macrophages. As shown in Fig. 6B, the cell percentages of IA/IE were increased at 1, 3 and 5 weeks p. i. ($p < 0.01$). However, when the infection from 5 weeks of the early stage to the acute stage (7 weeks p. i.), the IA/IE$^+$ cell percentages showed a significant declining trend, then lasting until the chronic stage ($p < 0.01$). After further analysis on the common expression of IA/IE and CD8α markers on macrophages, it revealed that with the duration of the infection, the percentages of IA/IE$^+$CD8α$^+$ macrophage reached the maximum at 1 week p. i., and then gradually declined, especially at 7 week p. i. to the minimum ($p < 0.01$) (Fig. 6C). The results showed the ability of antigen presenting and the Type1 immunity inducing on the splenic macrophages of mice is repressed due to the infection of *S. japonicum*, especially the worm eggs influence the most significantly.

Analysis of the expression trend of spleen lymphocyte labeled proteins of the infected T1/T2 double transgenic mice at different time points

To further analyze the relation between the trend of Type1/Type2 immune response and the macrophage transformation during the *S. japonicum* infection *in vivo*, we used specific T1/T2 double transgenic mice to detect the proportion of spleen helper T and effector B lymphocytes and their expression of Type1/Type2 surface markers of the infected mice at different time points. As shown in Fig. 7A, the experimental results showed the infected CD4$^+$ cell percentage increased more than that of the control group at 1 week and 3 week p. i. ($p < 0.05$); was not different from the control group at 5 week and 7 week p. i.; and reached the maximum of 37.5% at 9 week p. i. ($p < 0.05$); and then declined at Week 12 and Week 18 p. i. ($p < 0.05$). cells had no obvious change from Week 1 to Week 9 p. i., mainly maintained at 14–19%, and significantly declined to below 6% at Week 12 and Week 18 p. i. ($p < 0.01$). In addition, Fig. 7B showed B-cells gradually increased from 1 week p. i. to 44.1% of the top at 3 week p. i., and then maintained at about 40%, with the exception of a decline to 36.3% at 9 week p. i. It increased to 43.9% again at 12 week p. i., and again, declined to the same degree as the control group in 18 week ($p < 0.05$). As for Type1/Type2

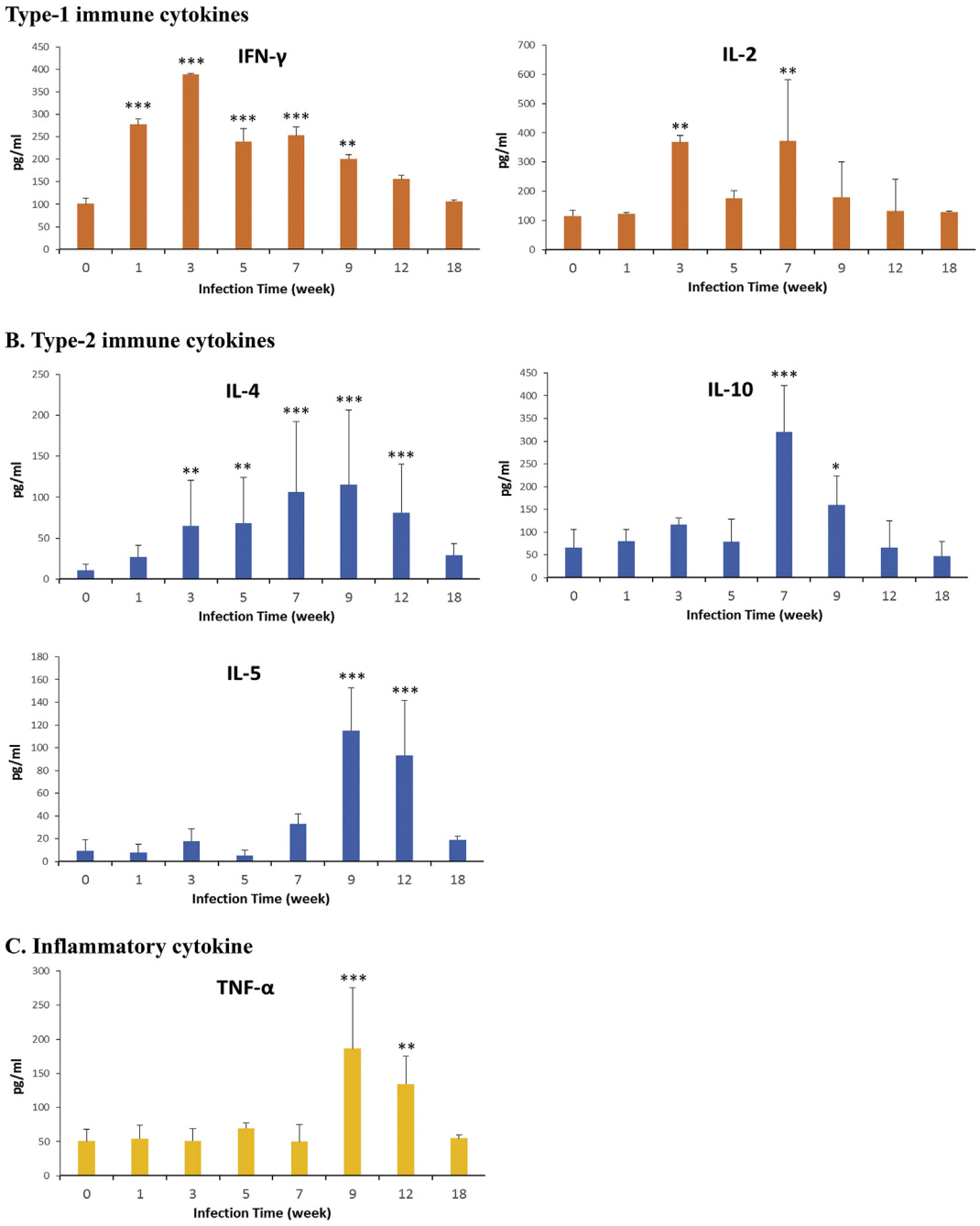

Fig. 5. Detection of serum cytokines secretion in mice infected with *S. japonicum* at different time points by ELISA kits analysis. The levels of cytokines released in the serum of mice with schistosome infection at different weeks were analyzed for (A) Type 1-immune cytokines (IFN-γ and IL-2), (B) Type 2-immune cytokines (IL-4, IL-10, and IL-5), and (C) Inflammatory cytokines (TNF-α) by ELISA assay. The X-axis shows different infection times, including the normal control group and the test groups 1, 3, 5, 7, 9, 12, and 18 weeks post-infection; the Y-axis shows the concentrations (pg/ml). Data are expressed as the mean ± SD from three separate experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, when compared to the control group from Week 0.

cell differentiation, Fig. 7C showed Th1/Th2 differentiation of $CD4^{+}$(helper T) cells, Th1 cells began differentiations in the first week p.i., and reached the maximum 7.87% of 3 week p. i., which was still apparently higher than the control group in 5 week ($p < 0.05$). However, it gradually returned to an insignificant difference state, and declined in 18 week of the chronic stage ($p < 0.01$). Th2 cells only increased slightly in the beginning of infection, then began to rise from 5 week and sharply reached the maximum of 16.13% at 7 week p.i., which is 6 times more than the control group ($p < 0.001$). Afterward, it maintained the high expression until 12 week, then declined to similar to the control group at 18 week p. i. ($p < 0.001$). On the other hand, the research showed that B-cells can also be differentiated into two subtypes populations, called the effector Type1/Type2 B-cells (Be1/Be2).[82] Fig. 7D showed Be1/Be2

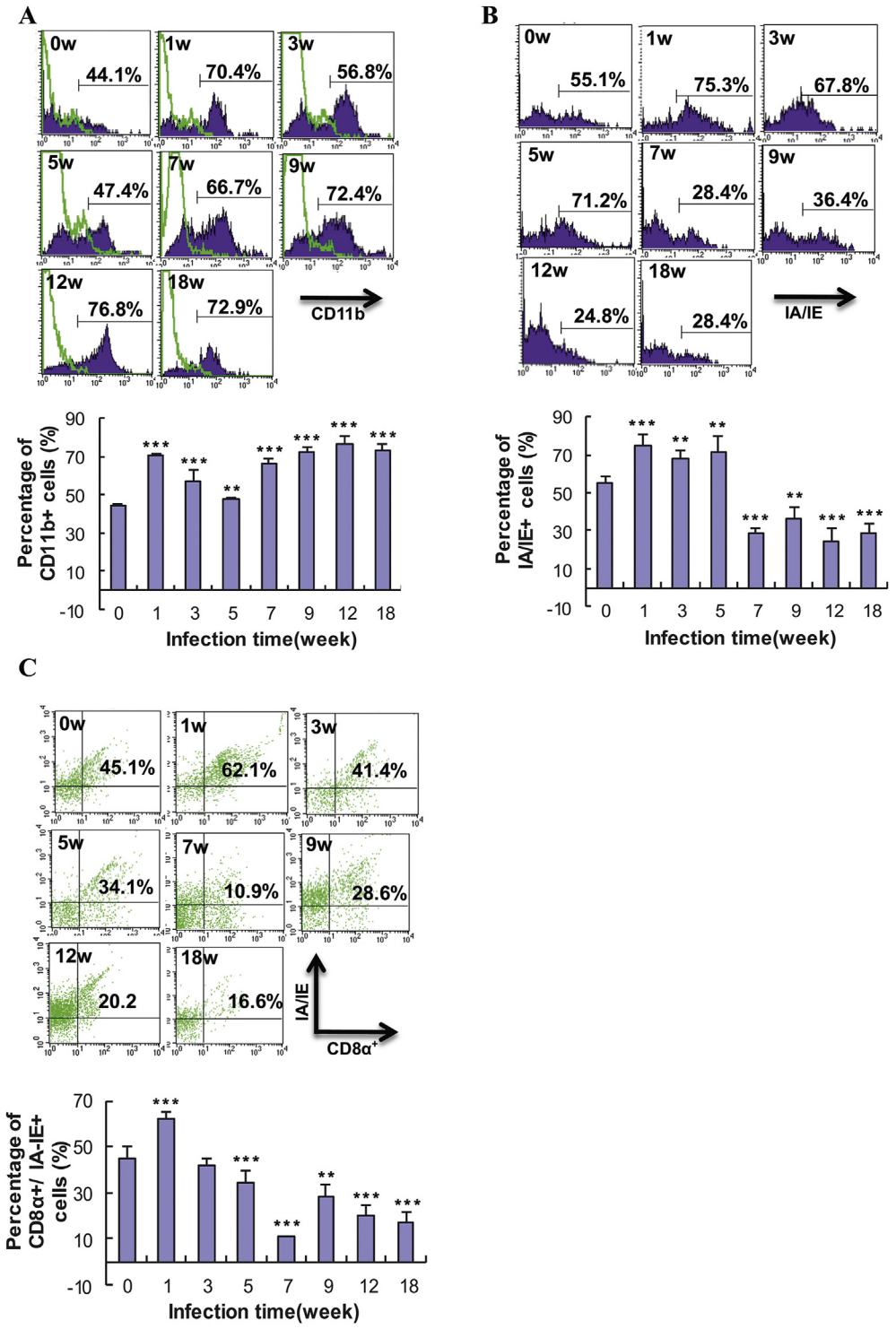

Fig. 6. Percentages of surface CD11c $^{+}$ expression on splenocytes and surface CD8α and MHC class II I-A/I-E marker molecule expression on the CD11b $^{+}$ splenocytes. Flow cytometry was used to measure the expression of (A) macrophage (CD11b^{+}) cells in the spleen, and (B) I-A/I-E marker expression on CD11b $^{+}$ cells that are shown as histograms and (C) both I-A/I-E/CD8α cell markers co-expression shown as dot-plots at different time points from the mice infected with *S. japonicum*. Numerals above the lines or on the right upper quadrant show the cell frequency of the positive frequency of CD11b cells or IA/IE or I-A/I-E/CD8α co-expressing cells from total CD11b $^{+}$ cells, respectively. The results shown are representative of typical results. The X-axis shows different infection times, including the normal control group and the test groups 1, 3, 5, 7, 9, 12, and 18 weeks post-infection; the Y-axis shows the cell percentage (%). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; p value is the comparison between each time point and the control group from Week 0.

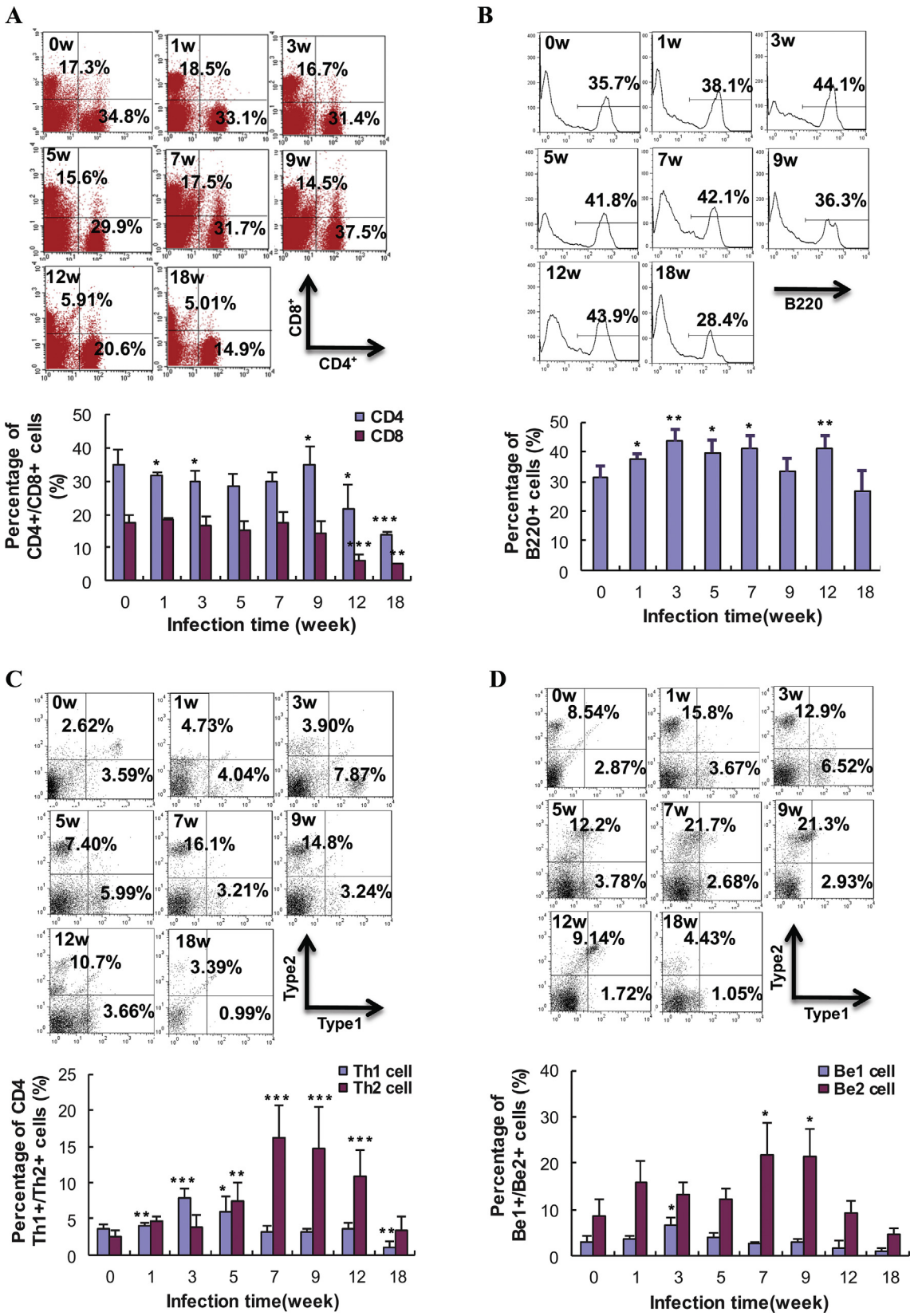

Fig. 7. Percentages of T and B cells on splenocytes and surface type 1 and type 2 marker molecule expression on CD4+ helper T cells and B cells, respectively. Flow cytometry was used to measure (A) CD4 and CD8α cells ratio and (B) B-cell ratio, and the respective percentages of Type 1/Type 2 cells on (C) CD4 and (D) effector B-cell in the spleen cells at different time points from T1/T2 transgenic mice infected with *S. japonicum*. Figure (A), (C), and (D) are shown as dot plots and Figure (B) is shown as a histogram; the results shown are representative of typical results. Numbers above the lines or in the right or upper quadrants show

differentiation of B-cells. Be1 cells were different from the control group only at 3 week p. i. of 6.52%, and there was no obvious statistical discrepancy as compared with the control group at other times. But the expression of Be2 cells was similar to Th2, it reached 21.75% of the peak at 7 week p. i., which was about 3 times than the control group; thus, showing a significant rising trend until it returned at 12 week p. i. ($p < 0.05$). The results showed that over the course of time, splenic Th cells and effector B (Be) cells changed their phenotypes from type 1 to type 2 immunity, synchronized to the timing of macrophage transformation in the mice infected with *S. japonicum*.

Discussion

The sensitivity and pathogenicity of the hosts infected with schistosoma are closely related to their genetic genotype.[83] This study found that these important immunoregulation may be triggered by macrophages, which leads to the subsequent immune response into a suitable environment for the development and survival of schistosoma.

Based on further analysis of the microarray data, the "switching point" between 4.5 weeks and 8 weeks p. i., which is an important boundary of the macrophage phenotypes was found. The results also indicated that the variance in gene clusters have greater differences, both before and after this point. Here we were focused on three conclusions as follows:

1. The gene clusters mainly on immune-related genes, such as CCLs and CCRs populations, which modulated inflammatory activity, cytotoxicity of macrophages to resist pathogens, were upregulated before the "switch point", and downregulated afterwards. It showed that repressing the ability of the host's macrophage to suppress the regulation of inflammation or cytotoxicity in the later stages, in order to prevent enormous destructive immune responses from damaging host tissues. Those that downregulated before the switch point and upregulated after the point were mostly about cell chemotaxis, adhesions, and tissue repair associated functions. For example, the coactions of CCL7 and CCR1. IL-11 and Spp1 factors were helpful to the formation of granuloma and tissue fibrosis. This data was consistent with the results of the flow cytometer experiment; that $CD4^+$ and $CD8^+$ cell proportion declined, while macrophages increased. Similar situations had also occurred in other schistosome infection tests.[14,19,22,84,85]
2. The regulation of schistosome infection on macrophages showed a significant inhibitory effect when the eggs appeared, as many of the upregulated gene clusters were negatively regulated factors, such as Lst1. The repressed immune gene clusters induced a downregulation of acquired immune response associated genes, especially Type1 immunity (IL-12, CD40, CD28, Icos).[13,86–88] It's shown the *S. japonicum* infection resulted in the immunoregulation of mice splenic macrophages, which were mainly repressing Type1 immune responses priority, rather than inducing Type2 immune response precedents. The schistosome antigens caused the macrophage decreased the abilities of antigens presenting and Type1 response inducing, in particular, the eggs had the most significant influence. This result was also consistent with that of the flow cytometry tests. The influence of macrophage typing on subsequent Th1/Th2 or other immune responses was proved in past studies **and our transgenic mouse experiments**.[29,44,89]
3. According to the microarray data, many of the upregulations in the immunoregulation-associated factors of macrophages, such as cytokines and chemokines when mice were infected with schistosoma, were not the gene expressions of the ligand itself, but contrarily, were the expressions of the large amount of genes of the receptor. This result inferred that the immunoregulatory response of macrophages to resisting schistosome, the specificity of the receptor accepting the factors is more important than the amount of secreted factors. Based on this, the modulation of the host's immune system may control limitations of responses in order to give the unique immunity for the characteristics of local tissues and specific organs, and not to cause systemic or other tissues' immune injuries. As for the schistosome infection initiating complicated immunoregulation, the response intensity and scale in different stages were modulated by mutual growth and decline between the ligands and the receptors in order to control the pathological changes.[14,15,44,90]

To sum up, this study observed phenotype changes in macrophages of the spleens of mice, CAMϕ was changed to AAMϕ between 4.5 weeks and 8 weeks after infection. IFN-γ and IL-12 would induce CAMϕ activation and present CCl5.[44,91] By analyzing the expression trends of these pro-CAMϕ inflammation related factors, we proved that the differentiation of CAMϕ was significantly repressed to downregulate after the switch point, which was negatively correlated to the activation of AAMϕ, and it was confirmed that the *S. japonicum* infection resulted in the key transition of CAMϕ to AAMϕ. However, IL-1α, which is also related to pro-inflammation, was repressed after being infected with larvae. Previous study had shown that IL-4 induces IL-1 receptor antagonists (IL-1ra) to repress IL-1 expression,[53] thus, IL-1α showed an downregulated expression when IL-4 showed a high expression. The qRT-PCR data also showed the same trends between the expressions of these two genes. The YM family only appears on AAMϕ, rather than on CAMϕ, thus, it can be used as the specific surface markers of AAMϕ.[73,74,92] According to YM1 and YM2 trends, significant rises occurred in the second stage after the switch point, and were increased with the

the positive frequency of CD4/CD8 or type 1/type 2 expressing cells from $CD4^+$ or B cells, respectively. The X-axis shows different infection times, including the normal control group and the test groups 1, 3, 5, 7, 9, 12, and 18 weeks post-infection; the Y-axis shows cell ratio (%). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; p value is the comparison between each time point and the control group of Week 0.

infection time, which showed that numerous AAMϕ were generated and played an important role in the acute and the chronic stages of *S. japonicum* infection. IL-4 and IL-13 are the determinant factors that promote AAMϕ phenotype differentiation. These two cytokines stimulate Arginase1 activation in macrophages to produce Proline, and influence granulomatous collagen generation and fibrosis development.[27,28,93] However, the experiments revealed that the expressions of IL-4 and IL-13 receptors (IL-4Rα and IL-13Rα1) were not coincident with YM, as it only rose in and after the late acute stage. IL-21R can enlarge the expressions of IL-4Rα and IL-13Rα1 to improve the active functions of IL-4 and IL-13[77,94]; and IL-27Rα is similar to IL-10, in that it can inhibit the expression of pro-inflammatory cytokines.[75] The expressions of these two receptors were unexpected, a change from rising to declining during the transformation of macrophages, which showed an opposite trend to YM. This interesting result may assume a meticulous regulation of change in the splenic macrophage gene expression profile during *S. japonicum* infection. When the CAMϕ of the first stage was transformed into the AAMϕ of the next stage, the regulatory effect of this critical change was begun in an earlier stage of infection, though the immune response was still mainly based on a Type1 response at this time. IL-21R and IL-27Rα upregulated between 3—4.5 week p. i., matching the highly expressed IL-4 to start forming a Type2 response inclined environment. In this transition period, the host's macrophages did not largely or quickly express IL-4Rα or IL-13Rα1, but showed a slow increased situation. It is possible to maintain a proportional balance CAMϕ and AAMϕ to avoid a drastic incline of the systemic immune response. The advantage of AAMϕ had appeared after the acute stage, thus, IL-21R and IL-27Rα may have been reduced to control the enlargement of Th2 responses. In addition, macrophages may improve the specificity of AAMϕ responses based on the upregulation of IL-4Rα and IL-13Rα1, in order to maintain its effect on specific tissues. mRNA expression also showed similar results, that is, most of the CAMϕ-related genes were suppressed, while the AAMϕ-related genes showed different growth and decline at different stages, respectively. This was also coincident with previous data that the immunoregulation intensity and scale on different stages of schistosome infection were modulated by the mutual growth and decline between the ligand and the receptor.[37] We have proven that in mice, during the course of infection with *S. japonicum*, cytokines in the serum exhibit a profile of immune-related changes from type 1 to type 2, which may be influenced by the transformation of type 1 macrophages (M1) to type 2 macrophages (M2). Previous reports have shown that galectins, eosinophils, and M2 macrophages may play crucial roles in the regulation of chronic immunopathology in murine *S. japonicum* infections.[95] Another study found significant correlations between egg counts and eosinophil counts and between egg counts and IL-1β or TNF-α concentrations, which may be positively associated with *Schistosoma*-related liver fibrosis.[96] This further explains the changes in related cytokines at different time points in our serum cytokine experiments, especially IL-5 and TNF-α, at 9—12 weeks.

Our results showed that the number of significant expressed genes from mice splenic macrophages increased with infection time. The proportion of macrophages increased greatly after infection with *S. japonicum*, and then declined suddenly somewhere around 5 week. In addition, two surface markers of the macrophage, the expressions of IA/IE and $CD8\alpha^+$ had large differences at this conversion stage, with a change from rising to a sharp decline. This study found that the cell masses that significantly present CD8α+ among the mice splenic antigen presenting cells were also the dominant populations that present the MHC class II I-A/I-E molecules.[97] Previous studies have suggested that $CD8\alpha^+$ DC cells of the host initiated the development of Th1 type cells, and contrarily $CD8\alpha^-$ DC cells induced Th2 type responses.[98—100] The *S. mansoni* experiment also proved that the co-expression of macrophage CD8α and MHC class II I-A/I-E molecules declined obviously after 8 weeks p. i., but the DC cells did not.[101] SEA stimulated DC can strongly induce Th2 polarization response of SEA specificity, but $CD8\alpha^+$ DC cells may produce IL-12 that affect the host's TLR ligand, and induce Th1 cell.[102] This may be because the schistosome antigens begin to change from worms to eggs during this period. The induced cell factors regulate macrophage immunostimulation to change phenotype from CAMϕ to the AAMϕ at the transitional stage. The cellular reaction may change from the original proliferation to the differentiation and migratory functions; this transformation also causes the host immunity to develop towards a Th2 rise, while Th1 declines.

Based on the test of specific T1/T2 double transgenic mice, in comparison to the whole spleen cell population, and T-cell declined and the B-cell increased in the infection stage of *S. mansoni*,[103] but the modulation of *S. japonicum* on these cells was obviously slower, the influence was also smaller. Previous studies reported that T-cell-dependent immunoresistance caused by *S. japonicum* was relatively slow.[7,104] But although the proportional difference was small, the activity of difference was more apparent. This study found that the differentiation mode of the acquired Type1/Type2 immune response inside the mice infected with *S. japonicum* strongly developed toward the Th2 type polarization, and a repressed Th1 response. The same result was found in other schistosome infection experiments.[93,105] According to the experimental results, the differentiation mode of $CD4^+$ T and B-cells exactly matched the time interval of macrophage stage conversion, the change of Th2 rose, and Th1 declined, which also happened at the "switching point" between 4.5 weeks and 8 weeks p. i.. The research showed that the schistosome can adjust B7 co-stimulatory molecule programmed death ligand 1 of macrophages upwards, thus, the function of T-cells was further weakened, as based on the mechanism initiated by the interaction effect with the T-cells.[88] When the mouse was infected with schistosoma, Th1 response would decline, by IL-10 acted on repressing the production of IL-12; and resulted in the polarization of Th2 reaction.[106] Ji et al. conducted microarray experiments and proved that, the *S. japonicum* repressed the Th1 type response after infection, and when a large amount of eggs appeared,

modulated the host's Th2 type response to high development.[107—109] The current study showed that *S. japonicum* cystatin could significantly activate the polarization of macrophages to the M2 subtype characterized by the expression of $F4/80^+$ with the elevated secretion of IL-10 and TGF-β.[110] Xiao et al. also revealed that *S. japonicum* infection modulates the differentiation of B cell subsets, which can affect the $CD4^+$ T cell response, and suggest that *S. japonicum*-induced B cells exhibit multiple regulatory phenotypes and play a pleiotropic role in schistosomal infection.[111] This study demonstrated that during *S. japonicum* infection, the different worm and egg antigens influenced the phenotype conversion of macrophages to regulate and control subsequent differentiation of Th and Be lymphocytes, thus skewing the whole immune response to type 2 responses.

Previous reports proved that the eggs are the primary cause initiating ideal Th2 responses and subsequent hepatopathy,[112] and developing AAMϕ is a response strategy of the host to the schistosome infection, which causes this Th2 dependent chronic inflammation mode. Herbert et al. proved that AAMϕ is necessary in the period of schistosome infection. Although they proved also that AAMϕ is not consequential to developing Th2 type responses, instead, Th1 and egg-induced inflammatory responses are reduced hereby to protecting organs and the survival of the host.[27] This is consistent with previous experimental results. Other parasite studies also showed that AAMϕ can reduce IL-12 generation, and is inclined to a Th2 response in chronic infection.[113] Many experiments have proved that the AAMϕ involved immunosuppression effect is indispensable for protecting the host from pathological injury in the schistosome infection, and it influences the size and degree of granuloma and fibrosis, respectively.[16,76,114,115] A study reported that prior infection with *Toxoplasma gondii* markedly reduced the granuloma size and collagen deposit in livers against *S. japonicum* infection by promoting a shift toward Th1 instead of Th2 immune response.[116] It also hints towards improved strategies for the intervention of M1/M2 immune response in *S. japonicum*-infected hosts. However, microarray experiments showed that IL-12α and other inflammatory factors had strong inducement expression at 18 week of the chronic stage. Moreover, neither the T or B cells showed proliferation or activation in this stage. Whether this means the AAMϕ induced the Th2 chronic inflammatory responses is only concerned with innate responses, but excludes acquired immune effects. The alternative activation pathway may be a spontaneous reaction to tissue injuries when lacking acquired responses[81]; however, further studies are required to clarify.

Dominant Th1 responses can effectively destroy immature worms and larvae of *S. japonicum*, which are the most sensitive stages to immunity,[45] whereas inducing a more ineffective Th2 response may be a delicate defense mechanism evolved by the schistosome. Research has shown that schistosomes can repress the generation of Type 1 $CD4^+$ T-cells by ensuring that Th2 and Be2 cells produce adequate regulatory cytokines or markers, such as IL-10, TGF-β, or PD-L1,[13,45,117] to escape the immune responses of the host. Therefore, how to effectively control the ratio between CAMϕ and AAMϕ phenotypes to maintain the balance of Th1/Th2 responses and avoid a drastic incline becomes the most important subject over the course of schistosomal infections. This study focuses on discussing the trends of the overall genotype changes based on macrophages in different stages of *S. japonicum* infection *in vivo*. The data obtained in this study may provide a helpful reference for the treatment of *S. japonicum* or the development strategies of the vaccine.

Declaration of competing interest

The authors do not have a commercial or other association that might pose a conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

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