Immunomodulatory effects of IL-12 released from poly-*N*-acetyl glucosamine gel matrix during *schistosomiasis* infection

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Abstract We have reported recently that Interleukin-12 (IL-12) released from poly-*N*-acetyl glucosamine gel matrix (F2 gel/IL-12) is more effective than free IL-12 to enhance vaccination of mice with *Schistosoma* soluble worm antigen preparation. The aim of this study is to evaluate the effect of F2 gel/IL-12 on the inflammatory responses in mice undergoing *schistosomiasis* infection in absence of vaccination. To achieve this, mice undergoing *Schistosoma mansoni* infection or cured from this infection, after treatment with praziquantil (PZQ), were treated with subcutaneous injection of IL-12 for 3 consecutive days or once with F2 gel loaded with IL-12 (F2 gel/IL-12). The treatment was started on day 35 days after infection. For infection, mice were infected with 100 cercariae of *S. mansoni* using tail immersion method. We found that treatment with F2 gel/IL-12 induced significant decreases in the egg burden with a moderate reduction in the size of granuloma and decrease in the cellular granulomatous reaction in the lung as

compared to infected mice treated with IL-12. These effects of F2 gel/IL-12 were more pronounced in infected mice previously treated with the anti-*schistosomal* drug PZQ. The total numbers of white blood cells in all treated mice showed similar profile. Treatment with IL-12 or F2 gel/IL-12, however, showed significant reduction in the number of mononuclear cells when compared with non-treated infected mice. In conclusion, this study showed the ability of IL-12 released from F2 gel to lower the inflammatory response to *Schistosoma* infection even in absence of vaccination.

Keywords Cytokine · IL-12 · Infection · Polymer · Slow release · Granuloma · *Schistosoma mansoni*

Introduction

Schistosomiasis is a disease caused by blood flukes of the genus *Schistosoma* with over 200 million people infected worldwide and causing an estimated 500,000 deaths per year and an additional 600 million people are at risk of acquiring this infection (Siddiqui et al. 2003). The control of this disease in human depends exclusively on treatment with the drug praziquantel (PZQ), which cures the host from adult worms but does not induce immunomodulatory effects on inflammatory response if it is injected after the establishment

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of the disease. In addition, acquisition of tolerance to PZQ has been reported (Savioli et al. 2004). Therefore, developing approaches that can modulate the inflammatory response to *Schistosoma* infection is of paramount significance to treatment of these diseases and other similar disease settings.

IL-12 is well-known for its essential role in bridging the innate and adaptive arms of immunity, in regulating inflammatory responses, innate resistance to infection, and adaptive immunity. The use of IL-12 protein or IL-12 gene as an adjuvant have been found to elicit significant levels of protective immunity against *S. japonica* challenge infection. Thus, IL-12 seems to significantly benefit the immunoprophylactic treatment of *schistosomiasis*. Clinical application of IL-12, however, has been failed due to its toxicity, largely due to the repeat dose regimens utilized, and modest clinical response (Salem et al. 2006).

Recently, we have developed a highly purified polysaccharide (Poly-*N*-acetyl glucosamine) that can be formulated into a stable gel designated F2 gel matrix, which can be formulated with antigenic peptides and adjuvant cytokines, creating a vaccine delivery system capable of providing the sustained release of the incorporated protein in vivo, establishing a potent micro-environment for antigen presentation (Maitre et al. 1999; Nguyen 2001; Nguyen et al. 2003; Salem et al. 2004). We have recently reported that F2 gel matrix can release low but functional levels of IL-12 during vaccination against tumor antigens and against *schistosomiasis* without induction of toxicity (Salem et al. 2010a, b).

The aim of the present study was to compare the adjuvant effects of F2 gel/IL-12 and free IL-12 on the development of granulomatous response in mice undergoing infection in the absence of vaccination.

Materials and methods

Mice and *Cercariae*

Adult male albino mice (*Mus musculus*; CD1 strain) weighing between 16 and 22 g were purchased from the Helwan Research Animal Center (Cairo, Egypt). The mice were housed at the animal facility center, Zoology Department, Faculty of Science, Tanta University in accordance with the internal guidelines of the use of experimental animals, Zoology Department,

Faculty of Science, Tanta University. Cercariae and viable eggs of *Schistosoma mansoni* were obtained from the Theodore Belhariz Institute (Giza, Egypt).

Reagents

Recombinant murine IL-12 was purchased from R&D Systems (Minneapolis, MN, USA) and reconstituted in 0.1 % Bovine Serum Albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA). Praziquantel (PZQ) was purchased from the Egyptian International Pharmaceutical Industries Company (EIPICO, Cairo, Egypt) and prepared as a suspension in 2 % Cremophor El vehicle and used as a standard treatment against *schistosomiasis* infection. Highly purified poly-*N*-acetyl glucosamine (p-GlcNAc) in a gel formulation was obtained from Marine Polymer Technologies Inc. (Boston, MA, USA).

Preparation of IL-12

The F2 gel matrix was prepared by chemical deacetylation of pGlcNAc natural nanofibers to 70 % with conversion of the polymer to a lactate salt (Maitre et al. 1999; Chan et al. 2000; Nguyen 2001; Salem et al. 2004). A final 15 % polymer concentration in a gel form was achieved by hydration. Next, 100 µl of this gel matrix was mixed with 100 µl of phosphate buffered saline (PBS) and IL-12 alone to form F2 gel/PBS, and F2 gel/IL-12, respectively. Mixing F2 gel with the reagents was performed using two 1 ml syringes connected by a four-way stopcock (Medex; Hilliard, OH, USA). The mixing process was performed on ice to prevent denaturation of IL-12. Mice were treated with s.c injection of 100 µl of the formulation at the base of the tail.

Infection with *S. mansoni* and treatment:

Mice undergoing infection or cured of infection (treated with PZQ 1 day before the vaccination) were treated with subcutaneous (s.c.) injection of IL-12 alone (0.5 µg) for 3 consecutive days or with a single treatment with F2 gel/IL-12 (100 µL F2 gel loaded with 1.5 µg IL-12) starting on day 35 after infection with 100 cercariae of *Schistosoma mansoni* by the tail immersion method as previously described (Prechatangkit et al. 1994). Then, all mice were sacrificed 1 week after the last treatment for the analysis listed below.

Induction of lung fibrosis

Fifty viable *S. mansoni* eggs were injected into naïve mice via tail vein injection. Ten days after egg injection, mice have received PBS, 3 s.c. injections of IL-12, single s.c. injection of F2 gel/PBS, or single s.c. injection of F2 gel/IL-12. Fourteen days after egg injection, mice were sacrificed and the lungs were harvested to assess focal egg-induced fibrosis by histological examination using Hematoxylin and Eosin (H&E) staining as it was previously described (Schulte and Wittekind 1989).

Evaluation of worm burden

The livers of infected and non-infected mice were perfused for worm recovery as previously described (Prechatangkit et al. 1994). All worms (male, female, or couples) from the perfusion fluid (mesentery and liver) were counted. The percentage of worm reduction (% protection) was calculated according to the following equation: % protection = $(C-I)/C \times 100$; where (C) is the mean worm recovery of non-immunized group, and (I) is the mean worm recovery of immunized group.

Evaluation of egg deposition (oogram)

Samples of the liver tissue weighing about 0.1 g were transferred to clean slides and covered with glass slips then pressed to spread the liver tissue homogeneously. Triplicate samples of each liver were prepared and the average number was expressed as number of eggs/g tissue. All viable and dead eggs were counted and classified according to the criteria previously described (Christensen 1984).

Evaluation of lung and liver fibrosis

For histological examination, lung and liver specimens of each mouse of each group were fixed in 10 % neutral formalin buffer. After dehydration, paraffin sections of 6 μm thickness were prepared and stained with the conventional H&E staining method. For morphometric measurements granuloma dimensions per liver section were measured using an ocular micrometer as previously described (Coutinho et al. 2003) based on the following equation: granuloma size (mm^3) = $4/3 \pi r^3$, where r is the radius of granuloma.

Statistical analysis

P values were calculated using student *t*-test and a significant difference among experimental groups was defined as a *P* value of <0.05.

Results

Effect of IL-12 on worm burden

Data in Table (1) showed no significant alteration in the worm burden in mice treated with IL-12 or with F2 gel/IL-12 (25.6 ± 8.4 and 21.3 ± 7.9 respectively) when compared with the untreated control mice (24.3 ± 6.9). As expected, pretreatment of *S. mansoni* infected mice with PZQ before IL-12 or F2 gel/IL-12 treatment displayed a 100 % reduction in worm burden (Table 1).

Effect of IL-12 on egg burden

The numbers of total, viable, and dead eggs in the liver from infected mice with or without treatment are shown in Fig. 1. The total liver egg count from infected mice without treatment was 522 ± 30.4 eggs/g liver tissues, of which 56.1 % were live and 43.9 % were dead. Treatment with F2 gel/IL-12 alone showed significant reduction in the total number of eggs 290 ± 23 , while IL-12 slightly, but not significantly, decreased the number of eggs deposited in the liver and intestine of mice undergoing infection. Treatment with PZQ alone before IL-12 or F2 gel/IL-12 treatment induced significant decreases in the total number of eggs load of 221.5 ± 2 , 302.5 ± 46.1 , and

Table 1 Worm burden in the liver of mice infected with *S. mansoni* and treated with PZQ followed but F2 gel/IL-12 or IL-12

Groups	Worm burden	
	Mean $\pm$ SD	% of change
Control	24.3 ± 6.9	–
F2 gel/IL-12	21.3 ± 7.9	12.3
IL-12	25.6 ± 8.4	5.3
PZQ	0.00	0.00
PZQ + F2 gel/IL-12	0.00	0.00
PZQ + IL-12	0.00	0.00

Fig. 1 Effect of IL-12 treatment on the egg burden in mice undergoing infection and in those cured of infection. Six groups of mice ($n = 10/\text{group}$) were infected with 100 cercariae of *S. mansoni* by tail immersion. (A) Mice were left untreated (control) or s.c. treated with injection of IL-12 alone (0.5 μg) for 3 consecutive days starting on 35 days of infection or with a single injection of F2 gel/IL-12 (100 μL F2 gel loaded with 1.5 μg IL-12)

202 ± 34.5 , respectively. Interestingly, combinatorial treatment with PZQ and F2 gel/IL-12 at day 35 after infection resulted in significant decreases in the total number of eggs with higher increase in the percentage of dead eggs in the liver and intestine (Fig. 1).

Effect of IL-12 on hepatic granuloma size

As shown in Fig. 2, the granuloma size was significantly decreased ($P < 0.01$) after treatment with F2 gel/IL-12 or IL-12 as compared with untreated control group in mice undergoing infection. In mice pretreated with PZQ, treatment with IL-12 or F2 gel/IL-12 showed insignificant reduction in the size of granuloma (0.25 and 0.3 mm^3 , respectively) when compared with mice treated with PZQ alone (0.36 mm^3).

Effect of IL-12 treatment on lung fibrosis

To evaluate the anti-fibrotic effects of IL-12 in the absence of the complications of the *S. mansoni* infection, we induced fibrosis in lung through intravenous injection of viable *S. mansoni* eggs. When

on day 35 of infection. (B) Infected mice were treated with PZQ (to cure mice from infection) followed by IL-12 and F2 gel/IL-12 treatments as above. The livers were harvested after 1 week of the last treatment. The numbers of the total (A and B) eggs in the liver of the mice with ongoing infection (A) and in the mice cured of infection (B). The numbers of dead eggs in the intestine of the mice with ongoing infection (C) and in the mice cured of infection (D)

compared with normal lung (Fig. 3A), histological examination of the lung sections of the egg-injected mice showed pronounced granulomatous reaction with inflammatory cellular infiltrations around the deposited eggs (Fig. 3B). Lung sections from mice treated with F2 gel alone (100 μL) showed a cellular granulomatous reaction around the ova (Fig. 3C). Lung sections from mice treated with IL-12 (0.5 μg for 3 days) showed a less cellular granulomatous reaction around the ova (Fig. 3D) as compared to the untreated control. Interestingly, however, mice treated with F2 gel/IL-12 (0.5 μg for 3 days) showed a much less cellular granulomatous reaction around the ova as compared to mice treated with IL-12 (Fig. 3E). These data indicate to the anti-fibrotic effect of IL-12 released from F2 gel in this lung fibrosis model.

Effect of IL-12 on the white blood cells count in mice undergoing infection and in those cured of infection

As shown in Fig. 4, the total number of WBCs cells was similar in non-treated infected mice and in mice

Fig. 2 Effect of IL-12 treatment on the liver granuloma size in mice undergoing infection and in those cured of infection. Six groups of mice ($n = 10/\text{group}$) were infected with 100 cercariae

of *S. mansoni* by tail immersion and treated as described in the legend of Fig. 1. All mice were sacrificed 1 week after the last treatment to assess granuloma size (mm^3) (* $P < 0.05$)

Fig. 3 Lung pathology of mice injected with *S. mansoni* eggs. Mice were injected with 100 eggs and sacrificed 3 weeks later to assess lung pathology by H&E staining: from normal mice (A), egg injected mice (B), as in B + F2 gel (C), as in B + IL-12

(D), as in B + F2 gel/IL-12 (E). The white arrows point to the inflammatory cellular infiltration around the deposited egg in the formed granuloma. The blue arrows point to the deposited eggs. (X10)

treated with IL-12 for 3 consecutive days (from day 35–37) or one with F2 gel/IL-12 on day 35 after infection. In addition, the mice treated with IL-12 + PZQ or F2 gel/IL-12 + PZQ showed similar WBC numbers when compared with the PZQ-treated mice. Figure 5 showed the mean total number of mononuclear cell count in the spleen, which showed significant reduction in the number of mononuclear

cells in mice treated with IL-12 or F2 gel/IL-12 when compared with the non-treated mice with infection. Interestingly, however, the mononuclear cell number was significantly increased in mice treated with IL-12 + PZQ but not after treatment with F2 gel/IL-12 + PZQ as compared to mice treated with PZQ alone.

The differential leukocyte counts are illustrated in Table 2. The total numbers of neutrophils were

Fig. 4 Effect of IL-12 treatment on the numbers of white blood cells (WBCs) in mice undergoing infection and in those cured of infection. Mice were infected with 100 cercariae of *S. mansoni* by tail immersion. Mice ($n = 10/\text{group}$) undergoing infection or cured of infection (treated with PZQ one day before cytokine treatment) were left as control or administered with s.c. injection

of IL-12 alone ($0.5 \mu\text{g}$) for 3 consecutive days starting on day 35 of infection, or with a single administration of F2 gel/IL-12 ($100 \mu\text{L}$ F2 gel) loaded with $1.5 \mu\text{g}$ IL-12) on day 35 of infection. All mice were sacrificed 1 week after the last treatment ($*P < 0.05$)

Fig. 5 Effect of IL-12 treatment on the numbers of mononuclear cells in spleen in mice undergoing infection and in those cured of infection. Mice were infected with 100 cercariae of *S.*

mansoni by tail immersion and treated as described in the legend of Fig. 4. All mice were sacrificed 1 week after the last treatment ($*P < 0.05$)

statistically significantly higher in mice infected with *S. mansoni* in comparison with mice treated with IL-12, F2 gel/IL-12, PZQ, IL-12 + PZQ, and F2 gel/IL-12 + PZQ. The numbers of lymphocytes in all treated groups were also higher and statistically significant in relation of those in the untreated control mice. Analysis of variance showed no significant differences in the number of monocytes, eosinophils, and basophils.

Discussion

Our group has established that the F2 gel matrix can release low but effective functional levels of IL-12, as evidenced by the enhanced post-vaccination responses of CD8^+ T cells and anti-tumor responses (Salem et al. 2004). Our recent studies demonstrated also that IL-12 released from F2 gel does not induce significant toxicity

Table 2 Differential leukocyte counts in the blood of mice infected with *S. mansoni* and treated with PZQ followed by IL-12 of F2 gel/IL-12

Groups	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils
Control	56 ± 5.06	43 ± 3 4.8	0.17 ± 0.4	0.5 ± 0.8	0
F2 gel/IL-12	29 ± 2 3.8	67.7 ± 5.1	0.33 ± 0.8	2.8 ± 2	0
(% of change)	(47.9)	(56.2)	(48.5)	(4.7)	
IL-12	46.8 ± 20	52.8 ± 20.5	0.1	0.4 ± 0.5	0
(% of change)	(−16.4)	(21.9)		(−20)	
PZQ	46.2 ± 18.8	52.3 ± (18.1)	0	13.2 ± 2	0.2 ± 0.4 (4.8)
PZQ + F2 gel/IL-12	39 ± 15.6	59.5 ± 16.4	0.3 ± 0.5	1 ± 1.3	0.3 ± 0.5
(% of change)	(−15.5)	(11.6)		(2)	(4.8)
PZQ + IL-12	40.2 ± 8.6	56.2 ± 6.6	0	2 ± 2.2	0.3 ± 0.5
(% of change)	(−12.9)	(7.4)			(94.1)

as compared with systemic IL-12 administration associated with similar levels of interferon-gamma (IFN- γ), tumor necrosis factor-alpha (TNF- α) and IL-2 after paracrine and systemic IL-12 treatments (Salem et al. 2010a). Furthermore, injection with F2 gel/SWAP/IL-12 induced a higher anti-*schistosomal* activity as compared with systemic IL-12 administration as evidenced by the decrease in the total liver egg counts and the reduction in fibrotic reaction and granuloma size. Collectively, the results of our previous studies pointed to the beneficial effects of F2 gel/IL-12 adjuvant system against *schistosomiasis* induced hepatic fibrosis without induction of toxicity. In this study, we extended our previous studies to evaluate the effects of IL-12 released from F2 gel to induce beneficial effects to the inflammatory responses to *S. mansoni* infection but in absence of vaccination. We found that IL-12 released from F2 gel can reduce the inflammatory responses in the liver to egg deposited after *S. mansoni* infection and in lung to the intravenous injection of *S. mansoni* eggs.

The granulomatous reaction in response to egg deposition depends on the number of inflammatory cells in circulation, which correlates with their infiltration into the liver at the site of egg deposition. Interestingly, we found that the less granulomatous reaction in the liver in the case of the F2 gel/IL-12 treatment was accompanied with more infiltration of inflammatory cells around the ova. Furthermore, we found that although IL-12 or F2 gel/IL-12 treatments had no effects on the total numbers of WBCs in the blood, F2 gel/IL-12 treatment decreased the numbers of neutrophils and eosinophils while increased the

numbers of lymphocytes. Meanwhile, both treatments decreased the numbers of splenocytes in infected mice. This might indicate that the effect of F2 gel/IL-12 to decrease the granuloma size might be due to the increase in the numbers of lymphocytes that produce Th1 cytokines, which we reported recently (Salem et al. 2006). In the blood, however, PZQ did not induce lymphopenia (Fig. 5) but induced decreases in the differential numbers of neutrophils associated with increases in the numbers of lymphocytes and monocytes (Table 2). Interestingly, PZQ induced lymphopenia in spleen, which was corrected after treatment with F2 gel/IL-12 and slightly after treatment with IL-12 (Fig. 5). These results point to the ability of F2 gel/IL-12 to increase the numbers of lymphocytes and are consistent with the results of our recent study showing the induction of IFN- γ by F2 gel/IL-12 as well as others' studies showing that IL-12 has potent IFN- γ stimulating activities on NK cells as well as on CD4⁺ and CD8⁺ T lymphocytes (Manetti et al. 1993; Trinchieri and Gerosa 1996).

Although, we have not studied the molecular mechanisms mediating the beneficial effects of F2 gel/IL12 toward granulomatous reaction, it could be mediated by its modulation of TH1/Th2 cytokine responses. Indeed, previous studies revealed that the predominant immune response to larval and adult *schistosome* worm antigens appears to be skewed toward the production of Th1 cytokines (Cheever 1993; Caldas et al. 2008). Although CD4⁺ T cells have been shown to be crucial for the development of egg-induced granulomas, this could explain why Th1

immune response with increased IFN- γ predominates in early infection and Th2 type predominates as egg production and tissue reaction begins and why the decrease in IFN- γ as infection progresses is accompanied by an increase in granuloma formation (Zhu et al. 2005). Thus, an enhanced Th2 cytokine synthesis was observed during the formation of granulomas in infected mice or in animals injected with *schistosome* eggs (Vella and Pearce 1992; Wynn et al. 1993). In contrast, the inhibition of egg pathology was observed after in vivo depletion of either IL-4 or IL-2 (Wynn et al. 1993), two cytokines required for the generation of Th2 responses. This dichotomy in the nature of anti-*schistosomiasis* Th1/Th2 responses is consistent with in vitro studies in human intestinal *schistosomiasis*. In these studies, when inflammatory cells collected at various stages of infection were stimulated by egg or adult worm antigens, antigen-specific Th1 cytokine responses predominated in the acute infection stage (Khalil et al. 1996). In line with these studies, we reported recently that copious amounts of the Th1 inflammatory cytokines IFN- γ and TNF- α rapidly were induced upon injection of F2 gel/IL-12 or IL-12. These high levels of IFN- γ and TNF- α could skew the immune response to Th1 type, and thus decrease the Th2-mediated granulomatous reaction. This could explain the decrease in granuloma size after F2 gel/IL-12 treatment (Fig. 1) which was consistent with the reduction in the numbers of viable eggs (Fig. 2).

In this report we have shown the beneficial effects of the F2 gel/IL-12 adjuvant system toward *schistosomiasis* induced liver fibrosis. We extended these effects using another model of fibrosis, which still depends on the injection of viable eggs of *S. mansoni* viable eggs that migrate to the lungs and cause local fibrotic foci. Indeed, we found that F2 gel/IL-12 is capable of reducing the granulomatous reaction in response to the egg deposition in the lung. This effect was due to the levels of IL-12 released from F2 gel since injection of F2 gel alone or IL-12 alone did not induce similar effects. The added beneficial effect of F2 gel to the adjuvant effects of IL-12 could be explained because F2 gel provides a danger signal via the innate immune system and at the same time serves as a sustained release delivery vehicle for IL-12 (Nguyen 2001; Salem et al. 2004). These anti-*schistosomiasis* and anti-fibrotic effects of the low levels of IL-12 released from F2 gel are better than those obtained after systemic IL-12 treatment, which

often induces toxicity. Therefore, F2 gel/IL-12 adjuvant system could be used as anti-pathology adjuvant for preventing granulomatous disease. Further studies, however, are required to confirm and expand the beneficial effects of the F2 gel/IL-12 adjuvant system and to evaluate its effects on different toxicity-related parameters.

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