Somatic Antigens of Tropical Liver Flukes Ameliorate Collagen-Induced Arthritis in Wistar Rats

Yasir Akhtar Khan¹, Sadiq Umar², Syed M. A. Abidi¹ *

¹ Section of Parasitology, Department of Zoology, Aligarh Muslim University, Aligarh, India, ² Department of Pharmaceutical Science, Collage of Pharmacy, Washington State University, Spokane, Washington, United States of America

* abbasabidi92@hotmail.com

Abstract

Parasitic helminths polarize immune response of their vertebrate hosts towards anti-inflammatory Th2 type and therefore it is hypothesized that they may suppress the inflammatory conditions in autoimmune disorders. The present study was undertaken to investigate in vivo immunomodulatory and therapeutic potential of somatic antigens (Ag) of liver infecting digenic trematodes [Fasciola gigantica (Fg) and Gigantocotyle explanatum (Ge)] in collagen-induced arthritic (CIA) Wistar rats. The CIA rats were administered subcutaneously with different doses (50 μg, 100 μg and 150 μg) of somatic antigens of Fg and Ge, daily for 21 days, the time period required to establish infection in natural host (Bubalus bubalis). Thereafter, the control, diseased and treated rats were compared for different parameters viz. hind paw thickness; serum interleukins, IL-4 and IL-10, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ); expression level of matrix metalloproteinases (MMPs) -2, -9, -13 and nitric oxide (NO) in knee joints and patellar morphology. The CIA rats treated with different antigens, Fg-Ag and Ge-Ag, show significant amelioration of the disease by down regulation of serum TNF-α and IFN-γ (p < 0.05) and upregulation of IL-4 and IL-10 cytokines (p < 0.05); inhibition (p < 0.05) of MMPs (-2,-9,-13) and NO in knee joints and improved patellar morphology with decreased synovial hypertrophy and reduced infiltration of plymorphonuclear cells. The activity of pro as well as active MMPs (-2 and -9) and active MMP-13 in knee joints of CIA rats was very high compared to the control and treatment groups, suggesting the extent of collagen degradation in CIA rats. Interestingly, the highest dose (150 μg) of Ge-Ag almost wiped out MMP-13 expression. The overall findings suggest that the somatic proteins of Ge-Ag appeared to be therapeutically more effective than Fg-Ag, reflecting interspecific molecular differences which could contribute to the ability of these worms to successfully ameliorate the pathology of CIA.
Introduction

Rheumatoid Arthritis (RA) is a systemic autoimmune disease of unknown etiology [1], affecting about 1% of the world population [2]. It is characterized by progressive damage in bone and cartilage that ultimately leads to disability [3, 4].

In RA, the involvement of CD4+ cells and matrix metalloproteinases (MMPs) are the hallmark for disease progression and degradation of cartilage [5, 6]. RA is mediated by a disequilibrium of Th1 subset derived cytokines including TNF-α and IFN-γ [7, 8] whereas the Th2 subset exerts antagonistic effect on Th1 subset by producing the anti-inflammatory cytokines including IL-4 and IL-10 [9, 10]. Such effect could be used as a therapeutic target in RA. The inflammatory cytokines such as IL-1β and TNF-α upregulate the expression of MMPs which degrade extracellular matrix proteins and play a central role in RA [11, 12]. The articular chondrocytes preferentially express MMP-13 (collagenase-3) which primarily cleaves type II collagen as well as targets type I, type III collagen and aggrecans [13, 14]. Further cleavage is achieved by the gelatinases, MMP-2 and MMP-9 which can also digest type I collagen and aggrecans [15, 16]. Therefore, the inhibition of different MMPs could also be one of the most effective therapeutic targets to prevent RA. Many synthetic inhibitors have been used to suppress expression of MMPs [17, 18]. The inhibition of cytokine suppressive binding protein / p38 kinase significantly ameliorates the disease in the CIA models, possibly through suppression of MMPs [19]. Besides, the IL-4 is known to inhibit IL-1 induced expression of MMPs while IL-10 suppresses the MMPs by upregulating the expression of TIMP (Tissue inhibitor of metalloproteinase) [20–24]. The protective role of Th2 cytokines such as IL-4 and IL-10 in arthritis has been shown earlier [25]. It has been reported that IL-10 inhibits paw swelling, pannus formation, suppresses pro-inflammatory cytokines and cartilage degradation in CIA rats [25]. Other key modulators in RA are reactive oxygen species (ROS), reactive nitrogen species (RNS) and nitric oxide (NO) which are triggered by pro-inflammatory cytokines, TNF-α, IFN-γ, IL-1 and IL-2 [26–28] while anti-inflammatory cytokines, IL-4, IL-8, IL-10, IL-13 and TGF-β suppress their synthesis [29–31].

The skewing of pro-inflammatory response towards an anti-inflammatory nature and thereby suppression of MMPs, could be a valid therapeutic strategy in RA and that could be achieved by the use of helminth antigens because it has been revealed that ES62 antigen derived from a filarial nematode suppresses TNF-α and IFN-γ in Collagen-induced arthritis [32]. The liver fluke, Fasciola hepatica infection polarizes a strong Th2 response leading to antibody class switching and not only inhibit the Th1 response [33], but also attenuate the experimental autoimmune encephalomyelitis through suppression of Th1 and Th17 response [34]. The Th2 response developed in Fasciola hepatica infection remains consistent even in chronic infection [35]. Fasciola hepatica derived Excretory / Secretory (E / S) products also induce strong Th2 response [36]. Further, these parasites also suppress Th1 derived immune response during Bordetella pertussis and Mycobacterium bovis infection and inhibit mast cells to mount Th1 response in mice [36–39].

Almost nothing is known about the immunomodulatory role of the tropical liver fluke, Fasciola gigantica and amphistome, Gigantocotyle explanatum antigens. Though, in our preliminary studies we found a strong Th2 type response against their antigens. But the present study was undertaken to investigate therapeutic efficacy of liver fluke antigens which modulate the cytokines (IL-4, IL-10, TNF-α and IFN-γ), MMPs (-2, -9 and -13), nitric oxide (NO) and patellar morphology in Wistar rats which were subjected to collagen-induced arthritis.

Materials and Methods

Animals

Female Wistar rats, weighing about 200g and 8 week old, were purchased from the Animal Facility at Jamia Hamdard Central University (JHCU), New Delhi, India under the license of the
Departmental Ethical Committee of Department of Biochemistry, Aligarh Muslim University, which reviewed and approved all the experimental work involving animals. The experimental animals were randomly divided into 4 groups viz. 1. Vehicle control, 2. Collagen-induced arthritis (n = 5 in each group), 3. Fasciola gigantica antigens treated and 4. Gigantocotyle explanatum antigens treated. The groups, 3 and 4 were further divided into three subgroups (n = 5 in each subgroup) for three different doses of antigens.

**Extraction of somatic antigens of liver flukes**

The adult live flukes of *Fasciola gigantica* and *Gigantocotyle explanatum*, obtained from the infected livers of freshly slaughtered Indian water buffaloes at the local abattoir, were thoroughly washed with Hanks’ saline (HBSS) (Fisher Scientific, U.S.A.) followed by a rinse with 0.1M phosphate buffered saline (PBS) (Fisher Scientific, U.S.A.), pH 7.4, containing 0.5% antibiotic / antymycotic solution (Himedia, India) and then incubated for 1 hour at 37°C to allow regurgitation of the gut contents. Thereafter, worms were homogenized in 0.1M PBS, pH 7.4; centrifuged at 10,000×g for 30 min in cooling centrifuge (Eppendorf, Germany) at 4°C. The cell free supernatants of *Fasciola gigantica* (Fg) and *Gigantocotyle explanatum* (Ge) were separately aliquoted and stored at —80°C for subsequent use as antigens (Ag) for treatment purpose.

**Induction of collagen-induced arthritis and treatment schedule**

The collagen-induced arthritis (CIA) was developed according to the previously described protocols [40] with some modifications. Briefly, female Wistar rats were intradermally immunized at the tail base avoiding the tail vain with 100 μg of chicken type II collagen (generous gift from Prof. Haqqi, Cleveland, U.S.A.) prepared at a concentration of 2mg/ml in 0.05M acetic acid, emulsified with an equal volume of Freund’s Complete Adjuvant (FCA) (Sigma, U.S.A.). Following CIA induction, three different doses (50 μg, 100 μg and 150 μg) of Fg-Ag and Ge-Ag were daily injected subcutaneously at the tail base up to 21 days in each rat from treatment groups. The rats in the treatment groups were primed with antigens one day (Day-1) before injection of collagen type II (Day 0), while the control group received only vehicle (PBS). On day 0 the rats were also injected with antigens 6 hours after the collagen injection. Finally, on day 22 the rats were anesthetized by anesthetic ether and immediately afterwards maximum amount of blood was collected by cardiac puncture for serum collection. Thereafter, patellae and knee joint tissues were collected for various studies. For better understanding, the treatment schedule is shown by the schematic diagram (Fig 1).

**Paw thickness measurement**

The paw thickness was measured using digital vernier caliper (Digital Caliper, India) by taking ankle joints of both the hind paws. The two independent observers having no knowledge of experimentation accomplished this task and pooled data was analyzed and subjected to statistical analysis.

---

*Fig 1. Schematic diagram for treatment schedule.* The figure showing timing and duration of arthritis induction and treatment of collagen (chicken type II) inoculated Wistar rats.

doi:10.1371/journal.pone.0126429.g001
Quantitation of serum cytokines

The level of serum anti-inflammatory cytokines, IL-4 and IL-10 and pro-inflammatory cytokines, TNF-α and IFN-γ of the control, CIA and Fg-Ag / Ge-Ag treated rats were analyzed according to the instructions provided with the CBA kit (BD Bioscience, U.S.A.). The data was acquired on LSR II Flow Cytometer at BD FACS Academy, JHCU, New Delhi, India and analyzed using FCAP ARRAY software version 3.0 (BD Bioscience, U.S.A.).

Preparation of cell free suspension of knee joints

The knee joints were macerated using a mortar and pestle, in 50mM tris- HCl buffer (Sigma, U.S.A.), pH-7.6 and 0.1% Triton X-100 (Sigma, U.S.A.) and then homogenized in Teflon Tissue Homogenizer (Yorco, India) while tissue was kept on ice. The crude extract was sonicated with 3 pulses of 10 seconds with a time gap of one minute, keeping the samples on ice, using Digital Ultrasonicator (Biogen Scientific, India). Homogenate was centrifuged at 10,000xg for 10 minutes at 4°C. Afterward, supernatant was aliquoted and stored at −80°C until use.

Zymographic detection of matrix metalloproteinases (MMPs) in knee joints

The substrate gel zymography was performed as previously described [41] with some modifications. The SDS polyacrylamide gels co-polymerized with gelatin (BioRad Cat. # 170–6537, U.S.A.) (1 mg/ml) were used to detect the gelatinolytic activity in the tissue extracts of knee joints. A total of 26 μg of protein from each sample was subjected to electrophoresis at a constant voltage of 100V. After electrophoresis, gels were incubated in 2.5% Triton X-100 (4 x 15 min changes) with gentle shaking and then incubated overnight at 37°C in activation buffer (50mM Tris-HCl, pH 7.4, containing 10mM Calcium chloride and 0.05% Brij 35 (Sigma, U.S.A.). Gels were then fixed and stained with Coommassie Brilliant Blue R 250 (Sigma, U.S.A.) prepared in fixative (methanol: glacial acetic acid: water:: 45:10:45) and images were acquired on Gel Doc XR+ (BioRad, U.S.A.). The pixel density / unit volume for each band of different active MMPs and ratio between pro / active MMPs for different experimental groups were calculated by using Image Lab software (BioRad, U.S.A.). The identical gels were incubated in activation buffer containing 20mM EDTA (Sigma, U.S.A.), for MMPs inhibition study.

Nitric Oxide assay in knee joints

Nitric oxide (NO), an important physiological messenger and effector molecule in immunological systems [42], was determined by a previously described method using the Griess Reagent System [43]. Briefly, the cell free suspension of the joint extract was prepared in 0.05M Tris-HCl buffer, pH-7.6 and then 50 μl of experimental sample was added in 96 well ELISA plate in duplicate followed by addition of 100 μl of Griess reagent (0.04g/ml) (Sigma, U.S.A.). Samples were incubated at room temperature for 10 minutes in dark. The reading was taken at 540nm on iMark Elisa Plate Reader (BioRad, U.S.A.). The level of NO (μM/ mg protein) in unknown sample was measured by comparing with standard curve prepared with sodium nitrite (Fisher Scientific, U.S.A.).

Scanning Electron Microscopy of patellae

The joints of interest i.e. knee joints were dissected out and patellae were carefully removed and fixed in 2.5% gultaraldehyde / paraformaldehyde (Sigma, U.S.A.) solution for 24 hours at 4°C. Further sample processing was done at the All India Institute of Medical Sciences (AIIMS) EM Facility, New Delhi, India, following standard protocol. Samples were visualized on Jeol
Scanning Electron Microscope, Model, JSM 6510 LV (USIF, AMU, Aligarh, India) and the images were saved.

Protein estimation
The protein content in extracts of *F. gigantica*, *G. explanatum* and knee joints was estimated by the dye binding method [44] as modified for smaller volumes [45]. The bovine serum albumin was used as the standard.

Statistical Analysis
The data was subjected to Tukey HSD (Tukey high significant difference) One way ANOVA test by using R software [R version 3.1.2 (2014-10-31) "Pumpkin Helmet"] for multiple comparison in all the parameters studied. The graphs for different parameters (cytokines, MMPs and NO) were plotted in the form of box plots which show median, quartile and interquartile range using R software. The paw thickness was plotted on Microsoft excel (Window 7.0) and Mean±SEM was calculated. The Spearman rank correlation coefficient (r²), for paw thickness measurement (carried out by two independent observers) was calculated by the above statistical software. The level of significance with the p value less than 0.05 was considered as statistically significant.

Results
Induction of Arthritis
Arthritis was developed promptly in rats inoculated with collagen emulsified with FCA. Clinical emblems of the disease were erythema of one or more ankle joints, followed by metatarsal and interphalangeal joints, first appeared in the hind paws between 8 and 9 days after injecting chicken type II collagen, with a 100% incidence by day 13±1. Swelling, redness and restriction in movement was also observed in CIA rats as compared to the controls (Fig 2A).

Clinical symptoms of arthritis were modified by liver fluke somatic antigens
The collagen-induced arthritic joints in Wistar rats showed remission of the disease following immunization with Fg-Ag and Ge-Ag. The Ge-Ag was found to be more effective than the Fg-Ag (Fig 2B) but the significant dose dependent response was not evident. The maximum disease suppression occurred at 150 μg of Ge-Ag treatment. From the very beginning the treated rats (except Fg50) consistently showed low to moderate symptoms (redness, swelling and restriction in movement) as compared to the CIA rats. In Fg50 treated rats, the symptoms lowered down significantly after 16th day. The Spearman rank correlation coefficient (r²) between data (paw thickness) recorded by two independent observers was: Control (0.932), CIA (0.981), Fg50 (0.938), Fg100 (0.987), Fg150 (0.986), Ge50 (0.989), Ge100 (0.994) and GF150 (0.996). The therapeutic significance level of different antigen treated rats in comparison to CIA rats was: Fg50 (p < 0.05), Fg100 (p < 0.05), Fg150 (p < 0.01), Ge50 (p < 0.01), Ge100 (p < 0.001), Ge150 (p < 0.001) and the order of disease remission was: Ge150 > Ge100 > Ge50 > Fg150 > Fg100 > Fg50.

Modulation of serum cytokines
The disequilibrium in inflammatory cytokines has a central role in the perpetuation of chronic inflammation and tissue damage during progression of RA. The level anti-inflammatory cytokines, IL-4 and IL-10 and pro-inflammatory cytokines, TNF-α and IFN-γ served as the marker...
for Th2 and Th1 environment in rat’s serum, respectively. Our results showed significant (p < 0.05) increase in the level of IL-4 at 150 μg of Fg-Ag and at higher doses (100 μg and 150 μg) of F. gigantica and G. explanatum somatic antigens. The therapeutic significance level of different antigens treated rats in comparison to CIA rats was: Fg50 (p < 0.05), Fg100 (p < 0.05), Fg150 (p < 0.01), Ge50 (p < 0.01), Ge100 (p < 0.001), Ge150 (p < 0.001). The values are represented as Mean ± SEM. The p value < 0.05 was considered as significant (A & B). (The data of all the antigens treated groups is compared with the CIA group)

doi:10.1371/journal.pone.0126429.g002

for Th2 and Th1 environment in rat’s serum, respectively. Our results showed significant (p < 0.05) increase in the level of IL-4 at 150 μg of Fg-Ag and at higher doses (100 μg and 150 μg) of Ge-Ag (p < 0.001) (Fig 3A). In case of Fg-Ag treated rats a significant (p < 0.05 and p < 0.001 for 100μg and 150μg doses, respectively) increase in IL-10 was observed in a dose dependent manner, while no significant change was recorded at 50 μg of Fg-Ag. In case of Ge-Ag treated rats, all the antigenic doses (50 μg, 100 μg, 150 μg) showed a significantly (p < 0.01, p < 0.001 and p < 0.05, respectively) increased IL-10 level in a dose independent manner with maximum level at 100 μg of antigen (Fig 3B).

A significant (p < 0.001) depletion of TNF-α in serum was observed with dose dependent manner in case of Fg-Ag treated rats, while Ge-Ag treated rats showed a significant (p < 0.001) dose independent depletion in TNF-α level (4A). The level of pro-inflammatory cytokine IFN-γ in CIA rats was significantly (p < 0.001) increased as compared to control rats, but declined significantly (p < 0.05) after treatment with Fasciola antigens in a dose independent manner at higher doses (100 μg and 150 μg of Fg-Ag). However, in case of Ge-Ag treated rats IFN-γ level decreased significantly (p < 0.01, p < 0.01 and p < 0.001 for 50 μg, 100 μg and 150 μg of Ge-Ag respectively) in a dose dependent manner (Fig 4B).
Down regulation of MMP-2, MMP-9 and MMP-13

Both the pro- (latent) and active-MMPs were detected in control, inflamed (CIA) as well as treated knee joints extracts (Fig 5A showing representative gel). A very significant (p < 0.001) increase in different MMPs expression was observed in CIA rats in comparison to control rats, showing the severe pathology and degradation of collagen after injecting type II collagen.

There was an overall significant (p < 0.001) inhibition of MMP-2 (Fig 5B), MMP-9 (Fig 5C) and MMP-13 (Fig 5D) in joint extracts of Wistar rats after treatment with different doses of Fg-Ag and Ge-Ag somatic antigens in comparison to the untreated CIA rats. It is evident from Fig 5A that the level of MMP-13 was suppressed more significantly than MMP-2 and MMP-9 by the fluke antigens and it vanished completely at the highest dose of Ge-Ag. In control rats pro MMPs (-2, -9 and -13) were not detectable while in rest of the treatment pro MMP-2 and MMP-9 could be measured except pro MMP-13. The ratio of pro and active MMPs is shown in Table 1. The ratio of pro and active MMP-2 and 9 was maximum (0.337 and 0.242, respectively) in CIA rats while minimum in Ge150 (0.103) and Fg150 (0.075) treated rats. However, the difference between MMP-2 and MMP-9 ratio was not significant. The gels which were incubated in the activation buffer containing EDTA did not show any gelatinolytic activity, hence confirming the presence of MMPs (S1 Fig).
Inhibition of Nitric oxide in joints

The NO level was sharply upregulated in CIA rats while treatment of diseased rats with parasite antigens (Fg-Ag and Ge-Ag) significantly (p < 0.001) brought down the level of NO. The effect of Ge-Ag appeared to be more pronounced, particularly at the highest dose (150 μg), which maintained NO level close to the control, reflecting dose dependent inhibitory effect of the Ge-Ag (Fig 6).

Patellar morphology and interaction with polymorphonuclear cells

The scanning electron microscopy revealed that the patellae of control rats appeared healthy and well orchestrated (Fig 7A–7C). Whereas, the CIA rats showed severe pathology with synovial hypertrophy, protrusion of synovial membrane, recruitment of inflammatory cells and prominent fissuring of articular cartilage. The overall disruption and shrinkage of patellae was clearly evident (Fig 7D–7F).

Though the patellae in Fg-Ag treated rats was not as healthy as control, but showed some degree of protection with slight synovial hypertrophy and presence of PMNCs at

Table 1. Ratio of pro and active MMPs (-2 and -9).

| MMPs | Control | CIA | Fg50 | Fg100 | Fg150 | Ge50 | Ge100 | Ge150 |
|------|---------|-----|------|-------|-------|------|-------|-------|
| MMP-2 | 0.00 | 0.337 | 0.206 | 0.178 | 0.154 | 0.265 | 0.144 | 0.103 |
| MMP-9 | 0.00 | 0.242 | 0.083 | 0.110 | 0.075 | 0.130 | 0.142 | 0.141 |

doi:10.1371/journal.pone.0126429.t001
synovium-cartilage junction without any cartilage damage that reflects disease remission (Fig 8A–8I), thus supporting our other results. The patellae of Ge-Ag treated rats showed not only a high degree of disease remission as compared to CIA rats, but also in comparison to Fg-Ag treated rats, thus providing a high level of protection. The highest dose of Ge-Ag (150 μg) resulted in negligible synovial hypertrophy and absence of PMNCs in the joints (Fig 9A–9I).

Discussion

Experimental infection in mouse models suggests that helminth infection can ameliorate autoimmune diseases [32, 34, 46–50] and provokes a major shift in understanding of T cell regulation. It has been reported that the Fasciola hepatica infection as well as its E/S products induce
polarized Th2 responses in mice and suppressed Th1 responses [36, 51]. Collagen-induced arthritis is a well known model of autoimmune disorder having many similarities with the rheumatoid arthritis. It is evident from the present results that there was considerable inflammation of joints in the CIA rats along with an increased MMP-2, MMP-9 and MMP-13 activity, which has also been known to be upregulated in humans [52]. The ratio of pro and active MMPs (-2,
-9) was maximum in knee joints of diseased rats, while pro MMP-13 was not detectable in diseased as well as in other experimental groups. The difference between the ratio of pro/active MMP-2 and MMP-9 was not significant. In the present study, upregulation of serum TNF-α and IFN-γ level while down regulation of IL-4 and IL-10 level, increased NO level and damages to the patellar morphology was observed in CIA rats. The treatment of arthritic rats with tropical liver fluke, Fasciola gigantica and the amphistome, Gigantocotyle explanatum derived somatic antigens resulted in significant remission of the disease as manifested by clinical symptoms (swelling, redness and restriction in movement), depletion in serum TNF-α and IFN-γ, increased serum IL-4 and IL-10 levels, under expression of MMPs, decline in NO synthesis and amelioration of patellae pathology. The reason behind this effect could be understood from the epidemiology of autoimmune disorders and helminthic infections that build the background for hygiene hypothesis which proposes that increased incidence of the inflammatory diseases is associated with the absence of appropriate priming of the immune system by parasitic helminths during the childhood [53].

In RA, the disregulation in Th1 immune response leads to the destruction of cartilage and bone through matrix metalloproteinases which disrupt the function of chondrocytes and osteoclasts and high oxidative stress [54, 55]. Furthermore, altered / activated macrophages secrete different cytokines including TNF-α, IL-1 and IL-6, which attract other cells towards the inflammation site [56] where TNF-α along with IL-1 in synovial tissues stimulate release of MMPs, which further enhance the damage [11, 12]. However, the helminth derived somatic products inhibited the Th1 type of immunity in the present study. It has been shown that the nematode secreted product, ES-62, ameliorates the arthritic symptoms [32]. This protective effect can be correlated with the inhibition of collagen specific pro-inflammatory cytokines—TNF-α, IFN-γ, IL-6 and suppression of IgG2a antibody [57]. It has been described earlier that the Fasciola infection induces a strong Th2 response leading to the antibody class switching and inhibiting the Th1 response [33]. Studies have also revealed that the F. hepatica infection can attenuate the experimental autoimmune encephalomyelitis through suppression of Th1 and Th17 immune responses [34]. Furthermore, the parasite derived products also induce strong Th2 response [36, 39]. The analysis of serum cytokines showed that the Fasciola gigantica and Gigantocotyle explanatum derived somatic antigens also elicited strong Th2 type of immune response in Wistar rats as reflected by high levels of serum IL-4 and IL-10 and low level of TNF-α and IFN-γ in CIA rats after treatment with Fg-Ag and Ge-Ag in the present study. Therefore, the immune suppression involving inhibition of different MMPs (-2, -9 and -13) by the somatic antigens that may also include the excretory / secretory precursors of liver flukes, might be one of the major mechanism that could be protecting the articular cartilage in experimentally induced auto-immune condition. The inhibition of MMP-13 was extraordinarily high in case of G. explanatum treated rats that may suggest the specificity of inhibition for MMP-13 by this parasite. Such specificity of inhibition may become a major shift in arthritis treatment, because the collagen degradation is mainly initiated by MMP-13 [13, 14]. The explanation of the rationale for such specific inhibition is not clear at this stage but it is well established that different MMPs can be selectively and non-selectively inhibited by different products [18, 58–60]. Therefore, it can be speculated that the G. explanatum antigens might be containing some molecules which strongly inhibited MMP-13 in particular and MMP-2 and MMP-9 in general, however, there was no significant difference between the cytokine profile of G. explanatum and F. gigantica treated rats. Furthermore, high level of NO is the outcome of the disease severity [55] which in turn stimulates the pro inflammatory environment and is an important mediator in central and peripheral pain; promote cartilage damage by modulating MMPs expression and apoptosis [61]. In the present study, inhibition of nitric oxide by the liver fluke antigens also suggests that the collagen mediated Th1 response and MMPs

---

[53] Helminth Antigens Ameliorate Collagen Induced Arthritis

PLOS ONE | DOI:10.1371/journal.pone.0126429 May 18, 2015 11 / 15
expression was suppressed in the Wistar rats. The destruction of articular cartilage, synovium hypertrophy, recruitment of PMNCs in patellae in CIA rats as evident from the SEM (Scanning Electron Microscopy) results reflect pro-inflammatory environment which was down regulated after treatment with different doses of Fg-Ag and Ge-Ag antigens. Despite of insignificant variation in IL-4, IL-10, TNF-α and IFN-γ level between Fg-Ag and Ge-Ag treated rats, the overall protection level was higher in rats treated with Ge-Ag, which could possibly be due to the interspecific molecular differences that remain a subject for further investigations.

**Conclusion**

Taken together, it is concluded that not only the tropical liver fluke, *Fasciola gigantica* but less known amphistome parasite, *Gigantocotyle explanatum* also ameliorates the experimentally induced arthritic symptoms by strong polarization of the immune responses towards the Th2 type. However, further studies are required to identify and characterize the active components of the two parasites to validate their anti-inflammatory potential by suppressing the MMPs and NO level as well as the signal transduction pathways which involve T cell polarization.

**Supporting Information**

**S1 Fig.** Matrix metalloproteinases (MMPs) inhibition study. Representative zymographic gel incubated in activation buffer containing EDTA.

**S2 Fig.** Schematic diagram showing summary of the work. MMPs; matrix metalloproteinases, IL-10; Interleukin-10, IFN-γ; Interferon-γ, CIA; Collagen induced arthritis, NO; nitric oxide

**Acknowledgments**

We are grateful to Professor Tariq Haqqi for his kind gift of Chicken type II Collagen, Dr Vinay Gupta, BD FACS Academy, JHCU, New Delhi, for his assistance in FACS and data analysis, Professor Aijaz Ahmed Khan, Department of Anatomy, JN Medical College, AMU Aligarh, for the demonstration of patella dissection and Professor Athar Ali Khan, Department of Statistics and Operational Research, AMU, Aligarh, for assistance in statistical analysis. We are also thankful to Mr. Azam and Mr. Sarfaraz for paw diameter measurements.

**Author Contributions**

Conceived and designed the experiments: YAK SU SMAA. Performed the experiments: YAK. Analyzed the data: YAK SU SMAA. Contributed reagents/materials/analysis tools: YAK SU SMAA. Wrote the paper: YAK SU SMAA.

**References**

1. Maradit-Kremers H, Nicola PJ, Crowson CS, Ballman KV, Gabriel SE. Cardiovascular death in rheumatoid arthritis: a population based study. Arthritis Rheum. 2005; 52(3): 722–732. PMID: 15751097
2. Akhter E, Bilal S, Kiani A, Haque U. Prevalence of arthritis in India and Pakistan: a review. Rheumatol Int. 2011; 31: 849–855. doi:10.1007/s00296-011-1820-3 PMID: 21331574
3. Pincus T. Long-term outcomes in rheumatoid arthritis. Br J Rheumatol. 1995; 34(2): 59–73.
4. Scott DL, Pugner K, Kaarela K, Doyle DV, Woolf A, Holmes J, et al. The links between joint damage and disability in rheumatoid arthritis. Rheumatol. 2000; 39(2): 122–132.
5. Fournier C. Where do T cells stand in rheumatoid arthritis? Joint Bone Spine. 2005; 72: 527–532. PMID: 16087382
6. Yasuda T. Cartilage destruction by matrix degradation products. Mod Rheumatol. 2006; 16: 197–205. PMID: 16906368
7. Schulze-Koops H, Kalden JR. The balance of Th1/Th2 cytokines in rheumatoid arthritis. Best Pract Res Clin Rheumatol. 2001; 15(5): 677–691. PMID: 11812015
8. Matsuno H, Yudoh K, Katayama R, Nakazawa F, Uzuki M, Sawai T, et al. The role of TNF-α in pathogenesis of inflammation and joint destruction in rheumatoid arthritis (RA): a study using a human RA/SCID mouse chimera. Rheumatol. 2001; 41: 329–337.
9. Spellberg B, Edward JE Jr. Type1/Type2 Immunity in Infectious Disease. Clin Infec Dis. 2001; 32: 76–102.
10. Maizels RM, Yazdanbakhsh M. Immune Regulation by helminth parasites: Cellular and Molecular Mechanism. Nature Rev Immunol. 2003; 3: 733–744. PMID: 12949497
11. Dayer JM, Beutler B, Cerami A. Cachectin/tumor necrosis factor stimulates collagenase and prostaglandin E2 production by human synovial cells and dermal fibroblasts. J Exp Med. 1985; 162: 2163–2168. PMID: 2999289
12. MacNaul KL, Chartrain N, Lark M, Tocci MJ, Hutchinson NI. Discoordinate expression of stromelysin, collagenase, and tissue inhibitor of metalloproteinases-1 in rheumatoid human synovial fibroblasts. Synergistic effects of interleukin-1 and tumor necrosis factor-alpha on stromelysin expression. J Biol Chem. 1999; 265: 17238–17245. PMID: 1698777
13. Fosang AJ, Last K, Knauper V, Murphy G, Neame PJ. Degradation of cartilage aggrecan by collagenase-3 (MMP-13). FEBS Lett. 1996; 380: 17–20. PMID: 8603731
14. Moore BA, Aznavoorian S, Engler JA, Windsor LJ. Induction of collagenase-3 (MMP-13) and stromelysin-1 (MMP-3) in human synovial fibroblasts. Biochimica et Biophysica Acta. 2000; 1502: 307–318. PMID: 11040455
15. Fosang AJ, Neame PJ, Last K, Hardingham TE, Murphy G, Hamilton JA. The interglobular domain of cartilage aggrecan is cleaved by PUMP, gelatinases, and cathepsin B. J Biol Chem. 1999; 265: 19470–19474. PMID: 1326552
16. Nguyen Q, Murphy G, Hughes CE, Mort JS, Roughley PJ. Matrix metalloproteinases cleave at two distinct sites on human cartilage link protein. Biochem J. 1993; 295: 595–598. PMID: 8049669
17. Shalinsky DR, Brekken J, Zou H, McDermott CD, Forsyth P, Edwards D, et al. Broad antitumor and antiangiogenic activities of AG3340, a potent and selective MMP inhibitor undergoing advanced oncology clinical trials. Ann N Y Acad Sci. 1999; 878: 236–270. PMID: 10415735
18. Gatto C, Rieppi M, Borsotti M, Innocenti S, Cerutti R, Drudis T, et al. BAY 12-9566, a novel inhibitor of matrix metalloproteinases with antiangiogenic activity. Clin Cancer Res. 1999; 5(11): 3603–3607. PMID: 10589777
19. Badger AM, Bradbeer JN, Votta B, Lee JC, Adams JL, Griswold DE. Pharmacological profile of SB 203580, a selective inhibitor of cytokine suppressive binding protein/p38 kinase, in animal models of arthritis, bone resorption, endotoxin shock and immune function. J Pharmacol Exp Ther. 1996; 279: 1453–1461. PMID: 8968371
20. Lacraz S, Nicod LP, Chicheportiche R, Welgus HG, Dayer JM. IL-10 Inhibits Metalloproteinase and Stimulates TIMP-1 Production in Human Mononuclear Phagocytes. J Clin Invest. 1995; 96: 2304–2310. PMID: 7593617
21. Borghaei RC, Rawlings PL Jr., Mochan E. Interleukin-4 suppression of interleukin-1 induced transcription of collagenase (mmp-1) and stromelysin 1 (mmp-3) in human synovial fibroblasts Arthritis Rheum. 1998; 41(8): 1398–1406. PMID: 9704637
22. Roth I, Fisher SJ. IL-10 is an Autocrine Inhibitor of Human Placental Cytotrophoblast MMP-9 Production and Invasion. Dev Biol. 1999; 205: 194–204. PMID: 9882507
23. Mostafa Mtairag E, Chollet-Martın S, Oudghiri M, Laguay N, Jacob MP, Michel JB, et al. Effects of interleukin-10 on monocyte/endothelial cell adhesion and MMP-9/TIMP-1 secretion. Cardiovasc Res. 2000; 49: 882–890.
24. Chambers M, Kirkpatrick G, Evans M, Gorski G, Foster S, Borghaei RC. IL-4 inhibition of IL-1 induced Matrix Metalloproteinase-3 (MMP-3) expression in human fibroblasts involves decreased AP-1 activation via negative crosstalk involving of Jun N-terminal Kinase (JNK). Exp Cell Res. 2013; 319(10): 1398–1408. doi: 10.1016/j.yexcr.2013.04.010 PMID: 23608488
25. Khalifeh MS, Al-Rukibat R, Hananeh W, Boumezzag A, Okour O. Investigation of the role of tumour necrosis factor-α, interleukin-1β, interleukin-10, nitric oxide and tumour necrosis factor-immunoglobulin M in a rat model of arthritis. Lab Anim. 2010; 44: 143–149. doi: 10.1258/la.2009.0080132 PMID: 19858163
26. Drapier JC, Wietzertin J, Hibbs JB Jr. Interferon-γ and tumour necrosis factor induce the L-arginine-dependent cytopoietic effector mechanism in murine macrophages. Eur J Immunol. 1988; 18: 1587–1592. PMID: 3142779
27. Kilbourn RG, Belloni P. Endothelial cell production of nitrogen oxides in response to interferon gamma in combination with tumor necrosis factor, interleukin-1 or endotoxin. J Natl Cancer Inst. 1990; 82: 772–776. PMID: 2109093

28. Hibbs JB Jr, Westenfelder C, Taintor R, Vavrin Z, Kablitz C, Baranowski RL, et al. Evidence for cytokine-inducible nitric oxide synthase from L-arginine in patients receiving interleukin-2 therapy. J Clin Invest. 1992; 89: 867–877. PMID: 1541678

29. McCall TB, Palmer RMJ, Moncada S. Interleukin-8 inhibits the induction of nitric oxide synthase in rat peritoneal neutrophils. Biochem Biophys Res Commun. 1991; 186: 680–685.

30. Oswald IP, Gazzinelli RT, Sher A, James SL. IL-10 synergizes with IL-4 and transforming growth factor-β to inhibit macrophage cytotoxic activity. J Immunol. 1992; 148: 3578–3582. PMID: 1588047

31. Doherty TM, Kastelein R, Menson S, Andrade S, Coffman RL. Modulation of murine macrophage function by IL-13. J Immunol. 1993; 151: 7151–7160. PMID: 7903102

32. Walsh KP, Brady MT, Finlay CM, Boon L, Chantler SE, Estes DM. Bovine CD4+ T lymphocyte clones specific for rhoptry-associated protein 1 of Babesia bigemina stimulate enhanced immunoglobulin G1 (IgG1) and IgG2 synthesis. Infect Immun. 1999; 67(1): 155–164. PMID: 9864210

33. Clery DG, Torgerson P, Mulcahy G. Immune responses of chronically infected adult cattle to Fasciola hepatica. Vet Parasitol. 1996; 62: 71–82. PMID: 8638395

34. O'Neill SM, Mills KH, Dalton JP. Fasciola hepatica cathepsin L cysteine protease suppresses Bordetella pertussis specific interferon gamma production in vivo. Parasit Immunol. 2001; 23(10): 541–547. PMID: 11696165

35. Brady MT, O'Neill SM, Dalton JP, Mills KH. Fasciola hepatica suppresses a protective Th1 response against Bordetella pertussis. Infect Immun. 1999; 67: 5372–5378. PMID: 10496919

36. Flynn RJ, Mulcahy G, Walsh M, Cassidy JP, Corbett D, Milligan C, et al. Co-Infection of cattle with Fasciola hepatica and Mycobacterium bovis: immunological consequences. Transbound Emerg Dis. 2009; 56: 269–274. doi: 10.1053/j.trans emerg.2009.01.007. PMID: 19575746

37. Vukman KV, Adams PN, Metz M, Maurer M, O'Neill SM. Fasciola hepatica tegumental coat impairs mast cells' ability to Drive Th1 Immune Responses. J Immunol. 2013; 190(6): 2873–2879. doi: 10.4049/jimmunol.1203011. PMID: 23418624

38. Heussen C, Dowdle EB. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulphate and copolymerized substrates. Anal Biochem. 1980; 102: 248–254. PMID: 942051

39. Bredt DS, Snyder SH. Nitric oxide: a physiologic messenger molecule. Annu Rev Biochem. 1994; 63: 175–195. PMID: 7526779

40. Sajad M, Zargan J, Chawla R, Umar S, Sadaqat M, Khan HA. Hippocampal neurodegeneration in mice by a polyphenolic fraction from green tea. Proc Natl Acad Sci USA. 1999; 96: 4524–4529. PMID: 10200295

41. Heussen C, Dowdle EB. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulphate and copolymerized substrates. Ana Biochem. 1980; 102: 196–102. PMID: 7188482

42. Haqqi TM, Anthony DD, Gupta S, Ahmad N, Lee MS, Kumar GK, et al. Prevention of collagen-induced arthritis in mice by a polyphenolic fraction from green tea. Proc Natl Acad Sci USA. 1999; 96: 4524–4529. PMID: 10200295

43. Walsh KP, Brady MT, Finlay CM, Boon L, Chantler SE, Estes DM. Bovine CD4+ T lymphocyte clones specific for rhoptry-associated protein 1 of Babesia bigemina stimulate enhanced immunoglobulin G1 (IgG1) and IgG2 synthesis. Infect Immun. 1999; 67(1): 155–164. PMID: 9864210

44. Heussen C, Dowdle EB. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulphate and copolymerized substrates. Ana Biochem. 1980; 102: 248–254. PMID: 942051

45. Spectro T. Refinement of the Comassie Blue method of Protein Quantitation. Anal Biochem. 1978; 86: 142–146. PMID: 653575

46. Cooke A, Tonks P, Jones FM, O'Shea H, Hutchings P, Fulford AJ, et al. Infection with Schistosoma mansoni prevents insulin dependent diabetes mellitus in non-obese diabetic mice. Parasite Immun. 1999; 21: 169–176. PMID: 10320614

47. LaFlamme AC, Ruddenklaus K, Backstrom BT. Schistosomiasis decreases central nervous system inflammation and alters the progression of experimental autoimmune encephalomyelitis. Infect Immun. 2003; 71: 4996–5004. PMID: 12933842

48. Mo HM, Liu WQ, Lei JH, Cheng YL, Wang CZ, Li YL. Schistosoma japonicum eggs modulate the activity of CD4+ CD25+ Tregs and prevent development of colitis in mice. Exp Parasitol. 2007; 116: 385–389. PMID: 17433300
49. Osada Y, Shimizu S, Kumagai T, Yamada S, Kanazawa T. *Schistosoma mansoni* infection reduces severity of collagen induced arthritis via down-regulation of pro inflammatory mediators. Int J Parasitol. 2009; 39(4): 457–464. doi:10.1016/j.ijpara.2008.08.007 PMID: 18835272

50. Song X, Shen J, Wen H, Zhong Z, Luo Q, Chu D, et al. Impact of *Schistosoma japonicum* Infection on Collagen-Induced Arthritis in DBA/1 Mice: A Murine Model of Human Rheumatoid Arthritis. Plos One. 2011 Aug 8; 6(8): e23453. doi:10.1371/journal.pone.0023453 PMID: 21858123

51. O’Neill SM, Brady MT, Callanan JJ, Mulcahy G, Joyce P, Mills KH, et al. *Fasciola hepatica* infection downregulates Th1 responses in mice. Parasite Immunol. 2000; 22: 147–155. PMID: 10672196

52. Reynolds SJ, Williams AS, Williams H, Smale S, Stephenson HJ, Amos N, et al. Contractile but not endothelial dysfunction in early inflammatory arthritis: a possible role for matrix metalloproteinase-9. Br J Pharmacol. 2012; 167(3): 505–514. doi: 10.1111/j.1476-5381.2012.01988.x PMID: 22506619

53. Sewell DL, Reinke EK, Hogan LH, Sandor M, Fabry Z. Immunoregulation of CNS autoimmunity by helmint and mycobacterial infections. Immunol Lett. 2002; 82: 101–110. PMID: 12008041

54. Feldmann M, Brennan FM, Maini RN. Role of cytokines in rheumatoid arthritis. Annu Rev Immunol. 1996; 14: 397–440. PMID: 8717520

55. Kamanli A, Naziroglu M, Aydilek N, Hacievliyagil C. Plasma lipid peroxidation and antioxidant levels in patients with rheumatoid arthritis. Cell Biochem Funct. 2004; 22(1): 53–57. PMID: 14695655

56. Gierut A, Perlman H, Pope RM. Innate immunity and Rheumatoid arthritis. Rheum Dis Clin North Am. 2010; 36(2): 271–296. doi: 10.1016/j.rdc.2010.03.004 PMID: 20510234

57. Hemett W, Hemett M. Immunomodulatory activity and therapeutic potential of the filarial nematode secreted products, ES-62. In: Fallon PG. Pathogen derived immunomodulatory molecules. Landes Bioscience + Springer Science + Business Media; 2009. pp. 89–104.

58. Thomas NV, Kim SW. Metalloproteinase Inhibitors: Status and Scope from Marine Organisms. Biochem Res Int. 2010 Dec 9; ID 845975, doi:10.1155/2010/845975 PMID: 21197102

59. Elkayam O, Yaron I, Shirazi I, Judovitch R, Caspi D, Yaron M. Active leflunomide metabolite inhibits interleukin 1β, tumour necrosis factor-α, nitric oxide, and metalloproteinase-3 production in activated human synovial tissue cultures. Ann Rheum Dis. 2003; 62: 440–443. PMID: 12685157

60. Wang L, Li X, Zhang S, Lu W, Liao S, Liu X, et al. Natural products as a gold mine for selective matrix metalloproteinases inhibitors. Bioorg Med Chem. 2012; 20(13): 4164–4171. doi:10.1016/j.bmc.2012.04.063 PMID: 22688537

61. Kang S, Araya-Secchi R, Wang D, Wang B, Huynh T, Zhou R. Dual Inhibitory Pathways of Metallofullerenol Gd@C82(OH)22 on Matrix Metalloproteinase-2: Molecular insight into drug-like nanomedicine. Sci Rep. 2014 April 24; 4: 4775, doi:10.1038/srep04775 PMID: 24758941