Soluble Tachyzoite Antigen Immunization Can Protect Mice from Experimental Autoimmune Encephalomyelitis Via Induction of programmed death-1.

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Abstract: Background: Multiple sclerosis (MS) is the most common autoimmune neurodegenerative disorder that affects young aged people causing many disabilities and financial burdens to patients. Immunotherapy is a main line of MS treatment. Induction of programmed death-1 (PD-1) proved effectiveness in many studies. This protein is induced in many protozoal chronic infections. **Aim:** assessment *of Toxoplasma gondii* soluble tachyzoite antigen (STAg) as an immunotherapeutic agent for experimental autoimmune encephalomyelitis (EAE) and the role of PD-1 in this protection. **Methods and results:** Four mice groups were used. Third and fourth groups represented the EAE model. Others served as controls. Fourth group was immunized by STAg. Disease condition was assessed clinically (by recording days of disease onset and mean clinical scores), immunologically (by measuring proinflammatory cytokines and antibodies) and histopathologically (by recording brain inflammation and demyelination scores). PD-1expression on immune cells was assessed by immunohistochemistry. It was found that STAg immunized EAE group had an improved condition with lower clinical, immunological and histopathological scores. PD-1 expression was higher in STAg-EAE group. It also correlated negatively with all other assessed parameters. **Conclusion:** STAg immunization can protect from EAE by induction of PD-1 on immune cells.
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1. Introduction

Multiple sclerosis (MS) is the most common disabling neurodegenerative disorder that affects young aged people especially women. More than two millions worldwide suffer from that disease. Its global prevalence ranges from 0.67 to over 200 per 100,000 people with annual incidence ranging from 1.4 to 12.2 per 100.000 people **[1].** Regarding distribution, 50% of MS patients were recorded in Europe **[2,3]** while the lowest prevalence and incidence rates were recorded in Africa. According to World Health Organization (WHO), MS is responsible for 11% of disability-adjusted life year (DALY) worldwide **[4].** The associating disabilities - that affect not only motor functions but may extend to involve sensory, visual and even cognitive functions - markedly burden economic conditions of patients and their families because they usually leave work in addition to the cost and side effects of treatment that cannot be forgotten **[5:7].**

Pathogenesis of MS is a result of immune dysregulation that leads to early demyelinating inflammation followed by gliosis, progressive neurodegeneration and axonal destruction. The disease process starts by abnormal phenotype of antigen presenting cells (APCs) that recognize the peripherally circulating myelin sheath proteins as 'non-self". Then, they cross the blood brain barrier and trigger activation of T helper (Th)-1 – with production of Interferon-gamma (INF-ɣ)– and Th-23 lymphocytes – with production of interleukin (IL)-17 –. This is followed by T cytotoxic lymphocytes and B lymphocytes activation. The resulting proinflammatory cytokines and autoantibodies subsequently damage myelin sheath and axons that present clinically as episodes of remission and relapse **[8:13].**

Normal individuals can tolerate myelin sheath proteins that reach their peripheral circulation because inflammatory reactions are suppressed by sets of immune cells called regulatory cells. Their immune-suppressive potential is mediated by either fork head box protein-3 (foxp3) or programmed death-1 (PD-1) expressed on activated cells. They act either by direct inhibition of effector cells or indirectly by inhibiting APCs. They also secrete many immune-suppressive cytokines e.g. IL-10, IL-35 and transforming growth factor (TGF)-β**.** These cells were found to be malfunctioning in MS patients especially during relapses **[14:18].**

Regulatory cells expressing PD-1 showed a more potent role in immune-suppression of autoimmune diseases and allograft rejection. They were even used as a trial therapy for cancer **[19].** Their surface marker,

PD-1 plays a very important immune downregulating role via its ligands. The PD-1/PD-L1 ligand is expressed on various types of immune cells and has an important role in protection against autoimmune diseases including experimental autoimmune encephalomyelitis (EAE) – an animal model that resembles human MS - **[20, 21].** Its ligand PD-1/PD-L2 is expressed on brain endothelial cells and partially mediates functional integrity of blood brain barrier against inflammatory cell infiltration. This ligand was found to be defective in MS patients **[22].**

Being an immune mediated disease, treatment of MS usually targets the immune response at its different levels to ameliorate symptoms and space between relapses. Unfortunately, many of these drugs have serious side effects. So, the need of new lines of treatment that target the immune-regulatory cells has emerged **[23].** Many studies that benefit from immune modulation induced by helminths were held and reported a significant immunotherapeutic role of many helminths in EAE. Their action was explained by skewing immune system towards Th2 response induced by helminths. Other studies reported a role of T-regs, B-regs and their immune-suppressive cytokines that are originally induced to limit damage caused by helminths and suppress autoimmunity as a double benefit effect **[24:27].** Protozoal infections that affect central nervous system (CNS) or their antigens e.g. *Plasmodium falciparum* and *Trypanosoma cruzi* were also effective in protecting mice from EAE. Their effects were also related to foxp3^{+}T-regs and their immune-suppressive cytokines **[28, 29].** Another CNS protozoan parasite that showed a negative correlation with MS is *Toxoplasma (T.) gondii* **[30].**

Toxoplasma gondii is a protozoan parasite that can induce CNS pathology. It can infect any vertebrate animal by any of its developmental stages. Upon countering infection, the parasite is transformed into rapidly replicating tachyzoites that are distributed to all body tissues and then lodge as bradyzoites in the host neural and muscle tissue for the life time of the host **[31].**

Chronicity of toxoplasmosis was found to be associated with progressive increased expression of PD-1 on lymphocytes that induces their apoptosis and suppresses the immune response with possible reactivation of latent infections in a phenomenon known as "T cell exhaustion". Even low levels of PD-1 expression can markedly suppress their INF-ɣ and can extend beyond CD8^{+}T lymphocytes to affect IL-2 production and subsequently Th cell expansion **[32, 33].**

In the present work, we hypothesized that induction of PD-1 expressing exhausted immune cells by chronic *T. gondii* infection can protect mice from developing severe EAE manifestations. Soluble tachyzoites antigen (STAg) was proved to provoke similar immune response to whole infection **[34]** so, it was used instead of whole infection to avoid the possible CNS pathology induced by *T. gondii* for better studying of immunological changes.

2. Materials and Methods

2.1. Ethics Statement:

All animal experiments were conducted at Theodor Bilharz Research Institute, TBRI (Giza, Egypt). Mice were kept under standard housing conditions in the animal house of TBRI and were maintained on a standard commercial pelleted diet in an air-conditioned room at 20-22°C. All experimental procedures were performed in accordance with international ethical guidelines after approval of the institutional ethical committee of TBRI.

2.2. Animals and Study Design:

Six weeks old female 18-22gram BALB/c pathogen free mice were divided among four groups. Group I (GI) represented the untreated negative control group. Group II (GII) received STAg immunization. Both groups consisted of 10 mice each. Group III (GIII) represented the untreated EAE model and group IV (GIV) represented the STAg immunized EAE model. The latter two groups consisted of 20 mice each.

2.3. Procedures:

2.3.1. Soluble Tachyzoite antigen preparation and animal immunization:

Tachyzoites of the virulent *T. gondii* RH strain were purchased from laboratory of Parasitology Department, Faculty of Veterinary Medicine, Cairo University. Mice were intraperitoneally injected with a volume of 50 µL of a suspension of RH *T. gondii* strain tachyzoites at 10^3-10^4 tachyzoites/ml. Peritoneal exudates were collected 72 hours post infection. Parasites were lysed by five cycles of freezing and thawing then sonication was done at 4ºC with 20 cycles/second for 10 minutes. The protein concentration of the resulting supernatant containing STAg was estimated by colorimetric method and the samples were aliquoted and stored at - 80°C **[35:37].** Mice of GII and GIV were subcutaneously injected by20 µg of STAg twice in the first and 7th days of experiment at the base of the tail **[38].**

2.3.2. Induction of EAE model and clinical assessment:

Mice of GIII and GIV were sensitized by subcutaneous injection of 200 µg of myelin basic protein (MBP), (Sigma-Aldrich, USA) in both flanks emulsified in CFA (Sigma-Aldrich, USA), once at day 30 of experiment. Mice were observed daily for another 30 days. Two parameters were examined to evaluate the severity of EAE, mean day of onset (the mean day that affected mice within a group first

developed clinical signs of disease) and mean clinical score (MCS) (the mean of clinical scores for all mice within a group at day 60 of the experiment). Clinical score was based on the following scale: 0 = normal; 1= limp tail; 2 = partial hind limb paralysis or ataxic gait; 3 = complete hind limb paralysis; 4 = partial or complete forelimb paralysis; and 5 = moribund or dead. Food was made accessible to immobile animals. Moribund animals were euthanized with a score of 5 **[39, 40].**

2.3.3. Cerebrospinal fluid (CSF) collection and euthanizing animals:

At day 60 of the experiment **[41]**, all mice were anesthetized by inhalant ether then CSF was collected as described by **Li et al. [42].** Samples were centrifuged, and the supernatants were collected and stored at – 80 °C for immunity studies. Anesthetized mice were then decapitated, brains were removed, rinsed with saline and preserved in formalin 10% for further procedures.

2.3.4. Assessment of CSF levels of IL-17, INF-ɣ and total anti-MBP antibodies:

Suppression of immune response was assessed by measuring CSF levels of the proinflammatory cytokines, IL-17 and INF-ɣ using sandwich ELISA kits (Abcam, USA) and total anti-MBP antibodies through an indirect ELISA kits (My BioSource, Inc., USA). Techniques were performed as described by the manufacturers.

2.3.5. Histopathological examination:

Brains were embedded in paraffin then, sections were stained with hematoxylin and eosin for assessment of inflammation and with Luxol Fast Blue for assessment of demyelination. Inflammation score was assessed as follows: 0 = none; 1 = a few inflammatory cells; 2 = organization of perivascular infiltrates; and 3 = increasing severity of perivascular cuffing with extension into the adjacent tissue. Demyelination score was assessed as follows: 0 = none; 1 = rare foci; 2 = a few areas of demyelination; and 3 = large (confluent) areas of demyelination **[41, 43].**

2.3.6. Assessment PD-1 expression on immune cells:

Immunohistochemical staining of paraffinized brain tissue sections was done using anti-PD-1 antibodies, (Abcam, USA) **[44].** Positivity was identified when the cell membrane alone or together with the cytoplasm showed brown staining. Immunohistochemical grading of PD-1 staining was determined by histo score (H score) where intensity of membrane staining was given a number from (0, 1+, 2+ or 3+). Percent of stained cells in each tissue was multiplied by the intensity of staining. [1 × (% cells 1+) + 2 × (% cells 2+) + 3 × (% cells 3+)]. Then, a score of 0-300 was given for each field followed by a mean score for all fields **[45].**

2.3.7. Statistical analysis

Data entry, coding, and analysis were conducted using SPSS (20), IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.

-Data of this study were of both quantitative and qualitative types. Quantitative data were expressed in Mean ($\bar{x}$), and Standard Deviation (SD) while qualitative data were expressed as number (frequency) and percent (%).

-Tests of significance used were as follows:

- t student test to estimate the difference between means of two quantitative variables.
- Chi^2 test to assess the relationship between qualitative parameters.
- ANOVA (Analysis of Variance) to estimate the difference between means of more than two quantitative variables. Post Hoc test was used to assess the difference in two means of two individual groups after a significant ANOVA. The interpretation of Post Hoc test is as follows: p1: comparison between the –ve control group (GI) and sole STAg group (GII). p2: comparison between the –ve control group (GI) and EAE group (GIII). p3: comparison between the –ve control group (GI) and STAg-EAE group (GIV). p4: comparison between sole STAg group (GII) and EAE group (GIII). p5: comparison between sole STAg group (GII) and STAg-EAE group (GIV). P6: comparison between EAE group (GIII) and STAg-EAE group (GIV).

-The level of significance of our data was 95%, so, p value >0.05 was considered a non-statistically significant difference, while p value < 0.05 was considered a statistically significant difference.

3. Results

3.1. Improvement of clinical condition of EAE after STAg immunization.

Clinical assessment of EAE revealed delayed onset of manifestations with STAg immunization. Mice of STAg-EAE group started to show manifestations around the 15^{th} day. This delay was statistically significant when compared to the non-immunized group that started their clinical presentation around the 9^{th} day after MBP sensitization ($p<0.001$). STAg effects didn't stop at delaying the days of disease onset, but they also affected its progression. This was detected on comparing MCSs of the studied groups that revealed a significantly lower MCS in STAg-EAE group (1.75±0.78) than sole EAE one (3.95±0.75) ($p<0.001$). [Table (1)]

Table (1): Comparison between the studied groups regarding mean days of onset and mean clinical scores of EAE.

	Group	N	Mean	SD	t – test	p-value
Day of onset	-ve control	10	.0000	.000	26.72	**<0.001 ***
	STAg	10	.0000	.00		
	EAE	20	9.50	0.68		
	STAg-EAE	20	15.60	0.75		
MCS	-ve control	10	.00	.00	9.001	**<0.001 ***
	STAg	10	.00	.00		
	EAE	20	3.95	0.75		
	STAg-EAE	20	1.75	0.78		

* statistically significant

3.2. Reduced inflammatory immune response after STAg immunization.

Study of some important indicators of inflammation ran along the same line with the clinical findings. Measures of the proinflammatory cytokines, IL-17 and INF-γ showed a statistically significant reduction in the STAg immunized EAE group (389±80.25 & 1520±439.91 pg./ml respectively) than the pure EAE one (612.5±77.92 & 2620±416.24 pg./ml respectively). (p6<0.05). STAg effects extended beyond cytokines to involve the humoral immune response. The mean total anti-MBP antibodies was reduced from 15.9±1.51 ng/ml in the pure EAE group to 11.5±1.57 ng/ml in STAg-EAE group. Their comparison was statistically significant (p<0.001). [Tables (2)].

Table (2): Comparison between mice groups regarding CSF measures of proinflammatory cytokines - IL-17 & INF-γ -and total anti-MBP.

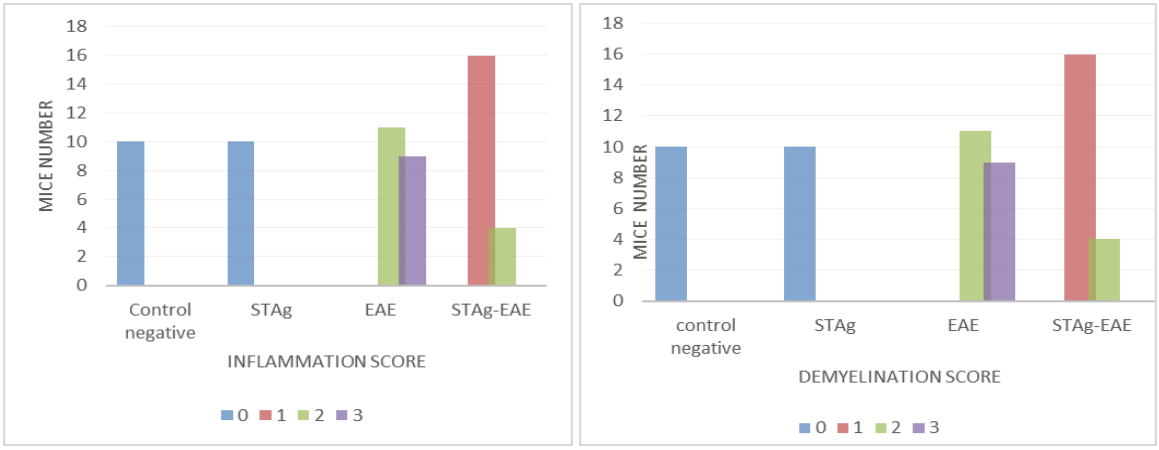
	Group	N	Mean	SD	Test of significance	p-value	Post Hoc test
IL-17 (pg/ml)	-ve control	10	18.00	4.58	249.35**	<0.001*	p1: <0.05 *
	STAg	10	118.00	17.51			p2: <0.05 *
	EAE	20	612.50	77.92			p3: <0.05 *
	STAg-EAE	20	389.00	80.25			p4: <0.05 *
							p5: <0.05 *
							p6: <0.05 *
INF-ɣ (pg/ml)	-ve control	10	53.18	10.06	176.304 **	<0.001*	p1: >0.05
	STAg	10	188.40	12.38			p2: <0.05 *
	EAE	20	2620	416.24			p3: <0.05 *
	STAg-EAE	20	1520	439.91			p4: <0.05 *
							p5: <0.05 *
							p6: <0.05 *
Anti MBP (ng/ml)	-ve control	10	.00	.00	9.001 #	<0.001 *	
	STAg	10	.00	.00			
	EAE	20	15.90	1.51			
	STAg-EAE	20	11.50	1.57			

* Statistically significant.
**ANOVA (Analysis of Variance) to compare the four groups.
T student test (to compare between EAE and STAg-EAE group).
N.B. The interpretation of Post Hoc test is as follows: p1: comparison between the –ve control group (GI) and sole STAg group (GII). p2: comparison between the –ve control group (GI) and EAE group (GIII). p3: comparison between the –ve control group (GI) and STAg-EAE group (GIV). p4: comparison between sole STAg group (GII) and EAE group (GIII). p5: comparison between sole STAg group (GII) and STAg-EAE group (GIV). P6: comparison between EAE group (GIII) andSTAg-EAE group (GIV).

3.3. Improved brain inflammatory and demyelination scores with STAg immunization.

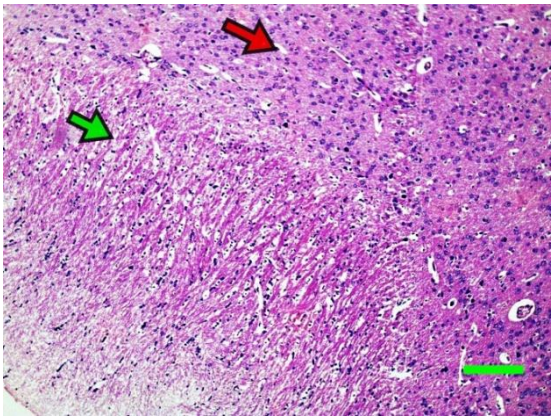
Examination of brains revealed decreased inflammation in STAg immunized EAE group than the pure EAE one. The highest inflammation score – score 3 – was totally absent in STAg-EAE mice while it extended to involve up to 45% of the pure EAE ones. Another sign of improvement was that 80 % of STAg-EAE mice had the score, 1 while the pure EAE group only ranged between the scores 2 and 3 with total absence of the mild inflammation score (score 1). Theses all differences were statically significant (p<0.001). [Figures 1a, & 2].

Similar results were recorded regarding axonal demyelination. Demyelination scores of the pure EAE mice ranged between scores 2 and 3 while 80% of the STAg-EAE mice were recorded with the mild demyelination score (score 1) with total absence of confluent demyelination (score 3). This comparison was also a statistically significant one ($p<0.001$). [Figures 1b, & 3].



(a) **(b)**

Figure (1): comparison between the studied groups regarding both inflammation (a) and demyelination (b) scores. Pure EAE scores ranged between 2 & 3 (green & purple bars) while STAg administration improved both scores to range between 1 & 2 (red & green bars).



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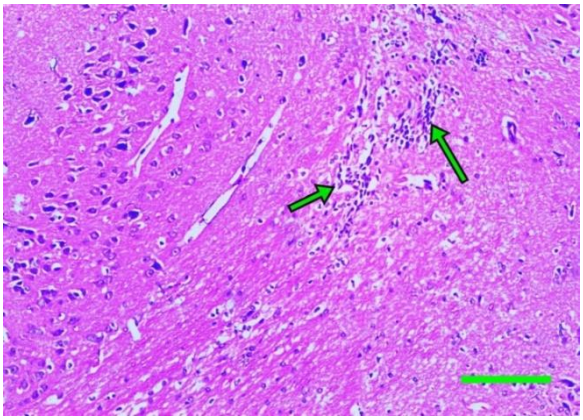

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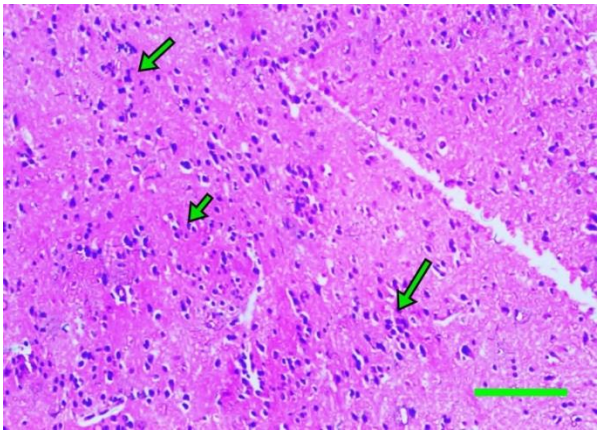
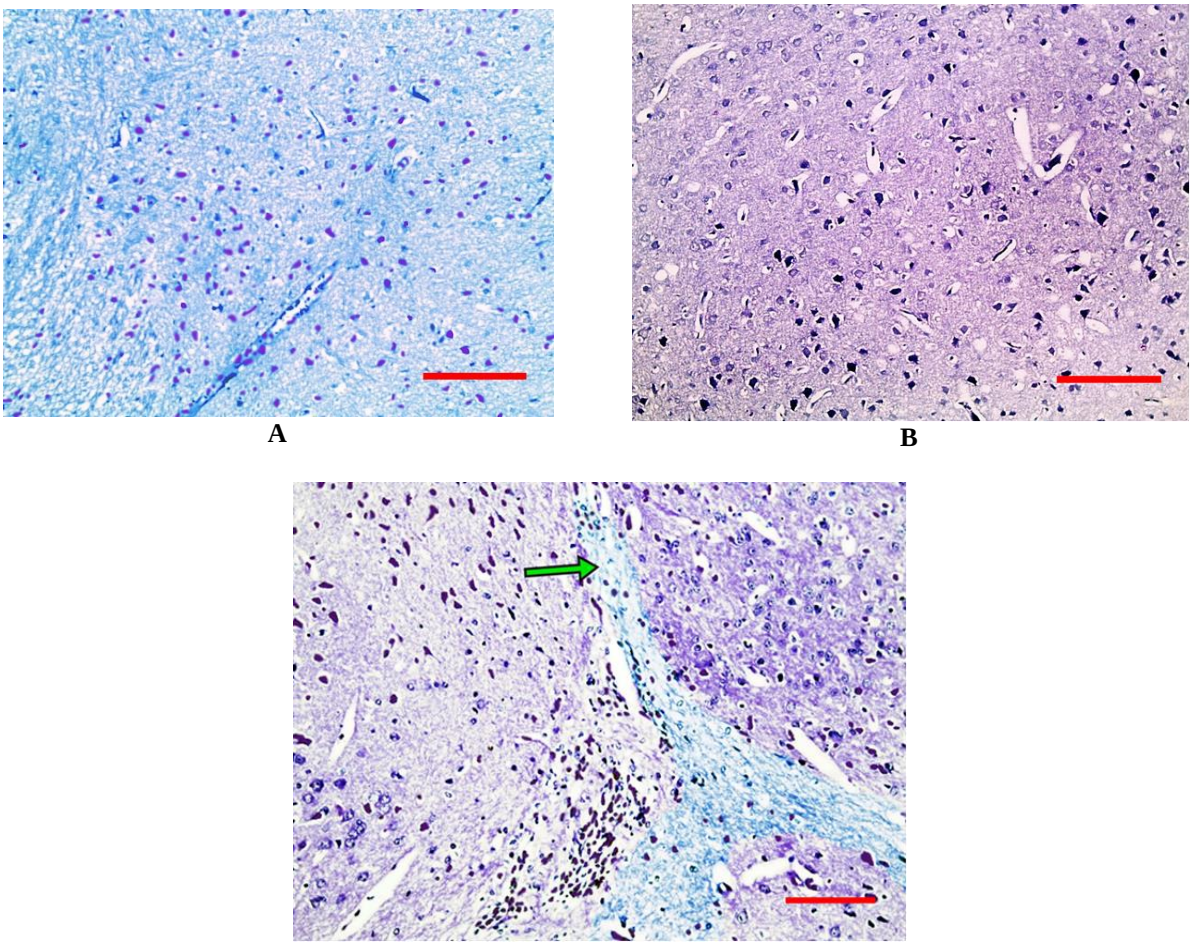


C

Figure (2). H & E stained brain tissue. (scale bar 100µ)

a) Normal brain tissue of the control negative group showing normally appearing white matter (illustrated by green arrow) with scattered oligodendroglia and nerve fibers. Gray matter (illustrated by red arrow) shows abundant neuropils surrounding large neurons.
b) Brain tissue of EAE group showing severe inflammation with diffuse lymphocytic infiltration (illustrated by green arrows).
c) Brain tissue of STAg-EAE group showing mild inflammation with a small area of inflammatory cell infiltration (illustrated by green arrows).



A **B** **C**

Figure (3). Luxol fast blue stained brain tissue. (scale bar 100µ)
a) Normal brain tissue of the control negative group showing blue stained myelin sheath surrounding purple axons.
b) Brain tissue of EAE group showing completely demyelinated area.
c) Brain tissue of STAg-EAE group showing a small demyelinated area (illustrated by green arrow) with normal myelination in the remaining brain tissue.

3.4. PD-1 expression is increased with STAg immunization.

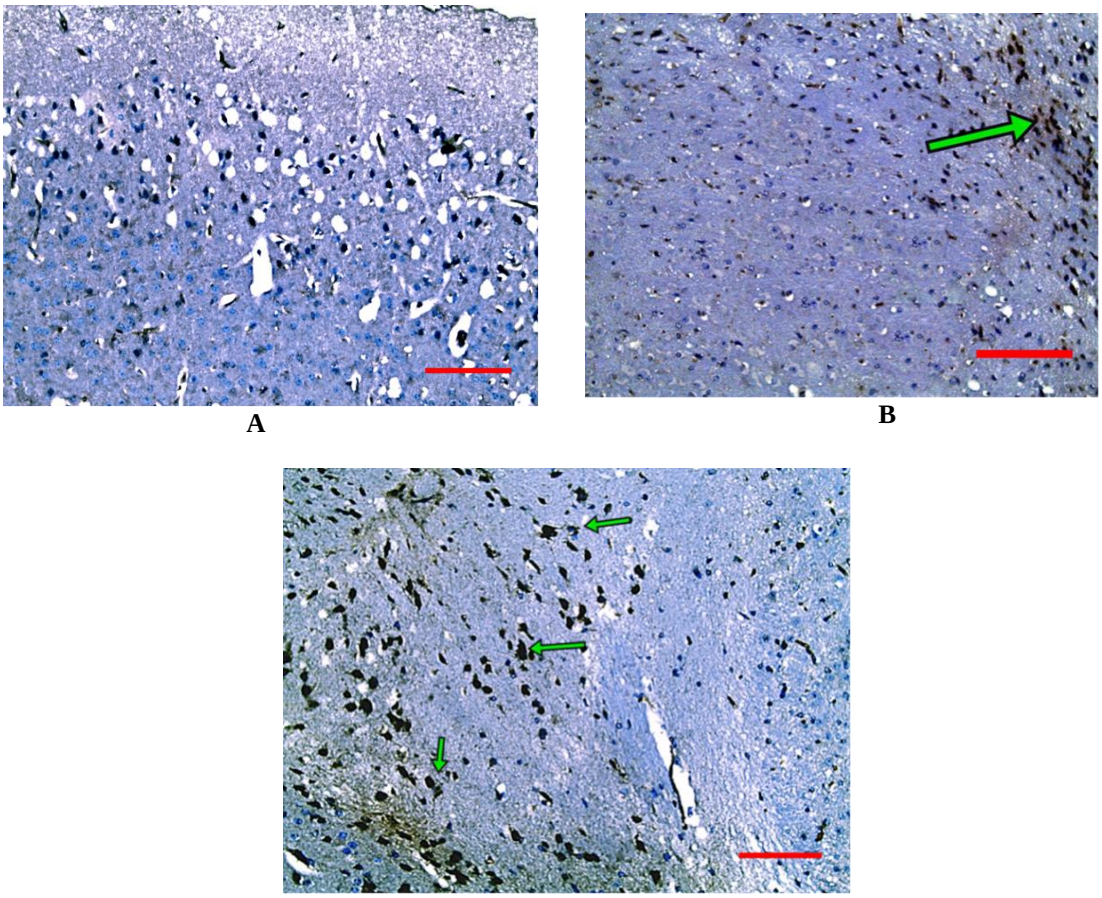
Comparison between sole STAg group (7.10±1.19) and negative control group (0±0) was statistically significant proving that PD-1 was induced by STAg immunization (p1 <0.05). Regarding both EAE groups, although the pure EAE group took the upper hand in inflammation and clinical disease parameters, the reverse was detected regarding the immune-suppressive PD-1. Immune staining of brain tissue revealed a statistically significant increase in PD-1 expression in STAg-EAE mice (mean H-score = 72.5±7.86) than the pure EAE ones (mean H-score =15.65±3.77) (p6<0.05). [Table (3) and figure 4].

Table (3): Comparison between the studied groups regarding mean H- score of PD-1 expression on immune cells.

	Group	N	Mean	SD	ANOVA	p-value	Post Hoc test
PD-1 H-score	-ve control	10	.00	.00	721.56	<0.001*****	p1: <0.05 *
	STAg	10	7.10	1.19			p2: <0.05 *
	EAE	20	15.65	3.77			p3: <0.05 *
	STAg-EAE	20	72.50	7.86			p4: <0.05 *
							p5: <0.05 *
							p6: <0.05 *

*****statistically significant

N.B. The interpretation of Post Hoc test is as follows: p1: comparison between the –ve control group (GI) and sole STAg group (GII). p2: comparison between the –ve control group (GI) and EAE group (GIII). p3: comparison between the –ve control group (GI) and STAg-EAE group (GIV). p4: comparison between sole STAg group (GII) and EAE group (GIII). p5: comparison between sole STAg group (GII) and STAg-EAE group (GIV). P6: comparison between EAE group (GIII) and STAg-EAE group (GIV).



A **B** **C**

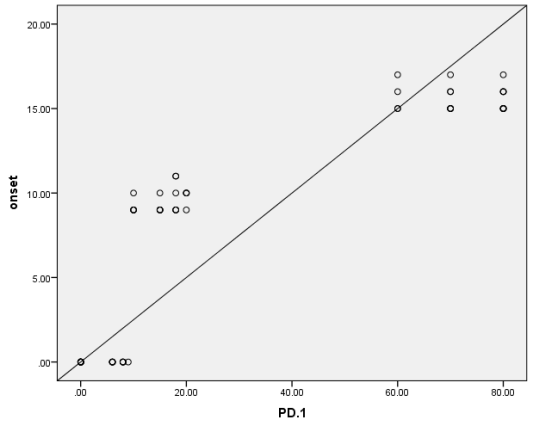
Figure (4). immune stain reaction for PD-1 in mice brain tissue (red arrows refers to positive PD-1 expression). (Scale bar = 100µ)

a) Normal brain tissue of control negative group with negative staining of PD-1.
b) Brain tissue of nonimmunized EAE group showing limited expression of PD-1.
c) Brain tissue of STAg immunized EAE group showing strong diffuse expression of PD-1.

3.5. PD-1expressionis negatively correlated with other assessed clinical, immunological and histopathological disease parameters.

To prove the immune-suppressive role of STAg induced PD-1, its H-score was correlated to the other assessed clinical, immunological and histopathological parameters of EAE. Negative correlation was detected

between its expression and disease onset, MCS, IL-17, INF-ɣ, anti-MBP, inflammatory and demyelination scores. [Figure 5].



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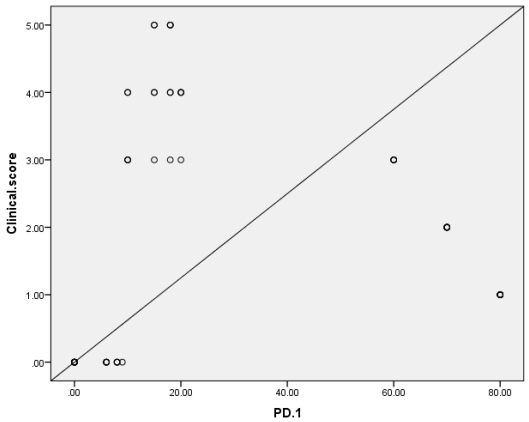

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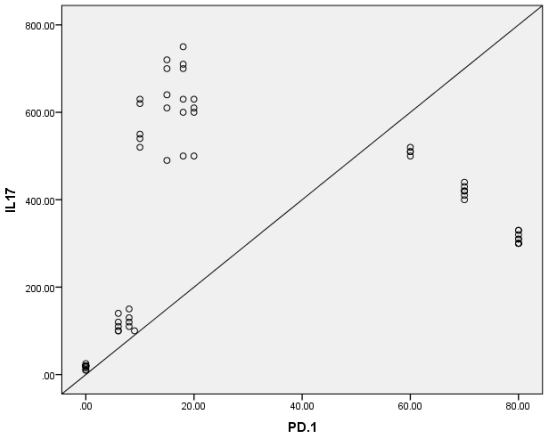

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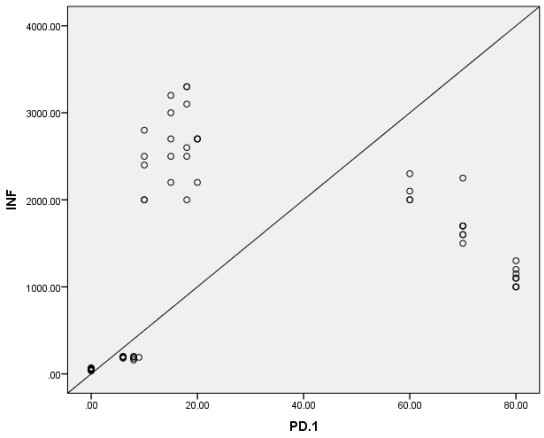

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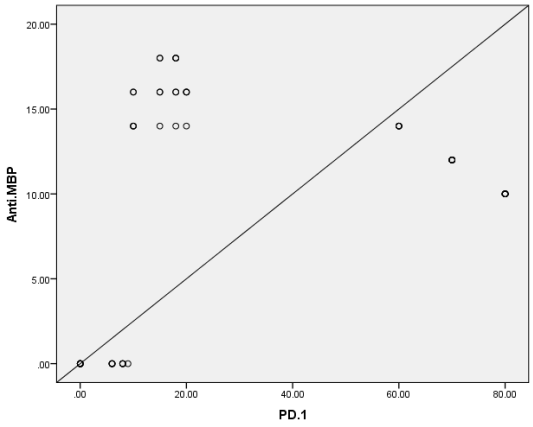

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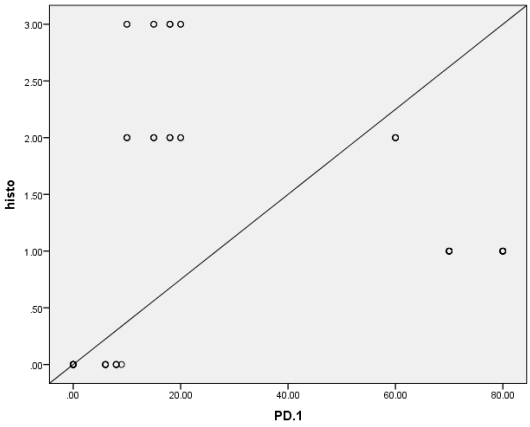

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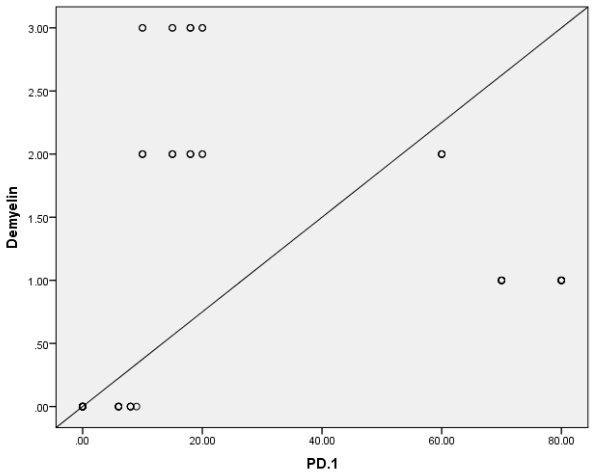

G

Figure (5): correlation between H- score of PD-1 and other assessed disease parameters showing negative correlation between its expression and: (a) day of disease onset. (b) mean clinical score. (c) CSF level of IL-17. (d) CSF level of INF-ɣ. (e) CSF level of anti-MBP antibodies. (f) inflammation score. (g) demyelination score.

4. Discussion

The present study aimed to assess the immunotherapeutic effect of *T. gondii* STAg in EAE model and the role of STAg induced PD-1 in this protection.

Comparing STAg immunized and nonimmunized EAE groups revealed a statistically significant reduction in day of disease onset and clinical progression of manifestations. Improvement extended beyond clinical presentation to involve the proinflammatory cytokines, - IL-17 and INF-ɣ -, anti-MBP antibodies and even inflammation and demyelination scores of brains.

In agreement with our results, **Stascheit et al. [30]** reported a negative correlation between presence of *T. gondii* IgG in serum and being a MS patient. Opposing point of view was presented by **Oruc et al. [46]** who recorded significantly higher incidence of *T. gondii* IgG seropositivity among MS patients than normal individuals.

Other intracellular CNS parasites also correlated negatively with severity of EAE. **Tadokoro et al. [28]** reported that infection with *T. cruzi* completely prevented EAE development and induced complete and lasting remission in mice that were infected with this parasite after they had developed clinical EAE manifestations. They related their results to increased nitric oxide and IL-10 that suppressed inflammatory cytokines e.g. INF-ɣ and IL-2. **Puentes et al. [29]** reported that repeated sequences of *P. falciparum* S–an antigen protein linked to self T cell epitopes – markedly protected mice from EAE and could even treat the ongoing disease. They related their findings to the physical linkage of the T cell epitope to the parasite structure, the action of anti-inflammatory cytokines like IL-10 and TGF-β and foxp3 induced suppression of tumor necrosis factor (TNF)-α.

STAg induced PD-1 production was proved by the statistically significant difference in PD-1 H-score between the pure STAg immunized group and the negative control group which showed a negative PD-1 expression. These results were similar to what was reported by **Bhadra et al. [32,33].** They reported that chronic toxoplasmosis was associated with progressive elevated expression of the inhibitory receptor PD-1 on effector $CD8^+$ T cells in both lymphoid and nonlymphoid tissue. PD-1 induces lymphocyte apoptosis with subsequent immune-suppression that lead to reactivation of the parasite. They also reported that, blockade of the PD-1/PD-L1 pathway caused the exhausted $CD8^+$ T cells to restore their function.

Similarly, $CD8^+$ and $CD4^+$ cell exhaustion was also detected with the intracellular protozoan parasite, *Leshmania infantum*. This was regarded to PD-1 upregulation on T lymphocytes and was proved by PD-1 blockage that induced upregulation of both $CD4^+$ and $CD8^+$ T cells in the study published by **Esch et al. [47].**

In the present work, the inhibitory role of STAg induced PD-1 was proved by its negative correlation not only with clinical presentation of disease but also the assessed immunological and histopathological findings.

Likewise, **Arruda et al. [48, 49]** related the prolonged clinical remission in MS patients with autologous hematopoietic stem cell transplantation to $CD8^+PD\text{-}1^+$cells and the inhibitory signals of their receptor, PD-1.

In agreement with our results, a negative correlation of PD-1 and its ligand PD-L1 with clinical

manifestations of MS was reported by **Javan et al. [50].** They reported significantly lower expression of PD-1 and PD-L1 in peripheral blood mononuclear cells from MS patients when compared with the healthy control group.

Accordingly, **Terrazas et al. [51]** regarded ameliorated immune response in EAE model to PD-1/PD-L2 expressed on alternatively activated macrophages induced by *Taeniacrassiceps*.

Also, **Shi et al. [52]** recorded high anti-PD-1 IgG antibodies in sera of newly diagnosed cases of systemic lupus erythematosus (SLE). Its level was significantly correlated with clinical manifestations and neurological involvement. In vitro examination showed that purified anti-PD-1 IgG obtained from SLE patients enhanced T cell proliferation when co-cultured with dendritic cells.

Regarding correlation with IL-17, results of **Carter et al. [53]** were in accordance. They reported production of significantly lower concentrations of proinflammatory cytokines – including IL-17 and INF-ɣ – in PD-1^+/PD-$L1^+$ animal models of EAE than PD-1^-/PD-$L1^-$ models. Similarly, **Wang et al. [54]** reported that IL-17 production was suppressed by PD-1 over-expression on $CD4^+$ and $CD19^+$ cells. They proved that estrogen induced suppression of EAE and the proinflammatory cytokine, IL-17 were PD-1 dependent.

As for PD-1 correlation with INF-ɣ and autoantibodies, similar results were reported by **Salama et al. [20].** They reported that PD-1 blockade resulted in EAE exacerbation with higher levels of serum INF-ɣ and anti-myelin oligodendrite glycoprotein antibodies. Also, **Schachtele et al. [55]** reported that inhibition of PD-1 expressed on *Cytomegalovirus* induced glial cells promoted INF-ɣ production from $CD8^+$ cells co-cultured with them. Contrarily, although **Trabattoni et al. [56]** reported that PD-1 expression on $CD8^+$ and $CD4^+$ lymphocytes was significantly higher in stable MS than in acute relapsing remittent MS patients, they reported that INF-ɣ producing cells showed no difference between groups.

5. Conclusion

All the above-mentioned results can give a conclusion that, STAg immunization could improve EAE at clinical, immunological and histopathological levels. This protection could be related to induction of PD-1 on immune cells which in turn down regulated all inflammatory reactions of EAE due to their immune-suppressive properties.

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