Unraveling *Ascaris Suum* Experimental Infection in Humans

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Research

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Abstract

Background: *Ascaris lumbricoides* and *A. suum* are two closely related parasites that infect humans and pigs. The zoonotic potential of *A. suum* has been a matter of debate for decades. Here we sought to investigate the potential human infection by *A. suum* and its immunological alterations.

Methods: We orally infected five healthy human subjects with eggs embraced by A. suum. The infection was monitored for symptoms and possible respiratory changes, by an interdisciplinary health team. Parasitological, hematological analyzes, serum immunoglobulin, cytokine profiles, and gene expression were evaluated during the infection.

Results: Our results show that *A. suum* is able to infect and complete the cycle in humans causing *A. lumbricoides* similar symptoms, including, cough, headache, diarrhea, respiratory discomfort and chest x-ray alterations coinciding with larvae migration in the lungs. We also observed activation of the immune system with production of IgM and IgG and a Th1/Th17 response with downregulation of genes related to Th1 and apoptosis. PCA analysis show that infection with *A. suum* leads to a change in the immune landscape of the human host.

Conclusion: Our data reinforce the zoonotic capacity of *A. suum* and bring a new perspective on the understanding of the immune response against this parasite.

Background

Ascaris is the most common human worm infection [1]. It is estimated that about 800 million people are infected or at risk of infection with *Ascaris* worldwide, especially in underdeveloped and developing countries [2, 3]. Although human ascariasis is associated with areas with poor sanitation and hygiene, increased travel and migration have made *Ascaris* infections more common in non-endemic areas, with cases also described in developed countries, being, for example, the most prevalent helminthic disease in United States [4].

The *Ascaris* genus present both *Ascaris lumbricoides* and *Ascaris suum*. Two closely relate parasites that are morphologically indistinguishable and infect human and pigs respectively [5, 6]. *A. suum* is a highly prevalent parasite in pigs and is associated to reduced growth, low feed conversion efficiency and liver condemnation incurring in significant economic losses [7]. The zoonotic potential of *A. lumbricoides* and *A. suum* has being a matter of debate for decades [8–10]. It has been demonstrated that cross transmission can occur, with some host preference [11]. It is also known that there is gene flow between the species [11]. Although human infection with *Ascaris suum* has been described both naturally and experimentally [5, 10, 12–14], little is known about the course of infection and immunological changes induced by the parasite in humans.

The better understanding of the infection dynamics of *A. suum* in humans including the migratory pattern, ability to establish in the small intestine, range of pathological effects and the immunological

response triggered by infection is crucial to unravel the zoonotic potential of *A. suum* and may help implement appropriate control programs in both human and pigs. Here we show that *A. suum* is capable to infect and complete the cycle in human hosts, leading to a polarization of the immune response towards a Th2/Th17 phenotype.

2. Material And Methods

2.1 Volunteers and ethics statement

Five healthy volunteers, legally capable, negative for intestinal worms and without alterations in clinical and laboratory exams were recruited. Individuals were clarified about the research and signed the term of free and informed consent. This work was approved by the Research Ethics Council of the Federal University of Minas Gerais, Brazil (CAAE,26945814. 1.0000.5149).

2.2 Parasites

Adult female *A. suum* worms were harvested from intestines of infected pigs that were discarded by a slaughterhouse at Contagem, Minas Gerais, Brazil. Adult worms were kept in PBS pH7,4 in the Laboratory of Immunology and Genomics of Parasites at the Universidade Federal de Minas Gerais, Brazil. The parasites were identified through morphological characteristics and molecular markers.

2.3 Egg embryonation assay

The embryonation of *A. suum* eggs was performed as described by Gazzinelli- Guimaraes, 2013 [15]. Briefly, the eggs were isolated from female worms by mechanical maceration and filtered in Tamis (100 mm). Eggs were placed in culture in 0.2 M sulfuric acid at a concentration of 25 eggs/µl to induce embryo formation and stored in B.O.D at 26□ C for at least 80 days for experimental infection and 110 days for antigen preparation. During incubation, egg suspension was oxygenated by shaking three times a week.

2.4 Purification of eggs for experimental infection

After incubation period, eggs were centrifuged at 800 g for 10 minutes at room temperature. Supernatant was discarded and eggs were resuspended in 5% (v/v) sodium hypochlorite and incubated at 37□ C and 5% CO_2 for 2 hours in order to rupture the outer egg membrane.

After incubation, fully embryonated eggs were washed three times with drinking water in order to remove the sodium hypochlorite. Purified eggs were counted in aliquots for use in experimental infections.

2.5 Antigen preparation

Crude extract of adult worm (AW) was obtained by mechanical maceration followed by sonication. The macerated extract, kept on ice, was sonicated at 60 Watts for 1 min with a 30 seconds interval for each

cycle, repeating 5 times. After sonication, soluble crude extract was centrifuged at 800 g for 10 minutes. Pellet was discarded and supernatant stored at -80 ºC until use.

Crude extract of adult worm tegument (AT) was preprocessed before maceration in order to remove internal structures of the parasite with the aid of scissors, tweezers, and scalpel followed by maceration and sonication.

Crude extract of infective larvae (EL) was obtained as described by Ponce-Macotela et al., 2011 with modifications. Briefly, once in hatch culture, purified larvar were collected and transferred to a 50 mL tube and centrifuged at 800 g for 10 min at room temperature. Supernatant was discarded washed once with saline. After centrifugation pellet was resuspended in 5 mL PBS and sonicated at 60 Watts for 1 min, with a 30 seconds interval each cycle, for a total of 10 cycles. After sonication, larvae extract was centrifuged at 800 g for 15 min at 4 °C and stored at -80 °C until use.

Using the BCA commercial kit (Pierce, USA), all antigenic preparations were quantified according to the manufacturer's instructions.

2.6 Experimental infection, clinical follow-up, and parasitological analysis

After purification and egg counts, each volunteer received orally a suspension of 50 viable *A. suum* eggs diluted in 200 mL of filtered water. Volunteers were followed with a multidisciplinary health team and received a spreadsheet to report daily symptoms. Chest x-ray were performed 12 days after infection (12pdi) to follow the pulmonary cycle of the larvae and changes caused by infection. After 30 days of infection, stool samples were collected once a week until confirmation of the patency, when subjects were treated with Ivermectin 200µg / kg in a single dose. Stool samples were submitted to spontaneous sedimentation method (Lutz, 1919). After treatment, stool samples were collected from the following 36 hours and packaged in 500ml containers for recovery of adult worms using the Tamis method.

2.5 Hematological analysis

Hematological profiles of infected individuals were evaluated during the different phases of the infection. Briefly, peripheral blood was collected at 0, 12, 66, and 88 days post infection with anticoagulant EDTA. Blood samples were sent to the laboratory for clinical analysis to perform the complete blood count. Percentages and absolute numbers of lymphocytes, monocytes, eosinophils, and neutrophils were further determined by optical microscopy in bloods smears stained with Giemsa.

2.6 Parasite-specific antibody reactivity and cytokine profile in sera

To determine the levels of IgG and IgM and cytokine profile in the serum, peripheral blood was collected from individuals at all experimental time points (0, 12, 66, and 88 days post infection). Blood was collected without EDTA, centrifugated and serum was collected and stored in -80 ºC.

The production of IL-4, IL-6, IL-10, IL-17A, IL-13, TGF-β and TNF-α was assessed by sandwich ELISA kit (R&D Systems, USA) according to manufacturer's instructions. The absorbance was determined by a VersaMax ELISA microplate reader (Molecular Devices, USA), and cytokine concentration (pg/mL) for each sample was calculated by interpolation from a standard curve.

In order to detect the levels of IgG and IgM against *Ascaris suum* antigens (AW, AT and EL), Elisa's assay was performed following the manufacturer's protocol. Antigens were used at a concentration of 1: 5000.

2.7 Evaluation of gene expression by real-time PCR

To evaluate the gene expression that regulates innate and adaptive immune responses of the infected individuals at the larval migration peak (12dpi), peripheral blood was collected and the peripheral blood mononuclear cells (PBMC) were obtained by centrifugation gradient using Ficoll-Histopaque (Sigma–Aldrich Co.) following the protocol proposed by Bueno, 2008 [16]. Cells were frozen in RNA-protect and subsequently subjected to RNA extraction using TRIzol (Ambion) following manufacturer's recommendations. Evaluation of gene expression was performed using the commercial kit RT² Profiler ™ PCR Array Human Innate & Adaptive Immune Responses (Qiagen), following manufacturer's instructions.

2.8 Statistical analysis

Statistical analyzes were performed using GraphPad Prism 5.0 software. Grubb's test was used to detect sample outliers. We compared our results using One-Way Anova or Kruskal-Wallis followed by Dunn's test, as indicated by the results obtained from running the Kolmogorov-Smirnov normality test. Differences that returned *p* values equal or less than 0.05 were considered statistically significant from one another. For the principal component analysis (PCA), we used Clustvis software [17].

3. Results

3.1 *Ascaris suum* infection and clinical manifestation in humans

We first sough to assess if *Ascaris suum* is capable to infect humans. In order to do this, five healthy individuals were oral infected with 50 embryonated *A. suum* eggs. We were able to recover adult worms from four individuals, confirming that *A. suum* is capable of infect humans. Individual "B" did not provide sample for analysis. *A. suum* was able to complete the cycle in four volunteers, with presence of eggs in feces (Figure 1A).

Volunteers were invited to record the clinical manifestation during the infection period. All volunteers (100%) presented cough, starting from the 10^{th} day post infection and persisting for up to three weeks. Four individuals (80%) showed respiratory discomfort starting as early as 7 days post infection and up to 33 dpi. All volunteers (100%) presented headache for a short period of time (less than a week). Four individuals (80%) presented diarrhea, starting 19 dpi during 4 to 9 days (Figure 1C).

Volunteers were submitted to chest x-ray at 12 dpi to evaluate the impact of larvae migration through the lungs. Volunteer “A” presented bilaterally diffuse peribroncovascular infiltrate, convex phrenic domes, free cost-phrenic sinuses, complete costal frame, heart and base vessels withing the limits of normality. Volunteers “B”, “C” and “D” presented bilaterally diffuse biliary-nodular infiltrate and cardiothoracic indices withing normality (Figure 1B). Individual “E” did not perform x-ray examination. All volunteers showed normal chest x-ray at day 75 post infection (data not shown).

3.2 Increase IgG and IgM levels induced by Crude extract of adult worm in experimental infection with *Ascaris suum*

Next, we investigate IgG and IgM levels induced by crude extract of adult worms (AW), infective larvae (EL) and adult worm tegument (AT). Figure 2 shows expression of IgG and IgM after each stimulus. It was observed an increase in IgG and IgM expression as early as 12 days post infection after AW stimuli (Figure 2 A and B). Levels remain high at 66 dpi and decrease at 88 dpi. AT stimuli did not change levels of IgG or IgM (Figure 2 C and D) and EL stimuli induced an increase in IgG after 66 dpi comparing to before infection. No difference was observed in IgM levels (Figure 2 E and F).

3.3 *A. suum* infection leads to increase expression of IL-4, TGF-β and IL-17 in human sera

We next analyzed the cellular immune response triggered by *A. suum*. Although we observed no difference in blood cellularity throughout infection (data not shown), the cytokine serum levels did change overtime. We observed an increase in IL-17 and IL-4 levels 66 days post infection (Figure 3 A and F). Interestingly, levels of IL-4 remain higher at 88 dpi, while IL-17 levels decrease. We also observed an increase in TGFβ levels at 88 dpi (Figure 3 E) No difference in levels of IL-10, IL-6 and TNF was observed throughout the infection (Figure 3 B, C and D).

3.4. Infection with *A. suum* induce downregulation of genes related to activation, Th1 phenotype and apoptosis

In order to evaluate the changes induced during larvae migration, we compared, through real time PCR, the expression of 84 genes related to the innate and adaptive immune response before infection and at 12 dpi. Results showed an upregulation of 4 genes and a downregulation of 13 genes comparing 12 dpi as the test group. Volcano plot shows downregulation of genes related to activation, Th1 and apoptosis response such as CD40LG, FASLG, IFNG, JAK2, MAPK8, TBX21, TRAF6, NFKB1, IFNGR1, TICAM1 and STAT1. We also observed an upregulation of CD4 and CD14 (Figure 4). Supplementary table 1 shows a list of all upregulated and downregulated genes (Sup table 1).

3.5 Principal Component Analysis (PCA) confirms change in the immune landscape of the host after *A. suum* infection

In order to evaluate how the expression of all cytokines throughout the infection change overtime we performed a dimensional reduction technique. Principal component analysis (PCA) shows how the cytokines expression change over time. Interestingly, cytokine expression in serum of volunteers at 88

days post infection (purple) cluster together and with a clear separation with all other time's of infection, specially day zero (red), suggesting that infection with *A. suum* does change the immune landscape of the host (Figure 5).

Discussion

Ascaris lumbricoides and *Ascaris suum* are two of the most common soil transmitted nematodes. Although these parasites infect pigs and humans respectively there is reports of cross-transmission between hosts. The zoonotic potential of *Ascaris* parasites has been a matter of debate for decades. Although described in the literature, human infection with *A. suum* are restricted with a few reports and poor knowledge about larvae migration and immunological response [5, 10, 13]. Here we show that *A. suum* is capable of complete the cycle in human hosts, inducing clinical symptoms. Infection also leads to IgG and IgM production and develops an immune response that changes the immune landscape of the host.

Differently from other authors [5, 10, 13], here we shown that *A. suum* was not only capable of infect the human host but also complete the cycle in 4 of the 5 volunteers, since we could recover eggs and adult worms after treatment. This raise the concern that *A. suum* could potentially infect humans not only through cross-transmission between pigs and humans, but also human – human infection.

Infected individuals presented a wide range of symptoms, including cough, respiratory discomfort, diarrhea and headache. Volunteers also presented clinical alteration in chest x-ray. All symptoms are also common symptomatology in human *A. lumbricoides* infection. These manifestations coincided with the period of larvae migration through the lung [18].

Regarding parasite-specific humoral immune responses, we observed an increase expression in IgG and IgM against the crude extract of adult worm (AW) as early as 12 dpi, coinciding with larvae migration. The IgG and IgM were maintained for up to 66 dpi but levels dropped after treatment (88dpi). The role of humoral response in *Ascaris* infection is still unclear. Some authors argue that the humoral response confers some degree of protection against reinfection [19] or indirect protection, due the inflammatory process [20]. Other authors believe that this is only a reflection of the cellular response against infection [21–23]. King and colleagues demonstrated that individuals re-infected with *A. lumbricoides* after 6 months of the first infection were not protected or showed decreased parasite load suggesting that humoral immunity may not be related to protection against reinfections [24].

We next sought to investigate the cellular response driven by the parasite. We observed an increase in IL-4 and IL-17a levels in serum at 66 dpi. Human ascariasis is characterized by a Th2 immune response [8, 9–29]. IL-4 and other cytokines related to the Th2 type response is known as protective during human ascariasis [25–28]. We already demonstrated that a Th2/Th17 mixed response plays a protective role during *Ascaris* re-infection in animal model [29]. Nevertheless, this profile was also related to tissue injury and remodeling, leading to a greater lung damage [29]. We also observed an increase in TGF-β after treatment (88 dpi). Principal component analysis (PCA) showed that *A. suum* infection leads to changes

in the immune landscape of the host, that did not return to homeostasis, clustering separate individuals before and after treatment (0 and 88 dpi).

Gene expression analysis comparing 12dpi with volunteers before infection showed upregulation of T-helper and monocytes (CD4 and CD14 genes), and downregulation of activation, Th1 and apoptosis related genes. Our results are in line with others that showed that *A. suum* downregulates inflammatory pathways in human dendritic cells *in vitro* [30, 31].

Taken together our data indicate that *A. suum* is capable of infect and complete the cycle in human hosts. Larvae migration leads to modulation of the immune response towards Th2/Th17 phenotype. Our data reinforce the zoonotic capacity of *A. suum* and bring a new perspective on the understanding of the immune response against this parasite, especially in the larval phase of infection.

Declarations

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Ethics approval and consent to participate

This study was approved by the Research Ethics Council of the Federal University of Minas Gerais, Brazil (CAAE,26945814. 1.0000.5149), all the volunteers agree to participate spontaneously..

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Conceived and designed the experiment: FSB, DSN, NMR, PHGG,. Performed the experiment: TES, FSB, DSN, NMR, CCA, ACGG. Analyzed the data: TES, FSG, LMDM, ASC . Contributed reagents/ materials/analysis tools: DCB, RTF, LLB. Wrote the paper: TES, LMDM, AGV. All authors read and approved the final version of this paper.

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Figures

(A)

	Eggs/g feces	Female worms	Male worms	Total Adult worms
A	0	0	3	3
B	ND	ND	ND	ND
C	12.011	6	3	9
D	15.869	12	3	15
E	20.307	24	19	43

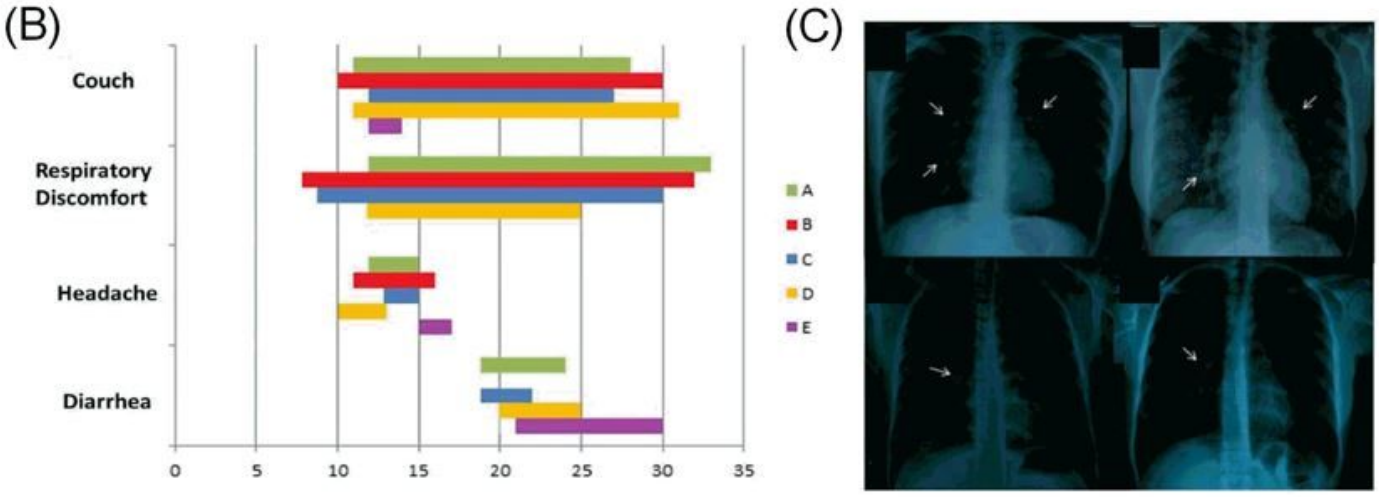

Figure 1

Ascaris suum infection and symptoms in humans. Five volunteers received orally a suspension of 50 viable A. suum eggs. Volunteers were followed with a multidisciplinary health team and received a

spreadsheet to report daily symptoms. (A) Table shows number of eggs per gram of feces and adult worms divided by gender recovered after treatment with Ivermectin (200µg/kg). (B) Symptomatology presented by experimentally infected subjects. (C) Representative Chest x-ray after 12 days of infection. Arrows indicate presence of cellular infiltrate in the peribroncovascular interstice due to larvae migration. ND = no data.

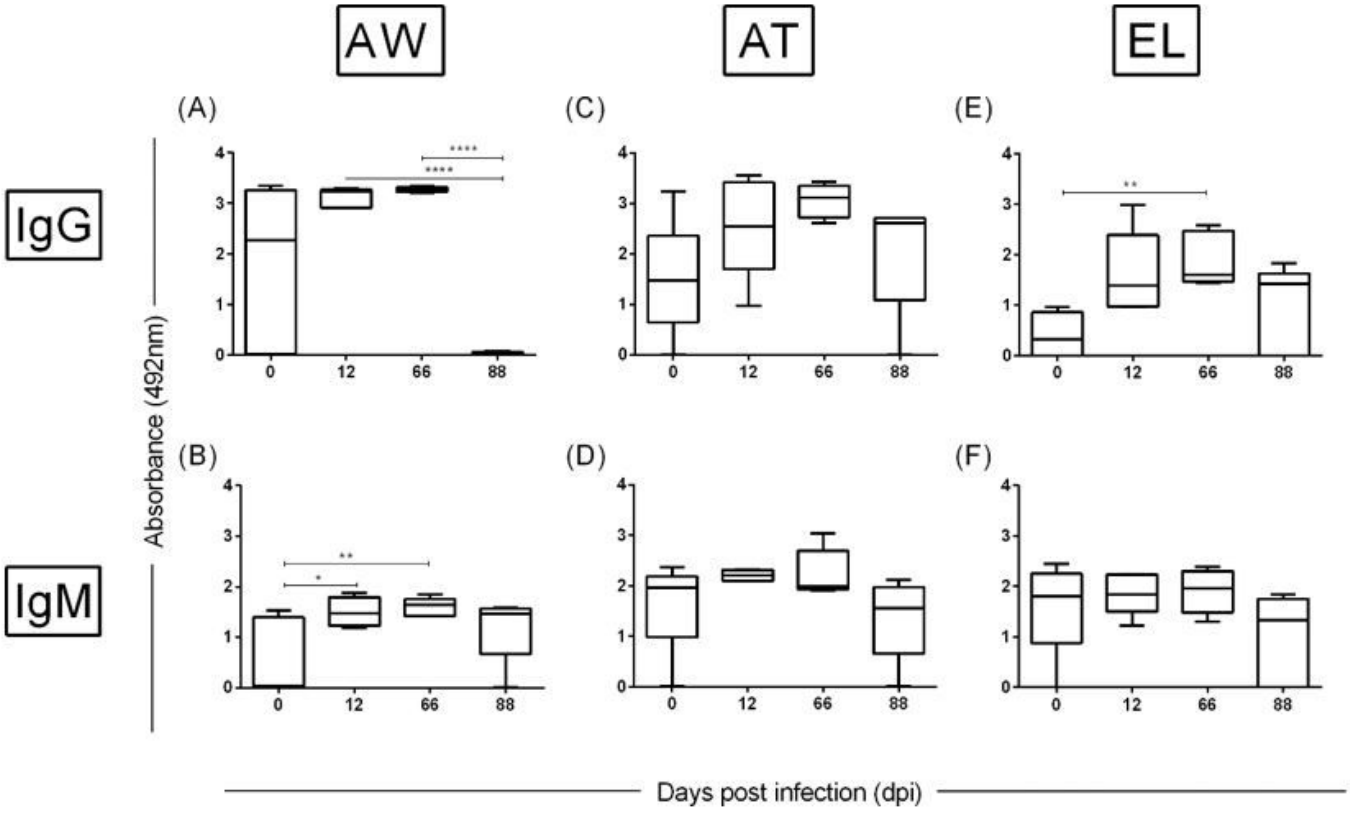

Figure 2

Increase IgG and IgM levels induced by AW in experimental infection with Ascaris suum. Peripheral blood was collected from all volunteers at 0, 12 66 and 88 dpi. After centrifugation, serum was collected, and ELISA performed to detect IgG and IgM serum. Crude extract of adult worm (AW), crude extract of adult worm tegument (AT) and crude extract of infective larvar (EL) antigens were used at a concentration of 1:5000. Figure show IgG (first row) and IgM (second row) against AW (A and B); AT (C and D) and EL (E and F). Results are expressed as box and whiskers plots showing minimum and maximum values. Comparison between groups were performed using One-Way ANOVA test as described in material and methods section. *p<0.05, **p<0.01 and ****p<0.0001.

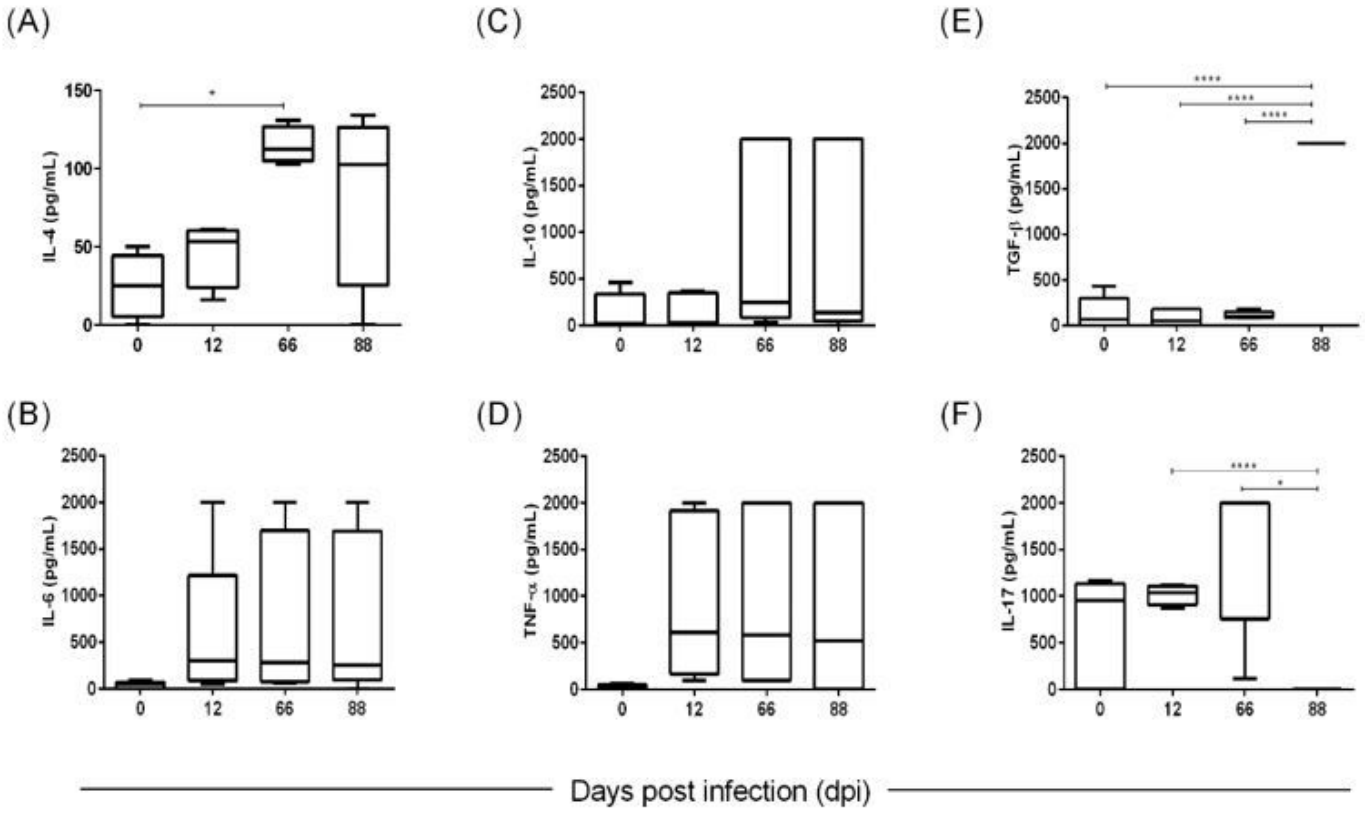

Figure 3

A. suum infection leads to increase expression of IL-4, TGF-β and IL-17 in human sera. Peripheral blood was collected from all volunteers at 0, 12, 66 and 88 dpi. After centrifugation, serum was collected, and cytokines were measured by ELISA. (A) IL-4; (B) IL-6; (C) IL-10; (D) TNF-α; (E) TGF-β; (F) IL-17. Results are expressed as box and whiskers plots showing minimum and maximum values. Comparison between groups were performed using One-Wal ANOVA test as described in material and methods section. *p<0.05 and ****p<0.0001.

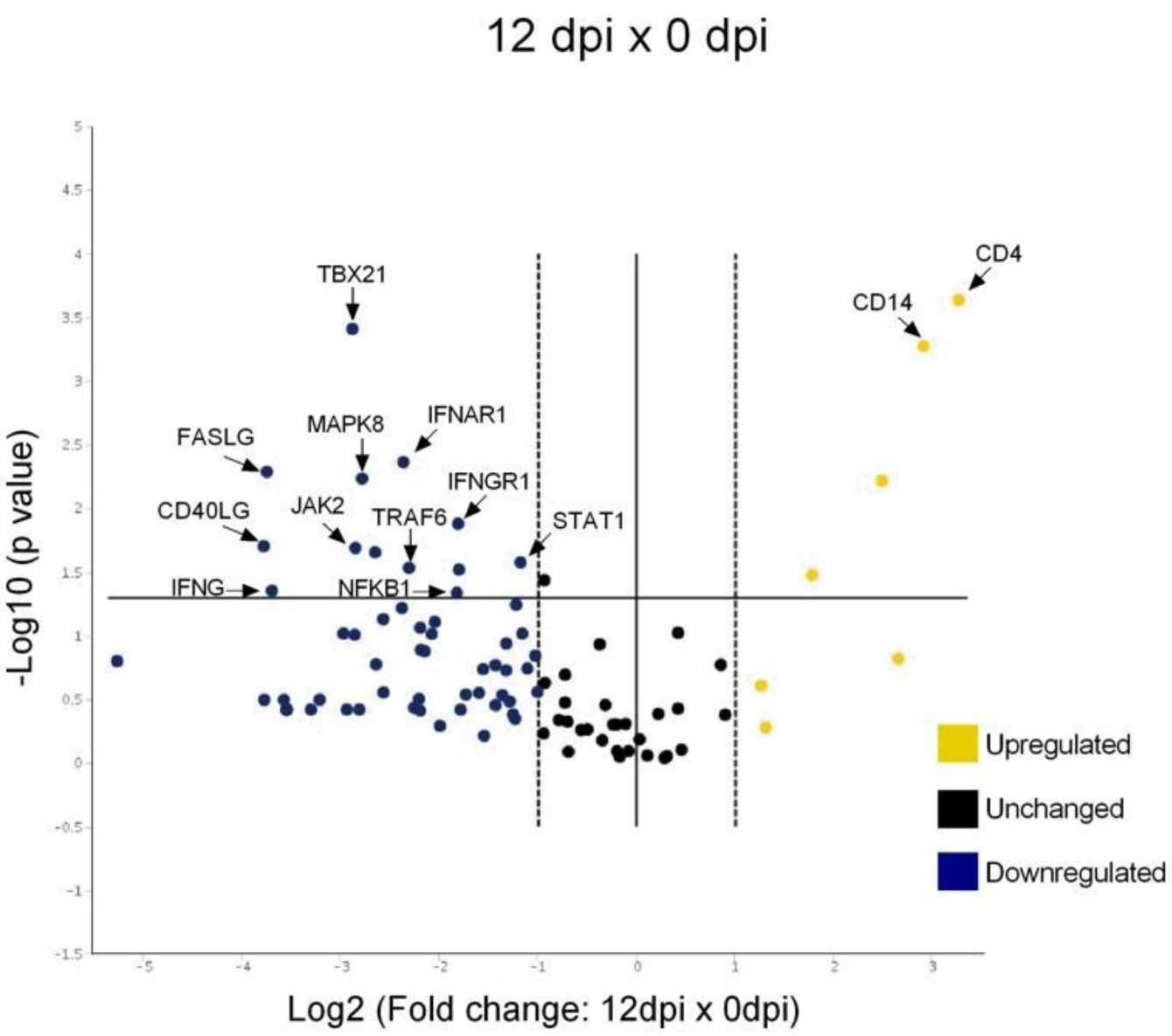

Figure 4

Gene expression change at 12 dpi. Evaluation of gene expression was performed comparing 0- and 12-days post infection. Volcano plot shows the global transcriptional changes in volunteers infected 12 dpi vs. 0 dpi. All genes were plotted. Each circle represents one gene. The log2 fold change is represented in x-axis. The y-axis shows -log10 p value. A p value of 0.05 and a fold change of 2 are indicated by dashed lines. Upregulated genes are showed in yellow dots, downregulated genes are showed in blue, unchanged genes are showed in black.

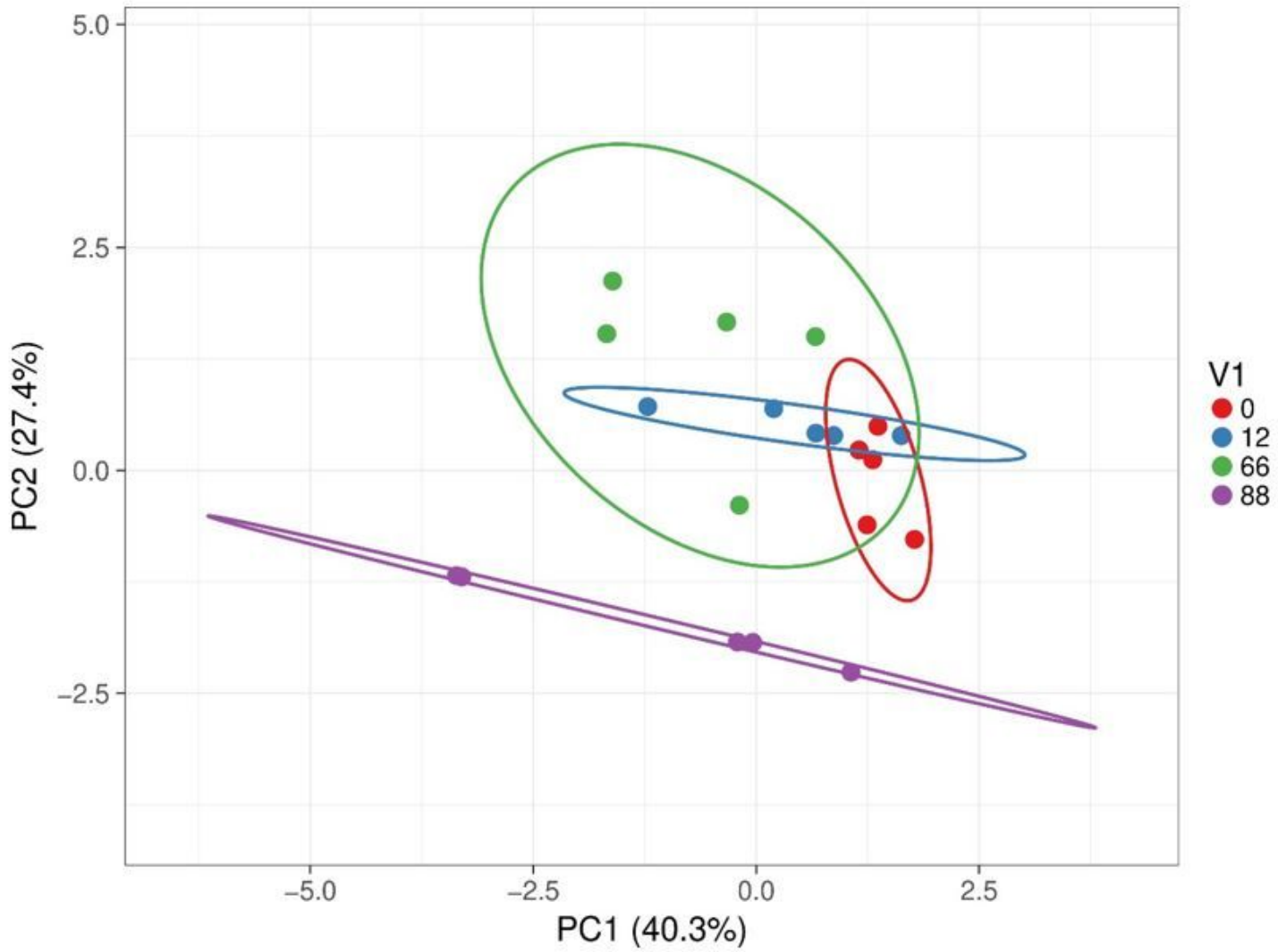

Figure 5

Principal Component Analysis (PCA) confirms change in the immune landscape of the host after A. suum infection. PCA was performed with values of cytokine expression measured by ELISA after 0 (red), 12 (blue), 66 (green) and 88 (purple) days post infection.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.docx