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Expression Patterns of TLR1 during *T. congolense* Infection in Mice

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Abstract:
After QTL mapping and physical representation of the particular chromosomal fragment spanning trypanosomosis resistance loci Tir2 and 3 in mice, possible candidate genes were selected. These appeared to be linked to the innate immune response. Plausible trypanotolerance candidate genes within the loci include TLR1 which is critical in the regulation of pro-inflammatory cytokine secretions. In an effort to find an association between Tircandidate genes and the disease, expression patterns of TLR 1 gene mapping to trypanotolerance QTL Tir1 was investigated using quantitative real time PCR.Susceptible and resistant mice infected with T. congolense portrayed diverse TLR expression patterns. Up regulation of TLR1 seemed to coincide with up regulation of IL-10 and TNF in susceptible and resistant strains respectively. T. congolense infection therefore induces a response characterized by changes in TLR 1 expression in liver and spleen tissues which appear to regulate cytokine profiles in mice. These phenomena may be responsible for the diverse disease pathology evident in different mouse strains and may account similar trends observed in livestock breeds.

1. Introduction

Trypanotolerance QTL mapping and physical representation of the particular chromosomal fragment spanning trypanosomosis resistance loci *Tir*2 and 3 in mice (Nganga *et al* 2010), possible candidate genes were selected. These appeared to be linked to the innate immune response. Plausible trypanotolerance candidate genes within the loci include TLR1, 5 and 6. TLRs are critical in the regulation of pro-inflammatory cytokine secretions. In an effort to find an association between the genes and the disease, expression patterns of TLR genes mapping to trypanotolerance QTL were investigated using quantitative real time PCR. Most mouse mutants with a defective immune system have a broad vulnerability to infections. The concerted effort of host and pathogen gene-expression profiling by microarray technology and mouse genetics will be the method of choice for *in vivo* induced gene analysis in the future. Many strains of mice are available while their ease of breeding enhances the chances of pinpointing the genes responsible for resistance through the expression studies. It is also possible to identify, remove and augment particular components of their immune system (Alsford *et al* 2013, Wakelin 1991).

Genetic factors that mediate differential susceptibilities to trypanosomes are poorly understood. However trypanosomes are mainly cleared from circulation through antibody-mediated phagocytosis by hepatic Kupfer cells and also by spleenic macrophages (Macaskill *et al.* 1980). Parasite growth, differentiation rates and host immune responses are similar for the first 5 days in resistant and susceptible mouse strains but proceed more rapidly with higher antibody titres in resistant than susceptible mice (Black *et al.* 1983). Susceptible and resistant mice initially develop similar levels of parasitemia, anemia, biochemical derangement, and immunosuppression early in the infection. After 8 days the susceptible strain sustain high levels of parasitemia until death, whereas the resistant strain shows a marked decrease in parasitemia by day 10 post infection. (Whitelaw *et al.* 1980). The resistant C57BL/6J mice are able to remove circulating trypanosome parasites with this ability persisting for several weeks after infection compared to the susceptible A/J (Whitelaw *et al.* 1983 andThoma-Uszynski *et al.* 2001 Roffê *et al* 2012).

Though trypanotolerance in C57BL/6J mice may depend on their more efficient antibody response (MacAskill *et al.* 1983), attempts to enhance the immune response of susceptible mice using either a variety of immunostimulants, simultaneous vaccination with irradiated parasites at the time of infection, passive immunization or reducing the number of parasites used for infection, failed to confer a level of protection comparable to that of C57BL/6J mice. Therefore, the basis of trypanotolerance, though immunological in nature, is associated with other factors especially those appearing early in the course of the infection (Noel *et al.* 2004).

Such factors include TLRs of the innate immune system which detect host invasion by pathogens and initiate early immune responses. The interaction of the different TLRs with distinct combinations of adapter molecules creates a platform to which additional kinases, transacting factors, and possibly other molecules are recruited, leading ultimately, to variety of gene expression patterns (Vogel *et al.* 2003). Activation of the innate host defences by TLRs leads to the recruitment of neutrophils and activation of macrophages, enhancing phagocytosis and killing of microbes. Trypanotolerance seems to be dependent on the early immune responses than late (Noel *et al.* 2004). This can therefore be seen to arise from early recognition of invasion by the innate immune mechanisms with TLRs being likely players in these responses. They recognize PAMPs and are coupled to signal transduction pathways that control macrophage activation (Figure 1) and hence the expression of a variety of inducible immune responses (Kopp and Medzhitov 1999).

TLRs are important for this induction of adaptive immune responses (Pasare and Medzhitov 2003b) hence their relative abundance within a cell is likely to play a critical role in their innate and adaptive responses.

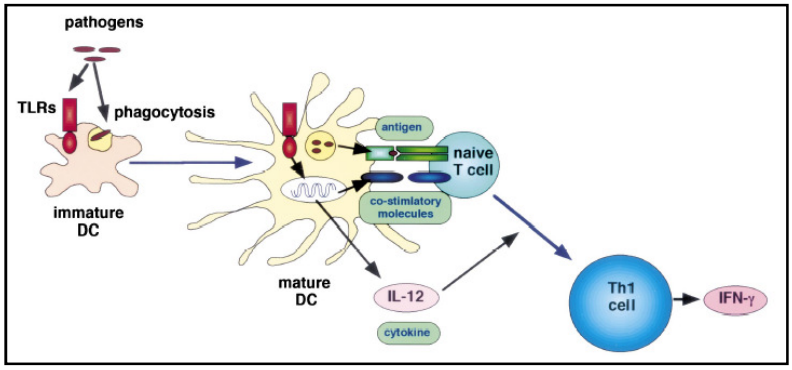

Figure 1: Involvement of TLR in linking innate immunity to adaptive immunity. Among the innate immune cells, immature DCs, which are capable of capturing pathogens by phagocytosis, express several kinds of TLRs. The immature DCs finally mature after the recognition of microbial components via TLRs (Takeda and Akira 2001).

Disease resistance or susceptibility is a complex phenomenon that is under the control of many genes or QTL. These quantitative differences in the expression of genes can be investigated using a functional genomics approach that involves a variety of quantitative PCR methods. QTL mapping may fall short in explaining the occurrence of an observed phenotype if not accompanied by an association study. This may be complex where genetic modification is apparent as observed in humans and mice where environmental factors and alleles of a disease gene cannot explain the modification of a phenotype (Nadeau 2001). The effects of modifier genes include dominance modification and expressivity (Nadeau 2001). Genetic analysis detects genes only when there is some allelic variation unlike expression analysis which deal directly with DNA and its translated information to identify genes even when they do not show allelic variation, provided the gene products can be identified.

Reverse transcriptase (RT) PCR based assays are the most common methods of characterizing and or confirming gene expression patterns and comparing mRNA levels in different sample populations (Bustin 2002). The method is based on reverse transcription of mRNA to make cDNA, followed by amplification by PCR also referred to as RT-PCR. These assays have evolved in the recent past to include internal standards, which are useful in the normalization of the relative gene expressions. Inclusion of target and competitor primers in RT-PCR has improved on the reproducibility and reliability of their conventional RT-PCR especially with small amounts of mRNA (Sato *et al.* 2003).

2. Materials and Methods

To determine the expression patterns of selected candidate genes mapped to trypanotolerance QTL quantitative and semi quantitative PCR methods were used. Total RNA from the susceptible A/J and BALB/c and the resistant C57BL/6J were extracted from spleen and liver as tissues associated with trypanotolerance. The tissues were harvested in the course of *T. congolense* infection for RNA extraction. Variation of gene expression was subsequently calculated as the amount of the target mRNA relative to β-actin as a house keeping gene.

2.1. Animals

The experimental populations were generated over two different time intervals. Mice were produced as pathogen-free, and kept in a conventional environment. All mice were housed at ILRI small animal facilities and were maintained in accordance with good animal care practices. Animals from each strain were challenged with *T. congolense* clone IL1180. Spleen and liver tissues from each strain were then harvested at different days post inoculation (PI). Total RNA was extracted from Spleen and liver tissues.

2.2. Experiment I

This experiment set to determine the expression patterns of TLR1in A/J and C57BL/6J in the course of *T. congolense* infection. Twenty healthy mice from each of the susceptible A/J and the resistant C57BL/6J were selected for this study. These were bred to maturity and challenged with *T. congolense*. In order to investigate the mode of expression of TLR1gene, groups of five mice from each of the strains were sacrificed at different time points during challenge and spleen and liver tissues collected for total RNA extraction. One group of mice was sacrificed prior to infection and considered as time zero of challenge while the rest of the mice were challenged with *T. congolense* clone IL1180. The other groups of five mice from each of the strains were sacrificed on day 4, 7 and 10 post challenge. Levels of TLR1 was subsequently determined as a ratio of target TLR gene normalized to β-actin using semi quantitative PCR.

2.3. Experiment II

Twenty five healthy mice from each of the susceptible A/J, BALB/c and the resistant C57BL/6J were selected for the study This experiment used quantitative real time PCR to estimate the expression levels TLR1. Five uninfected mice from each of the three

strains were sacrificed and their spleen and liver tissues collected. The rest of the mice were subsequently challenged with *T. congolense* clone IL1180 and the two tissues collected on day 3, 7, 13 and 17 post challenge. Liver and spleen tissues were also collected and used for the estimation of TLR1 expression levels in the course of the infection using quantitative real time PCR

Fresh tissues from healthy and infected mice were collected and snap frozen in 2 ml reaction tubes in liquid nitrogen and stored at -70°C. RNA was extracted using Trizol® Reagent (Invitrogen Life Technologies, MD, USA) as described on the manufacturers protocol. RNA for semi quantitative assays performed on Agilent 2100 bioanalyser depended on this type of tissue homogenization. In order to confirm the RNA quantity and quality, all RNA samples from experiment II were also quantified using RNA 6000 nano chips kit on the Agilent 2100 bio analyzer (Agilent Technologies, Germany) according to the manufacturer instructions. The 2100 expert software (Agilent technologies Germany) was used for data collection and analysis.

2.4. Quantitative RT-PCR

In order to design primers for multiplex reaction between the TLR target and β–actin as the house keeping gene, it was necessary to determine the exon location and intron size, so as to localize the potential priming site. These were identified using the mouse genomic DNA sequence from ENSEMBL at http://www.ensembl.org/Mus_musculus/.

In order to design oligonucleotide primers specific to the two mouse TLR genes and β–actin, published gene sequences from mouse genome data base (TLR1-ENSEMBL ENSMUSG00000044827, TLR6-ENSEMBL ENSMUSG00000051498 and β–actin-EMBL AK078935) were obtained from the ENSEMBL and EMBL data bases. β-actin and TLR1primers for multiplex semi-quantitative PCR were designed using the web based PRIMER3 computer program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) at *MIT Center for Genome Research* (Table 1).

Target sequence	Name	Primer Sequence (5'-3')	%GC Ratio	Tm in °C	Length in Bps	Product sizes
β -actin	β -AC fwd	ACCTCATGAAGATCCTGACCGAGC	54.17	67.1	24	576Bp
β -actin	β -AC rev	AACAGTCCGCCTAGAAGCACTTGC	54.17	67.0	24	
TLR 1	INTR' TLR1 rev	TTTGGGGGAAGCTGAAGACATC	50.00	60.3	22	643Bp
TLR 1	INTR' TLR1 fwd	GTGATCTTGTGCCACCCAACAG	54.50	62.1	22	

Table 1: β-actin, TLR1 primers, used for multiplex PCR reactions.

For the sake of this experiment, an improved method for determination of gene expression levels with RT-PCR was developed. The procedure was rapid and more reliable than the traditional densitometry analysis. Since the detection of individual transcripts was PCR-based, small amounts of tissue samples were sufficient for the analysis of expression patterns of the mapped TLR1 and 6 genes. While offering the accuracy of conventional semi-quantitative or competitive RT-PCR, the method allowed quick screening of a wide range of RNA samples with just a thermal cycler and the Agilent 2100 bio analyzer.

Semi quantitative PCR reactions were carried out using Ready-to-go RT-PCR beads (Amersham biosciences UK). Specific quantities were determined using Agilent 2100 bio analyser using 1ul of each of the PCR products. These were loaded on to 7500 nano chip and quantified on Agilent 2100 bio analyser according to the manufacturer's instructions (Agilent technologies Germany). Results were interpreted as a ratio of the concentration of the gene under study to β–actin expression quantified in nmol/µl. Mean relative expression and standard deviation (SD) at each time point was subsequently calculated for the resistant and susceptible mice. Analyses of variance within and between the resultant data sets were then calculated.

Quantitative PCR primers for used to amplify TLR1 and β–actin, were designed using the web based PRIMER3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) at *MIT Center for Genome Research* (Table 1).Two sets of primers were designed by PRIMER3 program using the sequenced mouse genome database but only best set is shown. The primers sets were selected from a preliminary reaction set to determine PCR and dissociation profiles of selected samples in order to define the amplification efficiency of the target relative to the house keeping gene. This ensured reproducibility of the results and specificity of the amplification. The sequences of the primer pairs used for amplification of TLRs and β–actin as the house keeping gene are shown on table 2. Except for β-actin where universal primers were used for both semi quantitative and quantitative real time PCR, target primers in this case were designed to give amplicon sizes ranging from 140-200 base pairs (Bp).

Target sequence	Primer Sequence (5'-3')	Length	% GC Ratio	T_m in °C	Product sizes (bp)
β -AC fwd	ACCTCATGAAGATCCTGACCGAGC	24-mer	54.17	67.09	576Bp
β -AC rev	AACAGTCCGCCTAGAAGCACTTGC	24-mer	54.17	67.03	
TLR 1 rev	ATTGCTGTGTGTAACACGTTCC	22-mer	44.45	59.96	169
TLR1 fwd	TACTCCATCCCTACCAATTACC	22-mer	44.45	57.02	

Table 2: β-actin, TLR1 PCR primers used in real time PCR reactions

Reverse transcription reactions were carried out in a total volume of 20 µl in duplicates using superscript reverse transcriptase kit (Perkin-Elmer) and its accompanying buffers as described by the manufacturers. Unlike the one step RT-PCR used in semi quantitative PCR, a two-Step RT-PCR was used with all real time PCRs.
TLR1 gene specific primers and β–actin primers were used to amplify their respective genes using total cDNA synthesized from known RNA concentration. SYBR green PCR mix (Applied biosystems UK) was used for all reactions according to the manufacturer's instructions.

2.5. Data analysis
A plot of the ratio of target to housekeeping gene was used as a measure of the expression patterns of the target genes over time. Alternatively, the results were presented as line graphs of the normalized expression values divided by the calibrator sample ± SD. Each value was analyzed for statistical difference using the Statistical Analysis System (SAS) program. These statistical data analysis for the expression differences between strains over time was carried out using Analysis of Variance (ANOVA) (Proc ANOVA of SAS release 9.1 (SAS Institute Inc., Cary, NC, USA. 2003)). Only those results with a p-value less than or equal to 0.05 ($P \leq 0.05$) were considered significantly different. However this depended on the spread of the data given as the standard deviation. Data were finally presented as the mean expression ± SD.

3. Results
TLR1 and β–actin gene expression profiles were determined initially using semi quantitative RT- PCR in the resistant C57BL/6J and susceptible A/J mice.The expression levels of specific PCR amplifications products from the target TLR gene and β–actin were obtained in nmol/ul. Ratios of quantities of the target TLR gene to β-actin were subsequently analyzed in spleen and liver tissues from resistant C57BL/6J and susceptible A/J mice. Each RNA sample represented a single time point for individual mice; but groups of five mice were used at each time point. The mean value and the SD were also calculated from each of the groups.Analysis of variance of the mean ratio of target gene expression to β-actin as the house-keeping gene revealed that the genes are regulated in a statistically significant fashion. Band intensities of all the PCR products were quantified and normalized against their respective β–actin quantity. The β–actin system was optimized to give almost constant band intensities relative to the target TLR gene. Therefore, this method of monitoring TLR mRNA expression might be a useful tool for the determination of the expression patterns of the genes involved in the immune response to trypanosomosis in mice.
Spleen tissue expressed detectable TLR1 quantities throughout the experimental period in both the resistant C57BL/6J and the susceptible A/J (Figure 2). The level of TLR1 increased between day 4 and day 7 in the resistant C57BL/6J while remaining constant in the susceptible strain over the same time in the spleen tissue. Expression on days 4 and 7 were not significantly different between the resistant and susceptible mouse strains. In the same tissue, the level of TLR1 gene expression was constant in A/J between day 0 and 4 (Figure 2).
The level of TLR1 in the resistant C57BL/6J spleen tissue was however comparable to the susceptible A/J between pre-infection and day 4. Though TLR1 expression level increased in spleen tissue during the next two time points, it remained constant but at a higher level in the resistant C57BL/6J than the susceptible A/J strains. Like all the other time points the difference in expression between the mean of the pre-infection samples and day 7 samples was not statistically significant ($P = 0.2013$). Expression patterns were therefore not time specific as there was no significant difference between the expression levels in spleen tissue across and between the two strains analyzed ($P = 0.4298$).

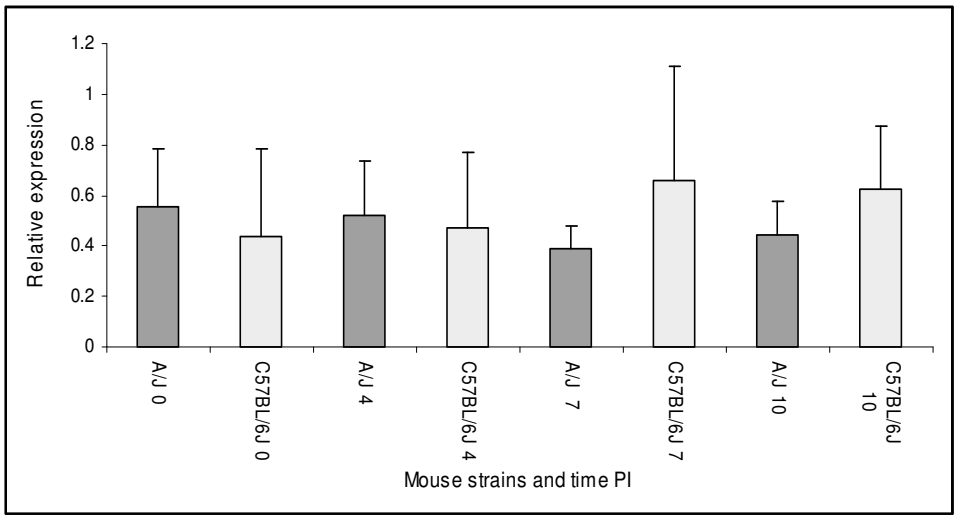

Figure 2: Ratio of quantities of TLR1 to β-actin in mouse spleen tissue. Results were expressed as a ratio of the TLR1 gene to β-actin in nmol hence referred to as the expression level. Standard deviations were also calculated and are shown by the error bars.

Liver tissue also expressed detectable TLR1 quantities late in the course of the experimental period. The level of TLR1 in the liver tissue was not detectable in both strains before challenge and even on day 4 post challenge. This could only be detected on day 7 and

10 in all liver tissues (Figure 3). TLR1 expression level on day7 post infection was slightly less in the resistant C57BL/6J than the susceptible A/J though the difference was not significant. During day7 to 10 period, the expression levels in the liver tissues from C57BL/6J and A/J were however almost the same though their standard deviations were overlapping. This difference was therefore not significant (P= 0.332) between resistant and susceptible mouse strains. The gene was equally expressed on day 10 in both strains (Figure 3).

TLR1 expression levels on day 10 was however far less than the expression level on day 7. This was observed in both strains with the difference being highly significant on day 7 ($P < 0.0001$) and day 10 ($P = 0.0001$). Though different between the two time points, TLR1 expression in the liver tissue was not significantly different between the two mouse strains ($P = 0.5921$).

Analysis of variance showed a significant difference in TLR1 expression between the two tissues over time (Figure 3 and 4) ($P < 0.0001$). The expression profiles of the two tissues showed different expression trends though the levels were essentially the same on day 7. Expression on days 0 and 4 were also significantly different between the two tissues (Figure 3 and 4).

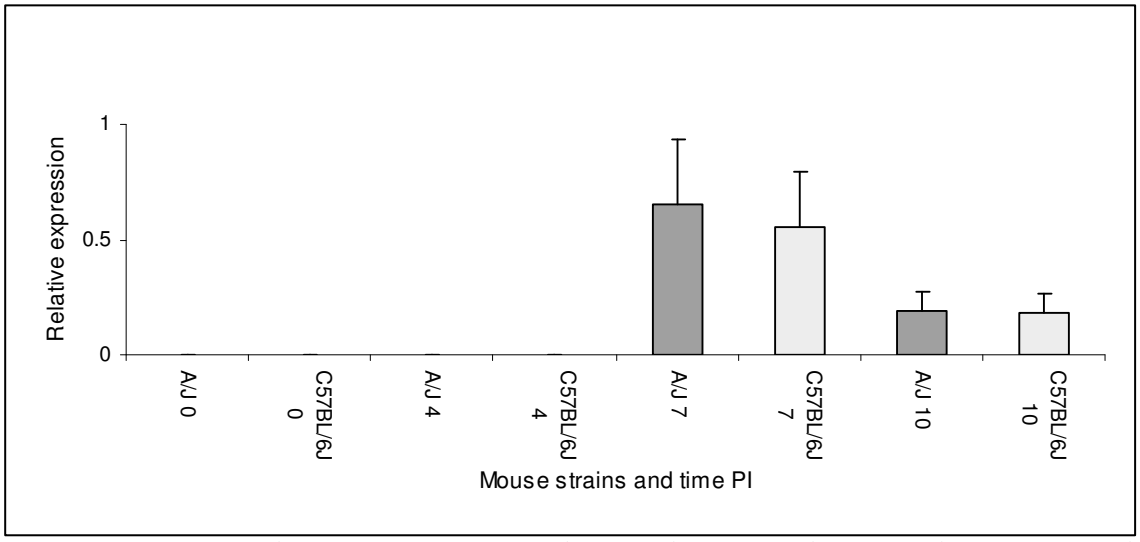

Figure 3: TLR1 mRNA expression in liver tissues from naive and T. congolense infected mice. Results were expressed as a ratio of TLR1 to β-actin in nmol hence referred to as the expression level. Bars show the standard deviation

The expression data was represented as the average relative expression level of the gene at a given time point. The data was obtained by calculating the mean and standard deviation of the indices of expression of each TLR in liver and spleen tissues obtained from five biological replicates in each mouse strain at each time point. Results of real time PCR assay also showed that the TLR gene was differentially expressed in susceptible and resistant mouse strains in the course of trypanosome infection. The gene was up-regulated or down-regulated in a time and strain specific manner. The pre-infection sample was used as the calibrator in each strain and tissue. Therefore the expression level at this time was equivalent to one unit in all cases.

TLR1 mRNA levels generally showed increased expression in spleen and liver tissues but the expression levels were much higher in the liver than spleen tissue (Figure 4). On day 3 PI the resistant C57BL/6J showed a slight increase in TLR1 expression in spleen tissues as opposed to the susceptible A/J and BALB/c mouse strains (Figure 4 (a)). This expression level was however constant in BALB/c and C57BL/6J while in A/J TLR1 expression was up regulated between day 7 and 17. The A/J strain had the highest expression levels at day 7 and 17 as opposed to the other two strains. On the other hand, BALB/c did not seem to show any change in TLR1 expression in spleen tissues throughout the experimental period. Though the susceptible A/J and the resistant C57BL/6J showed increased expression on day 17, the change in expression in the spleen tissue was not significant in all the strains analyzed throughout the experimental period as their standard deviations were overlapping for all the data points (Figure 4 (a)).

In the liver tissue, TLR1 expression showed a steady-state with no change in expression between pre-infection and day 7 PI (Figure 4 (b)) in all the strains analyzed. Within this period TLR1 gene expression was constant in the resistant and susceptible mouse strains with a very low and insignificant change in expression. A similar scenario had been observed with semi quantitative PCR where expression was only detectable after day 4 (Figure 2). However after day 7 this global fashion of gene expression was dramatically overturned in the liver tissue. The resistant A/J and BALB/c showed a very high expression level on day 13 with the resistant strain only showing a slight change in expression.

The expression level at this time point was significantly different between the resistant and the susceptible strains. Furthermore A/J had a significant and higher expression level on day 17 than the other two strains (Figure 4 (b)). At this time the expression level was much lower than the expression on day 13 for all the strains.

BALB/c and C57BL/6J had lower expression level than A/J with the standard deviation of their data points overlapping. This overlap in TLR1 gene expression was observed with the susceptible BALB/c and the resistant C57BL/6J strains on day 13 (Figure 4 (b)). TLR1 gene was therefore differentially expressed in the liver and spleen tissues being expressed at a higher level in liver tissue from susceptible mice with a clear demarcation between resistance and susceptibility being evident.

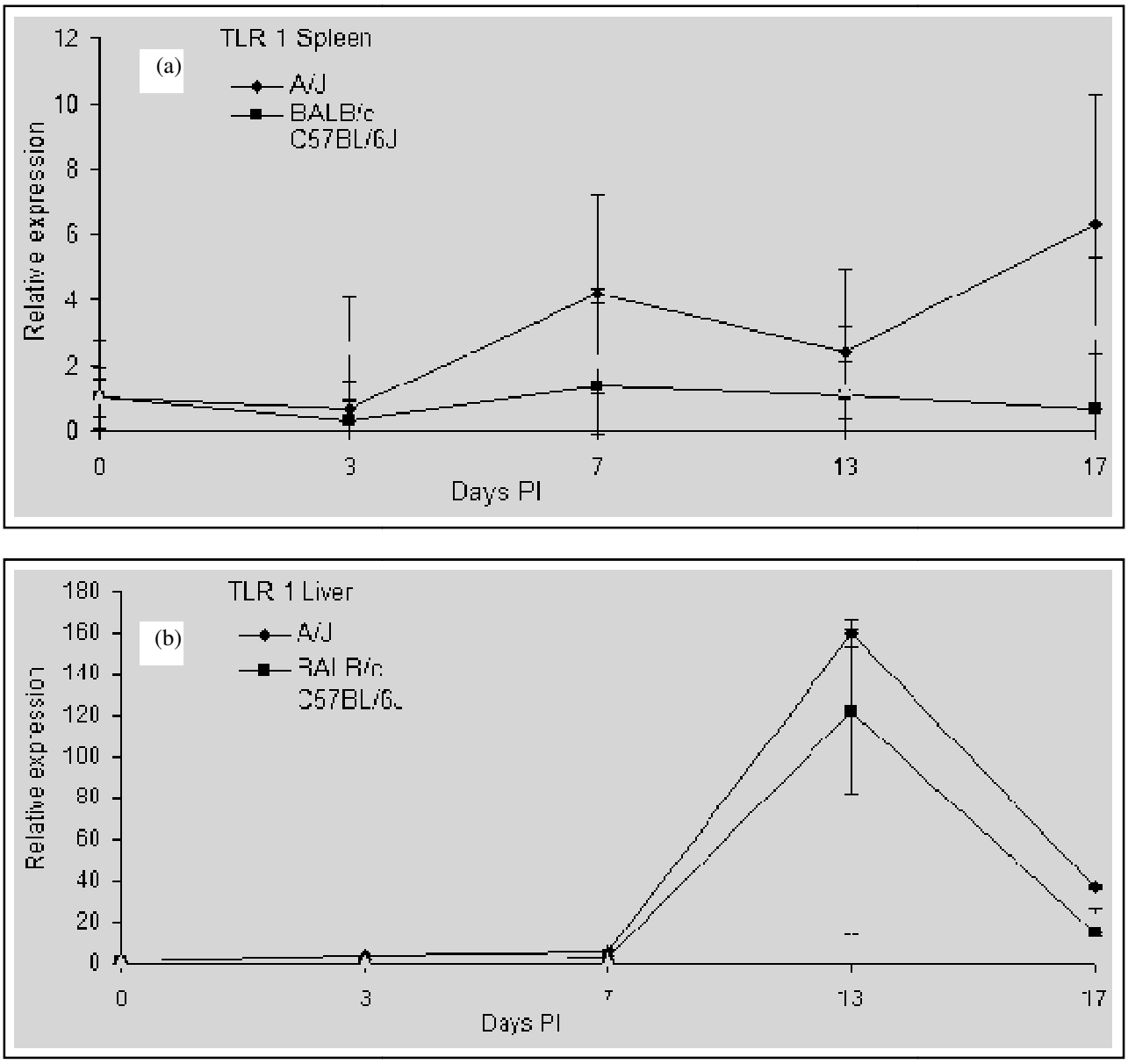

Figure 4: TLR1 mRNA levels in mouse tissues as estimated from real time quantitative PCR analysis of mRNA levels in mouse (a) spleen and (b) liver tissues. The data represents the average expression rate of TLR1 gene, obtained by dividing the mean of relative quantity of TLR1 with mean relative quantity of β-actin and calculating the mean standard deviation of the two data sets in spleen and liver tissues at each time point. The error bars represent the standard deviation at each time point.

5. Discussion

Quantitative and semi-quantitative RT-PCR were performed with constant and known amount of RNA otherwise, the results obtained would have shown unacceptable deviations between different individuals or between samples from the same individual. The sensitivity of the assays largely depended on the quality and quantity of RNA used. Though one step semi quantitative method was performed in a single tube while real time PCR was performed in two consecutive steps, in both cases all cDNA were synthesised from total RNA using poly $d(T)_{12\text{-}16}$ and subsequently amplified using gene specific oligo nucleotide primers. The main difference between the two methods was therefore at the point of quantification. For example, where TLR1 exhibited undetectable level of expression in uninfected animals and increased expression after day 7 PI using semi quantitative RT-PCR, real time PCR results only detected very low expression levels relative to other time points.

Quantification using real time PCR was found to be highly reproducible. Multiple measurements of single cDNA gave identical results. A major factor responsible for the accuracy of this method appears to be the determination of the Ct within the logarithmic phase of the amplification rather than the near endpoint determination used in the semi quantitative PCR. In semi quantitative PCR, the reactions were often characterized by tube-to-tube variations which lead to some being excluded from the analysis especially if the standard deviation of any of the experimental replicates was more than 20%. Minor differences in the composition of PCR assays may

have been responsible for the differential product accumulation, which were not always identical for the few samples. This exclusion did not however affect the final results as the reactions were replicated in each case. Semi quantitative PCR results were therefore confirmed by real time PCR while the latter was extended to cover day 17.

T. congolense challenge result with a change in TLR gene expression in both the resistant and the susceptible mouse strains. An overlap in the standard deviations of the expression levels of the genes that are differentially expressed was observed over time. These results can be rationalized by considering the high degree of cross-pathway regulation in TLR expression. For example, there is evidence for cross-pathway regulation between sampling of different pathogen epitopes by TLR and the concomitant signal transduction responses that up regulate cytokine secretion (Aderem and Ulevitch 2000,Applequist *et al.* 2002 andBarton and Medzhitov 2003).

High levels of TLR1 seem to confer susceptibility to *T. congolense* infection as opposed to low levels of both genes. Since trypanotolerance in mice depends on early clearance of parasitemia by kupfer and spleenic cells (Macaskill *et al.* 1980), than an established antibody response, this may depend on the TLR environments which influence macrophage activation. This subsequently results with activation of the adaptive immune response. The initial rapid defence mechanisms are provided through recognition of PAMPs by TLRs. Variation in expression patterns of TLR 1 between day 4 and 7 as a result of *T. congolense* infection suggests their role during the initial response to the infection.

TLR recognizes different microbial epitopes with TLR5 recognizing flagellin, TLR1 triacyl lipoproteins while TLR6 recognize diacyl lipopeptides during bacterial infections. Since no substantive sequence variation was reported between the susceptible and resistant mice on their TLR1 and 6 promoter or regulatory regions, it is possible that the elevated levels of TLR1in Balb/c may be as a result of stimulation by a set of PAMPs inherent of each mouse strains. A/J and C57BL/6J may on the other had sample the same parasite epitopes and mount variable immunogenicity or a different set of PAMPs just like BALB/c hence the observed variability in the expression of TLR1. The observed diversity of TLR1leucine rich repeats together with the differential expression reported here may influence the overall susceptibilities associated with this mice.

It is likely that the complex effects on gene expression described in this work are a direct consequence of the combinatorial regulation of gene expression. By comparing TLR1 expression levels from the resistant and susceptible livestock its their role in trypanotolerance can lead to the development of novel strategies against trypanosomosis in livestock. These findings illustrate the power of QTL mapping and candidate gene analysis approach when combined with the examination of gene expression. The functional role of the gene expression in different time points in the course of infection provides some impetus to the design of trypanosomosis control strategies in livestock.

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