INVESTIGATING THE ROLE OF INNATE IMMUNE SYSTEM RECEPTORS AND THEIR ASSOCIATED SIGNALING CASCADES IN MURINE MALARIA INFECTION IN VIVO

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ABSTRACT:

Plasmodium falciparum glycosylphosphatidylinositols (GPI) contributes to malaria pathology by inducing cytokine release. It has been shown to be recognized through Toll like receptors (TLR), TLR2 and to a lesser extent TLR4 *in vitro*. However, previous studies on the role of TLRs in parasite clearance or pathology *in vivo* are conflicting. Thus, we analyzed the role of TLR 9 and TLR 4 on protection using the *P. yoelii* (non lethal) infection model. It was found that, deficiency of TLR9 had no impact on parasitaemia. But, mice deficient for TLR4 were protected from malarial complication as hyperparasitemia and sever anemia during *P. yoelii* infection as they exhibited a low parasitaemia from the early phase of the infection and a rapid clearance of the parasite from blood. This phenotype was caused mainly by impaired Interlukines 12p 40 (IL-12p40) and high Interleukin (IL10) in the sera compared to wild-type positive control (+ve). Increased IL10 was basic for high IL10/TNFα ratio and low IFNγ/IL10 ratio. The imbalance between IL12p40 and IL10 led to direct the immune response towards Th2 and not Th1. This was indicated from high immunoglobulin titer (IgG) in different mice groups' sera. We conclude that TLR4 and not TLR9 had a role in malaria immune pathogenesis and complication through the pro-inflammatory response to *plasmodium Yoelii non lethal*.

KEY WORDS:

Plasmodium Yoelii non lethal (*PYNL*)
Inflammatory cytokines Toll like receptors (TLRs)

INTRODUCTION:

Malaria is still one of the major infectious diseases caused by infection with protozoan parasites of the genus *Plasmodium*, is a major global heath problem (Stevens and Riley, 2004). Half of the world's population is at risk of malaria, and an estimated 247 million cases led to nearly a million deaths in 2006, mostly of children under 5 years (WHO., 2008). Malaria infection involves injection of *Plasmodium* sporozoites from mosquito into humans. The sporozoites enter the blood circulation and then migrate to the liver, invade hepatocytes. At the end of this obligatory, clinically silent and relatively short hepatic multiplication step, merozoites are released into the bloodstream where they invade and multiply in red blood cells. During *Plasmodium* infection, parasite numbers are comparatively low in the liver stage (Prudêncio et al., 2006).

Clinical manifestations are exclusively linked to the blood stage of infection. Innate immune mechanisms have been shown to be important for early parasite control by initiating cytokine release and triggering cellular immune responses (Krishnegowda et al., 2005). Early Interferon gamma (INF-γ) and Tumor necrosis factor

(TNF-α) production plays a pivotal role in the host's immune defense reaction (Artavanis-Tsakonas, 2002). However, excessive and/or prolonged expression of pro-inflammatory cytokines contributes to disease pathogenesis associated with characteristic symptoms and conditions of severe malaria including severe anemia (Clark, 2004). Adaptive immune response through cell mediated immune response had been identified in parasite clearance, and it is the main orchestra factor or pathogenesis whiles the humeral immune response, many studies on human referred to its protective effect. The Immunoglobulin level was high in uncomplicated malaria **(**Leoratti et al, 2008).

Within the last years, the family of toll-like receptors (TLRs) has been identified as key host molecules in the induction of innate immune responses to microbial ligands. There are 11 or 13 TLR in human or mice respectively had been identified. TLR-2 (in synergy with TLR-1 and -6) and TLR-4 react to bacterial cell wall compounds. TLR-2 is activated by a variety of ligands, such as bacterial lipopeptides, as well as fungal and mycobacterial components. TLR-4 is activated not only by bacterial lipopolysaccharide but apparently also by other ligands, such as viral proteins (Akira et al, 2006). The contribution of this Pattern recognition receptor (PRR) to the pathogenesis of severe malaria is suggested by the following set of observations. Polymorphisms in human TLR4 (Asp299Gly/Thr399Ile) and TLR9 (T-1486C) are associated with susceptibility to severe malaria in children (Mockenhaupt et al., 2006) and with the outcome of Plasmodium infection during pregnancy (Mockenhaupt et al., 2006) respectively. Studies on the role of the TLR(s) in the development of severe disease (Coban et al., 2007) showed induction of(cerebral malaria) CM depend on TLR9 while (Lepenies et al, 2008) showed that induction of cerebral malaria during *P. berghei ANKA* infection is independent of TLR2/4/9.

The present study focused in investigating the role of TLRs (4 & 9) into two critical immunological aspects of malaria: (i) control of Parasitemia (ii) different serum cytokines levels.

MATERIAL AND METHODS:

1. Mice and parasites

Eight-week old female BALB/c mice were purchased from Clean, Japan. TLR4 and TLR9 deficient mice with BALB/C background were bred and maintained under specific Pathogen free condition.

Non-lethal *Plasmodium yoelii* strain (*PY17NL*) was prepared as described previously. Briefly, Py17NL was maintained by regular passage in BALB/c (Albino) mice (the infected blood kept in 0.9% NaCl, 4.6% sorbitol, and 35% glycerol) and stored as stock in liquid nitrogen, and then the stock used to infect a donor mouse which used to infect all the mice groups in this study. Mice were intraperitoneally (IP) infected with 10^5 *PY17NL*. All experiments were in accordance with local Animal Ethics Committee regulations. Parasitemia was assessed by counting 10 microscope fields from Giemsa-stained thin blood smears of tail blood prepared every other day started from day (Coban et al., 2 007).

2. Multiplexed Microsphere Cytokine Immunoassay

IL 12p40, INF γ, TNF α, IL4 and IL10 were evaluated in the different sera of 3 mice from each group at day

0, day 10 and day16 post infection using a commercially available multiplex bead-based cytokine assay coupled with the Luminex system (Austin, TX) and mice-specific magnetic beads Bio-Plex Cytokine Kit (Bio-Rad, Hercules, CA). Biological factor levels were measured using optimal concentrations of standards and antibodies according to the manufacturer's instructions. Briefly, plasma samples were diluted 1 : 4 in Bio-Plex sample diluents and an eight point broad range cytokine standard was prepared by serial dilution from a cytokine stock. Fifty micro liters from each diluted samples and each standard was transferred to 96-well filtration plate containing anti-conjugated beads. The plate was shaken for 30 seconds at 1,100 rpm then was shaken at 300 rpm with incubation for one hour at room temperature. After incubation the plate was washed. Detection antibody (2 mg/mL) was added, shaked and incubated for 30 minutes. After 4 times washes, streptavidin-PE was added and incubated for 10 minutes. Beads were mixed before reading on the Bio-Plex Suspension Array System. The data was analyzed using Bio-Plex Manager software with 5-parameter logistic (5PL) regression curves **(**Coban et al., 2 007**)**.

3. <u>ELISA for antibody detection</u> (Coban et al., 2007)

plates wells were Coated using 1X bicarbonate buffer containing Crude extract antigen 100 µl per well, kept at 4 C overnight. Then the content was dumped and blocked with block solution (5% skimmed milk and 0.5 Tween 20 in PBS) 200 µl per well for 2 hours. Dumping, washing and drying as ELISA for cytokines.

Prepare serum dilution (1/50), standard serial dilutions and blank using the block solution, put 50 µl per each well then cover with adhesive seal and wait for two hours at room temperature, the plate was washed and dried then add 50 µl per well from secondary antibodies as 4000 ng/ml for (IgG, IgG1, IgG2b, IgG2c and IgG3) cover with adhesive tape for 2hoursand wash 4 times. 50ul/well TMB liquid substrate (3,3, 5,5 tetramethlbenzidine) for 20 min. 50 µl/ well was added sulfuric acid used to stop the reaction. The blue color had changed to yellow. The optical density were measured at 450 λ (Coban et al., 2 007).

STATISTICAL ANALYSIS:

The statistical analyses (Mann-Whitney U-test or Student t- test) of the obtained data were performed by using sigma stat 3.0 software. $P< 0.05$ was considered statistically significant.

RESULT:

<u>Parasitemia level in TLR4$^{-/-}$, TLR9$^{-/-}$</u>

It is well established that TLR4 and TLR9 are two of the important innate immune receptors of the host immune response. BALB/c mice were infected with a non-lethal *P. yoelii* (*Py17NL*) strain to asses the role of each of these two receptors in the fate of malarial blood stage parasites. The parasitemia was measured by Giemsa stained thin blood smears (Table 1 & Fig 1)**.**

Table (1): Parasitemia level in different mice groups (TLR4$^{-/-}$, TLR9$^{-/-}$ and +ve control).

Days	WT/mean of 10 mice	±SEM	TLR4-/-/ mean of 10 mice	± SEM	TLR9-/-/ mean of 10 mice	± SEM
0	0	0	0	0	0	0
3	0.81	0.727	0.238246	0.226787	2.99	0
5	1.727	0.951	0.9799	0.9404	9.367	0
9	3.77	4.634	4.371	6.572	14.055	0.26
11	9.5	5.254	13.687	5.433	21.6	8.602874
13	20.7	10.503	14.128	13.403	23.65	15.48564
16	37.271	18.26	13.025*	9.232	31.35	27.5
18	45.4	20.36	13.425**	20.698	39.99	18.36
20	34.27	25.406	0	0	33.87	15.23
24	0.0666	16.03	0	0	9.969	9.6

(A)

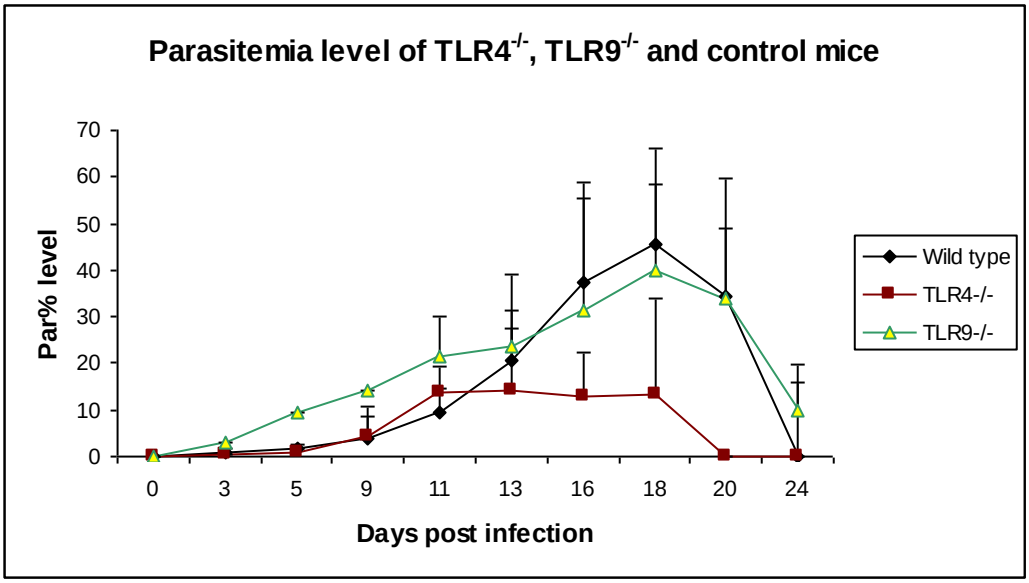


Fig. (1): Parasite levels of TLR4−/−, TLR9−/− and control + ve mice after infection with non-lethal *P. yoelii* (Py17NL). Data from the indicated number of mice (10 mice in each group) are presented as mean ± SEM. Statistical significance was tested using the Mann–Whitney U test. *P < 0.05; **P < 0.01; ***P < 0.005.

The parasitemia in different mice groups was approximately similar till day 13. The parasitemia of TLR4-/- was significantly lower than that of TLR4-/- and TLR9-/- on days 16 and 18 (TLR4$^{-/-}$ 13.25± 9.23 while in wild type 37.1±18.26 and TLR9$^{-/-}$ 31.35± 27.5 P=0.002), (TLR4$^{-/-}$0 vs. WT 45±20.83 and TLR9$^{-/-}$ 39.99± 18.36 P=0.01).

On day 20, TLR4$^{-/-}$ cleared the parasite while WT and TLR9$^{-/-}$ did not clear the parasite.

Cytokinemia during infection with *P. Yoelii* in mice deficient in specific TLRs (TLR4 and TLR9

Then the study was directed to evaluate the cytokine responses (Pro-inflammatory cytokines (IL12p40,

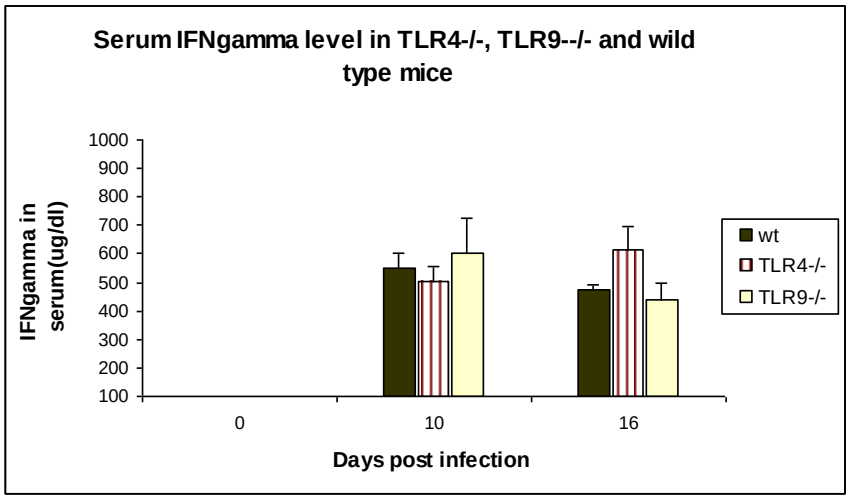
TNFα, IFNγ) and (anti-inflammatory cytokines (IL4 and IL10) of knockout mice and wild type at day 0, 10 and 16 post-infection in sera by Multiplexed Microsphere Cytokine Immunoassay.

Table (2): Serum levels of different cytokines in different mice groups

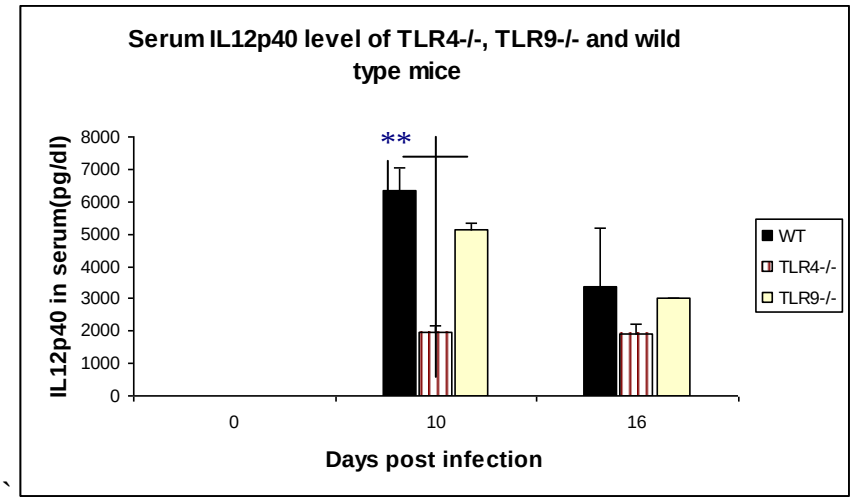
IFNγ	**Wt/ mean of 10 mice**	**±SEM**	**TLR4$^{-/-}$/ mean of 10 mice**	**±SEM**	**TLR9$^{-/-}$/ mean of 10 mice**	**±SEM**
Day 0	36	7	16	5	0	0
Day 10	548.5061	54.29643	502.36	50.73	604.36	121.5891
Day 16	472.1867	19.84	616.6267	78.718	436.06	58.97434
IL12p40						
Day 0	10	0	0	0	9	0
Day 10	6325.017	727.6559	1978.85***	199.7745	5120.849	202.7081
Day 16	3389.783	181.4891	1888.083	327.5847	3000	23.86757
IL10						
Day 0	0	0	0	0	0	0
Day 10	557.96	36.085	1870.12**	132.05	491.1	20.028
Day 16	389.586	26.304	4790**	524.423	320	16.3
TNFα						
Day 0	0	0	0	0	0	0
Day 10	716.0267	112.46	380.3867	44.725	610.14	11.10244
Day 16	416.32	33.602	336.0667	21.29	582.27	39.05768

Each value represented by the mean of three mice ±SEM. Statistical significance was tested using the Mann–Whitney *U* test. . *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$.

(A)



(B)



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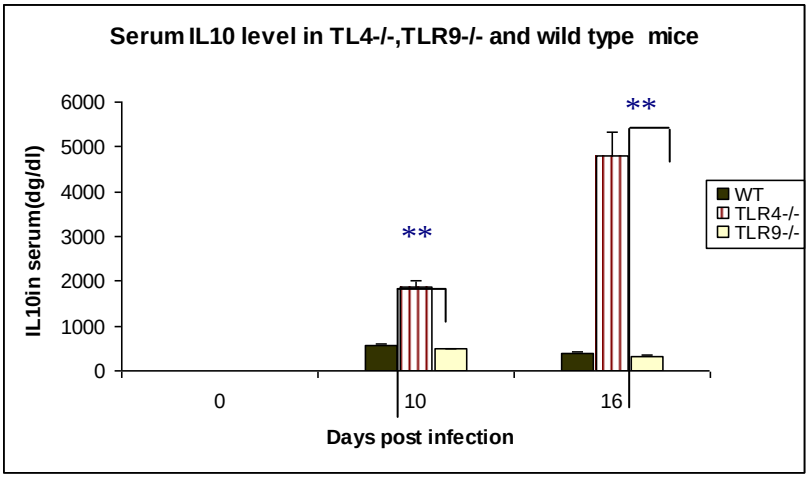

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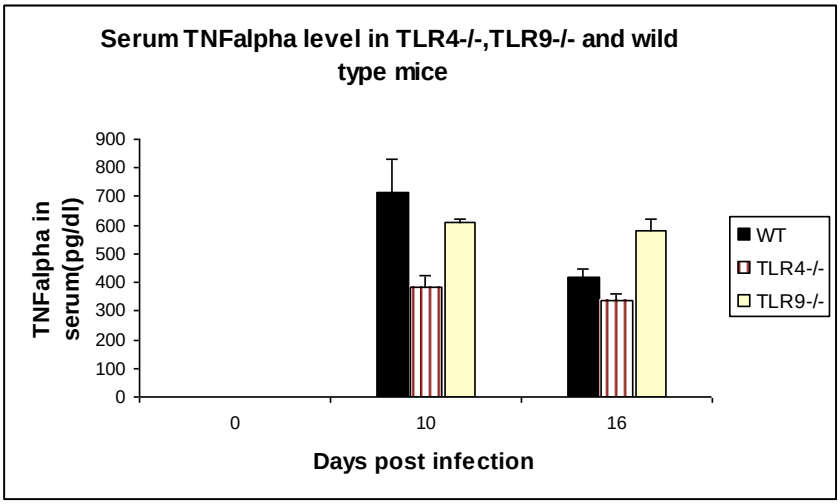


Fig (2): Serum cytokines were measured by Meltiplex in sera from different infected mice groups obtained on days 0, 10 and 16, IFNγ (A), IL-12p40 (B) , IL10 (C) and TNF (B). Data are presented as mean ± SEM for each group of mice. Statistical significance was tested using the Mann–Whitney *U* test. . **P* < 0.05; ***P* < 0.01.

IL12p40 in WT and TLR9$^{-/-}$ was, secreted on day 10 higher than on day 16, while on TLR4$^{-/-}$ mice IL12p40 was almost in the same level on both days but significant lower than wild type only on day 10 (WT 6325.01 ±727.65, TLR9$^{-/-}$ 5120.849±202.70 Vs TLR4$^{-/-}$1978.85±199.77 P 0.007). There was no significant difference in both TNFα and IFN γ between all study mice groups. IL10 in WT and TLR9$^{-/-}$ was secreted in minimal amount on day 10 and decreased on day 16 while, on TLR4-/- it was highly secreted on day 10 and continuous increased on day 16 this led to significant difference, on day 10 (TLR4$^{-/-}$1870±132.05 Vs. 557.96± 36.08 P0.007), on day 16 (TLR4$^{-/-}$ 4790± 524.42 Vs. WT 389.586±0.01 P 0.01). IL4 was under detectable amount in all study groups.

Table (4): Serum IL10/TNFα ratio in different mice groups.

	Day10			**Day16**		
WT	**IL10**	**TNFα**	**IL10/TNFα**	**IL10**	**TNFα**	**IL10/TNFα**
	491.52	1200	0.4096	507.88	569.92	0.8911
	458.8	637.48	0.7197	354.44	320.24	1.1068
	723.56	310.6	2.3296	306.44	358.8	0.8541
TLR4-/-						
	1547.8	563.52	2.7467**	5076.963	429.16	11.83**
	2479.7	371.6	6.6731**	2563.807	317.04	8.0867**
	1582.8	206.04	7.6822**	6729.732	262	25.686**
TLR9-/-						
	431.2	602.69	0.7154	330.89	551.03	0.6004
	416.8	596.55	0.6986	290.9	587.21	0.4953
	629.52	630.98	0.997	337.91	610	0.5539

Serum IL10/TNFα ratio in different mice groups, IL10/TNFα ratio was significant high in TLR4$^{-/-}$ mice compared with wild type and TLR9$^{-/-}$ on day10 and day16 (P value 0.02 on both days) . Statistical significance was tested using the Mann–Whitney *U* test. . *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$.

Table (5): Serum IFNγ/IL10 ratio in different mice groups was lowest value in TLR4$^{-/-}$ from TLR9$^{-/-}$ and wild type.

Day 10			**Day16**		
WT IFNγ	**WT IL10**	**WT IFNγ/IL10**	**WT IFNγ**	**WT IL10**	**WT IFNγ/ IL10**
446.48	491.52	0.908	506.32	507.88	0.996
797.92	458.8	1.739	427.64	354.44	1.206
401.12	723.56	0.554	347.6	306.44	1.13
TLR4-/- IFNγ	**TLR4$^{-/-}$ IL10**	**TLR4$^{-/-}$ IFNγ/IL10**	**TLR4$^{-/-}$ IFNγ**	**TLR4$^{-/-}$ IL10**	**TLR4-/- IFNγ/IL10**
642.48	1547.8	0.415*	454	5076	0.089***
594.96	2479.72	0.2399*	416.32	2563.8	0.162***
269.64	1582.84	0.17*	979.56	6729.68	0.14***
TLR9-/- IFNγ	**TLR9$^{-/-}$ IL10**	**TLR9$^{-/-}$ IFNγ/IL10**	**TLR9$^{-/-}$ IFNγ**	**TLR9$^{-/-}$ IL10**	**TLR9-/- IFNγ/IL10**
492.85	431.2	1.142973	440	330.89	1.329747
474.12	416.8	1.137524	420	290.9	1.443795
846.69	629.52	1.344977	450	337.91	1.331716

Serum IFNγ/IL10 ratio in different mice groups, IL10/TNFα ratio was significant low in TLR4$^{-/-}$ mice compared with wild type and TLR9$^{-/-}$ (P value 0.01 on day10 and 5.6E-5 on day 16) . Statistical significance was tested using the Mann–Whitney *U* test. . **P* < 0.05; ***P* < 0.01; ****P* < 0.005.

Antibody titer in serum:

To investigate the effect of imbalance between the pro inflammatory and anti inflammatory cytokines in the form of reduced IL12p40 level and high IL10 level in sera of TLR4$^{-/-}$ mice had effect on shifting the acquired immune response from Th1 to Th2; we measured the Total IgG antibody and IgG subclasses in the sera of the mice groups by ELIZA.

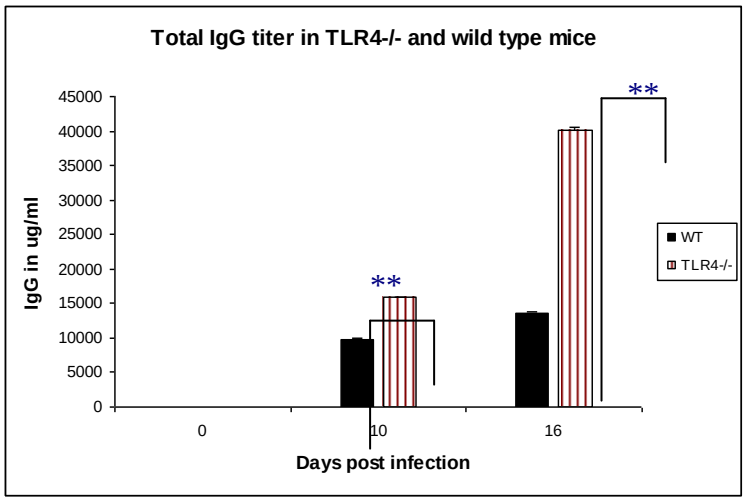
Table (6): Anti body titer in TLR4$^{-/-}$ and Wild type control

Total IgG	**Days0**	**±SEM**	**Day10**	**±SEM**	**Day 16**	**±SEM**
WT	0	0	9863.6	42.979	13675.9	122.454
TLR4-/-	0	0	15877.2***	76.64	40157.5**	300.39
IgG2a						
WT	0	0	2699.8	75.35	100095.8	276.059
TLR4-/-	0	0	7320.8*	140.49	25032.75**	331.59
IgG1						
WT	1805.94	17.02	1035.05	10.17	2497	28.45
TLR4-/-	2500.02	34.706	1081.9	21.63	4883.15*	94.66

Serum total IgG (A), IgG 2a (B) and IgG1 (C). Data is presented as mean ± SEM for each group of mice. Statistical significance was tested using the T test. **P* < 0.05; ***P* < 0.01; ****P* < 0.005.

Fig. (3): Serum level of total IgG, IgG2a and IgG1.

(A)



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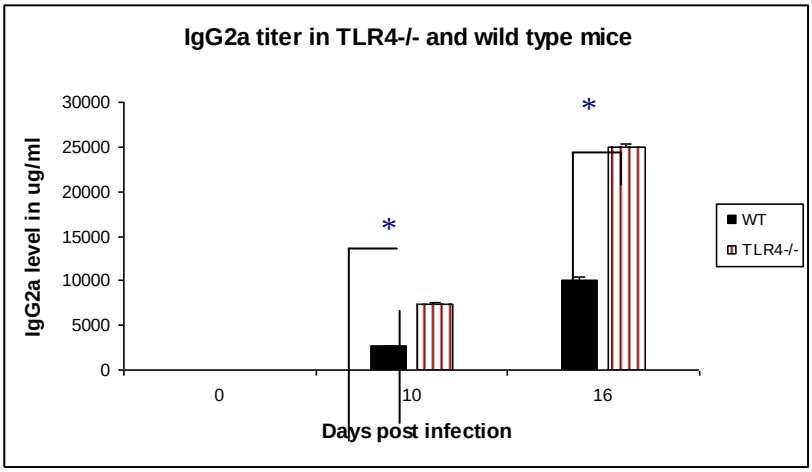

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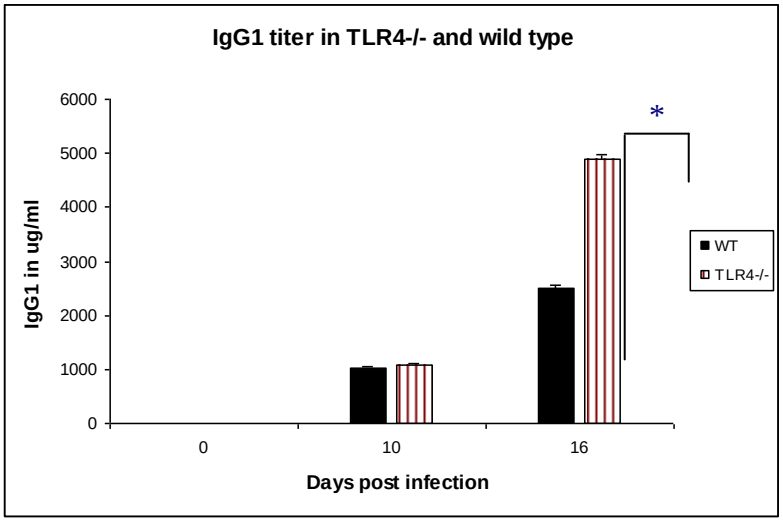

We found total IgG and IgG2a were highly secreted in TLR4$^{-/-}$ mice than control mice (Total IgG on day10 P 0.003 and on day 16 P0.001) while(Ig G2a on day 10 P 0.02 and on day 16 P 0.01) while IgG1 only showed significance difference on day 16 (p 0.02).

DISCUSSION:

Toll-like receptors (TLRs) are the first gate to innate immune system, which work against any invading microbes to eradicate infection. Studies on the role of TLRs had many conflicting results on both man and mice. $TLR4^{-/-}$ mice had lower parasite-emia and earlier parasite clearance than wild type (WT) mice. Parasite level and parasite clearance in $TLR9^{-/-}$ were comparable to WT mice. Jacob et al (2008), found that, TLR4, TLR9, or all of these three TLRs combined are dispensable for parasite clearance. Franklin et al (2007) found that parasitaemia was not different, when comparing $TLR4^{-/-}$ and $TLR9^{-/-}$ to WT mice, all the knockout mice, were capable of clearing parasitemia, at the same time of the WT mice. The previous two studies are in contrast with the present study, due to different mice back ground. The previous two studies had used C57BL/6 while the present study used BALB/c.

The present study had found the cytokines profile in the form of Low IL12p40, high IL10 in $TLR4^{-/-}$. TNFα was slightly decreased in $TLR4^{-/-}$ and no change in IFNγ. IL10/TNFα ratio is an indicator for malarial anemia, high ratio associated with escape from anemia. $TLR4^{-/-}$ had the highest ratio between all study mice groups. IFNγ/IL10 ratio indicates the malaria severity and complications, Low ratio means less severity and no complica-tions. $TLR4^{-/-}$ had the lowest ratio. Th2-type cytokines, such as IL-10, regulate Th1-cytokines and prevent severe forms of malaria in some animal models (Wilson et al, 2010). $IL10^{-/-}$ mice had a higher rate in pathology and mortality than wild type control, and this was subsequently shown to be mediated by excess production of pro-inflammatory cyto-kines (Li et al, 2003).This study agrees with the present study as the high level of IL10 is protective.

The effect of increased serum IL10 on $TLR4^{-/-}$ changed the immune response towards Th2. This indicated from high immunoglobulin (IgG, IgG2a and IgG1) titer in sera of $TLR4^{-/-}$ mice. Elgadir et al (2008) found lower prevalence of IgG antibodies against *P. falciparum* in individuals with severe malaria than in uncomp-licated. Stanisic et al, (2009) study done on human serum from both Guinea and Kenya found the strongest association with protection from symptomatic malaria. The previous two studies are in line with the present study that, antibody had a protective effect from sever malaria.

CONCLUSION:

TLR4, not TLR9 plays important role in pathogenesis and complications as hyperparasitemia and anemia during *Plasmodium Yoelii* non lethal. This may be due to the imbalance between Th1 and Th2 cytokines with shifted immune response to Th2 with high antibody titer in $TLR4^{-/-}$ mice.

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الملخص باللغــة العربيــة

دراسة دور مستقبلات جهاز المناعة الطبيعية ومسارات إشاراتها في عدوي الملاريا بالفئران

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مرض الملاريا من أكثر الأسباب التي تؤدى إلى وفاة الملايين من البشر .بالرغم من جهود العلماء على مر العصور لم يتم عمل لقاح ضد المرض . طفيل الملاريا له دورة حياة معقدة داخل الفقاريات وألا فقاريات .

سلاح المناعة الطبيعية يتعرف على المواد النشطة مناعياً المشنقة من كلاً من : الطفيل والعائل خلال مستقبلات للتعرف موجودة على غشاء البلازما مثل (TLR) أهم هذه المستقبلات موجودة في خلايا مناعية متخصصة مثل الميكروفاج، دندرتك والبى هذه الخلايا لها دور في التخلص من الطفيل .

فى هذه الدراسة تم دراسة دور TLR4 و TLR9 وتاثيرهما على مرض الملاريا باستخدام (*PYNL*) في الفئران

تم تقسيم الفئران إلى ثلاث مجموعات :

1- فئران لا تحتوى على TLR4
2- فئران لا تحتوى على TLR 9
3- الفئران الضابطة و تحتوى على (TLR4 – TLR9)

تم الحصول على النتائج التالية :

مجموعة الفئران التي لا تحتوى على TLR4 كانت نسبة الطفيل بالدم اقل وتم التخلص من الطفيل تماماً من الدم أسرع من المجموعتين الاخريتن .

انخفاض في مستوى IL12P40 وعدم تأثر مستوى كلا من TNFα و IFNγ ارتفاع في مستوى IL10 في الفئران التي لا تحتوى على TLR4 مقارنةً بالمجموعة التي لا تحتوى على TLR9 والمجموعة الضابطه وهذا ادى الى اتجاه الاستجابة المناعية ناحية (Th2) خلايا التي متخصصة في إنتاج الأجسام المضادة IgG IgG2a و IgG1 هذه الأجسام كان لها الدور بالقضاء على الطفيل بالدم.