Expression of non-TLR pattern recognition receptors in the spleen of BALB/c mice infected with *Plasmodium yoelii* and *Plasmodium chabaudi chabaudi* AS

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The spleen plays a crucial role in the development of immunity to malaria, but the role of pattern recognition receptors (PRRs) in splenic effector cells during malaria infection is poorly understood. In the present study, we analysed the expression of selected PRRs in splenic effector cells from BALB/c mice infected with the lethal and non-lethal Plasmodium yoelii *strains 17XL and 17X, respectively, and the non-lethal* Plasmodium chabaudi chabaudi *AS strain. The results of these experiments showed fewer significant changes in the expression of PRRs in AS-infected mice than in 17X and 17XL-infected mice. Mannose receptor C type 2 (MRC2) expression increased with parasitemia, whereas Toll-like receptors and sialoadhesin (Sn) decreased in mice infected with* P. chabaudi *AS. In contrast, MRC type 1 (MRC1), MRC2 and EGF-like module containing mucin-like hormone receptor-like sequence 1 (F4/80) expression decreased with parasitemia in mice infected with 17X, whereas MRC1 an MRC2 increased and F4/80 decreased in mice infected with 17XL. Furthermore, macrophage receptor with collagenous structure and CD68 declined rapidly after initial parasitemia. SIGNR1 and Sn expression demonstrated minor variations in the spleens of mice infected with either strain. Notably, macrophage scavenger receptor (Msr1) and dendritic cell-associated C-type lectin 2 expression increased at both the transcript and protein levels in 17XL-infected mice with 50% parasitemia. Furthermore, the increased lethality of 17X infection in Msr1 -/- mice demonstrated a protective role for Msr1. Our results suggest a dual role for these receptors in parasite clearance and protection in 17X infection and lethality in 17XL infection.*

Key words: malaria - spleen - macrophages - receptors - pattern recognition

Malaria remains the most devastating parasitic disease in the world: 100-300 million cases are detected annually and approximately one million malaria-related deaths occur each year in children less than five years old (who.int). Clinical studies and experimental rodent models have demonstrated the importance of the spleen in the development of immunity to malaria (Buffet et al. 2009). Indeed, the spleen removes infected red blood cells (iRBCs) from the circulation, acts as a major erythropoietic and haematopoietic site during embryogenesis and generates immune responses to pathogens (Engwerda et al. 2005, Urban et al. 2005a). In this way, splenic effector cells, which have the capacity to become rapidly activated to produce large amounts of pro-inflammatory cytokines, represent the first line of innate immune defence. Further, splenic cells phagocytose infected erythrocytes and help shape downstream adaptive immune responses (Stevenson & Riley 2004a, Urban et al. 2005b).

Macrophage activation is largely dependent on the interaction of microbial molecules or pathogen-associated molecular patterns (PAMPs) with Toll-like receptors (TLRs) and other pattern recognition receptors (PRRs) (Schofield & Hackett 1993, Tachado et al. 1996, Naik et al. 2000, Vijaykumar et al. 2001, Zhu et al. 2005, Areschoug & Gordon 2009). A great deal of research has focused on the role of TLRs in pathogen recognition (Krishegowda et al. 2005); however, the spleen is also rich in macrophage populations that express different sets of PRRs, reflecting the functional specialisation in splenic microenvironments under steady state conditions (Taylor et al. 2005).

Histopathological and physiological studies of the spleen during experimental infections of BALB/c mice with the reticulocyte-prone non-lethal *Plasmodium yoelii* 17X strain have revealed that major remodelling of this organ occurs through the creation of a blood-spleen barrier of fibroblastic origin. Infected reticulocytes then adhere to this barrier to avoid clearance (Weiss et al. 1986, Martin-Jaular et al. 2011). Strikingly, this remodelling and the formation of the barrier are not observed

Financial support: CNPq (to ARU), FAPESP (01/09401-0), European Community's Seventh Framework Programme (FP7/2007-2013 - 242095), Spanish Ministry of Science and Innovation (SAF2009-07760)
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Received 1 December 2010
Accepted 15 February 2012

when mice are infected with the normocyte-prone lethal *P. yoelii* 17XL strain (Weiss 1989). In the present study, we hypothesised that this unique remodelling of the spleen is accompanied by differential activation of splenic effector cell PRRs throughout the development of lethal and non-lethal *Plasmodium* infections. To test this hypothesis, we compared the transcript abundance of spleen PRRs during experimental infections of BALB/c mice with *P. yoelii* 17X and 17XL strains. In addition, to demonstrate the specificity of these results, we also compared the transcript abundance of these same PRRs in BALB/c mice infected with *Plasmodium chabaudi chabaudi* AS, a model in which mature parasite stages is sequestered from the circulation.

Mice and parasites - Female BALB/c mice were bred in a pathogen-free unit at the Institute of Biomedicine (São Paulo) in accordance with protocols approved by the Ethical Committee of Colégio Brasileiro de Experimentação Animal and the Comissão de Ética em Experimentação Animal (protocol 209/02). All mice were conventionally housed for experimental use and provided with sterile bedding, food and water. For experimental infections with *P. chabaudi chabaudi* AS and *P. yoelii* 17X and 17XL, the mice were routinely injected intraperitoneally (i.p.) with 10^6 iRBCs. Parasitaemia was monitored on a daily basis between days 2 and 20 post-infection (Supplementary data) by thin tail blood smears and Giemsa staining and the proportion of iRBCs among total RBCs was calculated (Langhorne & Simon 1989).

For transcript analysis, groups of BALB/c mice were infected with *P. chabaudi chabaudi* AS (n = 6) or *P. yoelii* 17XL (n = 6) or 17X (n = 6) and two mice of each strain were sacrificed when peripheral parasitaemias reached 1%, 10% and 50%. The experiment was performed twice (biological replicates), resulting in a *n* of 4 in each experimental group.

Macrophage scavenger receptor (Msr1) knockout (strain B6.Cg-*Msr1*^{tm1Csk}/J) and C57BL/6J wild-type female mice (The Jackson Laboratory, Bar Harbor, Maine, USA) were maintained with water and food *ad libitum* in the animal facilities of the Hospital Clinic, Barcelona. At 16-18 weeks of age, four-six animals of each group were injected i.p. with 10^6 parasites of either a lethal (17XL) or a non-lethal (17X) strain of *P. yoelii*. All of the experiments were performed according to the guidelines approved by the Ethical Committee of Animal Experimentation of the University of Barcelona.

Tissue preparation - Spleens from *P. yoelii* 17X and 17XL and *P. chabaudi chabaudi* AS infected mice were removed under aseptic conditions. To isolate circulating monocytes from the spleen, the mice were anaesthetised with 33% chloral hydrate solution and infected spleens were removed after perfusion with 0.9% saline solution and snap-frozen in liquid nitrogen or processed for flow cytometry analysis.

RNA extraction and cDNA synthesis for real-time polymerade chain reaction (RT-PCR) analysis - RNA was extracted from 50 mg total splenic tissue samples with Trizol reagent (Invitrogen). The samples were treated with DNase I (Invitrogen) and reverse transcription (First-Strand cDNA Synthesis, Invitrogen) was performed according to the manufacturer's instructions.

Quantification of macrophage receptor transcript abundance - We compared the expression profiles of different PRRs - mannose receptor C type 1 (MRC1), MRC type 2 (MRC2), Msr1, macrophage receptor with collagenous structure (MARCO), dendritic cell-associated C-type lectin 2 (Dectin2), EGF-like module containing mucin-like hormone receptor-like sequence 1 (F4/80), SIGNR1, sialoadhesin (Sn) and CD68) - with specific primers designed with Primer Express 3.0 software (Supplementary data). The samples were analysed in triplicate and the experiments were repeated twice. cDNA from non-infected mice (calibrator) was included in all PCR experiments. The relative quantification of transcript abundance was performed with SYBR green PCR master mix (Applied Biosystems) using an Applied Biosystems 7500 thermal cycler system. Actin was used as the endogenous control and non-infected mouse transcripts were used as the calibrator.

Data analysis - The relative expression level of each target gene was calculated from triplicate samples analysed in two independent experiments using $\Delta\Delta C_T$ method and normalised to the endogenous control. Fold changes were determined by comparing the relative expression levels in infected mice to those in the calibrator (non-infected BALB/c mice) (Livak et al. 2008). Data were analysed using GraphPad Prism software. Statistical significance was determined using two-way ANOVA and differences in parasitaemias in the same strain were evaluated by Bonferroni's post-test.

Flow cytometry analysis - Splenocytes were prepared from the spleens of mice infected with 17X and 17XL parasites when the mice reached 1%, 10% and 50% parasitaemias. The spleens were homogenised and passed through nylon mesh. Spleen cells were preincubated with Seroblock anti-Fc receptor antibody (Abcam, dilution 1/100) for 10 min and then incubated with an APC-conjugated antibody specific for murine Msr1 and a fluorescein isothiocyanate conjugated antibody specific for murine Dectin2 at 4ºC for 1 h and washed twice. 500,000 events were collected using a FACSCanto flow cytometer (BD Biosciences). The data were analysed using either one-way ANOVA with Dunnet's *post hoc* test or Student's *t*-test with GraphPad Prism software.

Microarray global expression analysis - Total RNA from spleens of mice infected with 17XL or 17X parasite was labelled using an Agilent Low RNA Input Fluorescent Linear Amplification Kit. mRNA from the spleens of each 17X or 17XL infected mouse was labelled with Cy3 or Cy5, respectively. Dual hybridisation was performed using the Agilent Whole Mouse Genome microarray according to the manufacturer's protocol. Microarray images were obtained using the GenePix 4000B scanner. Analysis of overrepresentation of functional terms had been done with GOstats tool, which finds statistically overrepresented gene ontologies within a group of genes. The program determined the annotated

GO terms and the GO terms that are associated (i.e., in the path) with genes analysed. The number of appearances of each GO term was then counted. Fisher's exact test was performed to determine whether the observed difference were significant. This resulted in a p-value for each GO category that was used to determine whether the observed counts could have occurred by chance. All data were deposited at GEO under accession GSE17603.

The expression profile of a variety of splenic effector cell PRRs was assessed in samples of total spleen tissue from BALB/c mice experimentally infected with the *P. yoelii* 17X and 17XL strains. Because of differences in the growth of these parasites, the analysis was performed when the mice reached 1%, 10% and 50% peripheral parasitaemia. To determine the specificity of the changes observed, the expression of these PRRs was also analysed in BALB/c mice infected with (the normocyte-prone non-lethal) *P. chabaudi chabaudi* AS strain, which in contrast to *P. yoelii*, sequesters in the deep capillaries of internal organs (Cox et al. 1987). Overall, the results revealed more dramatic increases in the expression of PRRs in mice infected with 17XL than in mice infected with 17X (Fig. 1).

Expression of most of the PRRs was lower in the spleens of *P. chabaudi chabaudi* AS-infected mice than in *P. yoelii*-infected mice, but these changes were not statistically significant (Fig. 1). These data support previous studies showing that *P. chabaudi chabaudi* AS is not cleared by the spleen due to its adhesion to internal organs. In contrast, the expression levels of most of the PRRs were significantly different in *P. yoelii* 17X and 17XL infected mice (Fig. 1).

The expression of the macrophage receptors MRC1 and MRC2 was decreased in the 17X-infected mice with 10% parasitaemia ($p < 0.001$), but the expression of both of these receptors was increased in 17XL-infected mice with the same degree of parasitaemia ($p < 0.001$). Similarly, the expression level of the F4/80 receptor in mice infected with the 17X strain was increased when the mice reached 1% parasitaemia (11.3-fold). This increased parasitaemia was maintained in mice with 10% parasitaemia (12.5-fold, $p > 0.05$) but decreased in mice with 50% parasitaemia (5.9-fold, $p < 0.001$). The expression of the three receptors fluctuated less than five-fold over the course of *P. chabaudi chabaudi* AS infection, with the exception of a slight increase in MRC2 expression when parasitaemia peaked at 50% ($p < 0.05$). Interestingly, MRC1/2 - and F4/80 - expressing macrophages are predominantly located in the red pulp of the spleen in the absence of infection (Taylor et al. 2005). We observed that 10% parasitaemia was achieved at approximately day 6 post-infection in mice infected with the 17X strain (Supplementary data).

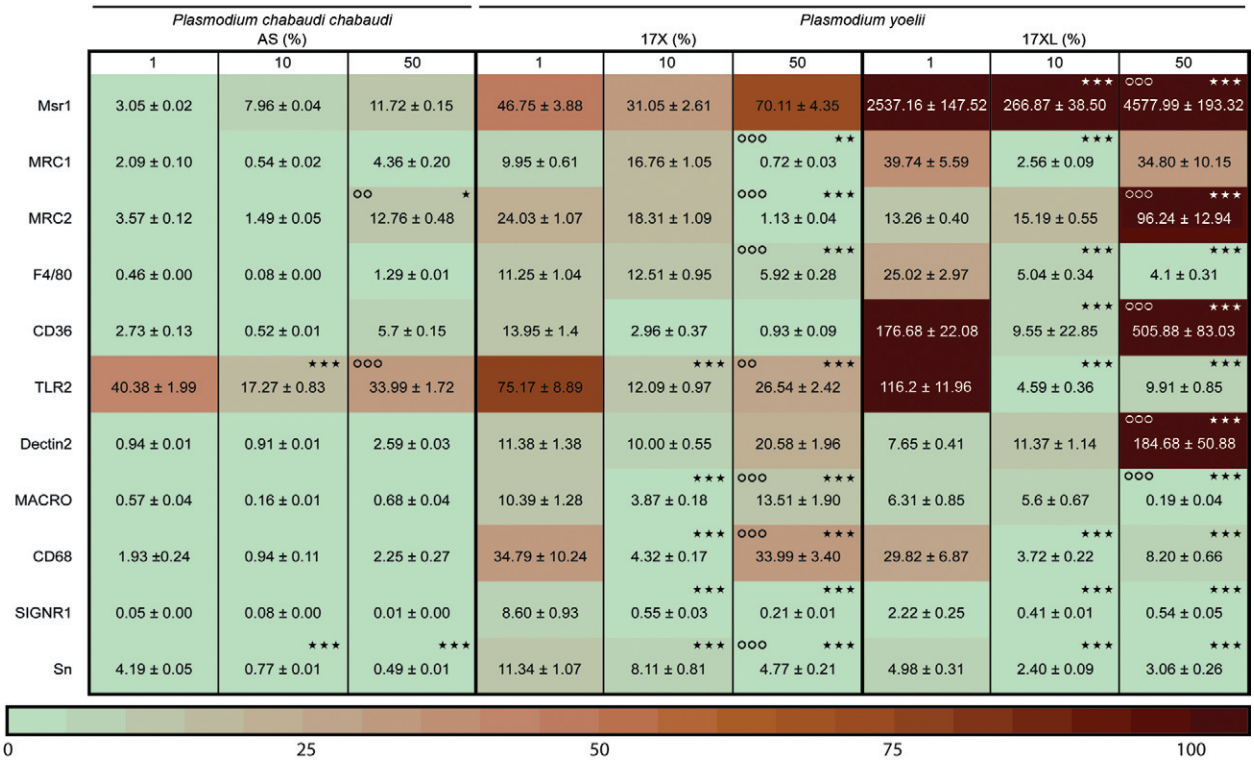

Fig. 1: the heat map represents colour-coded relative quantification values for expression levels (x-fold changes compared to non-infected control) of splenic effector cell pattern recognition receptors (PRRs) between *P. yoelii* 17X, 17XL and *P. chabaudi chabaudi* AS at 1%, 10% and 50% parasitaemias (n = 4 mice/group). Additionally, values for mean and standard deviation are included in the boxes and data was pooled from two independent experiments. Data was evaluated by a two-way ANOVA test of variance and Bonferroni post-test, vs. 1% infection (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$) and 10% infection (^{o}: $p < 0.01$; ^{ooo}: $p < 0.001$). Dectin: dendritic cell-associated C-type lectin; MARCO: macrophage receptor with collagenous structure; MRC: mannose receptor C; Msr: macrophage scavenger receptor; Sn: sialoadhesin; TLR: Toll-like receptor.

A recent study has reported that a blood-spleen barrier of fibroblastic origin forms during the initial days of 17X infection in BALB/c mice and prevents parasite contact with macrophages (Martin-Jaular et al. 2011). It is tempting to speculate that newly formed tissue barrier cells partially conceal 17X iRBCs from effector cells and, as a consequence, the expression of these receptors may be down-regulated at this level of parasitaemia.

Although statistically significant ($p < 0.001$) fluctuations in the expression levels of SIGNR1, Sn, MARCO and CD68 were observed, the low expression levels of these receptors were generally stable in mice infected with both strains of *P. yoelii.* Similar results were observed in biological replicates. These results suggest that these receptors are not likely to be involved in the process of innate immune activation initiated by malaria infection.

The analysis of more widely expressed PRRs showed that TLR2 was highly expressed in all strains at 1% parasitaemia (40.4-fold, 75.2-fold and 116.2-fold increases in mice with for *P. chabaudi chabaudi*, AS, 17X and 17XL, respectively) and its expression decreased with increasing parasitaemia. These data are in agreement with the perceived role of TLR2 in initiating host defence upon detection of PAMPs (Erdman et al. 2008). Likewise, following infection with each of the strains, the expression of CD36 increased when the mice reached 1% parasitaemia (increases of 2.7-fold, 14-fold and 176.7-fold for *P. chabaudi chabaudi* AS, 17X and 17XL, respectively); however, CD36 expression decreased when the mice reached 10% parasitaemia (decreases of 0.5-fold, 3-fold and 10-fold for *P. chabaudi chabaudi* AS, 17X and 17XL, respectively). CD36 expression levels in mice with 50% parasitaemia varied among the mice infected with different species (5.7-fold for *P. chabaudi chabaudi*, no change for 17X and 505.9-fold for 17XL). Previous studies have shown that TLR2 is activated by *Plasmodium falciparum* glycosylphosphatidylinositol in both human and murine macrophages, resulting in the production of tumour necrosis factor (TNF)-α, which appears to play a dual role in malaria infection. Some reports have suggested its importance in controlling parasites, while others have proposed a pathogenic role (Richards 1997, Krishnegowda et al. 2005, Zhu et al. 2011). In addition a role in mediating non-opsonic phagocytosis of iRBCs by macrophages and monocytes (McGilvray et al. 2000, Serghides & Kain 2002), CD36 has been reported to enhance TLR2 signalling by acting as a co-receptor for TLR2 in the induction of TNF-α (Patel et al. 2007, Erdman et al. 2009). Here, we observed a 177-fold increase in CD36 expression in mice infected with the 17XL strain with 1% parasitaemia.

Dectin2 has been shown to be involved in the recognition of several pathogens (McGreal et al. 2006, Sato et al. 2006, Robinson et al. 2009). Analysis of the expression of this receptor in mice infected with 17X revealed an increase in Dectin2 expression in mice with 1% parasitaemia. The expression level of Dectin2 remained steady in mice with 10% parasitaemia ($p > 0.05$) and further increased in mice with 50% parasitaemia. In contrast, expression of Dectin2 was highly correlated with increasing parasitaemia in mice infected with the 17XL strain ($r^2 = 0.988$). Although Dectin2 has been shown to be involved in the recognition of several pathogens, to our knowledge, this is the first time its expression has been described in malaria.

Although Msr1 -/- mice have been used for experimental infection studies, the role of Msr1 during malaria infection remains unclear and different groups have reported conflicting data (Nogami et al. 1998, Cunha-Rodrigues et al. 2006, Inoue et al. 2006). In our analysis, the expression of Msr1 was observed without significant changes in mice with 1% and 10% parasitaemia (increase of 46.8-fold and 31.1-fold respectively), but expression was much higher in mice with 50% parasitaemia (70.1-fold) following infection with the 17X strain. In addition, the expression of Msr1 was high throughout the course of 17XL infection, with peak expression at 50% parasitaemia (increase of 4.577-fold, $p < 0.01$). In contrast, expression of Msr1 in mice infected with *P. chabaudi chabaudi* AS reached a maximum level that was only 12-fold higher than that in uninfected mice. Moreover, transcriptional analyses of spleens from 17X and 17XL-infected mice with 1%, 10% and 50% parasitaemia using commercially available arrays identified this gene as one of the most variable (Supplementary data).

We next investigated whether Dectin2 and Msr1 protein levels also changed. To this end, BALB/c mice were infected with *P. yoelii* 17X and 17XL strains. Splenocytes were then obtained from animals with 1%, 10% and 50% parasitaemias, stained with antibodies specific for different receptors and analysed by flow cytometry. The number of cells expressing each PPR is shown as percentages of the total splenocyte population (Fig. 2). Phenotypic analysis of splenocytes revealed that the levels of Msr1 and Dectin2 proteins were also altered during infections; however, the changes in protein levels were not always positively correlated with changes in transcript levels (Fig. 2). The differences observed between transcript

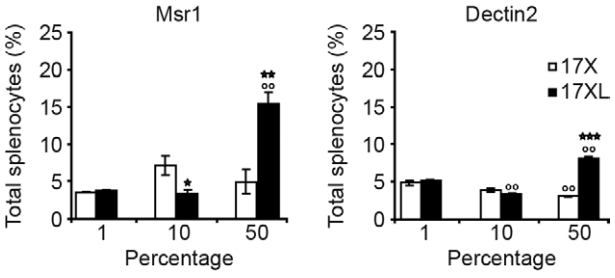

Fig. 2: flow cytometry analysis of splenocytes stained for macrophage scavenger receptor (Msr)1 and dendritic cell-associated C-type lectin (Dectin)2. Splenocytes of mice infected with *Plasmodium yoelii* 17X and 17XL parasites at 1%, 10% and 50% parasitaemias were labelled with conjugated antibody and analysed in a FACSCanto flow cytometer. Mean percentages ± standard error of the mean (SEM) of total splenocytes are shown from three mice per group. Percentages of $Msr1^+$ and $Dectin2^+$ cells were 1.41 ± 0.14 and 2.79 ± 0.26 for control animals (n = 3). Data was evaluated by analysis of variance vs. 1% infection for each strain separatelly (°: $p < 0.05$; °°: $p < 0.01$) (Dunnet *post hoc* test). Differences between 17X and 17XL strain were assesed using Student's *t*-test (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

and protein levels in mice with the same levels of parasitaemia may be due to either methodological constraints, such as RT-PCR values relative to the non-infected control, or differences in absolute numbers of splenocytes, or other variables, such as post-transcriptional regulation. The data obtained from the flow cytometry experiments, however, revealed changes in the levels of PRRs proteins and thus support a dual role of these receptors in parasite clearance and protection during 17X infection and in lethality during 17XL infection.

In order to further explore the role of the Msr1 receptor during malaria-induced innate immune responses in the spleen, we infected Msr1 -/- and C57BL/6J wild-type female mice with 17XL or 17X. We were unable to perform these experiments in Dectin2 -/- mice because they are not commercially available. Survival curves revealed a significant increase in lethality in Msr1-deficient mice infected with 17X (log-rank test $p < 0.01$). In contrast, Msr1-deficient mice appeared to survive longer than wild-type mice following 17XL-infection; however, this trend was not statistically significant (log-rank test $p = 0.06$) (Fig. 3). These findings suggest that Msr1 plays an important protective role during 17X infections that require increased expression at the transcript and protein levels (Figs 1, 2). In contrast, hyperactivation and uncontrolled immune responses during 17XL infections ultimately lead to the death of the host.

Severe malaria syndromes are thought to be due at least in part to the overproduction of pro-inflammatory cytokines by cells of the innate immune system (Stevenson & Riley 2004, Schofield & Grau 2005, Urban et al. 2005b, Clark et al. 2006, Bakir et al. 2011). Consistent with a role for such responses in the pathogenesis of sepsis and other hyperacute inflammatory states, there also appears to be a fine line between the beneficial role of inflammatory responses and the pathogenic side-effects that can result from immune hyperactivation (Clark et al. 2006). Accordingly, it has been postulated that in mild malaria the pro-inflammatory response may be effectively down-regulated, whereas inflammation may remain unbalanced and uncontrolled in severe and fatal malaria cases (Malaguarnera & Musumeci 2002). Interestingly, analysis of the 10 most variables genes revealed changes in different functional pathways between mice infected with the 17X and 17XL strains: endocytosis and phosphate transport were most significantly affected in 17X-infected mice and cell death and apoptosis were most significantly affected in 17XL-infected mice (Supplementary data). Remarkably, Dectin2 and Msr1 expression was increased at the transcript and protein levels in 17XL-infected mice compared to 17X-infected mice with 50% parasitaemia. These differences in expression ultimately resulted in parasite clearance and recovery in 17X-infected mice and elevated parasitaemia and death in 17XL-infected mice strain. Therefore, our results suggest that Dectin2 and Msr1 play dual roles in the development of innate inflammatory responses. Both PRRs seem to be involved in pathogenic responses in 17XL-infected mice, whereas they play a role in the protective response in 17X-infected mice. These data also indirectly support the previous observation that a cellular barrier forms in spleen during non-lethal *Plasmodium* infection (Weiss et al. 1986, Martin-Jaular et al. 2011) and suggest that this barrier prevents hyperactivation of pro-inflammatory responses that lead to pathogenic effects and eventually the death of the host.

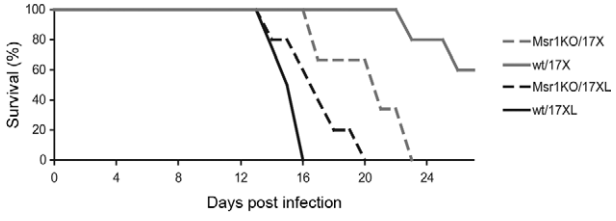

Fig. 3: survival curve of macrophage scavenger receptor (Msr)1 knockout (KO) and C57BL/6J wild-type (wt) female mice infected with 17XL or 17X strain of *Plasmodium yoelii*. Results correspond to four-six animals per group. Differences among Msr1 -/- and wt mice infected with the same strain were evaluated by long-Rank test.

ACKNOWLEDGEMENTS

To the anonymous reviewer, whose suggestions have improved significantly the quality of this paper, to Dr Leanne Robinson, for her critical review, to Dr Sergio Rodriguez, for discussions, and for the training in handling infections of BALB/c mice and rodent malaria species and Marcio Massao Yamamoto, for technical assitance.

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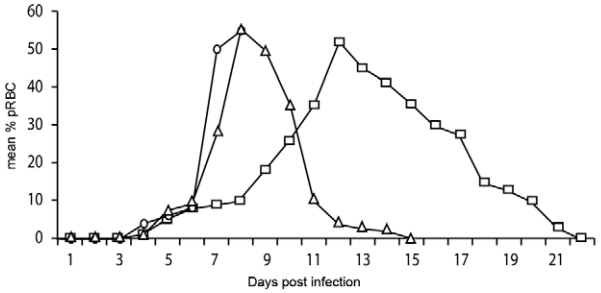
			tttgcaagctgtgacggc
MARCO	NM_010766	tgaagatgcgggtgtggaa	tgatgacctctcgggctcc
Dectin2	NM_020001	cctcaacaatggtgcaggaa	ttggacactgggacatcgg
Sn	NM_011426	cctgctcattccctgcatct	ttggacactgggacatcgg
CD68	NM_009853.1	tggcggtggaatacaatgtg	tgaatgtccactgtgctgcc
CD36	NM_007643	aaccagtgctctcccttgattc	cgatcacagcccattctcct
TLR2	NM_011905	atccccttccctcacttcca	tctgggcaccagcctagg
Actin	NM_007393	gcgggcgacgatgct	agggcggcccacgat

gene specificity of all primers was confirmed using BLAST (ncbi.nlm.nih.gov/BLAST/). Dectin: dendritic cell-associated C-type lectin; MARCO: macrophage receptor with collagenous structure; MRC: mannose receptor C; Msr: macrophage scavenger receptor; Sn: sialoadhesin; TLR: Toll-like receptor.

Analysis of over-representation of functional terms using the tool Gostats of the 10 most variable genes in lines 17X (Cy3) and 17XL (Cy5) obtained from the microarray expression analysis of the spleen of BALB/c mice infected with *Plasmodium yoelii* 17X and 17XL strain at 1%, 10% and 50% parasitaemia

GO term	CY3 X p and genes	CY5 XL p and genes
Taxis GO: 0042330	0.00101 ccl2 ccl5 ifng	0.000934 ccl2 ccl5 ifng
Phosphate transport GO:0006817	0.00841 marco msr1	-
Immune response GO:0006955	0.01 ccl2 ccl5 ifng	0.00157 ccl2 ccl5 ifng ptprc
Endocytosis GO:0006897	0.0145 mrc1 msr1	-
Apoptosis GO:0006915	-	0.00778 ifng tnfrsf18 ptprc
Cell death GO:0008219	-	0.0085 ifng tnfrsf18 ptprc



Course of parasitaemia in BALB/c infections with *Plasmodium yoelii* 17X and 17XL strains and with *Plasmodium chabaudii chabaudi* AS. Groups of six BALB/c mice were challenged with 1 x 10^6 *P. yoelii* 17X (squares), 17XL (circles) and *P. chabaudi chabaudi* AS (triangles) infected red blood cells (iRBCs) and course of parasitaemia was monitored by enumerating iRBCs in tail blood thin smears stained with Giemsa.