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Transcriptomic analysis of the dendritic cell regulation by malaria; production of fever mediators in the absence of maturation

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INDEX

Abbreviations 9

Abstract 11

Resumo 13

Keywords/Palavras-chave 18

Acknowledgments/Agradecimentos 19

Chapter I – General Introduction 23

1.1 Introduction to malaria infection 24

1.1.1 Life cycle of *Plasmodium* species 24

1.1.2 Global distribution and epidemiology of malaria infections 29

1.1.3 Animal models of malaria 28

1.1.4 Strategies for the control of malaria 30

1.2 Immunity to blood-stage malaria 32

1.2.1 Pathology and immune processes of malaria blood stages 32

1.2.2 Natural immunity to malaria 36

1.2.3 Innate immunity to malaria blood stages – cytokine production and their involvement in pathology & fever 39

1.2.4 The relevance of opsonization and phagocytosis of infected erythrocytes 42

1.3 Biology of dendritic cells 45

1.3.1 Dendritic cells link innate and adaptive immunity 45

1.3.2 DC plasticity and regulation of immunity 47

1.3.3 DC receptors and their signaling pathways 50

1.3.4 Evasion of immunity through modulation of DCs 52

1.4 Role of dendritic cells in Immunity to blood-stage malaria – PhD Significance & Objectives 54

1.4.1 DC response to blood-stage malaria 54

1.4.2 Significance and Objectives – Why study the role of DCs in blood-stage malaria infection? 56

Chapter II – Materials and Methods 63

2.1 Mice and Infections 64

2.2 Magnetic bead extraction of CD11c+ dendritic cells (DCs) from mouse spleen 64

2.3 Total RNA extraction 65

2.4 Global transcriptomic analysis of spleen DCs in *P. yoelii* infection 65

2.4.1 RNA processing and Genechip® array hybridization 65

2.4.2 Genechip® data analysis 66

2.5 Real-Time quantitative PCR 66

2.6 DC generation from bone marrow precursors 67

2.7 Culture of GM-CSF transfected cells 68

2.8 Purification of non-infected and *P. yoelii*-infected erythrocytes 68

2.9 *In vitro* culture of DCs with erythrocytes and drugs 68

2.10 Flow cytometry analysis of DC membrane expression of co-stimulatory molecules 69

2.11 Enzyme Immunoassays 70

2.11.1 Enzyme-linked immunosorbent assay (ELISA) for quantification of extracellular cytokines 70

2.11.2 Enzyme Immunoassay (EIA) for quantification of extracellular Prostaglandin E2 71

2.11.3 Enzyme Immunoassay (EIA) for quantification of intracellular cyclic AMP 71

2.12 Erythrocytes staining with CFSE 72

2.13 Immunocytochemistry studies of DC interaction with *P. yoelii*-IEs 72

2.13.1 Membrane staining of co-stimulatory molecules 72

2.13.2 Intracellular staining of NF-κB transcription factors 73

Chapter III – Analysis of spleen dendritic cell transcriptomic responses to *P. yoelii* 75

3.1 General analysis of *P.yoelii*-induced transcriptomic regulation in spleen DCs using Genechip® technology 76

3.2 Pathways, biological processes and molecular functions with significant *P.yoelii*-induced regulation 81

3.3 *P. yoelii* modulation of genes with defense functions 83

3.4 Comparison of the *P.yoelii*-induced transcriptomic regulation of DCs with the regulation by other pathogens 83

3.5 Quantitative PCR analysis of *P.yoelii*-regulated genes 87

3.6 Flow cytometry analysis of the DC expression of co-stimulatory molecules during *P. yoelii* infection 88

3.7 Discussion 90

Chapter IV – Prostaglandin E2-induced signaling in dendritic cells triggered by *Plasmodium*: pathway for *il-6* expression 95

4.1 *P. yoelii* regulates expression of signaling genes in spleen dendritic cells 96

4.2 *P.yoelii* induces the increase of intracellular cyclic AMP in DCs; role of cyclooxygenase activity 97

4.3 Production of IL-6 by DCs is activated by *P. yoelii in vitro* and *in vivo*, and requires cyclooxygenase activity 99

4.4 Involvement of intracellular cyclic AMP, PKA and p38-MAPK in *P.yoelii*-induced IL-6 100

4.5 Discussion 103

Chapter V – Modulation of dendritic cell (DC) maturation and Toll-like receptor-mediated signaling by *Plasmodium*; single-cell analysis of the DC response to *P. yoelii* 107

5.1 Do *P. yoelii* infected erythrocytes (IEs) induce Toll-like receptor (TLR)-dependent signaling and DC maturation? 108

5.1.1 DC responses *P. yoelii* IEs 108

5.1.2 Is TLR-dependent signaling activated by *Plasmodium*? 111

5.2 Characterization of *Plasmodium* –mediated blockage of DC maturation in response to TLR stimulation 113

5.2.1 *P. yoelii* inhibits responses to all TLRs – expression of co-stimulatory molecules 113

5.2.2 *P. yoelii* inhibits responses to all TLRs – IL-12 secretion 115

5.3 *P. yoelii* inhibits Nf-κB transcriptional activity 116

5.4 Visualizing phagocytosis of *Plasmodium*- IEs and its regulation of DC maturation 119

5.5 Discussion 122

Chapter VI – General Discussion & Conclusions **129**

6.1 Is cyclooxygenase (COX)-dependent increase in IL-6 production related to TLR-mediated signaling? **130**

6.2 Is PGE2 production associated with erythrocyte phagocytosis and inhibition of DC maturation? **134**

6.3 Is inflammation compatible with cellular immunity? **135**

6.4 Conclusions and Perspectives **137**

Bibliography **141**

Appendixes **155**

Appendix I – Genechip® data analysis: transcripts significantly regulated in spleen dendritic cells by *P. yoelii* infection 155

Appendix II – Genechip® data analysis: annotation of *P. yoelii*-modulated genes according to Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) 177

Appendix III – Comparison of data obtained from Genechip® analysis of transcriptomic regulation of spleen DCs by *P. yoelii* with data from the literature 183

Appendix IV – Manuscript published in *Cellular Microbiology* (Carapau 2007) 195

ABBREVIATIONS

DC – Dendritic cell

APC – Antigen-presenting cell

GM-CSF – Granulocyte/macrophage colony-stimulating factor

NE – Non-infected erythrocyte

IE – Infected erythrocyte

Py-IE – *P. yoelii*-infected erythrocyte

Pf-IE – *P. falciparum*- infected erythrocyte

PfEMP1 – *P. falciparum*- erythrocyte membrane protein 1

PAMP – Pathogen-associated molecular pattern

PRR – Pattern recognition receptor

TLR – Toll-like receptor

Treg – Regulatory T lymphocyte

NF-κB – Nuclear factor of κ light polypeptide gene enhancer in B-cells

PGE2 – Prostaglandin E2

COX – Cyclooxygenase

IL-6 – Interleukin-6

cAMP – Cyclic adenosine monophosphate

PKA – Protein kinase, cyclic AMP-dependent

MAPK – Mitogen-activated protein kinase

EPAC – Exchange protein directly activated by cyclic AMP

Esta Tese é dedicada aos meus Pais

Maria dos Anjos e João António

ABSTRACT

Malaria is an infectious disease responsible for ca. 1 Million deaths each year in the Developing World; nearly all victims of malaria are children and pregnant women from sub-Saharan Africa.

Our current understanding of the cellular and molecular immune mechanisms of the host responses to the parasite – which contribute to clear infection but also to the pathologies associated with malaria – is far from being extensive, despite decades of research.

Dendritic cells (DCs) are considered the most relevant class of antigen-presenting cells (APC) given their capacity to activate naïve T cells and also modulate CD4+ T cell polarization in response to each pathogen.

In these studies we describe various molecular responses of DCs to *Plasmodium* parasites, using a mouse model of malaria infection, *P. yoelii*. We found that malaria infection in mice regulates the expression of a high number of genes in DCs at the mRNA level (ca.700) using a whole-genome transcriptomic analysis. We identify specific gene families, metabolic pathways, cell functions and signaling pathways whose genes have their expression regulated in spleen DCs during *P. yoelii* infection.

Furthermore, we describe a new *Plasmodium*-induced signaling pathway in DCs which requires cyclooxygenase activity (responsible for PGE2 production), triggers production of cyclic adenosyl-monophosphate (cAMP) and leads to expression of interleukin-6, a mediator of inflammation and lymphocyte stimulation.

Given the relevance of Toll-like receptors (TLRs) in recognizing pathogen-associated molecular patterns (PAMPs) and triggering innate immunity, and also their recent association with malaria-induced inflammation, we describe how TLR-dependent

signaling for gene expression leading to DC maturation is impaired in the context of malaria.

Finally, we use a fluorescence labeling method for *Plasmodium* blood-stage parasites to visualize their interactions with DCs. We demonstrate that phagocytosis of infected or non-infected erythrocytes can induce a blockage of DC maturation, what might contribute to the induction of tolerance to *Plasmodium*.

RESUMO

A malária é responsável actualmente por 1 milhão de mortes por ano nos países em vias de desenvolvimento, sendo a maioria das vítimas mortais crianças e mulheres grávidas da África sub-Sariana.

O nosso conhecimento actual dos mecanismos moleculares e celulares que compõem a resposta imunitária do hospedeiro contra os parasitas do género *Plasmodium*, os quais são também responsáveis pela patologia associada à infecção, está longe de ser extensivo, apesar das várias décadas de pesquisa nesta área.

Neste estudo abordámos os papéis das células dendríticas ("dendritic cells", DCs) do sistema imunitário no contexto da fase sanguínea da infecção por *Plasmodium*. As DCs são uma das várias classes de células apresentadoras de antigénio ("antigen presenting cells", APC), tal como os macrófagos e as células B, com a particularidade de serem as únicas capazes de promover a activação primária de células T. Por outro lado, exibem uma grande flexibilidade no modo como reagem a diferentes agentes infecciosos, o que se traduz na produção variada de citoquinas e também em diferentes interacções com os receptores das células T; as DCs podem assim polarizar as células T CD4+ em diferentes fenótipos ("T helper 1", "T helper 2", células T reguladoras), além de modular a activação das células T CD8+.

Até há pouco, o conhecimento sobre as respostas das DCs ao parasita da malária era muito reduzido e limitava-se ao estudo dos marcadores de maturação. Com vista a um conhecimento mais vasto sobre os processos moleculares envolvidos na interacção entre as DCs e a fase sanguínea da malária – aquela em que os processos imunitários são acelerados, tal como os patológicos – propusemo-nos fazer as seguintes caracterizações:

1) Qual a extensão da regulação da expressão génica das DCs em resposta à infecção por *Plasmodium*? Para tal procedemos a uma análise global da regulação transcriptómica por Genechip® das DCs do baço em ratinhos infectados com *P. yoelii* (modelo de malária de roedores) em duas fases da infecção;
2) Dado a relevância da febre e dos processos inflamatórios nesta doença, qual o papel das DCs na produção de moléculas pirogéneas, tais como prostanóides e citoquinas, no contexto da malária? São estas moléculas induzidas a nível da expressão génica? Para responder a estas perguntas procedemos a uma análise mais detalhada dos dados obtidos pela análise de Genechip® com vista à identificação de genes relevantes, e também de vias de sinalização e regulação da transcrição, assim como os segundos mensageiros envolvidos.
3) Dada a relevância dos receptores "Toll-like" (TLRs) no reconhecimento de micróbios e na indução da maturação das DCs, será que esta família de receptores participa na indução da maturação das DCs em resposta ao *Plasmodium*? São as vias de expressão génica dependentes dos TLRs activadas pelo *Plasmodium*? Para este objectivo procedemos a uma análise dos critérios associados à maturação das DCs na presença do parasita *P. yoelii*, além da utilização de células transfectadas com TLRs individuais e de células que não transmitem determinados sinais dependentes de TLRs (MyD88-/-).
4) Dada a capacidade das DCs de fagocitarem e eliminarem micróbios, estará a fagocitose pelas DCs de eritrócitos infectados (EIs) com *Plasmodium* relacionada com o facto de desencadearem ou não a sua maturação? Para responder a esta pergunta desenvolvemos um processo de marcação por fluorescência dos EIs e estudámos a sua fagocitose *in vitro* por DCs, assim como o estado de maturação das DCs nesse sistema.

Neste trabalho descrevemos os resultados obtidos para cada uma das diferentes abordagens às interacções moleculares entre as DCs (ora do baço no caso *in vivo*, ora derivadas de progenitores hematopoiéticos nos sistemas *in vitro*) e a fase eritrocítica da malária.

Através da análise por microarray de oligonucleótidos, conhecidos como Genechip®, pudémos caracterizar a resposta transcriptómica das DCs à infecção por *P. yoelii* à escala do genoma. Perto de 700 genes foram identificados cuja expressão pelas DCs sofre uma alteração significativa durante a infecção por *P. yoelii*, seja ao dia 5 pós-infecção (pi), ao dia 10 pi ou em ambos. A análise dos processos moleculares e vias metabólicas associadas aos genes regulados pelo *P. yoelii* permitiu concluir que as principais funções celulares reguladas nas DCs do baço são: ciclo celular, imunidade, vias de sinalização e regulação da transcrição, metabolismo de purinas e pirimidinas e glicólise. Uma análise comparativa com estudos anteriores sobre a regulação da expressão génica em DCs por outros patogéneos permitiu concluir que as semelhanças entre o parasita da malária e outros agentes infecciosos são inferiores a 50%, o que indica que o *Plasmodium* exerce uma regulação em larga medida única e que carece de caracterização mais detalhada.

Os dados obtidos por análise de Genechip® de vários genes foram confirmados por PCR quantitativo, garantindo assim a qualidade dos resultados obtidos. Também os resultados para os marcadores de maturação das DCs (CD40, CD80, CD83, CD86) foram confirmados por citometria, observando-se a ausência de maturação *in vivo.*

Os dados de análise transcriptómica sugerem uma regulação de um elevado número de genes implicados em vias de sinalização molecular. Visto que a Prostaglandina E2 (PGE2) é um dos metabolitos produzidos pelas DCs em resposta ao *P. yoelii*, investigámos a sinalização dependente de PGE2 e a sua influência na expressão da interleuquina 6 (IL-6), uma das citoquinas cuja produção pelas DCs é estimulada nesta

infecção. Determinámos que a actividade da cicloxigenase (COX), responsável pela produção de prostanóides, entre os quais a PGE2, é responsável por: acumulação intracelular de adenosil-monofosfato cíclico (AMPc), activação das cinases de proteína PKA e p38-MAPK, e ainda a secreção de IL-6 pelas DCs em resposta a eritrócitos infectados (EIs) com *P. yoelii*.

Caracterizámos ainda a activação dos receptores "toll-like" (TLRs) no contexto da infecção por *P. yoelii*. Não foi detectada qualquer activação de expressão génica em resposta ao parasita em linhas celulares transfectadas com TLRs. Pudemos também observar uma inibição generalizada das respostas mediadas por TLRs em DCs na presença de EIs, sugerindo uma supressão dos mecanismos de sinalização partilhados entre as vias dependentes de TLRs. Investigámos ainda as interacções *in vitro* entre DCs e EIs marcados com moléculas fluorescentes; conclui-se que à fagocitose de EIs está associada uma forte inibição da maturação das DCs, para além de uma inibição das respostas a estimulação secundária via TLRs.

As principais conclusões desta Tese são:

i) A infecção por *P. yoelii* induz uma vasta regulação transcriptómica das DCs do baço, sendo que uma larga parte desta regulação é distinta daquela exercida por outros agentes patogénicos;

ii) Os eritrócitos infectados com *P. yoelii* induzem a expressão de dois importantes agentes pró-inflamatórios e pirógeneos pelas DCs – PGE2, IL-6 – sendo que a produção de IL-6 depende de uma via que envolve actividade de cicloxigenase (responsável pela produção de PGE2), produção de AMP cíclico, e activação das cinases PKA e p38-MAPK;

iii) Apesar da produção de agentes pró-inflamatórios, as DCs não apresentam marcadores de maturação, tanto *in vitro* como *in vivo,* e sofrem uma inibição

generalizada das vias dependentes de TLRs; a fagocitose de EIs é um dos factores que leva à inibição da maturação das DCs.

Estes estudos pretendem esclarecer algumas das incongruências existentes na literatura acerca das respostas das DCs à fase sanguínea da malária.

Apresentamos dados que revelam a extensão da regulação da expressão génica das DCs num modelo de malária de roedores, o que possibilitará estudos mais detalhados no futuro sobre os vários processos em que as DCs participam no contexto da malária.

Apontamos novos papéis para a produção de agentes potenciadores da febre pelas DCs e quais os agentes moleculares envolvidos; ao mesmo tempo, as DCs vêem a sua maturação impedida, o que parece estar relacionado com um contacto directo com os EIs através de fagocitose. A inibição da maturação, mas também a produção de mediadores solúveis como a PGE2 ou a IL-6, são factores que condicionam seriamente a capacidade das DCs activarem células T, o que sugere um papel para as DCs como indutoras de tolerância ao *Plasmodium*, como é discutido no final deste trabalho.

Esperamos com estes estudos contribuir para esclarecer em mais detalhe os papéis das DCs nos processos inflamatórios, febris e de imunidade adaptativa em resposta ao *Plasmodium* e assim apontar novos caminhos para intervenções terapêuticas que controlem as patologias associadas à malária.

Keywords

Malaria, *Plasmodium yoelii*, Dendritic cells, Maturation, Transcriptomic analysis, Prostaglandin E2, Interleukin-6, Toll-like receptors, Phagocytosis

Palavras-chave

Malária, *Plasmodium yoelii*, Células dendríticas, Maturação, Análise transcriptómica, Prostaglandina E2, Interleuquina 6, Receptores “Toll-like”, Fagocitose

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Chapter I

GENERAL INTRODUCTION

1.1 Introduction to malaria infection

1.1.1 Life cycle of *Plasmodium* species

Malaria infections are caused by eukaryotic unicellular parasites of the *Plasmodium* genus. Infection of the vertebrate host starts with the bite of an infected female mosquito of the *Anopheles* genus; about 70 out of the ca. 420 *Anopheles* species can transmit *Plasmodium* parasites. (*Malaria – Molecular and Clinical Aspects*, Hardwood Academic Publishers 1999). Different *Plasmodium* species infect different animal hosts, varying from birds to reptiles and mammals, while also having preference for one or more species of *Anopheles* mosquitoes as its vector.

The malaria parasites infectious to humans are *P. falciparum, P. vivax, P. malariae* and *P. ovale*; nearly all of the deaths caused by malaria infections (ca. 1 Million/year) are caused by *P. falciparum,* with a marginal percentage caused by *P. vivax.*

The complex life cycle of malaria parasites is depicted in fig. 1.1. The parasite cycle in the human host starts with injection of sporozoites by the mosquito into the dermis of the host. From there, sporozoites are able to travel within a few minutes to the liver of the host by way of the bloodstream. Once in the liver, the sporozoite traverses the sinusoid layer and starts migrating through hepatocytes by breaching their plasma membranes, until it invades a particular cell with the formation of a parasitophorous vacuole that allows for its replication (Mota et al. 2001); the liver stages of infection, also know as exoerythrocytic, last from 5 to 14 days in the human host, and ca. 42h in rodents (Parasitic diseases, Apple Trees Production). During that time the sporozoite asexually replicates into thousands of unicellular parasites called merozoites, which can be as many as 30,000/infected hepatocyte. Recently, the mechanism allowing for the release

of merozoites from hepatocytes to the bloodstream that contributes for evasion of immune recognition of the parasites has been described (Sturm et al. 2006).

Human-infective *Plasmodium* species vary in their ability to establish dormant parasites in the liver called hypnozoites, which are responsible for relapses of the disease. These are characteristic of both *P. vivax* and *P. ovale*.

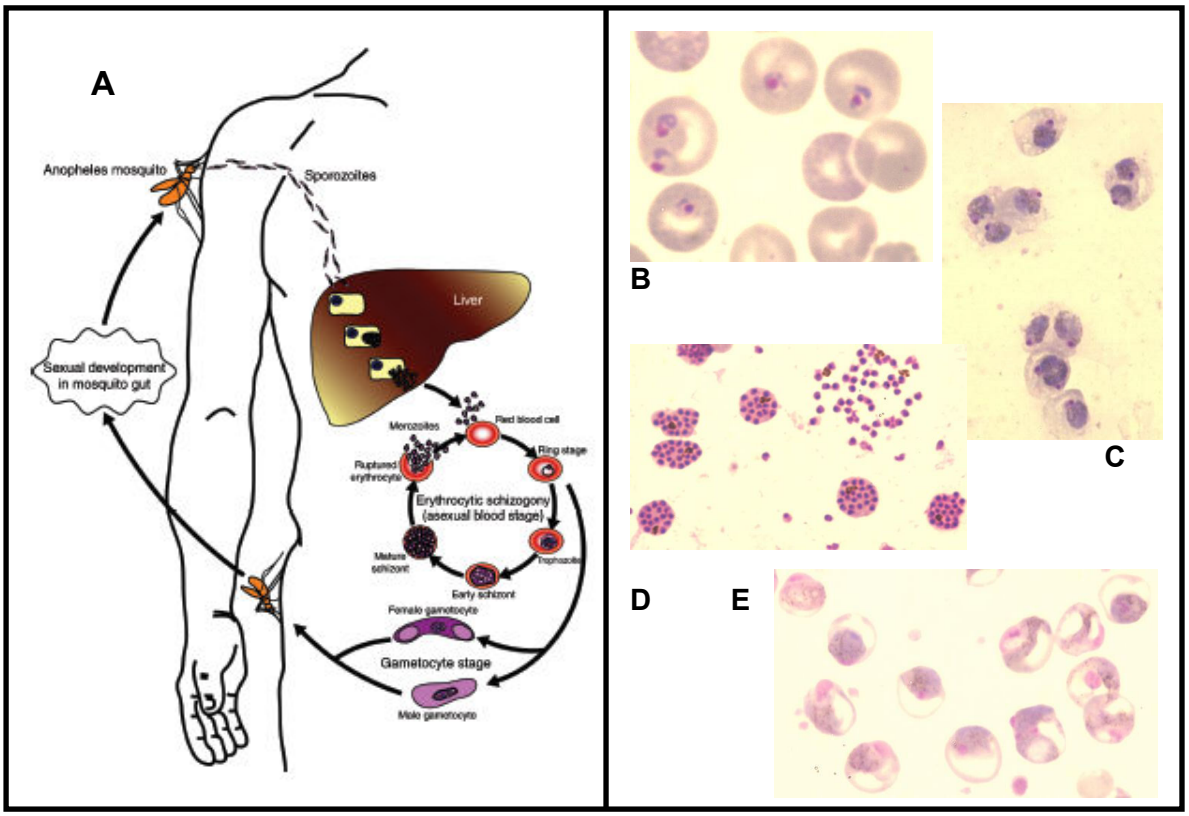


Figure 1.1 – (A) Life cycle of *Plasmodium* parasites (from Struik, Riley, Imm. Reviews 2004). **Detail of microscopic analysis of GIEMSA-stained *P. falciparum*-infected erythrocytes grown *in vitro***: (B) ring forms, (C) trophozoites, (D) schizonts, (E) male and female gametocytes (images from Leiden University, available at http://www.lumc.nl/1040/research/malaria/model05.html)

Merozoites are infective to the erythrocytes of the host, and are directly delivered to the bloodstream upon extrusion from the infected hepatocyte (Sturm et al. 2006); inside erythrocytes, *Plasmodium* undergoes asexual replication, evolving through different parasite stages called rings, trophozoites (early and late) and schizonts (early and late),

as depicted in figure 1.1. The mature schizont is comprised of 8 to 12 new parasites, which are released upon the rupture of the infected erythrocyte (IE) that is caused by the disintegration of the IE cytoskeleton (Glushakova et al. 2005).

Fever is usually associated with each round of erythrocytic replication; the periodicities associated with each cycle (48h for *P. falciparum, P. ovale* and *P. vivax*; 72h for *P. malariae*) are known as 'tertian' and 'quartan' (Parasitic diseases, Apple Trees Production). In rodents, the erythrocytic cicle takes close to 24h (Lamb et al. 2006).

Blood-stage infection is responsible for the severe symptoms of malaria, including severe malaria anemia (SMA), cerebral malaria (CM) and others.

After several rounds of replication within erythrocytes, the parasite life cycle can be continued when some of the parasites differentiate into gametocytes, a process still not clearly understood. Male and female gametocytes need to be uptaken by a mosquito, the definitive host; once in the mosquito lumen, gametocytes originate gametes, which can then undergo fertilization by merging into a non-motile, dyploid zygote. The obligatory mosquito stages of the *Plasmodium* life cycle therefore include sexual replication that allows for mitotic and meiotic recombination of parasite genomes, enhancing *Plasmodium* diversity, as reviewed in (McConkey et al. 1990) and (Baton and Ranford-Cartwright 2005). The zygote evolves into a motile ookinete that crosses the mosquito midgut to form an oocyst. This step is prevented in mosquitoes that are refractory to malaria since they are able to encapsulate ookinete by melanization. In susceptible mosquitoes, the oocyst will mature and give rise to several hundreds of motile sporozoites; these will migrate into the salivary glands of the mosquito and from there will be able to be transmitted to a new vertebrate host, and continue the life cycle of the malaria parasite (*Malaria – Molecular and Clinical Aspects*, Hardwood Academic Publishers 1999).

1.1.2 Global distribution and epidemiology of malaria infections

Malaria infections are still spread throughout all tropical and sub-tropical regions, from South America to Africa, Middle East and Asia, as depicted in Figure 1.2. Sixty percent of clinical episodes of malaria and 80% of global malaria-related deaths take place in sub-Saharan Africa, causing a drop in as much as 2% of GDP every year in this region (WHO Malaria Report 2005, in http://rbm.who.int/wmr2005/html/1-2.htm, Sachs 2002).

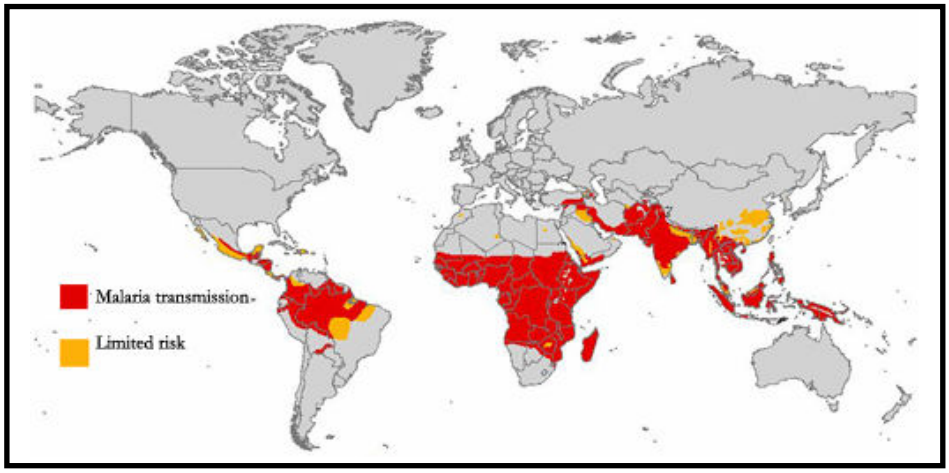


Figure 1.2 – Worldwide distribution of malaria in 2002. Source: World Health Organization.

Estimates of malaria infection rates and mortality have a high degree of uncertainty, varying between 5 and 10% of the World's population, and 1 to 3 million deaths/year, respectively. Using epidemiological, geographical and demographic data, the number of clinical *P. falciparum* episodes in 2002 is estimated to have been over 500 Million (Snow et al. 2005). A different study which also comprises different estimating methods concluded that the most likely figure of deaths caused by malaria in the year 2000 was ca. 900,000 (Rowe et al. 2006).

In 2002, *Plasmodium* was the third single infectious agent with highest impact on global mortality (2.1% of all deaths, 1.2Million), after HIV (4.9%, 2.8Million) and *Mycobacterium tuberculosis* (2.8%, 1.6 Million) (The World Health Report 2003, WHO – http://www.who.int/whr/2003/en/). In the same year, malaria was responsible for 11% of children deaths in developing countries.

Malaria infection can assume different epidemiological presentations: from hyper-endemic to low but stable, to not stable/epidemic transmission. Each presentation will have different profiles of parasite and disease prevalence among age groups, which are depicted in figure 1.3 (adapted from (Struik and Riley 2004)).

In areas of stable transmission, severe disease – usually in the form of severe malaria anemia (SMA) or else cerebral malaria (CM) – is almost exclusively prevalent in children and also pregnant women. Excluding the latter group, the maximum age where severe disease is detected varies from 10 years of age, in areas of low transmission, to only 5 years areas of higher transmission intensity. SMA and CM tend to predominate in different age groups, with SMA having higher prevalence at younger ages than CM. If malaria transmission is epidemic then the prevalent form of disease in children is SMA.

The fact that, in a stable transmission context, immunity to severe disease is commonly acquired within the first 5 years of age suggests that such process of immunity is faster to evolve than immunity to mild disease; the latter is associated with control of parasite density since the two decline simultaneously (fig. 1.3). Estimates from epidemiological data suggest that the number of infections required for induction of immunity to non-cerebral severe malaria disease is as low as one infection (Gupta et al. 1999). It is therefore likely that immunity to severe disease depends on conserved domains, rather than variable antigens. The latter is probably the case for immunity that controls parasite densities, explaining why this type of protection takes longer time to be obtained (10 to 20 years of exposure to malaria).

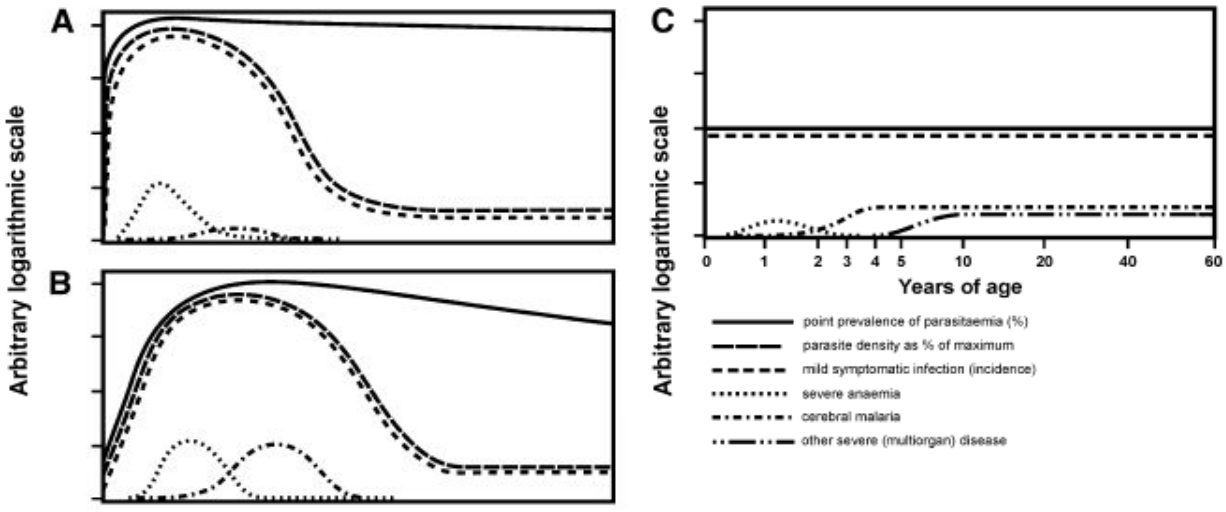


Figure 1.3 – Representation of malaria infection and disease by transmission intensity. (A) Area with transmission rate of 300 infective bites/person/year. (B) Area with 1 to 10 infective bites/person/year. (C) Area with unstable, epidemic transmission (<1 infective bite/person/year). From (Struik and Riley 2004).

1.1.3 Animal models of malaria

Animal models of malaria have been used by researchers for several decades, providing extensive characterization of the immune responses to *Plasmodium*, as reviewed in (Lamb et al. 2006)). Four different *Plasmodium* species infect rodents: *P. yoelii*, *P. berghei*, *P. chabaudi* and *P. vinckei*; the first three constitute widely used models for animal experiments.

Like human parasites, the four rodent-infective species have major differences in their erythrocyte preferences: *P. berghei* ANKA and *P. yoelii* 17X (non-lethal) preferentially invade reticulocytes, while *P. chabaudi* and *P. vinckei* prefer mature erythrocytes. *P. yoelii yoelii* 17XL and YM (both lethal) invade both kinds of erythrocytes and are therefore in this respect the most similar to *P. falciparum*.

Different combinations of the parasite species and mice strains provide different outcomes of the disease, allowing for studies of the different pathologies observed in

infected humans. *P. berghei* ANKA infection is the only one that can cause CM in certain genetic backgrounds, while several host/parasite combinations lead to hyper-parasitemia and SMA. Infections with *P. yoelii yoelii* 17XNL or *P. chabaudi chabaudi* are resolved by laboratory mouse strains.

Each of the three main rodent-infective *Plasmodium* species has similarities and differences with *P. falciparum* in regard to the immune responses that are triggered, and also with the molecular basis of pathology. On the other hand, genome-wide studies have shown that the *P. falciparum* and *P. yoelii* genomes are extensively similar, with extensive conservation of gene synteny (Carlton et al. 2002). Two examples of similarities between the two species are the response of dendritic cells (DCs) to the two parasites (Urban et al. 1999) (Ocaña-Morgner et al. 2003), and also the induction of regulatory T cells (Hisaeda et al. 2004) (Walther et al. 2005). Still, none of mouse-infective *Plasmodium sp.* should be considered the most accurate equivalent to *P. falciparum*, but instead the species (either one or more of them) that respond in the most similar way to *P. falciparum* in regard to the pathogen/host interaction under study should be the one considered for a given experimental setup.

1.1.4 Strategies for the control of malaria

Given the absence of a vaccine for malaria infection, the strategies that have been able to reduce the burden of malaria have been of two different natures:

i) those that control the spread of the *Anopheles* vector, such as insecticide spraying and insecticide-treated bed nets (ITNs);

ii) prophylaxis and chemotherapy with anti-malarial drugs such as chloroquine and, more recently, artemisinin.

Resistance to both anti-vector and anti-parasite chemicals which were used for several decades, like DDT and chloroquine, has emerged throughout large areas of malaria prevalence, rendering those chemicals inefficient. New drugs that have become recently available, the more important being artemisinin, have been adopted exclusively as part of combination therapies (Artemisinin Combination Therapies, ACT), as a way of preventing the development of resistant parasites.

Even if ITNs and ACT can improve the situation of malaria infections, a vaccine for malaria would be a major breakthrough in preventing the disease. Research on malaria vaccines has intensified since 1967 with the discovery of the irradiated sporozoite as a vaccine model (Nussenzweig et al. 1967).

So far only one vaccine candidate (RTS,S/AS02A) has shown long-term (2-year) moderate protection (50% reduction of severe disease) in children from endemic areas (Alonso et al. 2005); these recent trials have shown that it is possible to have some level of protection with a vaccine based on only one antigen, even if *Plasmodium* can express up to 5,300 genes (Gardner et al. 2002). The antigenic complexity of the parasite is a likely cause for the fact that a malaria vaccine has remained elusive to researchers for so long. The low efficacy of RTS,S/AS02A in protection from infection (29%) reminds us of the multitude of immune mechanisms at stake that are required for control of *Plasmodium*. Many of these mechanisms have been established from analyses of epidemiological data, experimental animal models, and also human clinical trials. These will be described in the next section in further detail.

1.2 Immunity to blood-stage malaria

1.2.1 Pathology and immune processes of malaria blood stages

The life cycle of *Plasmodium* parasites is considered a complex one, given the diverse morphological changes that occur throughout the cycle. Therefore, the immune system of the host needs to cope with constant changes occurring within the same infectious agent by constantly adapting its mechanisms so they are able to control each stage of the disease (*Malaria, Molecular and Clinical Aspects*, Hardwood Academic Publishers).

The work presented here will focus on the blood stages of infection, which is the phase when symptoms develop and also when immunity assumes special relevance in inducing both parasite killing and pathology, as reviewed in (Schofield and Grau 2005).

The regulation of immune responses is critical to achieve a balance that allows for the control of parasite levels without risking the life of the host; this is also beneficial for the parasite since transmission cannot be achieved in the short term (Riley et al. 2006). Immune responses to liver stages and gametocytes are also helpful for parasite control and for inhibiting transmission, but those mechanisms will not be described here.

Blood-stage malaria consists of the asexual replication of merozoites inside the host erythrocytes, leading to the release of new merozoites from the infected erythrocyte (IE) within 2 to 3 days (Kwiatkowski 1995). The rupture of mature schizonts in both *P. falciparum* and *P. vivax* malaria is synchronous, inducing a wave of inflammatory mediators that will cause the periodic symptoms (fever, shivers, pallor, anemia) that are characteristic of this disease (Schofield and Grau 2005) (Maitland and Marsh 2004).

A major consequence of blood-stage infection is severe malaria anemia (SMA), which results not only from the lysis of IEs but also the enhanced removal of uninfected

erythrocytes, but also from reduced erythropoiesis, as reviewed in (Chang and Stevenson 2004). Other pathologies of blood-stage infection are cerebral malaria (CM), placental malaria, respiratory distress and metabolic acidosis and others, as presented in Table 1.I. (Schofield and Grau 2005)

Syndrome	***Clinical features***	***Possible mechanisms of disease***
Severe malaria anemia (SMA)	Hemoglobin level of 4 to 6g/10ml, pallor, lethargy	Increased RBC destruction, erythropoiesis suppression by cytokines (TNF-α, IFNγ versus IL-10, PGE2), toxins; enhanced erythrophagocytosis, splenic hyperphagism.
Cerebral malaria (CM)	Sustained impaired consciousness, coma, long-term neurological sequelae	Cerebral parasite sequestration; cytokine and chemokines production (TNF-α, LT-α, IFN-γ); monocyte, macrophage and neutrophil infiltration; NK, CD4+, CD8+ and $\gamma\delta$ T cells; hypoxia; platelet and fibrinogen deposition.
Placental malaria (PM)	Placental insufficiency, premature delivery, low birth weight, fetus loss	PfEMP1-mediated binding to placental endothelium and syncytiotrophoblast through CSA and hyaluronic acid; cytokine production; chemokine-mediated recruitment and infiltration of monocytes
Metabolic acidosis	Respiratory distress, deep (Kussmaul) breathing, hypovolaemia	Mechanism unknown. Probably parasite sequestration, toxins, increased vascular permeability, anaemia, pulmonary airway obstruction, increased glycolysis, hypoxia.
Shock-like syndrome	Shock, haemodynamic changes, impaired organ perfusion, disseminated intravascular coagulation	Biocative toxins, Th1 cytokines, Acute-phase reactants.

Table 1.I – Description of severe disease syndromes observed in malaria infections and possible mechanisms of disease (adapted from (Schofield and Grau 2005)). PfEMP1 – *P. falciparum* erythrocyte membrane protein 1; IFN-γ - Interferon-γ; TNF-α - Tumor necrosis factor α; LT-α - Lymphotoxin α; IL-10 – Interleukin 10; PGE2 – Prostaglandin E2; CSA – Chondroitin Sulfate A.

Manifestations of malaria disease are usually a consequence of two important processes that stimulate each other, and that are characteristic of this infection.

The first process of disease is cytoadherence, which is common in *P. falciparum* infections and consists of the enhanced adhesion of the IE to multiple cell types, including uninfected erythrocytes (rosetting) and endothelial cells of microvessels (sequestration), causing to the arrest of the IEs in different organs (Schofield and Grau

2005). Cytoadherence is mediated by parasite-secreted proteins that are encoded by families of highly variable genes (*var, rif, stevor*), and that localize to the outer membrane of the IE. The most common of these proteins is known as *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which contains binding domains to a variety of receptors of the host cells (Sherman et al. 2003); the affinities of IE proteins for different host cell receptors determine which organs will have enhanced sequestration (Mota et al. 2000).

Both sequestration and rosetting induce not only an impaired blood flow and hypoxia, but also endothelial cell activation and extensive inflammation, including cytokine production and leukocyte infiltration, and might lead to the disruption of the brain blood barrier that is associated with CM (Schofield and Grau 2005).

It is generally accepted that cytoadherence fulfills the objective of avoiding clearance of IEs by the spleen, the organ responsible for erythrocyte recycling, what should compensate for expression of proteins that might induce immune responses against *Plasmodium*. Other mechanisms might also stimulate cytoadherence such as faster parasite replication rates in low oxygen environments, enhanced gametocyte survival and the modulation of immunity through binding to dendritic cells (DCs) (Sherman et al. 2003) (Urban et al. 2001).

The second pathology-inducing process of blood-stage infection is the excessive production of acute phase reaction and inflammatory mediators such as TNF-α, IL-1β, IFN-γ, LT-α and IL-6 (Clark et al. 2006). Inflammation can stimulate cytoadherence due to the cytokine and platelet-mediated induction of adhesion molecule expression in endothelial cells, which stimulates further sequestration (Schofield and Grau 2005). The fact that inflammation and cytoadherence promote each other might lead to the uncontrolled inflammatory process that is typically frequent in the first rounds of malaria

infection in non-immune individuals, and therefore most if not all of the pathologies associated with malaria (Schofield and Grau 2005). The roles of inflammatory cytokines in innate responses to malaria will be addressed further on in section 1.2.3.

Even if they might contribute for pathology, innate immunity and inflammation also have important roles in controlling parasite densities through macrophage-mediated IE phagocytosis, activation of NK and $\gamma\delta$ T cells, and the early production of chemokines and cytokines that are necessary for parasite killing (Stevenson and Riley 2004).

One of the roles of inflammation and innate responses is to trigger adaptive immunity, which is mainly comprised of antibodies and CD4+T cells against IEs in the case of blood-stage malaria. Since cytotoxic CD8+T cells do not recognize mature erythrocytes due to the absence of class I MHC, CD4+ T cells are essential for helping B cells produce antibodies that will inhibit erythrocyte invasion by merozoites and enhance opsonization of *Plasmodium*-IEs (Good et al. 2005).

The role of antibodies in the control of blood-stage malaria was shown by Cohen in the 1960s with the observation hat passive transfer of antibodies from malaria-exposed individuals induces protection to blood-stage infection (Cohen et al. 1961). The same was later reproduced in rodent and monkey models (Brown and Phillips 1974), (Gysin et al. 1982). Protection was not related to a direct effect of antibodies on inoculated parasites, but rather by inducing cellular responses from serum-treated humans or mice (Brown and Phillips 1974) (Bouharoun-Tayoun et al. 1990).

The roles of cellular immunity to blood-stage malaria are reviewed in (Good et al. 2005). The interactions between antigen-presenting cells (APCs) and adaptive immunity cells in malaria infection and their locations are depicted in Figure 1.4 (adapted from (Good et al. 2005)).

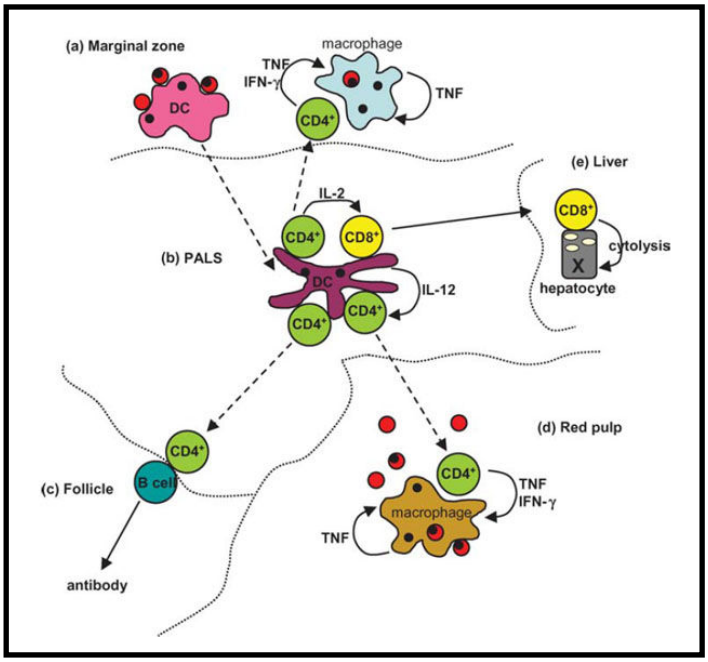


Figure 1.4 – Cellular interactions between the innate and adaptive immune cells in response to *Plasmodium*-infected erythrocytes (from (Good et al. 2005)). PALS – Periarteriolar lymphoid sheath

1.2.2 Natural immunity to malaria

The antibody and cellular immunity to malaria present in asymptomatic individuals living in endemic areas is referred to as 'natural immunity' to malaria. Serum transfer is a way of transferring this kind of immunity between individuals, as already mentioned (Cohen et al. 1961). Even if many studies have addressed the components of such protection, it still remains elusive which factors determine why individuals living in the same area, that might have similar levels of parasite density, have completely different clinical conditions, which vary from asymptomatic to mild malaria, SMA, CM and death. It is likely that a variety of host genetic factors, parasite diversity, environmental conditions and others determine the outcome of infection in each individual (Struik and Riley 2004).

The process of 'natural immunity', which evolves during a long period of the exposed individuals' life, until infection is in fact resolved or at least maintained under non-pathogenic levels, can tells us how the immune system achieves a compromise between killing the parasite and avoiding excessive responses that are prejudicial to the host. In the case of malaria, such a compromise takes decades to achieve (Marsh and Kinyanjui 2006). Many factors contribute to this slow development of immunity: the genetic variation of the parasite; the limited number of ways that allow for clearance of IEs; the detrimental effects on the host of excessive production of parasite-killing cytokines; the induction of immune-suppressive or immune-regulatory mechanisms by the parasite, among others (Struik and Riley 2004).

Many have argued that another reason for the lack of control of malaria parasites by the host during years of infection is that memory to malaria is short-lived; however, it remains to be understood if this is a direct effect of the parasite on memory cells, or else if it is simply a consequence of parasite persistence (Struik and Riley 2004).

It is assumed that immunity that controls parasite levels depends on highly variable antigens. This is suggested by the long time that it takes to establish parasite control in each host, but also by the correlation between antibodies to PfEMP1, and protection from disease in adult age (Marsh and Howard 1986). The roles of antibodies to variable genes in natural immunity are reviewed in (Bull and Marsh 2002).

Oppositely from immunity that controls parasite levels, immunity that prevents from severe disease, other than CM, can be achieved very early on, even after only one infection (Gupta et al. 1999). This explains why severe disease is only prevalent in young infants when there is high transmission (Struik and Riley 2004). It is assumed that this kind of immunity respond to non-variable parasite antigens, which have not been identified so far (Gupta et al. 1999).

More recently, it has been suggested that severe disease associates with some families of variable (*var*) genes, as reviewed in (Kraemer and Smith 2006), suggesting a new model for induction of immunity to severe disease other than conserved *Plasmodium* patterns.

A coherent theoretical framework that describes how the responses to variable genes of *Plasmodium* lead to natural immunity to severe, non-severe and pregnancy malaria has been recently proposed by Hviid (Hviid 2005). It is known for some time that once immunity to PfEMP1 forces the parasite to switch to the next variant (Hommel et al. 1983); this has been the basis for the immune evasion argument for antigenic variation. It is proposed that the host will achieve maximal protection when enough rounds of infection with different variants confer immunity to a large spectrum of variable antigens, including each one of the *var* gene families (Hviid 2005).

Independently of the mechanisms that support the development of "natural immunity", many cells of both innate and adaptive immunity participate in a wide array of immune mechanisms directed at the *Plasmodium*-IE, some of which are depicted in a Figure 1.5.

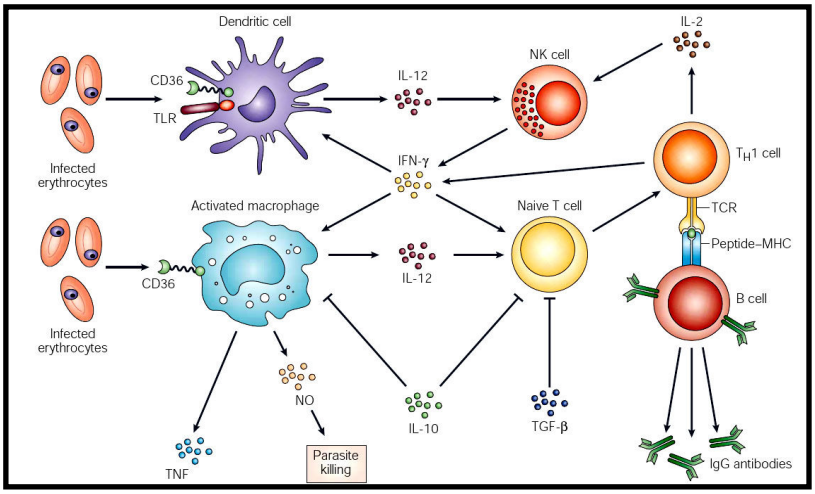


Figure 1.5 – Interactions between immune cells and cytokine production in response to *Plasmodium*-infected erythrocytes (from (Stevenson and Riley 2004)).

In the next sections we will briefly discuss the cells and molecules involved in innate immunity to *Plasmodium* blood stages, since the work presented in this Thesis will focus on that class of immune responses. First we will discuss how cytokines are induced and their involvement in pathology (1.2.3), and then the interactions of antigen-presenting cells (APCs) with the IE (1.2.4). These are early events that are crucial for the induction and regulation of the subsequent immune strategies of the host against *Plasmodium* parasites.

1.2.3 Innate immunity to malaria blood stages – cytokine production and their involvement in pathology & fever

The multiples roles of innate immunity in the control of blood-stage malaria have been studied more intensely in recent years, as reviewed in (Stevenson and Riley 2004).

Innate immunity was considered for some time to be simply a mechanism for the early control of infections; it would reduce damages to the host until adaptive immunity would be available and ready to clear infection. Innate responses were considered to have little flexibility, using similar mechanisms to control a wide array of pathogens; this would result from the absence of highly variable receptors that recognize antigens with high specificity, which are exclusive of the adaptive system (Roitt et al. 1989).

With the characterization of the toll-like receptors (TLRs) and how these are able to regulate different signaling pathways (Akira and Takeda 2004), it has become evident that innate responses differ considerably between pathogens, since antigen-presenting cells (APCs) detect a large number of pathogen-associated molecular patterns (PAMP) through TLRs, but also other PAMP-recognition receptors (PRR), triggering the more

adequate response to each infection (Janeway and Medzhitov 2002) (Kapsenberg 2003).

In malaria, all different classes of APCs, including dendritic cells (DCs), macrophages and B cells, play a role in the detection of IEs in the blood and modulating the adaptive responses to *Plasmodium* (depicted in Figures I.3, I.4). These events take place in the spleen, the organ responsible for erythrocyte clearance, but also in other lymphoid organs (Good et al. 2005) (Engwerda et al. 2005).

In the case of malaria, a variety of cytokines and other mediators released by APCs can trigger considerable inflammatory reactions and malaria pathology, including fever, a typical symptom of malaria; some cytokines, usually considered pro-inflammatory, help to control parasite and induce pathology (TNF-α, IFN-γ, IL-1, LT-α, IL-6, NO), while others counteract their effects (IL-10, TGF-β) (Clark et al. 2006) (Ndungu et al. 2005).

So far, two PAMPS of Plasmodium that trigger cytokine production by APCs have been identified: DNA (+hemozoin) (Parroche et al. 2007) and glycophosphatidilinositol (GPI) (Krishnegowda et al. 2005). Whether the two identified PAMPs are responsible for the induction of inflammatory mediators in the context of infection is still not clear; however, the observations that both GPI and hemozoin induce cytokine production when injected into mice suggest that they are involved (Coban et al. 2005) (Schofield et al. 2002).

The roles of individual cytokines on malaria pathogenesis have been explored for some time, especially those of TNF-α, as reviewed in (Hunt and Grau 2003); however, it has been recently observed that roles attributed to TNF-α could be instead due to the action of lymphotoxin-α (LT-α) (Engwerda et al. 2002). Other cytokines have been shown to play contribute to CM (IFN-γ) (Amani et al. 2000), but also to protect from liver stage infection (IFN-γ) (Schofield et al. 1987) or from severe disease (TGF-β, IL-10) (Omer and

Riley 1998) (Li et al. 1999), and also to induce regulatory T cells (TGF-β) (Walther et al. 2005), to mention some examples of cytokine-mediated regulation of malaria immunity.

It is likely that the production of cytokines that determine adaptive responses to malaria, and pathology associated with the infection, is induced very early on during blood-stage infection by cells of the innate system. Two examples are the production of IFN-γ by NK cells (Artavanis-Tsakonas et al. 2003), as well as the association of early TGF-β release – probably from activated platelets – with higher parasite replication rates and lower production of pro-inflammatory cytokines (Walther et al. 2005).

One cytokine whose role in the context of malaria infection remains somewhat unexplored is the acute phase mediator IL-6; this molecule is known for some time to associate with malaria infection, and even correlate with parasite density the same way as TNF-α does (Kern et al. 1989), and also to associate with hypoglycaemia, one of the pathologies associated with malaria (Clark et al. 1992). Given the multiple roles of IL-6 as a regulator of adaptive immunity (Jones 2005), but also fever (Dinarello 2004), it is required a better understanding of how this molecule is induced by *Plasmodium*.

Among the cells that secrete cytokines in the early phase of infection, DCs play a relevant role given their ability to secrete a large spectrum of soluble molecules that regulate virtually every aspect of immunity; DCs have therefore a special ability to modulate adaptive responses (Kapsenberg 2003). The general roles of DCs in immunity, as well as in malaria infection, will be discussed in Sections 1.3 and 1.4.

Cytokine induction of fever

The same “toxins” (GPI, DNA/hemozoin) that activate cytokine production in APCs are thought to be responsible for the synchronous fever reaction that is typical of malaria. Fever is induced through the action of endogenous pyrogens on the hypothalamus

thermoregulatory center; it is associated with innate responses, since they usually include production of pyrogenic cytokines such as IL-1, TNF-α and IL-6. Fever is now considered a consequence of either cytokine signaling or TLR-mediated signaling, but in both cases is mediated by cycloxygenase-2 (COX-2) activity and prostaglandin E2 (PGE2) (Dinarello 2004).

Malaria-associated fever is synchronous, being induced after each cycle of asexual replication within the erythrocyte, with a frequency that is specific of each *Plasmodium* species (*Malaria, Molecular and Clinical Aspects*, Hardwood Academic Publishers 1999) as discussed before (see chapter 1.1). Factors from the IE known as "malaria toxins" are released upon IE bursting, that will induce production of pyrogens from immune cells. A minimum number of IEs is required for the induction of fever, number that is known as the fever threshold and varies between *Plasmodium* species: while *P. falciparum* has a threshold of ca. 10,000/μl, *P. vivax* induces fever at a 100-fold lower density (100/μl) (Kwiatkowski 1995). Whether these intrinsic differences accounts for the severity of disease caused by each species remains to be determined.

1.2.4 The relevance of opsonization and phagocytosis of infected erythrocytes

APCs play a critical role in modulating the functions of both innate and adaptive immune cells into the responses more adequate to resolve, or at least dampen, infections (Janeway and Medzhitov 2002). On the other hand, APCs might also be a target for parasite evasion of the immune system (Palucka and Banchereau 2002).

One of the relevant processes in innate immunity to malaria in which APCs play a major role is the phagocytosis of *Plasmodium*-IEs, which might be mediated by antibodies

(opsonization) or not (Good et al. 2005). Together with complement-mediated lysis, IE phagocytosis is a major mechanism of parasite destruction (Waitumbi et al. 2000). Macrophages destruction of *Plasmodium*-IEs by opsonization is known for a long time (Criswell et al. 1971) (Shear et al. 1979); further studies in mouse models have supported the relevance of antibodies in controlling malaria infection by stimulating opsonization (Mota et al. 1998) (Yoneto et al. 2001).

One of the consequences of IE uptake by macrophages is the production of the macrophage migration inhibitory factor (MIF), which has been linked to malaria-induced anemia together with TNF-α and IFN-γ (Martiney et al. 2000) (McDevitt et al. 2006). IE phagocytosis has the benefit of reducing parasite load, but when excessive cannot be compensated with new erythrocyte generation, which is defective in malaria, therefore contributing to severe anemia (Chang and Stevenson 2004).

IE uptake is necessarily associated with the accumulation of hemozoin inside the phagocyte, which is common in monocytes and macrophages (Pichyangkul et al. 1994) (Schwarzer et al. 1992).

Reports on the consequences of hemozoin upload on host cells are often contradictory, varying from inhibition of cytokines such as IL-12 and TNF-α (Keller et al. 2006) (Taramelli et al. 2000) and inhibition of antigen presentation (Schwarzer et al. 1998) to the induction of inflammatory cytokines (TNF-α, IL-1β, IL-6, NO) (Coban et al. 2005) (Keller et al. 2004) (Pichyangkul et al. 1994). The latter activities, which rely on TLR9, have now been shown to depend on *Plasmodium* DNA, which usually contaminates hemozoin preparations, rather than hemozoin itself (Parroche et al. 2007).

Two of the genetic disorders that associate with malaria protection – sickle trait and β-thalassemia – have been reported to associate with higher uptake of ring stage parasites

(Ayi et al. 2004), suggesting that the rate of uptake of IEs is a determinant of disease severity.

IE phagocytosis is likely to take place in the spleen, the organ responsible for erythrocyte clearance (Engwerda et al. 2005). The assumption that the spleen eliminates *Plasmodium*-IEs has been used as the central argument to explain the intricate mechanisms used by *Plasmodium* for antigenic variation, and therefore sequester in organs other than the spleen (Sherman et al. 2003). However, a recent report describes an inhibition of IE entry into the spleen is during peak parasitemia in a mouse model of infection, suggesting that spleen removal cannot be the main mechanism for parasite destruction (Krucken et al. 2005). IE uptake in organs other than the spleen must therefore be accounted for a significant role in parasite control.

More recently, it has been noticed that macrophages and DCs can destroy IEs even in the absence of antibodies (McGilvray et al. 2000) (Ayi et al. 2005). This is due to the interactions between the *Plasmodium*-secreted proteins such as PfEMP1 in the membrane of the IE and receptors of host cells (Sherman et al. 2003).

In the context of APCs, the most relevant host receptor for PfEMP1 is CD36. This molecule, also known as SR-B, belongs to the family of scavenger receptors, which are common PRRs involved in immunity but also lipoprotein removal (McGuinness et al. 2003). CD36 is essential for phagocytosis of *P. falciparum*-IEs by macrophages (McGilvray et al. 2000) (Patel et al. 2004), while the interaction of *Pf*-IEs with CD36 on the DC surface associates with an inhibition of the DC ability to induce T cell activation, similarly to that exerted by apoptotic cells (Urban et al. 2001); this shows that phagocytosis is relevant not only for parasite clearance but also determines how immune responses to malaria are regulated by DCs and macrophages.

1.3 Biology of dendritic cells

1.3.1 Dendritic cells link innate and adaptive immunity

Dendritic Cells (DCs) are a specialized class of Antigen-Presenting Cells (APCs) that are capable of activating naïve T cells and therefore initiate adaptive immunity, as reviewed in (Banchereau et al. 2000). On the other hand, DCs play a central role in the induction of peripheral tolerance (Steinman et al. 2003).

DCs are considered the linkers of innate and adaptive immunity since they not only activate innate cells through production of soluble mediators (cytokines and chemokines) in response to foreign antigen, but also migrate from sites of infection to lymphoid organs, where they promote T cell activation.

DCs express receptors that recognize pathogen-associated molecular patterns (PAMPs) on invading microbes. Upon engagement, pathogen recognition receptors (PRRs) start a profound genetic and functional transformation of DCs which is known as maturation, or else terminal differentiation (Banchereau et al. 2000), (Janeway and Medzhitov 2002).

The ability of DC to discriminate between self and non-self molecules is the basis of the immunology theory proposed by Janeway in 1992, which is based on the principle of self vs. non-self discrimination (Janeway 1992). This model, updated several times since it was proposed (Medzhitov and Janeway 2002), claims that germ-line encoded receptors – identified later as the Toll-like receptor (TLR) family – expressed on DCs and other cells are responsible for identifying non-self molecules or entities.

A second theoretical framework of how immunity is triggered is the “Danger model”, which was proposed by Matzinger in 1994 (Matzinger 1994). The “Danger model” proposes that DCs respond to “Danger signals” of various molecular forms

independently of the presence of foreign antigen (Matzinger 2002). A vast amount of experimental data can support both models since DCs mature in response to non-self molecules found in microbes by PRR engagement, but also 'danger signals' from the microenvironment that are generated by a variety of cells, including granulocytes, epithelial cells and others, as a consequence of either tissue damage or infection; those signals include cytokines like IL-1 and TNF-α, but also heat-shock proteins or uric acid (Matzinger 2002) (Shi et al. 2003).

Whatever model of immunology is considered, DC maturation is an obligatory step in the activation of naïve T cells, and is characterized by the following changes in DCs:

- migration to lymph nodes, mediated by expression of chemokine receptors;
- antigen processing and presentation through peptide/MHC (class I and II) complexes and glycolipid/CD1 molecules;
- increased expression of co-stimulatory molecules (CD40, CD80, CD83, CD86);
- production of soluble immune regulators (cytokines and chemokines).

The changes in DC gene expression occur while the immature DC migrates from the place of infection to the closest lymphoid tissues; there, the mature DC can provide three different signals to T cells: MHC-antigen complex that binds to TCR; co-stimulation through ligation of CD80/CD86 with CD28 and CD40 with CD40L; and finally soluble molecules that vary according to the maturation stimulus. The nature of the soluble signal and also the presence or absence of the first two signals will together determine the nature of the T cell responses that are triggered by DCs (Kapsenberg 2003).

Either through direct receptor engagement in response to microbes or by indirect reaction to endogenous molecules/danger signals, the process of DC maturation and migration to lymphoid tissues will determine how they interact with T cells, B cells, NK cells and other members of the immune system, as depicted in figure 1.6.

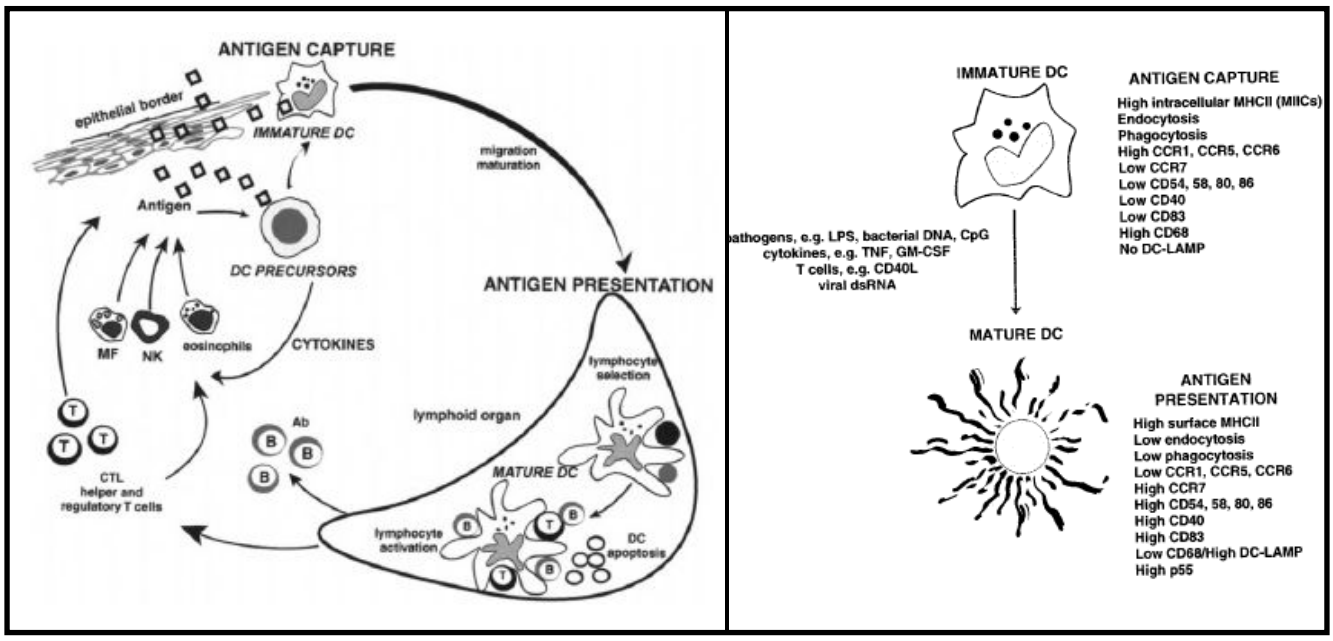


Figure 1.6 – The process of dendritic cell (DC) maturation. (A) Role of DCs among the processes of innate and adaptive immune reactions to microbes; (B) Differences between immature and mature DCs (adapted from Banchereau 2000).

Each one of the functions associated with DC maturation can be targeted for inhibition by the different pathogens as a way of modulating both innate and adaptive responses, and can even induce a general immunesuppression of the host (Palucka and Banchereau 2002). Strategies for microbe evasion of immune responses through manipulation of DCs will be discussed further on.

1.3.2 DC plasticity and regulation of immunity

For some time, DC maturation was considered a rigid program; however, many advances on the ability of DCs to respond differently to diverse pathogens have been reported in recent years, as reviewed in (Kapsenberg 2003).

The property by which the same DC population can trigger diverse signals in response to activation of different receptors is called “DC plasticity”. Alternatively, the induction of different T cell responses by DCs might be explained by differences among DC subsets – “DC selection” (Palucka and Banchereau 2002).

The three main types of T cell phenotypes that are induced from naïve CD4+T cells upon contact with DCs are: T helper 1(T_H1), T helper 2 (T_H2) and Regulatory T cells (Treg), as depicted in figure 1.7.

T_H1 and T_H2 are two different phenotypes of effector T cells that either target intracellular infections through cytotoxic responses (T_H1), or extracellular infections using antibodies (T_H2). Tregs have the ability to suppress effector T cells, either T_H1 or T_H2. Tregs can be beneficial to the host by controlling excessive inflammation and avoiding tissue destruction, but may also benefit infectious agents by inducing a generalized state of immune suppression (Kapsenberg 2003) (Belkaid and Rouse 2005).

DCs react to different maturation stimuli by releasing soluble molecules that polarize naïve CD4+ T cells into each of the different phenotypes. The factors that regulate DC maturation and production of the cytokines released by DCs are divided into three classes according to their polarizing effect on T cells (fig 1.7); those factors are PAMPs that bind to TLRs and other PRRs, or else tissue factors such as heat-shock proteins, extracellular matrix components and eicosanoids, which can all regulate DC maturation. The ability of PAMPs and tissue factors to polarize T cells is mediated by products of DCs that are able to regulate T cell functions: IL-12, IL-23, IL-27, type I IFNs (α,β) and ICAM-1 for T_H1; MCP1/CXCL2 and OX40L for T_H2; and IL-10 and TGF-β for Treg. These polarizing factors are usually weakly or transiently expressed, but can be enhanced by T-cell crosstalk, such as the CD40L-induced IL-12 production (Kapsenberg 2003).

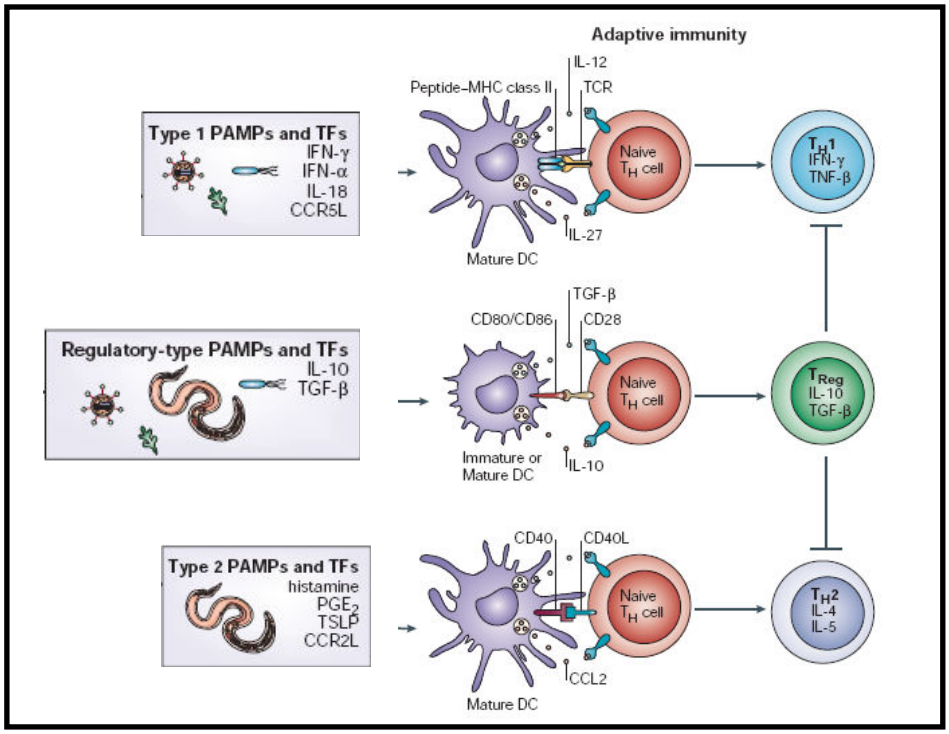


Figure 1.7 – Polarizing signals provided by DCs to naive CD4+T cells and the respective pathogens and tissue factors that trigger each phenotype (adapted from (Kapsenberg 2003))

The model of DC plasticity, which argues that a given DC population has the ability to induce different T cell responses to different stimuli, is supported by a large number of functional studies that show how activation of different TLRs in the same DC population results in expression of different cytokines (Akira and Takeda 2004). Using a different approach, studies at the gene expression level with microarray technology have determined how global DC gene expression is rearranged in response to diverse pathogens, from virus and bacteria to parasites (Huang et al. 2001) (Chaussabel et al. 2003). These studies show that DC maturation is comprised of a common program of transcriptomic regulation that is triggered in response to all kinds of microbes; such program is part of a larger common host response to infection (Jenner and Young 2005). Nevertheless, individual responses to each pathogen include the regulation of genes that are not shared with other responses and constitute the specific “signature” of that

microbe. The extent of this pathogen-specific signature in the whole transcriptomic responses is variable but considerable (Huang et al. 2001).

The fact that DCs respond differently to different infections also relies on the fact that DCs are a heterogenous population comprised of subsets with different receptor expression. The main classes of DCs are the myeloid ($CD11c^{hi}$) and the plasmacytoid (lymphoid lineage, $CD11c^{low}$) (Shortman and Liu 2002). Besides varying in their receptor expression, DC subsets can respond differently in the gene expression triggered by the same receptor. An example is TLR7, which triggers IL-12 production in myeloid DCs and IFN-α in plasmacytoid DCs, both enhancing T_H1 response (Kapsenberg 2003).

Both the ability of a DC population to discriminate between infections and the differences in DC subsets account for the variability of DC responses to pathogens (Palucka and Banchereau 2002).

1.3.3 DC receptors and their signaling pathways

Many PRRs are now characterized that can bind PAMPs of bacterial, viral and parasitic origin (Janeway and Medzhitov 2002) (McGuinness et al. 2003). The best characterized family of PRRs is the Toll-like receptor (TLR) family; other examples are the C-type lectins (macrophage mannose receptor/MMR, DC-specific intercellular adhesion molecule-grabbing nonintegrin/DC-SIGN), the scavenger receptors (MARCO/SR-A, CD36/SR-B) and the complement receptors (Janeway and Medzhitov 2002).

An extensive number of ligands have been identified for the 11 TLRs, ranging from molecules of pathogens to synthetic compounds and also host molecules such as oligosaccharides and heat-shock proteins (Akira and Takeda 2004). While most TLRs

are located at the cell membrane, a few TLRs (3, 7, 8 and 9) are targeted to the endosome, where they recognize ligands that have been internalized via phagocytosis.

TLR activation triggers diverse signaling pathways, as reviewed in (Akira and Takeda 2004). Two main independent signaling pathways are activated by TLRs: MyD88-dependent and TRIF-dependent. Together, the two pathways control nuclear translocation of NF-kB transcription factors, which regulate expression of a large number of genes including inflammation mediators such as IL-1α, IL-1β, TNF-α and IL-6. Both MyD88 and TRIF pathways can be triggered by a given TLR, but with different kinetics, resulting in two consecutive waves of NF-kB-dependent gene expression. A subset of TLR-dependent pathways activates a third pathway that relies on the Interferon Regulatory Factor 3 (IRF-3) for the expression of type I IFNs (IFN-α and -β), as well as IFN-inducible genes.

Signaling pathways for other PRRs besides TLRs are not known in great detail, even if some of those receptors are known to have profound effects on DC functions.

CD36 binds PfEMP-1 in malaria-infected erythrocytes, but also phosphatidilserine on apoptotic bodies; it has been suggested to be an inducer of tolerance in both situations by enhancing IL-10 production (Urban et al. 2001). Still, the mediators of gene expression in response to CD36 activation were not identified.

The role of C-type lectins in pattern recognition has been explored for some time, as reviewed in (Geijtenbeek et al. 2004). One important C-type lectin is DC-SIGN; it has been identified as a receptor for yeast zymosan (Taylor et al. 2004), a mediator of DC invasion and inhibition by *Mycobacterium tuberculosis* (Geijtenbeek et al. 2003) and as a facilitator of HIV infection (Geijtenbeek et al. 2000). Dectin-1, another C-type lectin, mediates IL-10 production in response to yeast zymosan, through a mechanism that requires Syk kinase and is independent from cooperation with TLRs (Rogers et al.

2005). These studies suggest that non-TLR receptors can use distinct signaling pathways from those of TLRs that are also able to regulate cytokine expression.

1.3.4 Evasion of immunity through modulation of DCs

Pathogens have devised a large number of mechanisms for the inhibition of both innate and adaptive immunity, as reviewed in (Finlay and McFadden 2006), and in (Sacks and Sher 2002) for the case of parasitic infections. Many of these strategies rely on the modulation of DC functions, given their prominent role in shaping adaptive immunity (Palucka and Banchereau 2002).

Some pathogens can interfere with the number of available DCs by inhibiting their generation from bone marrow precursors, by modulating DC migration to the site of infection, and by interfering with DC survival and apoptosis. One example is the infection of monocytes by the human T cell leukemia virus type I, which inhibits the differentiation into DCs (Palucka and Banchereau 2002). DC migration is targeted by poxviruses and herpesviruses that encode chemokine antagonists, but also by parasites such as *Schistosoma* and *Leishmania* (Palucka and Banchereau 2002) (Moll 2003).

Other strategies for immunity evasion include direct interference in one or more events associated with DC maturation such as antigen presentation, expression of co-stimulatory molecules, and cytokine production; targeting these processes will impair DC maturation and inhibit the development of anti-microbe effector T cells, since all three signals are required for optimal adaptive responses. Some common strategies for modulation of DC maturation are: inhibition of MHC/peptide complexes (human cytomegalovirus/HCMV), inhibition of co-stimulatory molecule expression (*P. falciparum, T. cruzi*), blockage of IL-12 production (measles virus, Mycobacteria, *T. gondii*) and

enhanced IL-10 production (Epstein-Barr virus, *Bordetella pertussis*). The usual outcomes of an impaired DC maturation are the induction of microbe-specific tolerance, what seems to happen in early HIV infection, and also the generation of regulatory T cells (Tregs). Many infectious agents can interact with DCs in such a way to induce Tregs, as reviewed in (Belkaid and Rouse 2005); those agents include viruses (HIV, cytomegalovirus, hepatitis C), bacteria (*Helicobacter*, *Listeria*), eukaryotic parasites (*Leishmania*, *Schistosoma*) and yeast (*Candida albicans*).

Besides inducing Tregs, pathogens can induce DCs to promote direct T cell death, phenotype known as “killer” or “cytotoxic DCs” (measles, HCMV).

Understanding the mechanisms used by pathogens to subvert DC functions to their advantage may lead us to new strategies against infectious agents that induce chronic infection or general immunesuppression such as *Plasmodium*, the causative agent of malaria (Greenwood et al. 1972) (Ho et al. 1986).

1.4 Role of dendritic cells in immunity to blood-stage malaria– PhD Significance & Objectives

1.4.1 DC response to blood-stage malaria

Malaria is the leading cause of parasitic-associated mortality, with 500 million new infections and 1 million deaths each year (Snow et al. 2005). To understand how malaria is widespread as ever in the tropical regions it is crucial to understand all mechanisms involved in the immune responses from the human host, as well as those that are actively suppressed by the parasite.

As described previously, malaria has special characteristics when compared to other infections in regard to the time it subsists in the human host and the lack of efficient induction of immune memory (Struik and Riley 2004); malaria immunesuppression includes the inhibition of adaptive responses to *Plasmodium* itself (Ho et al. 1986), increased susceptibility to co-infections such as HIV (Abu-Raddad et al. 2006), and reduced responses to vaccination (Williamson and Greenwood 1978).

DCs assume a central role in the initiation and modulation of adaptive immunity, besides interacting and regulating other cells of innate immunity (Banchereau et al. 2000) (Kapsenberg 2003). DCs are therefore key players in the response to any infection, including malaria. The way that DCs respond to *Plasmodium*-infected erythrocytes (IEs), which are the stages of the infection that contribute to immunity but also pathology, has been a subject of controversy (Stevenson and Urban 2006).

The first study on the interaction between IEs and DCs reported that human DCs fail to mature in response to *P. falciparum*-IEs; DCs were also unable to respond to a secondary stimulus such as lipopolyssacharide (LPS) (Urban et al. 1999). A later study

showed similar responses of mouse DCs to the rodent-infective species *P. yoelii* (Ocaña-Morgner et al. 2003). In this study, DCs were identified as agents of suppression of CD8+ T cells against the liver stages of malaria when DCs are previously exposed to Py-IEs, explaining why malaria liver infection that does not progress to blood stages (either through drug cure or by infection with γ-irradiated sporozoites) induces protective T cell responses, while the normal course of infection does not.

The data on malaria-induced DC inhibition has since then been challenged; DCs were found to mature *in vitro* in response to *P. chabaudi*-infected erythrocytes (Seixas et al. 2001), and also during *P. yoelii* (Perry et al. 2004) and *P. chabaudi* infections (Leisewitz et al. 2004).

These and the above studies differ in some of the methodologies used, including the method of *in vitro* DC generation, the time and load of infection at which DCs are extracted from infected mice, and the number of parasites used to start the infection; which of these could explain the differences observed has not been determined.

More recently, inhibition of DC functions in malaria has been challenged by data supporting that two molecules of *Plasmodium* induce DC maturation and cytokine production in the context of Toll-like receptors (TLR)-mediated signaling: DNA (+hemozoin) (Parroche et al. 2007) (Coban et al. 2005) and glycophosphatidilinositol (GPI) anchors (Krishnegowda et al. 2005).

Hemozoin is the heme-derived polymer that results from parasite-induced hemoglobin degradation. It was first shown to induce DC maturation by a TLR9-dependent mechanism by Coban and colleagues, including up-regulation of CD40 and CD86, and secretion of TNF-α, IL-12 and IL-6 (Coban et al. 2005). Until then, TLR9 was only known to bind CpG motifs in DNA of foreign origin. More recently it was shown that *Plasmodium*

DNA is the true ligand for TLR9 (Parroche et al. 2007); still, DNA localization to TLR9+ endosomes seems to require its conjugation with hemozoin.

GPI anchors are common in membrane proteins of mammalian and yeast cells, but also in parasites, with structures and immune activities that vary between parasite species (Ikezawa 2002) (Tachado et al. 1999). *Plasmodium* GPI has been proposed to be the main malaria toxin: GPI would be the main factor responsible for cytokine production and pathology in malaria, and a GPI-based vaccine would protect from disease (Schofield et al. 2002). Recently, the mechanism by which GPI induces secretion of TNF-α, IL-12 and IL-6 by macrophages was described to be mediated by TLR2 and TLR4 (Krishnegowda et al. 2005) (Zhu et al. 2005).

Even if DNA and GPI have been identified as inducers of DC maturation and cytokine production, whether the events triggered by those molecules will take place in response to whole infected erythrocytes and also during infection is still not clear.

1.4.2 Significance and Objectives – Why study the role of DCs in blood-stage malaria infection?

DCs are at the cornerstone of innate and adaptive immunity, modulating immune response to all kinds of pathogens (Banchereau et al. 2000). *Plasmodium* is a parasite of high complexity at the genetic, antigenic and life cycle levels, with many of its strategies to modulate host cells still to be unraveled.

One of the apparent paradoxes of the host response to this parasite is the way it induces waves of extreme inflammation without the (Baratin et al. 2005)concurrent induction of parasite control, even if it is able to vary its antigens that are exposed to the immune system of the host. Although immunity to severe disease is quickly established (Gupta et

al. 1999), parasite loads are maintained at high levels throughout infancy and adolescence in large fractions of the population in endemic areas (Struik and Riley 2004). It is clear that a balance between the beneficial and detrimental effects of immunity to *Plasmodium* takes years to be achieved; as a result, parasites persist for long periods of time in a given host.

DCs have the ability to control the extent of inflammation and also the nature of T cell responses (Kapsenberg 2003). In the case of malaria, DCs should promote inflammation in a way that enhances parasite killing without excessive tissue destruction. While some laboratories show roles for DCs in inducing Th1-like responses in malaria (Seixas et al. 2001) (Perry et al. 2004), others support the idea of DCs as agents of malaria-induced suppression of cellular immunity (Urban et al. 1999) (Ocaña-Morgner et al. 2003).

More recently, it has been proposed that the conflicting sets of data are the result of the maturation of DCs during the initial phase of infection, at low parasitemia, probably through TLR-dependent signals triggered by GPI and/or DNA; later in infection DCs are suppressed in their ability to respond to *Plasmodium* or other stimuli (Perry et al. 2005) (Urban and Todryk 2006). This model is similar to the mechanism known as “endotoxin tolerance”, by which DCs activated with LPS cannot respond to a new LPS stimulation, and that seems to apply to many TLR-dependent pathways (Fan and Cook 2004).

Strong evidence is still lacking today to support that DCs other than those of the plasmacytoid subset (Pichyangkul et al. 2004) are activated by *P. falciparum* –IEs; it is not clear if activation by GPI or DNA+hemozoin is triggered *in vivo* in the context of the whole parasite. In the case of GPI it was reported that it can be degraded by the macrophage surface phospholipases into inactive species, providing a mechanism for host regulation of GPI activity (Krishnegowda et al. 2005). *Plasmodium* DNA seems to be able to activate TLR9 if conjugated with hemozoin (Parroche et al. 2007), but it is still

not clear if DCs that express TLR9 (Kadowaki et al. 2001) are the ones that encounter *Plasmodium*- infected erythrocytes (IEs).

The major organ for generation of immunity to malaria is the spleen (Engwerda et al. 2005), which contains diverse populations of resident DCs which are likely to play a role in the responses to *Plasmodium*-IEs; however, the role of spleen DCs in blood-stage malaria remains a controversial subject.

To investigate how the malaria parasite induces strong inflammatory responses while inhibiting adaptive responses, we propose that the interactions between *Plasmodium*-infected erythrocytes and DCs are crucial for the understanding of such phenomena. The molecular interactions, and specially gene regulation of DCs, will determine not only the early control of parasite by innate immunity, but also how T cells are instructed by DCs to respond – or not – to *Plasmodium*.

In order to clarify the knowledge on DC responses to *Plasmodium* –IEs, which was limited in 2002, when the work presented here was first planned, to only two reports (Urban et al. 1999) (Urban et al. 2001), we proposed to address the following objectives, so that the variety of molecular interactions between DCs and *Plasmodium*-IEs that take place in malaria infection begin to unravel:

Aim 1 – Dissect *in vivo* gene regulation of DCs by *Plasmodium* through microarray analysis

This aim comprises the following studies:

i) **Whole-genome transcriptomic analysis** using Affymetrix Genechips®, the most generally used method for microarray analysis of gene expression. For this purpose we will use *P. yoelii*, a relevant mouse model for immunity studies that was shown to induce similar *in vitro* responses from DCs as *P. falciparum*

(Ocaña-Morgner et al. 2003). In two different times after infection spleen DCs will be isolated and RNA extracted for analysis with the MOE430Av2.0 Genechip®, an oligonucleotide microarray containing ca. 14,500 well characterized *Mus Musculus* genes.

ii) **The identification of signaling pathways that regulate gene expression** in DCs in response to *Plasmodium* blood stages; from the data obtained with the microarray analysis, we will determine which genes are significantly regulated by *P. yoelii*, but also signaling pathways responsible for that regulation

Among the genes for which we will study their transcriptional regulation and the signaling pathways responsible for it, are the cytokine genes. Cytokines were chosen for their relevance in determining responses from other cells of the immune system; in particular in the case of malaria, several cytokines are known as promoters or inhibitors of the various pathologies (Stevenson and Riley 2004) (Schofield and Grau 2005). Up to 2003, the only two studies that addressed DC cytokine production in response to malaria were restricted to TNF-α, IL-12 and IL-10, which were not detected in response to *P. falciparum* or *P. yoelii* (Ocaña-Morgner et al. 2003) (Urban et al. 2001).

Aim 2 – Characterization of DC maturation and Toll-like receptor (TLR)-mediated signaling in the context of blood-stage malaria

This aim is designed to address the following questions:

i) Do *Plasmodium*-infected erythrocytes (IEs) inhibit responses to a variety of TLR-dependent stimuli, or is this restricted to LPS?

It was described that both *P. falciparum* and *P. yoelii* blood stages inhibit DC maturation in response to LPS (Urban et al. 1999) (Ocaña-Morgner et al. 2003), but it has not been addressed whether this phenomenon is restricted to LPS, or else is part of a general suppression of TLR-dependent DC maturation. For this purpose, we will investigate DC responses to a variety of TLR ligands in the context of secondary stimulation after DC incubation with *P. yoelii*-IEs, either in respect to expression of co-stimulatory molecules (CD40, CD80, CD86) or else production of relevant cytokines such as IL-12 or others.

ii) If confirmed, is the lack of DC maturation in responses to TLR stimulation a result of a defect in TLR-dependent gene expression, or else a result of a mechanism similar to endotoxin tolerance?

In case it is detected a general suppression of TLR-dependent DC maturation in response to *Plasmodium*, we hope to clarify the mechanism leading to that inhibition: is it caused by a molecular defect in the pathways regulating TLR-dependent gene expression, or is it caused by a *Plasmodium*-mediated DC maturation (not detected in previous studies) which suppresses the response to a secondary stimulation? The latter case would be similar to “endotoxin tolerance” (Fan and Cook 2004).

To address such questions we plan to clarify if *P. yoelii*-IEs alone are capable of inducing DC maturation in a number of different experimental conditions. If maturation is detected, we plan to study the mechanism behind this endotoxin tolerance-like situation; if DC maturation is not observed, we plan to detect which levels of TLR-dependent gene expression do not respond to stimulation after DC pre-incubation with *P. yoelii*-IEs.

iii) What is the role that DCs play in phagocytosis of *Plasmodium*-IEs and how does it relate to DC maturation?

So far, studies on the phagocytosis of *Plasmodium*-infected erythrocytes (IEs) have focused on the role of macrophages and monocytes. We envisage to characterize the ability of DCs to capture *P. yoelii*-IEs *in vitro*, but also *in vivo* (even if DC numbers are low when compared to macrophages), and how IE phagocytosis regulates DC functions, specially maturation, as defined by expression of co-stimulatory molecules, and if it is related to inhibition of secondary stimulation of DC maturation with TLR ligands.

Extended studies on the regulation of dendritic cell gene expression by *Plasmodium*, but also on other molecular and cellular interactions between DCs and the malaria parasite, such as phagocytosis, will clarify some of the roles of DCs, both in promoting the excessive inflammation associated with malaria infection, but also in regulating adaptive immunity; the modulation of DC functions by the parasite might play a role in the context of the immune suppression associated with malaria, with implications in the progression of infection, but also vaccinations and co-infections.

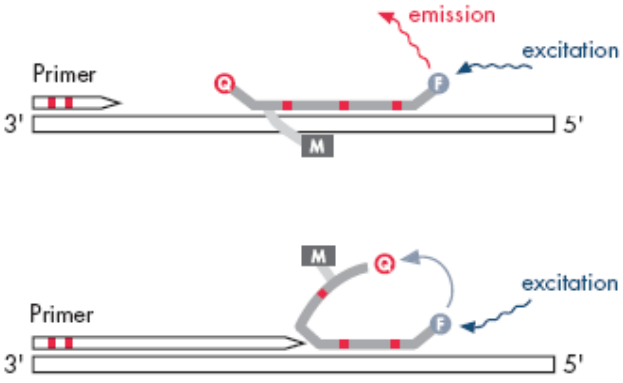
It might seem counterintuitive that DC might simultaneously be agents of inflammation and immune-suppression; however, the fact that, in recent years, some reports focused on *Plasmodium*-induced DC maturation and production of various cytokines, while others show *Plasmodium*-induced inhibition of DC functions, suggests that DCs play ambivalent roles, depending on the functions being assessed, but also the times and loads of infection being studied, among other factors. It is also well-characterized that several cytokines that are produced in response to inflammatory stimuli have inhibitory effects for T cell function (Haring et al. 2006). In particular prostaglandin E2 (PGE2),

whose effects are analyzed in our studies, is not only the universal mediator of fever, but also a potent modulator of T cell responses (Harris et al. 2002).

The continuing research on the interactions of malaria parasites with the host immune system will provide a better understanding of the mechanisms involved, so that better strategies for reducing the mortality and morbidity caused by malaria can be pursued.

Chapter II

MATERIALS AND METHODS



2.1 Mice and infections

BALB/C, C57BL/6 and Swiss Webster mice were purchased from NIH/NCI or Taconic, and housed in the Animal Facility of the New York University Medical Center according to protocols approved by IACUC. The strain of *Plasmodium* used was the non-lethal parasite *P. yoelii* 17XNL. For *in vitro* experiments, Swiss Webster mice were infected by mosquito bite. For *in vivo* experiments, BALB/C mice were infected with $5x10^6$ *P. yoelii*-infected erythrocytes, which were injected intra-peritoniously.

2.2 Magnetic bead extraction of CD11c+ dendritic cells (DCs) from mouse spleen

Total splenocyte suspensions were obtained from either non-infected or *P. yoelii*-infected mice by mechanical disruption with glass sides and filtering cells through a 100μm nylon cell strainer. Cells were then resuspended in ammonia buffer/ACK (155mM NH_4Cl, 1mM $KHCO_3$, 0.1mM Na_2EDTA) for lysis of erythrocytes. CD11c+ DCs were obtained by incubation with magnetic beads coupled to anti-mouse CD11c (Miltenyi Biotech) at 6 – 10 C for 15 minutes. Excess beads were then washed by centrifugation (200g, 10 min). The cell pellet was resuspended in PBS containing 0.5% FBS and 2mM EDTA and passed through a syringe coupled to a magnetic holder. Non-labeled cells were removed by washing. Labeled cells were eluted by removing the syringe from its holder. Cells in both labeled (CD11c+) and non-labeled fractions were washed, counted and equal numbers of each population were then used for either *ex vivo* cell culture, RNA extraction or FACS analysis. The amount of cells expressing high levels of CD11c in the CD11c+ fraction is typically between 80% and 90%.

2.3 Total RNA extraction

Total RNA from bone marrow-derived DCs, or else Spleen CD11c+ (myeloid DCs) and CD11c- cells was obtained from 2 x10^6 to 5 x10^6 cells using RNeasy reagents (Qiagen). DCs were lysed in a guanidine thiocyanate buffer with 1% (v/v) mercaptoethanol; lysates were then used for direct RNA extraction, or else stored at -80ºC. Total RNA was obtained from cell lysates by adding one volume of ethanol 70% in RNAse-free water and spinning at 10,000g for 15s on a tube containing a silica-gel membrane which retains the RNA. The membrane was washed with a guanidine hydrochloride buffer, followed by 2 washes with an ethanol solution. RNA was eluted with RNAse-free water. The procedure used allows for exclusion of RNAs smaller than 200 nucleotides (mostly transfer or ribosomal), therefore resulting in samples enriched in messenger RNAs.

RNA concentration was determined by optical densitometry at 260nm. Protein content was evaluated by the A260nm/A280nm ratio (consistently over 1.5).

2.4 Global transcriptomic analysis of spleen DC in *P. yoelii* infection

2.4.1 RNA processing and Genechip ® array hybridization

Each GeneChip® experiment was performed with one of the 6 independent RNA samples (2 replicate samples for each of the 3 conditions). RNA was processed for use on Affymetrix's (Santa Clara, CA, USA) Mouse Expression MOE430Av2.0 GeneChip®, according to the manufacturer's One-Cycle Target Labeling Assay.

2 µg of total RNA containing spiked in Poly-A RNA controls (GeneChip Eukaryotic Poly-A RNA Control Kit) were used in a reverse transcription reaction (One-Cycle DNA synthesis kit) to generate first-strand cDNA. After second-strand synthesis, double-stranded cDNA was used in an *in vitro* transcription (IVT) reaction to generate

biotinylated cRNA. Size distribution of the cRNA and fragmented cRNA was assessed using an Agilent 2100 Bioanalyzer with a RNA 6000 Nano Assay.

10 µg of fragmented cRNA was used in a 200-µl hybridization containing added hybridization controls. 130 µl of mixture was hybridized on arrays for 16 h at 45°C. Arrays were scanned on an Affymetrix GeneChip scanner 3000. All quality parameters for the arrays were confirmed to be in the recommended ranges.

2.4.2 Genechip ® data analysis

Results of expression levels detected with the MOE430A Genechip® in the form of .Cel files were loaded into ArrayAssist Software (Stratagene). A normalized master data table was created consisting of a matrix of expression (signal) values extracted from all the CEL files using the sequence-enhanced Robust Multi-Array Average (GC-RMA) algorithm (Wu and Irizarry 2004). Results were then analyzed by Multiclass testing method (p<0.01) for comparison of average expression levels within each pair of replicate arrays, and by cluster analysis using Pearson Correlations. Gene Ontology and KEGG pathway terms were obtained using the DAVID Functional Annotation Tools (http://david.abcc.ncifcrf.gov/).

2.5 Real-time quantitative PCR

1µg of each sample of total RNA was used for reverse transcription with MuLV reverse transcriptase (Applied Biosystems). 200ng of cDNA from each sample was used for real-time PCR amplification of target cDNA sequences using the Rotor 3000 (Corbett Robotics) in the presence of the fluorescent dye SYBR Green I. 1µl of cDNA (200ng) was mixed with “Quantitect SYBR Green PCR Master mix” (Qiagen), which includes the HotStarTaq® DNA Polymerase, PCR Buffer, SYBR Green I, dNTP (including dUTP),

and 5mM $MgCl_2$. Primers (designed by Qiagen) were added at 10ng/ml. PCR cycling was as follows: hot start at 95C for 10min, then 40-50 cycles of 95C, 10s (denaturation)/ 56C, 30s (annealing)/ 76C, 30s (extension). Acquisition of fluorescence was performed in the annealing steps of each cycle. Data analysis was performed using Rotor-Gene 6 (Corbett Research).

2.6 DC generation from bone marrow precursors

The culture of bone marrow precursors in the presence of Granulocyte/Macrophage Colony-Stimulating Factor (GM-CSF) is a common method for the *in vitro* generation of large amount of myeloid (CD11c+) DCs (Stockinger et al. 1996).

Bone marrow precursors were harvested from the tibia and femurs from sacrificed BALB/C mice by flushing the bones with complete DMEM containing 10% Fetal Bovine Serum (FBS) and also penicillin 100U/ml, streptomycin 100µg/ml and glutamine 2mM.

Cells were spinned down (400g, 5 min) and the pellet resuspended in ammonia buffer/ ACK (155mM NH_4Cl, 1mM $KHCO_3$, 0.1mM Na_2EDTA), which induces osmotic lysis of erythrocytes. After washing, the remaining cells were cultured in complete DMEM supplemented with 20% of conditioned medium from GM-CSF transfected cells. Medium was changed every 48h and cells split whenever confluent. After 8 days of culture, the cells in suspension grown this way are typically ca. 85% CD11c+.

2.7 Culture of GM-CSF transfected cells

Cells of the Ag8653 line transfected with GM-CSF were developed in the lab of Dr. Gray (Stockinger et al. 1996). Cells were cultured for 3 days in complete DMEM (10% FCS, penicillin, streptomycin and glutamine) with geneticin/G418 0.5mg/ml. Confluent cells were then harvested and transferred to complete DMEM without G418 at 10^7 cells/100ml

of medium. After 4 days in culture, cells were centrifuged and supernatant was passed through a 0.22μm filter.

The sterile medium thus obtained was stored up to 4 months at 4°C.

2.8 Purification of non-infected and *P. yoelii*-infected erythrocytes (IEs)

Swiss Webster mice, either non-infected or infected with *P. yoelii* 17XNL, were sacrificed and blood collected from thoracic cavity after cutting the heart. Blood was passed through a 100 μm nylon cell strainer, diluted in PBS and centrifuged at 800g for 3 min. Buffy coat was removed by pipetting from cell pellet, and erythrocytes washed with PBS. Total erythrocyte suspension from infected mice was reconstituted to 5ml per mouse.

Purification of *P. yoelii* schizonts was performed by layering 5 ml of total IE suspension onto 2ml of a 60% solution of Accudenz® (Accurate Chemical and Scientific Corp.) in PBS. The mixture was centrifuged at 200g for 25 min at 20°C. Using this method, an intermediate and a lower cell fraction are obtained; the intermediate fraction, which contains *P. yoelii*-IEs predominantly of the schizont stage (over 85%), was pipetted to a new tube, and cells washed with PBS, followed by centrifugation.

Blood from uninfected mice was also separated on a similar gradient, and erythrocytes that accumulate in the bottom fraction washed in PBS. Both uninfected and *P. yoelii*-IEs were counted and used for *in vitro* incubation with bone marrow-derived DCs.

2.9 *In vitro* culture of DCs with erythrocytes and drugs

Bone marrow-derived DCs were plated in either 24- or 96-well plates at 10^6 DCs/ml in complete DMEM with 5% conditioned medium. DCs were pre-incubated for the times provided with the indicated drugs (or DMSO) before addition of uninfected erythrocytes

or else purified *P. yoelii*- IEs (schizont stage), at a ratio of 30/DC, unless otherwise indicated. In the case of ELISA experiments, medium was collected and centrifuged at 800g for 3min and supernatants stored at -20C.

Bone marrow-derived DCs were stimulated with the following TLR ligands (Invivogen, except LPS): LPS (lipopolysaccharide) from *Escherichia coli* at 1μg/ml (Sigma), ODN-CpG (synthetic oligonucleotides containing unmethylated CpG dinucleotides) at 1μg/ml, zymosan from *Saccharomyces cerevisiae* at 50μg/ml, polyI:C (Polyinosinic polycytidylic acid) at 25μg/ml, flagelin from *Salmonella thyphimurium* at 100ng/ml and loxoribine (guanosine analogue) at 50μM.

Purified bioactive PGE2 (Cayman Chemicals) was used at 200pg/ml.

Forskolin from *Coleous forskohlii* (Calbiochem), which activates adenylate cyclase, was used at 0.1mM. The following cyclic AMP analogs (Calbiochem) were also used: 500μM 8-Br-cAMP, 20μM 8-CPT-2OMecAMP (specific activator of Exchange Protein directly Activated by cAMP – EPAC). Inhibitors (Calbiochem) used were: 5μg/ml indomethacin (cyclooxygenase inhibitor), 10μM H89 (Protein kinase cAMP-dependent inhibitor), 10μM Rp-8Br-cAMPS (cyclic AMP antagonist), 10μM SB203580 (p38-MAPK inhibitor), 10μM SB203474 (inactive analog of SB203580).

2.10 Flow cytometry analysis of DC membrane expression of co-stimulatory molecules

After stimulation, DCs were incubated in FACS buffer (PBS+3% FBS) with 1/100 dilution of “Fc block” (CD16/CD32, BD/Pharmingen) for 30 min at 4C for blockage of non-specific binding and Fc receptors. DCs were stained with one of the following mouse monoclonal antibodies at 1/100 dilution in FACS buffer at 4C for 30 min: FITC anti-

CD40, FITC anti-CD86 (all from BD/Pharmingen) and FITC anti-CD83 (Biolegend). PE anti-CD11c was also added to all samples at 1/100 dilution. Antibodies were washed 3 times with FACS buffer. Cells were then incubated with 7-amino-actinomycin (7-AAD, BD/Pharmingen) for 10 min before fluorescence acquisition in a Becton-Dinckinson FACScan. Cells were gated on expression of CD11c and absence of 7-AAD staining for exclusion of both non-viable and non-DC cells.

2.11 Enzyme Immunoassays

2.11.1 Enzyme-linked immunosorbent assay (ELISA) for quantification of extracellular cytokines

Concentrations of IL-6, IL-10, IL-12p70 and TNF-α in the culture medium of DCs were determined using the respective mouse BD Opteia systems (BD Biosciences) according to the manufacturer's instructions. 96-well plates were plated with coating anti-mouse antibodies for 16h. Plates were washed 3 times with PBS + 0.05%Tween (PBS-T). 200μl of PBS with 10% FBS was added for 1h at room temperature (RT). Plates were washed 3 times. 100μl of cell culture supernatant, cytokine standards, or else DMEM was added to each well for 2h at RT. Plates were washed 5 times. 100μl of developing mix containing biotinylated anti-mouse antibody and enzyme (streptavidin-horseradish peroxidase conjugate) was added for 1h at RT. Plates were washed 7 times. 100μl of substrate (tetramethyl-benzidine/TMB + H_2O_2) was added per well for 30min at RT, followed by addition of 50μl of H_2SO_4 2N. Optical density was measured at 450nm within 30 min.

2.11.2 Enzyme Immunoassay (EIA) for quantification of extracellular Prostaglandin E2 (PGE2)

PGE2 concentrations in the culture medium of DCs were determined using an Enzyme Immunoassay (Cayman Chemicals). 50μl of cell culture supernatant or PGE2 standard was added to the wells of a 96-well plate coated with goat polyclonal anti-mouse IgG. In addition to samples or standards, 50μl of PGE2 monoclonal antibody and 50μl of PGE2 tracer (PGE2-linked acetylcholinesterase) were added to all wells (except wells for evaluation of non-specific binding) and incubated for 1h at room temperature (or else 18h at 4C). Wells were washed 5 times with PBS-T. 200μl of substrate (acetylthiocholine+5,5'-dithio-bis-2-nitrobenzoic acid) was incubated at RT. Optical density was measured every 30 min at 405nm (detection of 5-thio-2-nitrobenzoic acid) until absorbance in the non-specific binding controls was higher than 0.3AU.

2.11.3 Enzyme Immunoassay (EIA) for quantification of intracellular cyclic AMP

Intracellular cyclic adenosine-monophosphate (cAMP) in bone marrow-derived DCs was determined by competitive ELISA of cell lysates using reagents from GE Healthcare. Briefly, DCs were plated at 10^6/ml in 96-well plates. After stimulation, culture medium was removed and 200μl/well of lysis buffer was added to cells. Cells were agitated by pipetting and horizontal shaking for 10min at RT. Lysates were stored at -80C for posterior quantification of cyclic AMP. After thawing lysates, 100μl of each one was transferred to a 96-well plate coated with donkey anti-rabbit IgG. Also 100μl of each cAMP standard was added to other wells. 100μl of anti-serum (rabbit anti-cAMP) was added to all wells (except non-specific binding controls) and incubated for 2h at approx. 4C. 50μl of cAMP-horseradish peroxidase conjugate was then added to all wells and

incubated for another hour at 4C. Wells were washed 4 times with PBS-T. 150μl of enzyme substrate (tetramethyl-benzidine+H_2O_2) was added and incubated for 1h at RT. 50μl of H2SO4 2N was added and optical density at 450nm was measured within 30 min.

2.12 Erythrocyte staining with CFSE

For single cell fluorescence analysis of interactions between DCs and erythrocytes (*P.yoelii*-infected or uninfected), the latter were stained with a solution of carboxyfluorescein diacetate, succinimidyl ester (CFSE). This reagent passively diffuses into cells, but only becomes fluorescent in the cell cytoplasm after cleavage of acetate groups by intracellular esterases, yielding carboxyfluorescein succinimidyl ester.

Erythrocytes were incubated in PBS+ 10μM CFSE for 15 minutes at 37C, at a density of $2x10^7$erythrocytes/ml. Erythrocytes were centrifuged (800g, 3 min) and resuspended in complete RPMI (10% FBS, penicillin 100U/ml, streptomycin 100μg/ml and glutamine 2mM) at the same density as before, and incubated another 15min at 37C. Excess CFSE was washed by two rounds of centrifugation and resuspension with RPMI. CFSE-labeled erythrocytes were then resuspended in complete RPMI and used for *in vitro* culture with bone marrow-derived DCs.

2.13 Immunocytochemistry studies of DCs

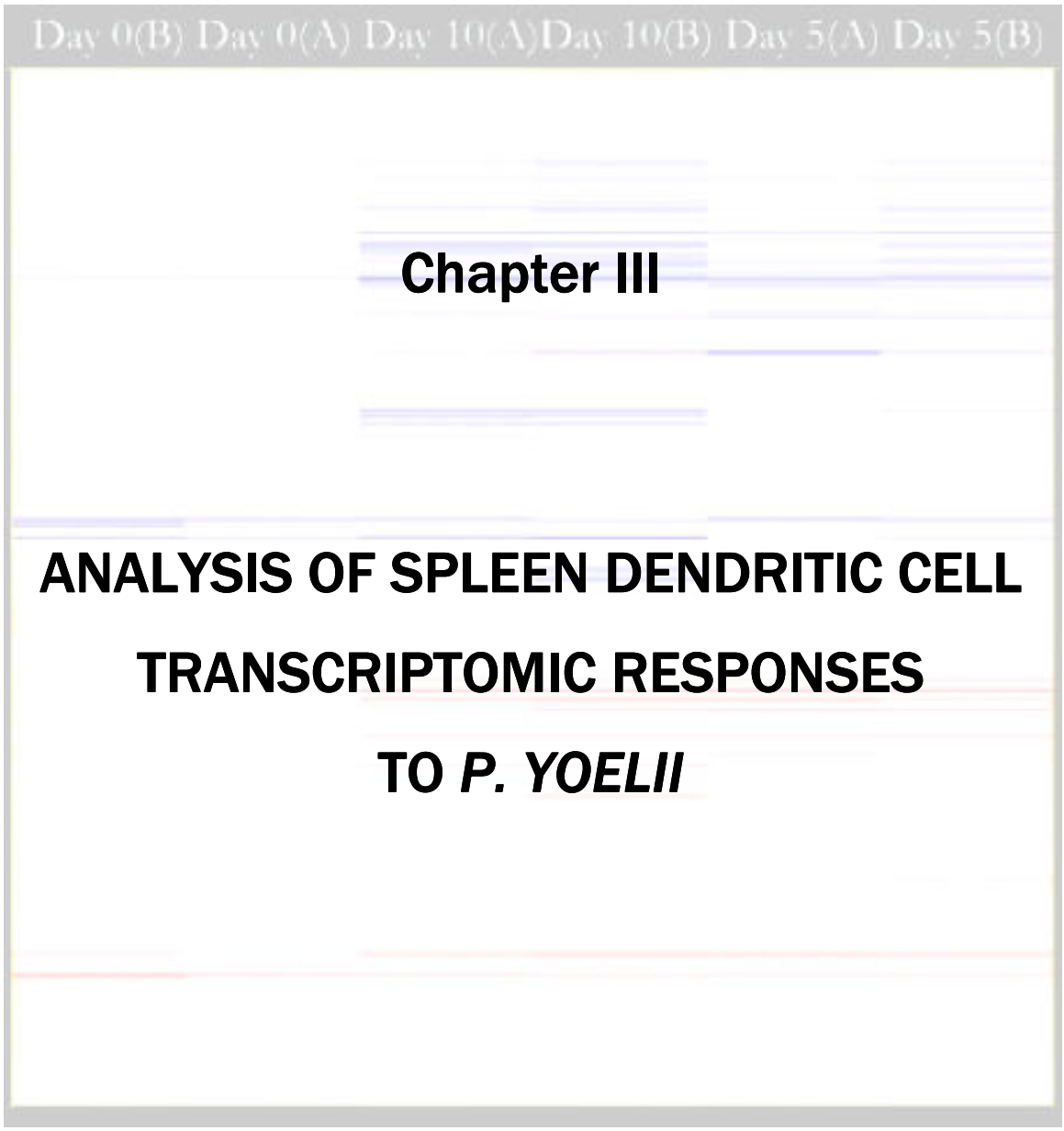
2.13.1 Membrane staining of co-stimulatory molecules

DCs plated on 24-well plates containing glass coverslips at $5x10^5$/ml; DCs were stimulated and then fixed with PFA 2% for 15min. Cells were blocked for 30 min with 10% goat serum in PBS with 100mM glycine (non-permeant blocking solution) + “Fc block” (BD/Pharmingen), and then stained for 30min in PE-anti-CD40 or PE-anti-CD86 in

blocking solution. Excess antibody is washed with PBS. Coverslips are incubated for 5min on DAPI 10µg/ml (4',6-diamidino-2-phenylindole dihydrochloride, Sigma) for nuclei staining, washed in PBS and mounted on a drop of Gel/mount (Biomeda). Fluorescence analysis is done on an OlympusIX70 microscope using the Metamorph v4.5 software.

2.13.2 Intracellular staining of NF-κB transcription factors

DCs plated on 24-well plates containing glass coverslips at $5x10^5$/ml; DCs were stimulated and then fixed with PFA 4% for 30min. Cells were blocked for 30 min with 10% goat serum in PBS with 100mM glycine and 0.2% saponin (permeant blocking solution) + "Fc block" (BD/Pharmingen), and then stained for 30 min in blocking solution with anti-mouse relA (Santa Cruz). After washing in PBS, FITC anti-mouse IgG (Sigma) in blocking solution is added for another 30 min. Excess antibody is washed with PBS. Coverslips are processed and fluorescence analyzed as described before.



Chapter III

ANALYSIS OF SPLEEN DENDRITIC CELL TRANSCRIPTOMIC RESPONSES TO *P. YOELII*

3.1 General analysis of *P. yoelii*-induced transcriptomic regulation in spleen DCs using Genechip® technology

Plasmodium blood-stage parasites have been shown to either induce or else inhibit Dendritic Cell (DC) maturation, according to different reports that look at the expression of a restricted group of proteins, the co-stimulatory molecules, that are associated with the standard DC maturation response (Stevenson and Urban 2006) (Urban and Todryk 2006).

In order to gain a larger understanding of the interactions between *Plasmodium*-infected erythrocytes (IEs) and DCs, we decided to extend studies on the interactions of DCs with blood stage malaria to the whole genome of the host. For that purpose, we used the Genechip® technology from Affymetrix to analyze the global transcriptomic responses of DCs extracted from Spleens of uninfected BALB/C mice (from hereon designated as 'day 0'), or else from Spleens of *P. yoelii*-infected BALB/C mice at two different times post-infection (pi), with considerable differences in the load of infection: day 5 pi (8.1±0.2% parasitemia) and day 10 pi (28.3±2.6%). The parasitemia profile of *P. yoelii yoelii* 17XNL in BALB/C mice is show in fig. 3.1. Infections were started with an intraperitoneal injection of $5x10^6$ *P. yoelii*-IEs.

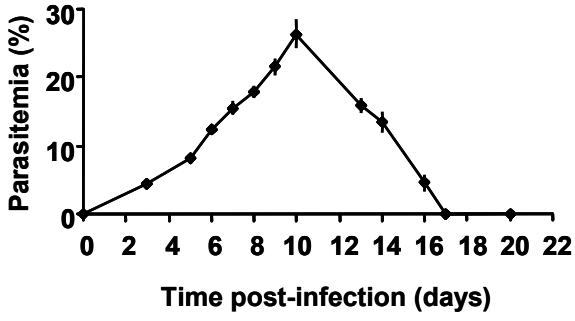


Figure 3.1 – Parasitemia over the course of *P. yoelii 17XNL* infection in BALB/C mice used for microarray analysis of spleen dendritic cells (CD11c+).

For each time of infection (day 0, day 5, day 10), two samples of RNA of CD11c+ Spleen DCs were obtained in two independent experiments, adding up to a total of six hybridizations onto the Mouse Expression Array MOE430Av2.0; the Genechip® used contains eleven pairs of probes selected from the 3' end of each of ca. 14,500 well characterized *Mus Musculus* genes, therefore allowing for a robust quantitation of transcription at the genome scale.

There was a high reproducibility between replica samples as measured by the high Pearson correlation coefficient between samples from the same time point (0.992-0.995), when compared to different time points: Day 0 vs. Day 5 (0.970-0.972), day 0 vs. day 10 (0.959-0.970), and day 5 vs. day 10 (0.984-0.990). The similarities between replicate arrays can be observed by scatter plots of expression values for all probesets in any given pair of samples (Fig. 3.2).

The same relationships are easily visualized by cluster analysis, which highlights the extensive similarity between replicate arrays, but also between arrays from the different samples from infected mice (day 5pi and day 10pi), compared to samples from uninfected mice (Fig. 3.3).

Using significance analysis of multiclass testing type, and a cutoff of $p<0.01$, we found that *P. yoelii* regulates a total of 698 number of unique genes or ESTs at the transcription level with 2-fold or higher difference from DCs of uninfected mice. This was obtained considering both times post-infection, and both induced and repressed transcripts (Table 3.1). The total number of genes was obtained after exclusion of redundant probesets (sequences from the same given gene/EST).

The distribution of the 698 *P. yoelii*-regulated transcripts was found to have an extensive overlap of the genes regulated at day 5 and day 10 (514 transcripts, 90% and 81% of regulated transcripts at day 5 and day 10, respectively). This overlap was also found when induced and repressed genes were analyzed separately (Table 3.I, fig. 3.4).

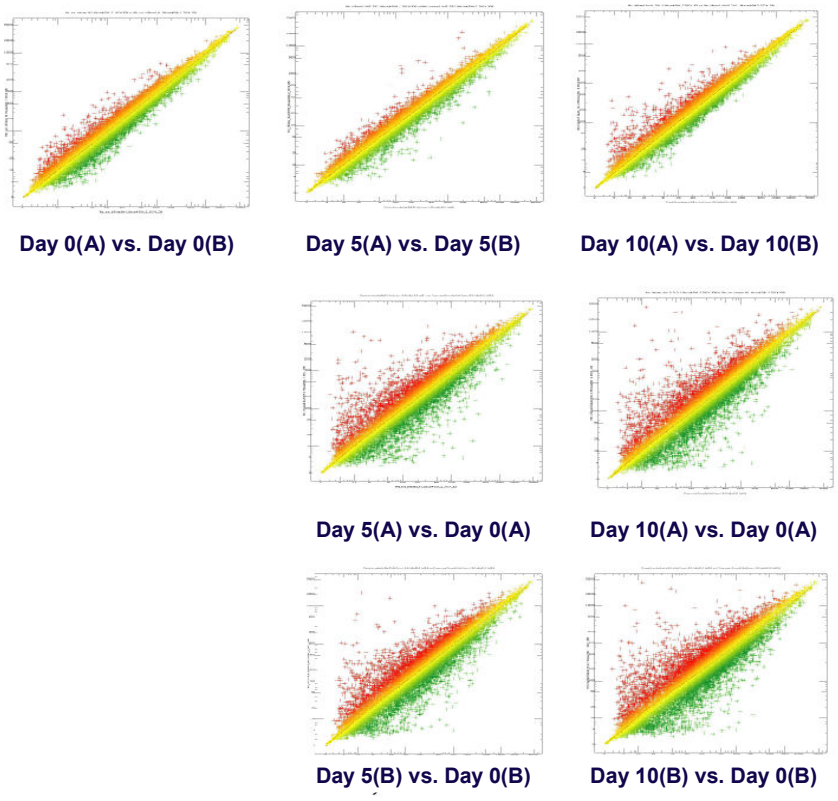


Figure 3.2 – Scatter Plots of Expression Values for all probesets in the MOE430Av2.0 Genechips ® for different pairs of individual hybridizations, either duplicates of the same experimental condition (first row) or between samples of different experimental conditions (second, third rows).

Samples compared	UNIQUE GENES/ESTs		
	Induced	Repressed	Total
D5/D0 (A)	276	298	574
D10/D0 (B)	293	341	634
Common A, B	242	272	514
D10/D5 (C)	51	56	107
Total of transcripts	**330**	**368**	**698**

Table 3.I – Total number of MOE430v2.0 GeneChip® unique genes or ESTs regulated by *P. yoelii* infection in spleen DCs. Total numbers of genes induced or repressed are presented for all comparisons of any two experimental conditions: between uninfected and day 5 pi (D5/D0), uninfected and day 10 pi (D10/D0), and between days 5 and 10 pi (D10/D5), as well as the number of common transcripts between the first two comparisons.

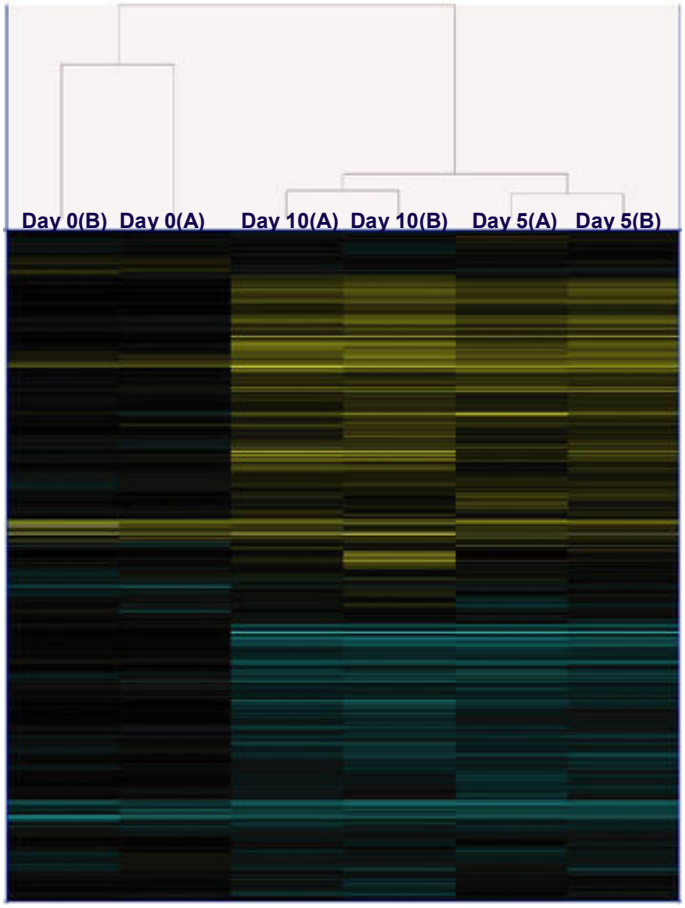


Figure 3.3 – Cluster analysis of genes detected by microarray analysis of spleen DCs at different times of *P. yoelii* infection. Six mRNA samples from CD11c+ splenocytes obtained from Balb/C mice at different times of infection were hybridized onto MOE430A v2.0 Genechip. A cluster analysis (Pearson correlation, average linkage method) of the 6.646 genes detected as 'Present' in at least one of the samples, and with max-min signal values in the six samples above 1 (2 fold change) is shown. Yellow and green indicate induced and repressed genes, respectively.

We segregated the 698 differentially regulated transcripts between those that are: a) induced upon infection (327 genes, 46.8%); b) repressed upon infection (367 genes, 52.6%); c) differentially regulated only when the two times post-infection are compared between each other (4 genes, 0.6%). No genes were found to have opposite regulation at day 5 and day 10 pi when each one is compared to DCs from uninfected mice. Examples of individual genes following different expression profiles, including genes with functions in Defense Responses, are shown in Fig. 3.5. Complete lists of the 698 *P. yoelii*-regulated transcripts in Spleen DCs can be found in Appendix I.

The data suggests that the universe of genes regulated in DC of the spleen during *P. yoelii* infection is very similar at these two different times of infection, which correspond to significantly different levels of parasitemia. Still, a limited number of genes were found

to be regulated specifically at one or the other time of infection, possibly reflecting changes in the response of DCs over time of infection.

The high numbers of transcripts found to have significant modulation in Spleen DCs at the two times of *P. yoelii* infection studied suggest that malaria infection cause a profound genetic reprogramming of DCs, which will very likely affect diverse metabolic pathways, biological processes and DC functions related to their role in immunity. We next investigated which cellular functions have the highest number of transcripts modulated by *P. yoelii* infection in Spleen DCs.

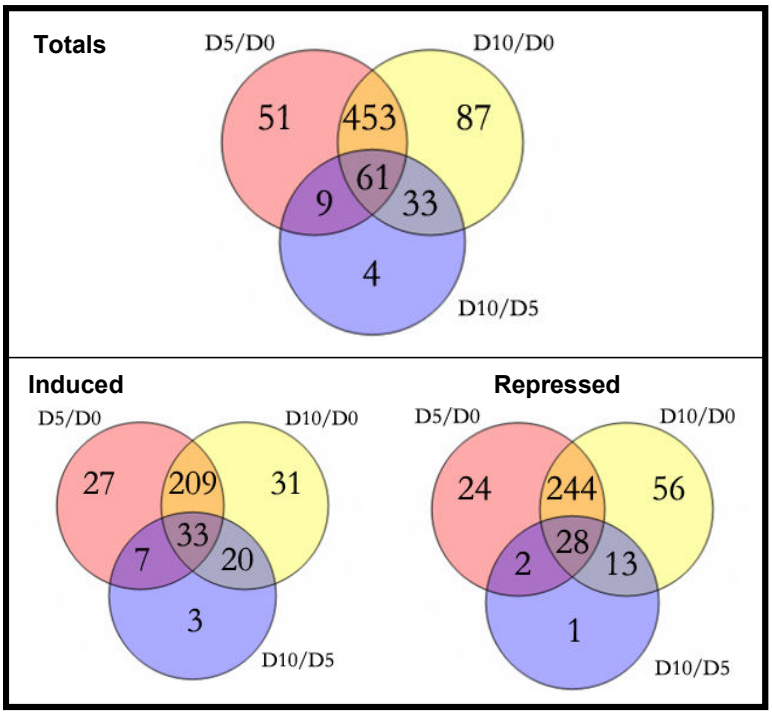


Figure 3.4 – Total numbers of unique genes regulated at the transcription level in spleen DCs by *P. yoelii* infection. Venn diagrams show the numbers of total, induced and repressed transcripts with differential regulation (over 2-fold) during *P. yoelii* infection at either day 5 or day 10 post-infection ($p<0.01$). Expression levels for each pair of replica arrays: uninfected (D0), Day 5 (D5) and Day 10 (D10) were compared using multiclass significance analysis.

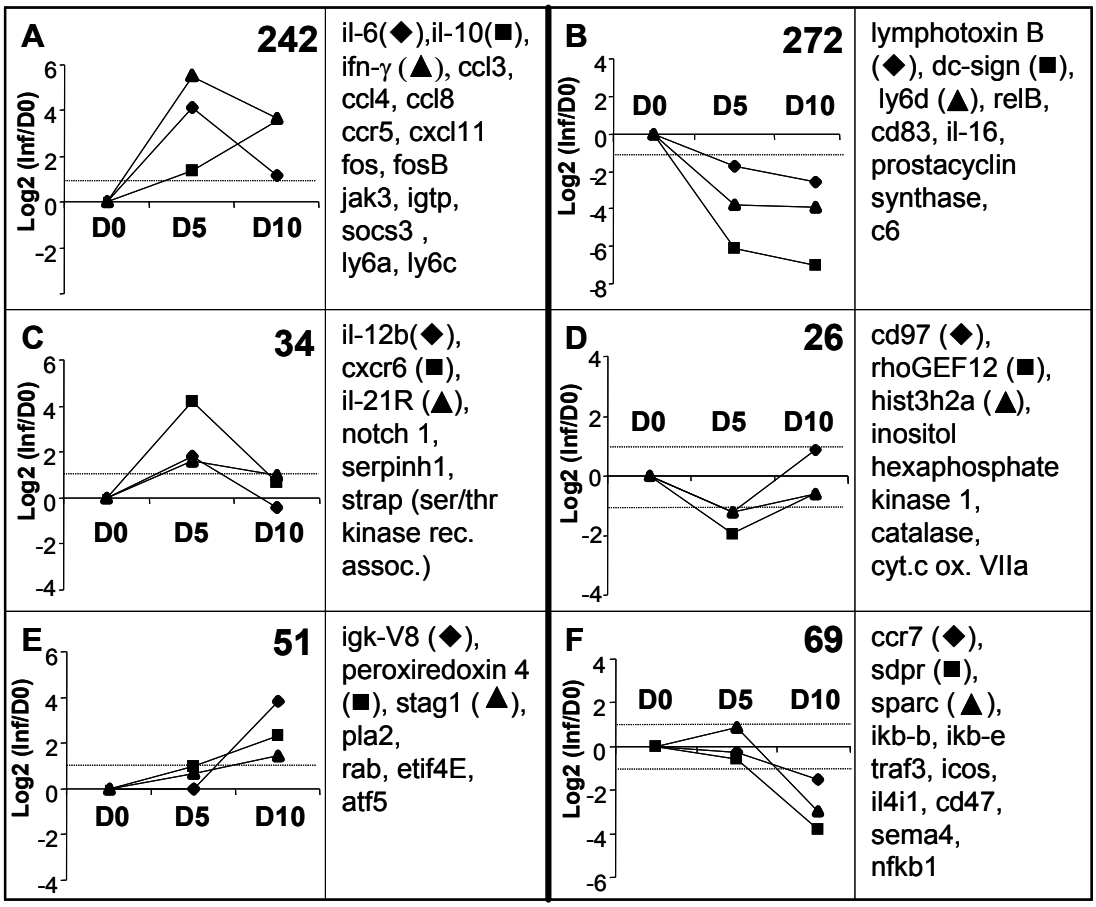


Figure 3.5 – Numbers and examples of transcripts regulated in spleen DCs at the transcription level by *P. yoelii* infection, according to their expression profile over time of infection. Expression levels for each pair of replica arrays: uninfected (D0), Day 5 (D5) and Day 10 (D10) were compared using multiclass significance analysis. Genes with differential expression (over 2-fold, $p<0.01$) between different times of infection were segregated according to expression profiles (induced, repressed or not changed between consecutive days). Numbers represent the total number of genes in each profile. Examples of genes in each profile are provided in the right side panels.

3.2 Pathways, biological processes and molecular functions with significant *P. yoelii*-induced regulation

Using the Gene Ontology (GO) systems of classification and the Kyoto Encyclopedia of Genes and Genomes (KEGG), we analyzed gene annotation for the total unique genes or ESTs that were regulated in the comparison between any two conditions

(Supplemental data 4-7). We found that the main cellular functions under parasite regulation are: 'cell cycle' (45 genes), 'defense responses' (44), 'transcription regulators' (32) and 'apoptosis' (18) (Appendix II). Transcripts associated with immune responses which are regulated by *P. yoelii* will be described in the following section.

Besides the classification according to biological processes, GO also annotates genes according to their molecular functions (Appendix II). The genes regulated by *P. yoelii* in DCs preferentially fall within the following functions: ATP binding (56), kinase activity (34), serine-type endopeptidase (11), rhodopsin-like receptor activity (8) and GTP binding (8). The data for the latter two categories, together with the high number of genes with kinase activity, suggests that *P. yoelii* modulates the activity of signaling pathways that are dependent on G protein-coupled receptors and also involve kinases.

Using the KEGG system for annotation of metabolic pathways, we found that *P. yoelii* preferentially regulates the following pathways in Spleen DCs: glycolysis and gluconeogenesis, purine and pyrimidine metabolism, and protein biosynthesis and catabolism (Appendix II).

Altogether, the data on functional annotation of *P. yoelii*-regulated transcripts of DCs shows that the parasite exerts pressure on many cell functions including diverse metabolic pathways – glycolysis and purine & pyrimidine metabolism – but also the survival of DCs – cell cycle and apoptosis – the ability of DCs to regulate their gene expression – mediators of signaling pathways and gene transcription – as well as their functions as part of the immune response. *Plasmodium* infection has therefore a broad and significant impact on many of DC processes. This large amount of data requires a more detailed approach, and also confirmations of *P. yoelii*-mediated regulation of specific genes using other methods, which we will describe later. For now we will focus on the regulation of genes involved in immune responses.

3.3 *P. yoelii* modulation of genes with defense functions

The class of 'defense response' includes both induced (28) and repressed (16) genes. Some examples of induced genes are chemokines (*ccl8, ccl4, ccl3, cxcl11*) and their receptors (*ccr5, cxcr6*), cytokines such as *il-6, il-10, il-12p40* and *ifn-γ*, and also members of the immunoglobulin super-family (Table 3.II). These data suggest that DCs are actively engaged in interactions with the immune system that might contribute to parasite killing. However, transcripts such as *cd40, cd80, cd83, cd1d1* and *icos*, which can interact directly with co-receptors in NK and T cells, are repressed by *P. yoelii*, suggesting an impaired capacity of DCs to communicate with other cells of both the innate and adaptive arms of the immune system. Other *P. yoelii*-repressed transcripts include *ly6d, nfkb1, il-16* and *ltb/tnfsf3* (lymphotoxin b) (Table 3.III).

The *P. yoelii*-mediated induction of cytokines at the transcription level will be the subject of a more detailed analysis using quantitative PCR (chapter 3.5), while the modulation of DC maturation markers will be studied by flow cytometry (chapter 3.6).

3.4 Comparison of the *P. yoelii*-induced transcriptomic regulation of DCs with the regulation by other pathogens

To have a better understanding of how the global transcriptomic regulation of DC by *Plasmodium* overlaps with the response to other pathogens, and how similar it is to a regular DC maturation process, we compared a list of genes that are simultaneously regulated by three diverse pathogens (a bacteria, a virus and a fungi) in human DCs (Huang et al. 2001) to the genes regulated in *P. yoelii* infection.

Class	Gene	Gene ID	Log2 (D5/D0)	Log2 (D10/D0)	Log2 (D10/D5)	DC Maturation
Chemokines and chemokine receptors	ccl8/mcp-2	20307	+8.15	+8.06	ns	Induced
	ccl4/mip1-β	20303	+5.25	+4.55	ns	Induced
	ccl3/mip1-α	20302	+5.14	+4.86	ns	Induced
	cxcl11/i-tac	56066	+3.33	+2.73	ns	Induced
	cxcr6	80901	+4.18*	ns	-3.52	Not regulated
	ccr5	12774	+3.26	+2.7	ns	Not regulated
Pattern Recognition	msr/sr-A	20288	+5.68	+4.33	-1.35	Not regulated
Transcription factors	fos	14281	+5.05	+5.19	ns	Not regulated
	fos b	14282	+5.3	+6.37	+1.07	Not regulated
Subset marker	cd8 b1	12526	+5.07	+1.53	-3.54	Not regulated
Cytokines and receptors	il6	16193	+4.12	+1.16	-2.97	Induced
	il10	16153	+1.37	+3.56	+2.19	Induced
	il12b	16160	+1.79	ns	-2.22	Induced
	il21r	60504	+1.58	ns	ns	Not regulated
IFN response	ifng#	15978	+5.5	+3.65	-1.85	Induced
	socs3	12702	+4.23*	+4.29*	ns	Not regulated
	jak3	16453	+1.78	+1.74	ns	Not regulated
	igtp	16145	+2.01	+2.13	ns	Not regulated
Prostaglandin signaling	ep4	19219	+1.1	+1.23	ns	Not regulated
Cytotoxicity & Complement	gzmb/ctla-1	14939	+9.73	+8.16	-1.57	Not regulated
	gzmk	14945	+6.14	+4.15	-1.98	Not regulated
	c4/c4b/FDC	12268	+4.66	+5.84	+1.18	Not regulated
Hummoral immunity	igh-1a	380793	+9.07	+11.75	+2.68	Not regulated
	fcgr2b/CD32	14130	+2.83*	+2.86*	ns	Not regulated
	fcgr3a	246256	+2.69	+3.4	ns	Not regulated
	igh-VJ558	16061	+1.54	+4.32	+2.78	Not regulated
	Igk-v8	384422	+1.46*	+4.03*	+2.57	Not regulated
Antigen presentation	ly6a/tap	110454	+4.22	+3.22	ns	Induced
	ly6c	17067	+1.6	+2.24	ns	Not regulated

Table 3.II – Examples of Immunity-related genes that are induced upon *P. yoelii* infection. Genes listed are induced over twofold ($P < 0.01$) in at least one of the times of *P. yoelii* infection, when compared with expression in DCs from uninfected mice. DC maturation indicates whether a gene is induced, repressed or not regulated in the study of Huang *et al*, 2001. ns, not significant. * Log2 values which are averages of two or more probes for the same gene. # Genes with $0.05 > P > 0.01$.

Class	Gene	Gene ID	Log2 (D5/D0)	Log2 (D10/D0)	Log2 (D10/D5)	DC Maturation
Co-stimulation	cd80#	12519	-2.25	-3.04	ns	Induced
	cd83	12522	-1.84	-1.99	ns	Induced
	cd86#	12524	-1.17	-1.85	ns	Induced
	icosl	50723	-1.62	-2.73	-1.1	Not regulated
Antigen presentation	cd1d1	12479	-1.3	-2.36	-1.06	Not regulated
	ly6d	17068	-3.82	-3.94	ns	Not regulated
	h2-Oa/hla-doa	15001	-2.03	-2.92	ns	Not regulated
	c2ta	12265	ns	-1.64	ns	Not regulated
Nf-kb-dependent transcription	ikb-b/nfkbib	18036	ns	-1.02	ns	Not regulated
	ikb-e/nfkbie	18037	ns	-1.32	ns	Not regulated
	relB/nfkb1	19698	-1.11	-2.33	-1.22	Induced
	traf3	22031	ns	-1.39	ns	Not regulated
Cytokines and cytokine signaling	ltb/tnfsf3	16994	-1.71	-2.56	ns	Not regulated
	il16	16170	-1.88	-1.49	ns	Not regulated
	il4i1	14204	ns	-2.38	-1.43	Not regulated
	il11ra1	16157	-2	-2.08	ns	Not regulated
	il13ra1	16164	-2.25*	-2.58*	ns	Not regulated
	ltbp2	16997	-2.31	-2.29	ns	Not regulated
	tgfb1i4/tsc22d1	21807	-1.87*	-2.25*	ns	Not regulated
Chemokine receptors	ccr7	12775	ns	-1.51	-1.26	Induced
Pattern Recognition	cd209a (DC-SIGN)	170786	-6.1	-7.02	ns	Not regulated
	cd209b (SIGNR1)	69165	-2.89	-2.87	ns	Not regulated
	cd209d (SIGNR3)	170779	-3.62	-4.34	ns	Not regulated
Complement	C6	12274	-4.62	-4.19	ns	Not regulated
Prostaglandin metabolism	ptgis	19223	-3.58	-3.56	ns	Not regulated
Other receptors	cd163	93671	-5.22	-4.83	ns	Not regulated
	cd33	12489	-1.08	-1.09	ns	Repressed

Table 3.III – Examples of Immunity-related genes that are repressed upon *P. yoelii* infection. Genes listed are repressed over twofold ($P < 0.01$) in at least one of the times of *P. yoelii* infection, when compared with expression in DCs from uninfected mice. DC maturation indicates whether a gene is induced, repressed or not regulated in the study of Huang et al, 2001. ns, not significant. ***** Log2 values which are averages of two or more probes for the same gene. **#** Genes with $0.05 > P > 0.01$.

There are essential differences in the experimental systems used for this comparison, as Huang *et al*. use human DCs incubated *in vitro* with the different pathogens. However, we have also compared our results to another comparative analysis that includes microarrays performed *in vivo* and *in vitr*o, and which also found a defined common host transcriptional response (Jenner and Young 2005).

To avoid errors resulting from comparing studies that use different statistical methods, we ranked the all the genes analyzed by their expression change in infected versus uninfected mice, from maximum increase to maximum decrease, regardless of their statistical significance. Genes with more than twofold regulation are considered up-regulated, and are found within the first 1453 of all the microarray probes.

From 49 human genes with homologs in the mouse, 21 (43%) were also induced by *P. yoelii* (Table 3.IV, Appendix III). The bulk of the common core of genes induced by different pathogens in DCs is either not significantly modulated by *P. yoelii* (20, 41%), or else repressed by the parasite (8, 16%). In an extensive comparison of transcriptomic analysis of host immune cells responses to different pathogens, a set of 197 genes was found to be exclusively regulated in DCs (Jenner and Young 2005). Comparison of this group of genes with their mouse homologs regulated by *P. yoelii* revealed that only 16% (29) of genes belonging to the common DC response to pathogens are regulated by the malaria parasite (Table 3.IV, Appendix III).

Transcriptomic analysis of DCs responses to three different intracellular parasites (*Toxoplasma gondii, Leishmania major* and *Leishmania donovani)* identified a cluster of genes that are simultaneously induced by all three (Chaussabel et al. 2003). From the 43 genes that have mouse homologs, we found that *P. yoelii* induces 24% (10) of them, while a large majority (76%, 31) are either repressed or not significantly regulated (Table 3.IV, Appendix III).

The data suggest that the transcriptomic response of DCs to *P. yoelii* is largely different not only from non-parasitic pathogens, but also from other intracellular parasites such as *Leishmania* and *Toxoplasma*. The implications of a "divergent" kind of DC maturation in terms of gene expression in response to *Plasmodium* compared to other pathogens will be discussed further on.

Reference	Set of genes used for comparison	Genes with **same regulation** by *P. yoelii*	Genes **not regulated** by *P. yoelii*	Genes with **opposite regulation** by *P. yoelii*
Huang et al. 2001	49 genes related to immunity induced in human DCs by *E.coli*, *Influenza* and *C. albicans*	43% (21)	41% (20)	16% (8)
Jenner & Young 2005	186 genes regulated in human DCs but not other immune cells in response to different pathogens	16% (29)	84% (157)	N/A
Chaussabel et al. 2003	43 genes induced in human DCs by *Toxoplasma gondii, Leishmania major* and *L. donovani*	23% (10)	63% (27)	14% (6)

Table 3.IV – Comparison of *P. yoelii* transcriptomic regulation of dendritic cells (DCs) with the regulation mediated by other pathogens. Similarities between the regulation in spleen DCs *during P. yoelii* infection (according to Genechip® data) of genes identified in three studies from the literature: the "DC maturation core" (Huang *et al* 2001), the common DC response to pathogens (Jenner & Young 2005), or else the common DC response of to intracellular parasites (Chaussabel *et al* 2003). N/A: not attributable.

3.5 Quantitative PCR analysis of *P. yoelii*-regulated genes

Transcriptomic analysis shows that DCs expression of cytokine genes such as *il-6, il-10* and *ifn-*γ is induced by *P. yoelii* infection. To confirm modulation of these cytokines at the mRNA level, we performed quantitative real-time PCR analysis (Fig. 3). We also included in this analysis the cyclooxigenase 2 (*cox2/pgts2)* gene, which is responsible

for the inflammation-inducible production of PGE2 an important mediator of immune functions (Harris et al. 2002) and inducer of fever (Dinarello 2004). As an example of a gene that is repressed during *P. yoelii* infection, we analyzed expression of the regulatory subunit of PKA (*prkar*), an important signaling molecule and regulator of gene expression.

Opposite to the cytokines studied, which are examples of how *P. yoelii* shares gene expression features of the general DC maturation program, we also analyzed mRNA expression of three genes that so far had not been found to be regulated in the DC response to pathogens, at least at the level of transcription; these are *c-fos*, *msr/sr-a* and *c4b/fdc*. These genes are example of the specificity of the transcriptomic response of DCs to *Plasmodium*.

Our detailed analysis of the expression of these genes throughout infection confirms the results obtained with the microarray analysis. Quantitative PCR analysis also reveals that the increase in expression of genes with immunity functions can be detected as soon as 3 days after infection, and is still significant at late times (day 22) when parasitaemia is undetectable in the blood. The response at later time points appears to be similar in trend but lower in magnitude compared with responses before or during the peak of parasitaemia.

3.6 Flow cytometry analysis of the DC expression of co-stimulatory molecules during *P. yoelii* infection

DC maturation is commonly determined by the increase in membrane expression of four proteins know as co-stimulatory molecules: CD40, CD80, CD83 and CD86.

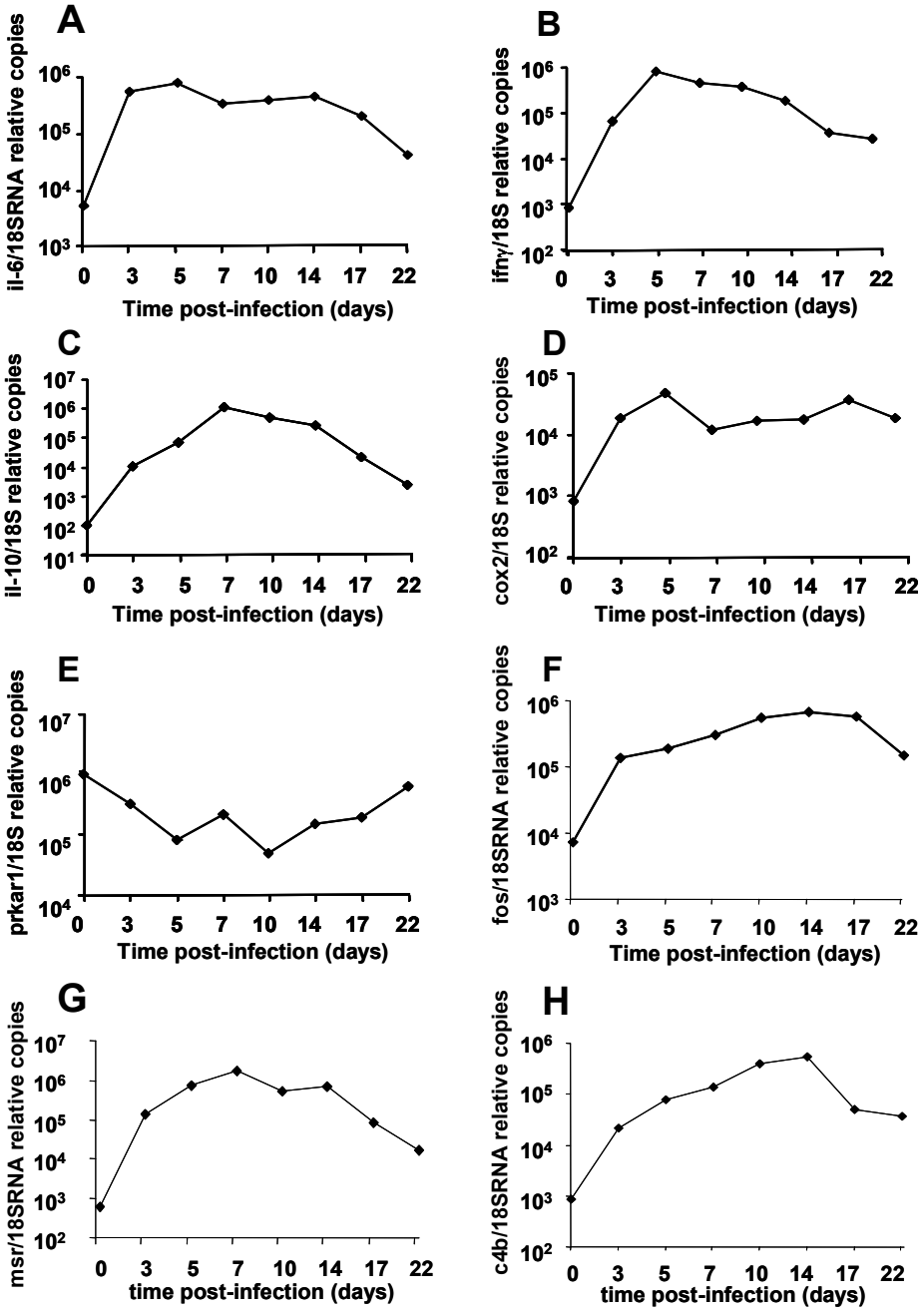


Figure 3.6 – Quantitative PCR analysis of expression in spleen DCs of selected genes over time of *P. yoelii* blood stage infection. A group of transcripts which showed differential regulation in microarray analysis was selected for quantitative real-time PCR analysis of mRNA in spleen DCs collected at different times of *P. yoelii* infection. Genes selected were: *il-6* (A), *ifn-γ* (B), *il-10* (C), *cox2/pgts2* (D), prkar1 (E), fos (F), msr/sr-a (G) and c4b/fdc (H). All genes were normalized to the expression of mouse 18S ribosomal RNA. Results show average of duplicated samples.

Transcriptomic analysis of spleen DCs during *P. yoelii* infection indicates that the genes for the co-stimulatory molecules CD80, CD83 and CD86 are down-regulated in both days of infection analyzed (Table III). CD40 was not significantly regulated in infected mice when compared to uninfected.

The level of surface expression of co-stimulatory molecules is commonly used to determine the maturation state of DCs. In the case of blood-stage malaria, two early studies detected an absence of *in vitro* DC maturation in response to both *P. falciparum*- (Urban et al. 1999) and *P. yoelii*- infected erythrocytes (Ocaña-Morgner et al. 2003).

To confirm that these molecules are not induced by malaria infection, as suggested by *in vitro* studies and our microarray analysis of DC gene expression in *P. yoelii* infection, we analyzed their surface expression on DCs from spleens of uninfected or *P. yoelii*-infected mice at day 5 and 10. We found that the surface expression of all four co-stimulatory molecules analyzed was not increased in DCs upon infection (Fig. 3.7).

Once again, the data presented suggest that *Plasmodium* infection modulates DC gene expression and surface proteins in a manner unlike the standard DC response to pathogens known as DC maturation.

3.7 Discussion

Global transcriptomic analysis of the transcriptomic modulation of DCs by *P. yoelii*-infected erythrocytes shows that blood stage parasites exert an extensive regulation of gene expression of DCs during malaria infection. Gene regulation is very diverse, including different cell functions and metabolic pathways. Some of these have been previously identified through microarray analysis of different models of malaria infection using whole mouse spleen (Schaecher et al. 2005) or brain (Delahaye et al. 2006), and

mononuclear blood cells in infected humans (Ockenhouse et al. 2006) and monkeys (Ylostalo et al. 2005), including the glycolysis pathway, kinase activities, ATP binding, transcription regulators, cell cycle and immunoglobulin genes. However, we found that genes with functions related to cell cycle regulation and defense responses have even a larger number of genes regulated in DCs compared to whole spleen (Schaecher et al. 2005) suggesting these two functions have a more extensive regulation in DCs than other cell types residing in the spleen.

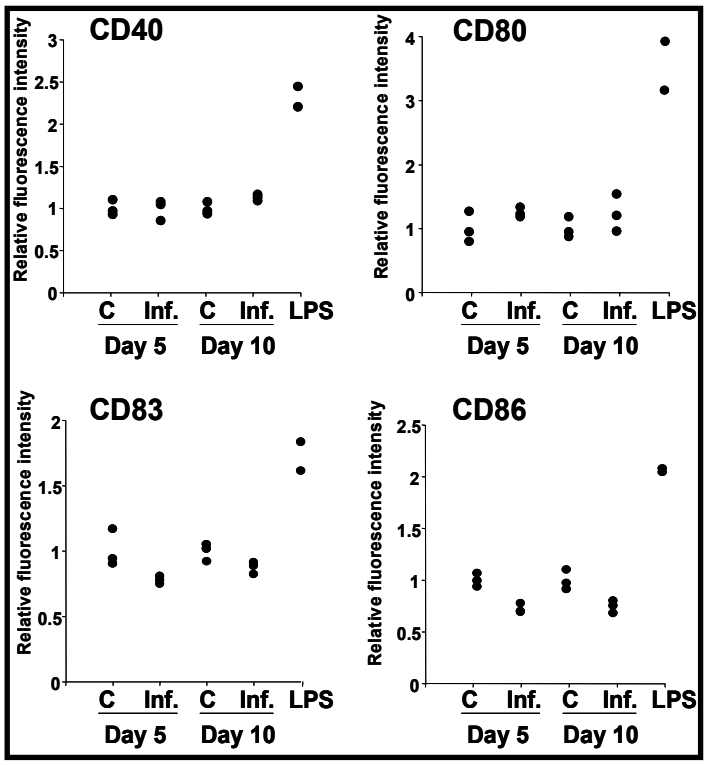


Figure 3.7 – Expression of co-stimulatory molecules on the surface of spleen DCs from *P. yoelii*-infected mice. CD11c+ DCs were obtained from the spleens of groups of three mice control (C) or infected with *P. yoelii* (Inf.) at either 5 or 10 days of infection. Co-stimulatory molecules were stained with fluorescently labelled specific antibodies and expression levels were analysed by FACS. Results are expressed as relative fluorescence intensity for the DCs of each mouse compared with the average of DCs from control uninfected mice. As positive control, two mice were injected with LPS 24 h before analysis.

DCs incubated with *P. yoelii*-infected erythrocytes have extended viability when compared to classic maturation stimuli (Ocaña-Morgner et al. 2003), which is likely to be related to the extensive regulation of genes with functions in cell cycle (45 transcripts) and also apoptosis (17 transcripts). Such gene regulation might be responsible for the increases in the numbers of DCs in both red pulp and marginal zones of the spleen in

spleens of malaria infected individuals compared to sepsis control (Urban et al. 2005). In particular, the induction of cyclins A2, B1 and B2 suggests that DCs may have increased proliferation rates (Schwartz and Shah 2005). On the other hand, the genetic regulation of apoptosis includes both the induction (fas) (Dutta et al. 2006) and repression (ask1/mekk5) (Ichijo et al. 1997) of pro-apoptotic genes, as well as modulation of transcripts with both pro and anti-apototic activities (relB, nfkb1/p50, c-fos, and traf3) (Dutta et al. 2006) (Liebermann et al. 1998), suggesting a rather complex regulation of the survival pathways.

It is noteworthy that a large majority of genes (over 70%) that are significantly induced, or else repressed, after 5 days of infection have the same modulation after 10 days. However, we found a significant number of genes (180) regulated specifically at only one of the time points post-infection, which may reflect the progression of the immune response from early to late infection.

A quantitative and more extensive kinetic analysis of specific cytokine genes revealed similar profiles of regulation over the course of infection, with some genes have maximal induction as soon as day 3 p.i. (*il-6*), others at day 5 p.i. (*ifn-γ, cox2*), while others show a gradual increase over time up to day 10 p.i. (*il-10, fos, msr, c4b*).

The transcriptomic profile is complex with some genes that are typically up-regulated during DCs maturation being also induced by *P. yoelii* (such as *il-6, cox2, ccl3, ccl4, cxcl9, cxcl10, cxcl11)*, while others are not regulated (such as *tnfα, il1-ß, ccl19, icam-1*) or even repressed (such as *cd86, cd83, nfkb1, nfkb2*). Conversely, a large number of genes modulated by *P. yoelii* are not typically associated with DC maturation, including induced genes such as *fos, msr, c4b* (confirmed by real time PCR)*, ccr5, cxcr6, gzmb, gzmk, fcgr2b, fcgr3a*, and repressed genes such as *cd209a/dc-sign, c6, cd1d1* and *traf3*.

We confirmed induction of three transcripts with relevance in immune responses: fos is a transcription factor that has been associated with Th2-inducing stimuli (Agrawal et al. 2003); msr/SR-A, a scavenger and pattern recognition receptor (McGuinness et al. 2003); and C4b, a marker of follicular DCs (Taylor et al. 2002).

The induction of *fos* is of special interest given the fact that in *Plasmodium* rodent infections Th2 responses are gradually induced over time (along with a reduction of Th1), and fos molecule has been shown to be induced by TLR agonists that modulate DCs into inducers of T cell responses of the Th2 type (Agrawal et al. 2003).

The differences between *P. yoelii* and the common maturation response are also reflected when gene regulation is analyzed by biological processes as defined by Gene Ontology. While defense response genes are extensively regulated by both *P. yoelii* and other microbes, other categories such as cell cycle, protein phosphorylation and protein catabolism are extensively modulated by *P. yoelii* but are not highly represented in the common maturation program (Huang et al. 2001).

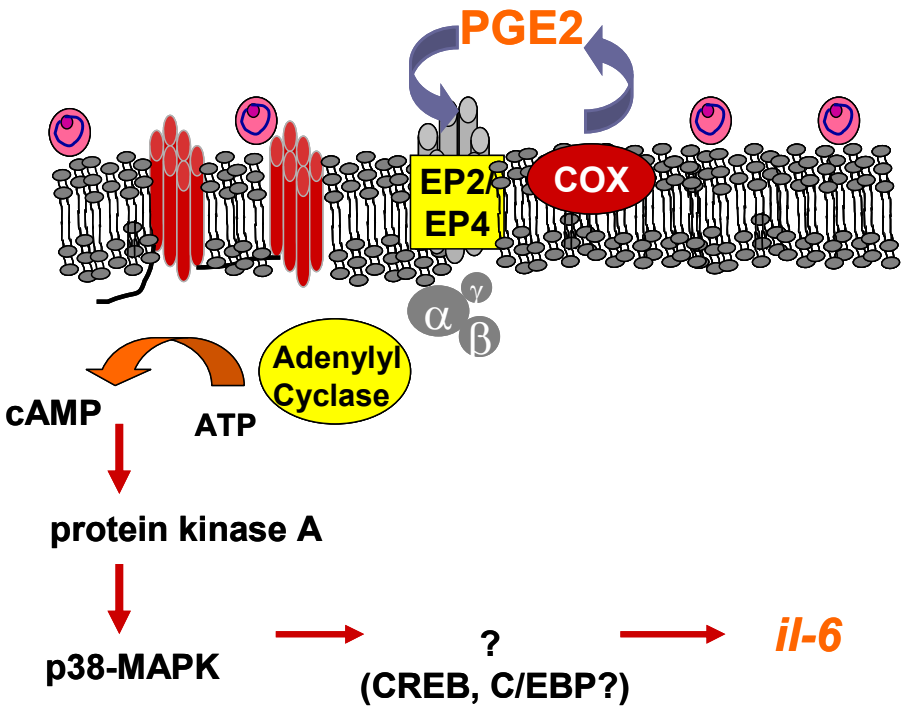
These data suggests that the DC responses to *P. yoelii* might have significant differences from the standard maturation program. In particular, we found that the increase in co-stimulatory molecules, which is a hallmark of maturation, is not present in DCs from *P. yoelii*-infected mice. We propose that the response to *Plasmodium* is a 'divergent' response from what is known as the 'standard' maturation response. A *Plasmodium*-related parasite, Toxoplasma (McKee et al. 2004) and also helminth antigens (Kane et al. 2004) induce atypical responses in DCs suggesting that these pathogens also modulate the host immune response. However, the DC transcriptional responses to helminthes are very limited (Kane et al. 2004) (Chaussabel et al. 2003) while *Plasmodium* induces extensive DC gene regulation. On the other hand a comparison of *P. yoelii* and Toxoplasma-induced transcripts (Table 3.4) revealed a low degree of similarity.

In conclusion, the DC response to *Plasmodium* infection is to a great extent unlike the response of DCs to a variety of pathogens, from virus and bacteria to other eukaryotic parasites. This data requires that more attention is focused on how DCs respond to malaria so that the specificities required to respond to this parasite – or else the modulations of DC functions that *Plasmodium* acquired throughout evolution – are understood in more detail, and extend besides the traditional "immature vs. mature" debate about the status of DCs.

Since a great number of genes are regulated, as we show here, there is plenty of work to be done to characterize DC gene expression in malaria. Therefore, DCs are not mere targets for inhibition and are passive bystanders, but instead undergo an extensive transcriptional reprogramming in many of the cellular processes that include its survival, participation in defense responses and signaling, transcription and metabolic pathways. Which of those regulations contribute or instead down-regulate inflammation or adaptive immunity needs more detailed laboratory work as the one presented in the following chapter.

Chapter IV

PROSTAGLANDIN E2-INDUCED SIGNALING IN DENDRITIC CELLS TRIGGERED BY *PLASMODIUM*: PATHWAY FOR IL-6 EXPRESSION



4.1 *P. yoelii* regulates expression of signaling genes in spleen dendritic cells

Activation of specific signaling pathways can be detected by whole-genome transcriptomic analysis using microarrays, as signaling events frequently result in feedback regulatory mechanisms that modulate the expression of the genes involved in each particular pathway. One notorious case is the reaction known as endotoxin tolerance, by which LPS-mediated activation of Toll-like receptor-4 signaling induces a period of tolerance to the same stimulation, a regulatory mechanism shared with other TLRs (Fan and Cook 2004). One of the molecular basis of endotoxin tolerance is the transient suppression in expression of TLR4 (Nomura et al. 2000), which obviously constitutes an impediment for LPS-induced signaling and gene expression.

Our transcriptomic analysis of regulation of DCs by *P. yoelii* infection indicates that:

i) signaling pathways that involve kinase activities are extensively regulated by *P. yoelii* infection (34 transcripts), and might include G-protein coupled receptors (8 transcripts) (Appendix II);

ii) several genes involved in prostaglandins synthesis and signaling are regulated in DCs by *P. yoelii* infection: *cox2/ptgs2* (cyclooxygenase 2, an enzyme that synthesizes PGE2), *ep4* (a PGE2 receptor) and *ptgis* (prostacyclin synthase), suggesting that *P. yoelii* may be activating this signaling pathway.

A strong up-regulation of the *cox2/ptgs2* gene, which is the inflammation-inducible cyclooxygenase, has been confirmed by quantitative PCR (fig. 3.6).

The data on regulation of prostaglandin synthesis genes is of special interest given the previous findings in the laboratory that detected the quick release of PGE2 by DCs in response to *P.yoelii*-infected erythrocytes (Ocana-Morgner et al. 2007). It was previously

shown that *P. falciparum* induces prostaglandin release (Kilunga Kubata et al. 1998), and that PGE2 levels correlate inversely with disease severity (Perkins et al. 2001) and cerebral malaria (Perkins et al. 2005).

Prostaglandin (PG) receptors are coupled to G-proteins, which can trigger production of cAMP, a second messenger with multiple effects on gene regulation (Harris et al. 2002). Altogether, the data on transcriptomic regulation of genes involved in Prostaglandin (PG) generation, PG-mediated signal transduction (*ep4*) and G-proteins, which may or may not be involved in PG signaling, suggest that these inflammatory mediators may be triggered in DC and playing a role in the modulation of gene expression through PG-dependent signaling pathway.

4.2 *P. yoelii* induces the increase of intracellular cyclic AMP in DCs; role of cyclooxygenase activity

We have previously observed that PGE2 is released in high amounts by DCs as fast as 30 minutes after the start of incubation with *P.yoelii*-infected erythrocytes (Ocana-Morgner et al. 2007).

There are PGE2 receptors of four different types (EP1-4), each one being associated with a different class of G-proteins ($G_{q/p}$, G_s and G_i): while $G_{q/p}$ proteins regulate PKC and IP3 and Ca2+ levels, G_s and G_i regulate cyclic AMP-dependent pathways (G_s activates adenylate cyclase and induces increases in intracellular cAMP levels, while G_i inhibits AC) (Harris et al. 2002). Since we detected regulation of *ep4* gene at the transcription level and this receptor is associated with G_s proteins, we investigated if *Plasmodium*-induced PGE2 activates the production of the second messenger cyclic AMP.

Incubation of bone marrow-derived DCs with *P. yoelii*-infected erythrocytes specifically induces increases in intracellular cAMP that are not observed after incubation with non-

infected erythrocytes (fig. 4.1A). Cyclic AMP levels were also observed to increase with the time and numbers of infected erythrocytes added to DCs (fig. 4.1B, 4.1C).

To study whether the cAMP increase is mediated by PGE2, we analyzed the effect of indomethacin, a cyclooxygenase inhibitor, on *P. yoelii*-induced cAMP production by DCs. Pre-incubation of DCs with indomethacin resulted in a significant inhibition of *P. yoelii*-mediated increase in cAMP (fig. 4.1C), suggesting that PGE2 contributes to the increase in this second messenger in response to the parasite.

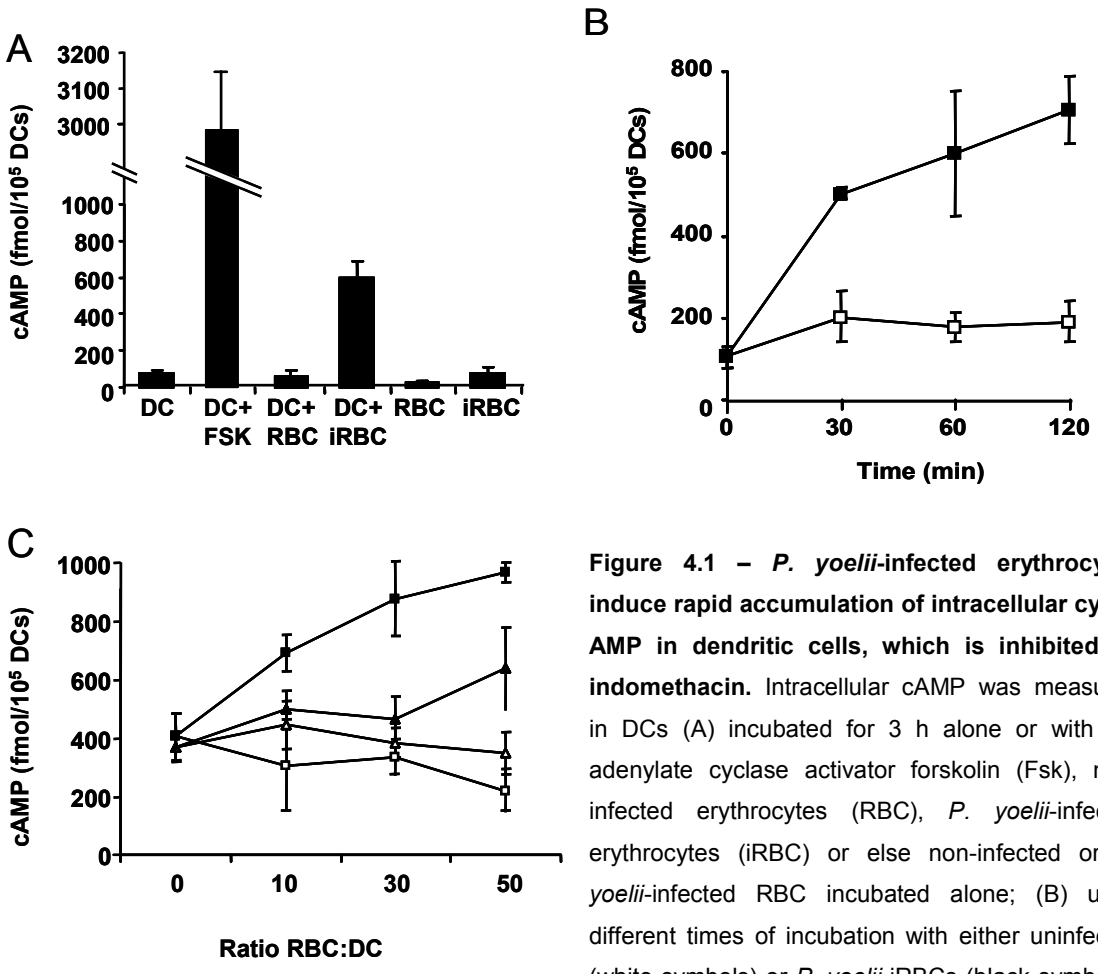


Figure 4.1 – *P. yoelii*-infected erythrocytes induce rapid accumulation of intracellular cyclic AMP in dendritic cells, which is inhibited by indomethacin. Intracellular cAMP was measured in DCs (A) incubated for 3 h alone or with the adenylate cyclase activator forskolin (Fsk), non-infected erythrocytes (RBC), *P. yoelii*-infected erythrocytes (iRBC) or else non-infected or *P. yoelii*-infected RBC incubated alone; (B) upon different times of incubation with either uninfected (white symbols) or *P. yoelii*-iRBCs (black symbols); (C) incubated for 3 h with different doses of uninfected (white symbols) or *P. yoelii*-iRBCs (black symbols), with the cycloxygenase inhibitor indomethacin (5μg/ml) (triangles) or DMSO as negative control (squares). (continued from legend of fig. 4.1) Standard deviations of triplicated samples are shown. Results are representative of three independent experiments.

These results suggest that *P. yoelii*-infected erythrocytes induce PGE2-mediated cAMP signaling in DCs. As cAMP regulates numerous signaling pathways in DCs, this finding may have broad implications for the understanding of DC/*Plasmodium* interactions.

4.3 Production of IL-6 by DCs is activated by *P. yoelii in vitro* and *in vivo,* and requires cyclooxygenase activity

IL-6 is an important mediator of the acute phase response, with multiple effects on immune cells, such as resolution of inflammation, B cell stimulation, Th2 polarization of CD4+ T cells, and also preventing induction of regulatory T cells (Jones 2005).

We found that IL-6 mRNA is strongly induced in splenic CD11c+ cells during *P. yoelii* infection. To confirm that spleen DCs secrete this cytokine during malaria infection, we isolated both CD11c+ (DCs) and CD11c- splenocytes from *P.yoelii*-infected mice (days 5 and 10 pi) and analyzed IL-6 released from these cells. DCs from both days of infection produced high levels of IL-6 compared to uninfected controls or to CD11c- spleen cells, which did not produce detectable levels of IL-6 (fig. 4.2A).

We next characterized IL-6 secretion by DCs *in vitro*. IL-6 is released in response to *P. yoelii*-infected erythrocytes as early as 3 h after incubation, with maximal secretion at 6 h (fig. 4.2B). IL-6 production is highly dependent on the dose of infected erythrocytes (fig. 4.2C). Since PGE2 is quickly released in response to *P.yoelii* (Ocana-Morgner et al. 2007) and PGE2 increases IL-6 gene expression in DCs (Rubio et al. 2005), we investigated the role of this immune mediator in *P. yoelii*-induced IL-6 release. We found that pre-incubation of DCs with indomethacin inhibited IL-6 production in response to infected erythrocytes and brought secretion down to background levels (fig. 4.2C),

suggesting that PGE2 mediates the enhanced IL-6 production of DCs in response to *P.yoelii*.

The implications for a PGE2-mediated increase in IL-6 production, which are both pyrogenic molecules, among other functions, will be discussed further on.

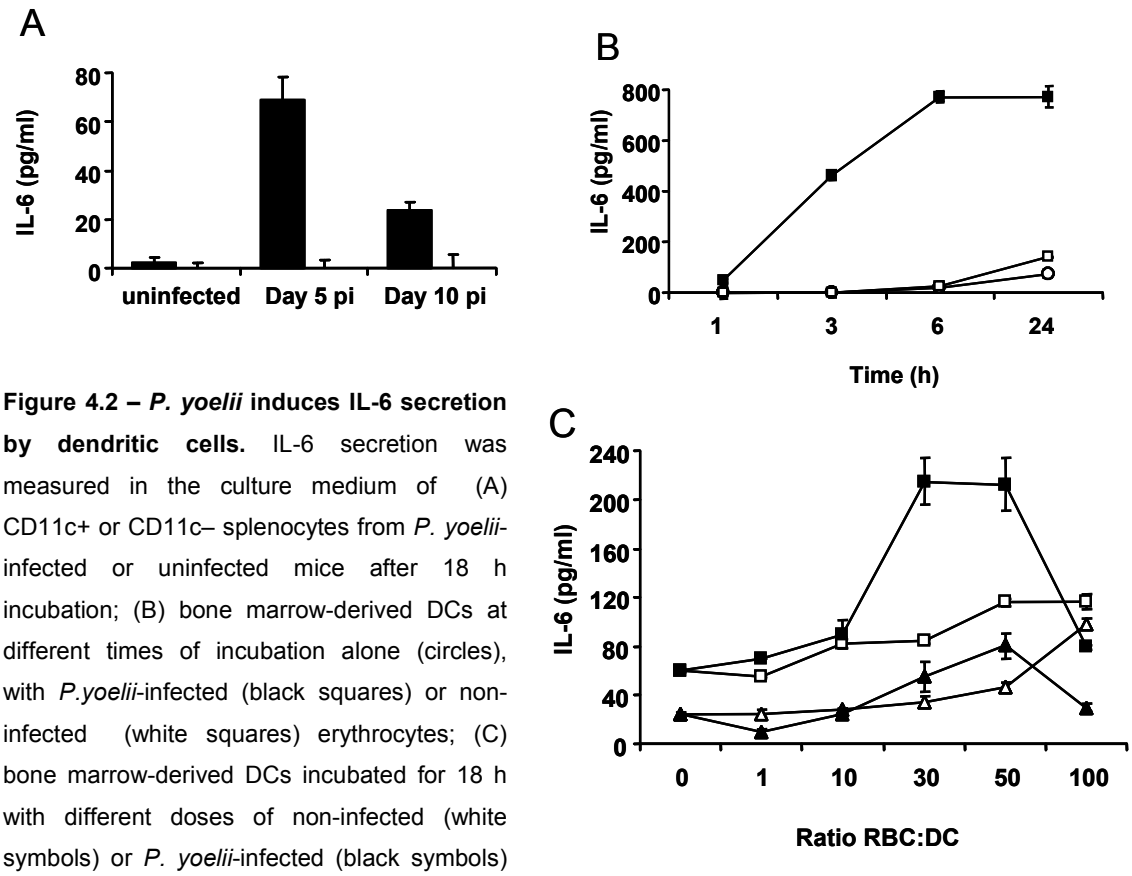


Figure 4.2 – *P. yoelii* induces IL-6 secretion by dendritic cells. IL-6 secretion was measured in the culture medium of (A) CD11c+ or CD11c– splenocytes from *P. yoelii*-infected or uninfected mice after 18 h incubation; (B) bone marrow-derived DCs at different times of incubation alone (circles), with *P.yoelii*-infected (black squares) or non-infected (white squares) erythrocytes; (C) bone marrow-derived DCs incubated for 18 h with different doses of non-infected (white symbols) or *P. yoelii*-infected (black symbols) erythrocytes, with the cycloxygenase inhibitor indomethacin (5μg/ml)(triangles) or DMSO as negative control (squares). Standard deviations of triplicated samples are shown. Results are representative of at least two independent experiments.

4.4 Involvement of intracellular cyclic AMP, PKA and p38-MAPK in *P.yoelii*- induced IL-6

Our findings indicate that *P. yoelii*-induced PGE2 contributes to the observed increases in intracellular cAMP concentrations, as well as to the release of IL-6 by DCs. Since

cAMP can increase expression of IL-6 in different cell types through activation of either PKA-dependent (Chen et al. 2006) (Chio et al. 2004) or independent pathways (Yin et al. 2006), we first investigated if these pathways are also functional in DCs. Addition of synthetic PGE2 or 8-Br-cAMP, a cell permeable analog of cAMP, trigger secretion of IL-6 in DCs (Fig. 4.3). Furthermore, PGE2-derived IL-6 could be inhibited by H89, a PKA inhibitor, suggesting that DCs can activate IL-6 production by a PGE2/cAMP/PKA pathway. cAMP signal transduction pathways can also activate the Exchange Protein directly Activated by cAMP (EPAC), which acts independently of PKA, and might also induce IL-6 (Yin et al. 2006). However, a specific activator of EPAC (8-CPT-2'OMe-cAMP) (Enserink et al. 2002) did not induce IL-6 secretion in DCs, suggesting that this pathway is not involved in the production of IL-6 by DCs.

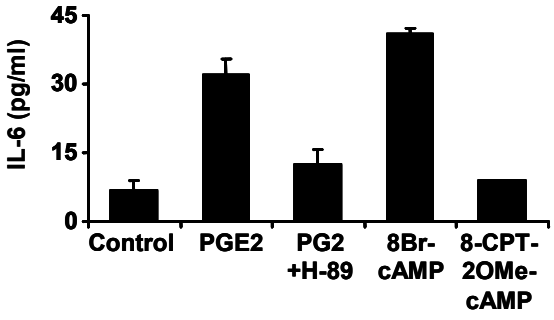


Figure 4.3 – A cyclic AMP/PKA pathway for PGE2-mediated production of IL-6 in dendritic cells. IL-6 was measured in the culture medium of DCs incubated for 6 h alone, with PGE2 200pg/ml (in the presence or absence of 10μM of the PKA inhibitor H89), a permeable cAMP analog (8-Br-cAMP, 500μM) or a specific activator of EPAC (8-CPT-2OMecAMP 20μM).

We next investigated if cAMP mediates the production of IL-6 in response to *P. yoelii*-infected erythrocytes. DCs were pre-incubated with a cAMP derivative (Rp-8Br-cAMPS), which acts as a competitive inhibitor of cAMP-dependent pathways. We found a significant inhibition of IL-6 secretion induced by *P.yoelii*-infected erythrocytes in the presence of Rp-8Br-cAMPS. Similar results were observed when DCs were pre-incubated with the PKA inhibitor H-89 (Fig. 4.4A).

Downstream effects of cAMP and PKA are frequently mediated by p38-MAPK, a sub-class of mitogen-activated protein kinases, in response to different stimuli and in different cell types (Delghandi et al. 2005) (Pomerance et al. 2000). p38-MAPK was also found to contribute to IL-6 expression in response to *Plasmodium*-derived GPI (Zhu et al. 2005). Pre-incubation of DCs with a specific p38-MAPK inhibitor (SB203580) completely abrogated *P. yoelii*-induced secretion of IL-6, while a chemical analog of this inhibitor that does not interfere with p38-MAPK activity (SB203474) did not have any effect on IL-6 secretion (Fig. 4.4B).

These experiments suggest that the *P. yoelii*-induced production of IL-6 in DCs is mediated by increases in intracellular cyclic AMP and requires the kinase activities of PKA and p38-MAPK.

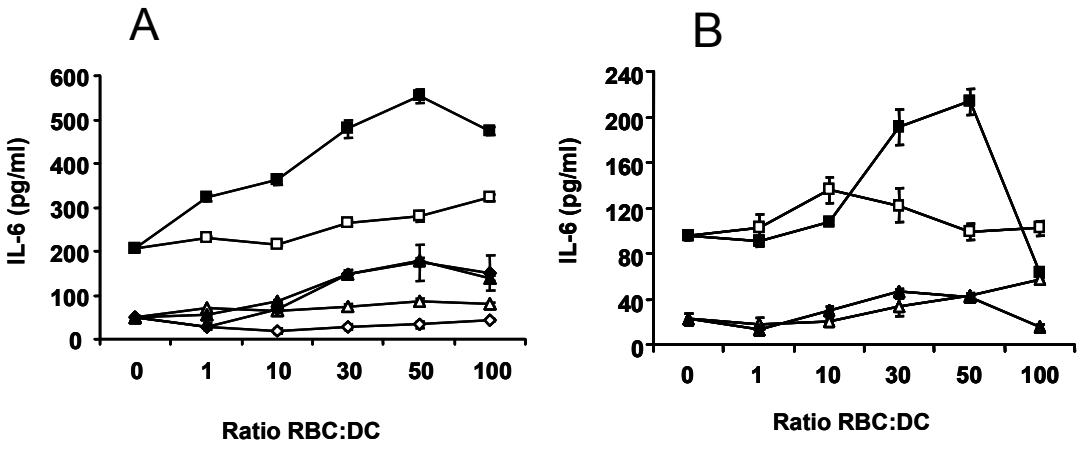


Figure 4.4 – Cyclic AMP, PKA and p38-MAPK mediate *P. yoelii*-induced IL-6 by dendritic cells. DCs were incubated for 18h with different doses of non-infected (white symbols) or *P. yoelii*-infected (black symbols) erythrocytes, (A) in the presence of H89 10μM (rhomboids), Rp-8Br-cAMPS 1μM (triangles), or DMSO as negative control (squares); (B) in the presence of the p38-MAPK inhibitor SB203580 10μM (triangles) or an inactive chemical analog of the same inhibitor SB203474 10μM as control (squares). Standard deviations of triplicated samples are shown. Results are representative of at least two independent experiments.

4.5 Discussion

In recent years, malaria researchers have looked at the relationships of cyclooxygenase activity and PGE2 levels with malaria pathogenesis; it has been shown an inverse association with disease severity in general (Perkins et al. 2001) and with malaria anemia (Keller et al. 2006) and cerebral malaria (CM) in particular (Xiao et al. 1999) (Ball et al. 2004) (Perkins et al. 2005). It was earlier observed that *P. falciparum* had the ability to induce the production of prostaglandins D2, E2 and F2α from arachidonic acid by a mechanism which is insensitive to aspirin and indomethacin, common inhibitors of mammalian cyclooxygenases (COX) (Kilunga Kubata et al. 1998). However, the PGE2 detected in our studies of DC iRBC co-cultures is inhibited by indomethacin, indicating that it is not of parasite origin (Ocana-Morgner et al. 2007). On the other hand, it has been proposed that hemozoin is able to reduce COX-2 activity and PGE2 production by blood mononuclear cells (Keller et al. 2004).

PGE2 is usually induced as part of the inflammatory response by increased expression of COX2 (Harris et al. 2002) and is necessary for induction of fever (Dinarello 2004); on the other hand, it can considered a mediator of immune suppression, including the case of malaria, as detected back in 1989 (Riley et al. 1989). The latter aspect of PGE2 is usually associated with its ability to induce IL-10 (Harizi et al. 2002), but might also relate to a suppression of IL-12 or TNF-α (Harris et al. 2002).

The production of PGE2 in response to the mouse malaria parasite *P. yoelii* has been studied in the laboratory; the data suggests that both CD4+ and CD8+ T cells might be suppressed by DC-derived PGE2 (Ocana-Morgner et al. 2007).

Here we have described how PGE2 contributes to IL-6 production in DCs, not unlike previous studies on the macrophage responses to Toll-like receptor-2 (TLR2) stimulation

with peptidoglycan (PGN) (Chen et al. 2006). However, the regulation of IL-6 by PGE2 has been shown to vary between systems under study: PGE2 is able to inhibit IL-6 production in DCs by an IL-10-dependent mechanism (Harizi and Norbert 2004), while it usually induces IL-6 in macrophages (Marcinkiewicz 1991) (Chen et al. 2006).

Our finding that *Plasmodium*-induced PGE2 contributes to the production of IL-6 by DCs has consequences for the understanding of the mutual regulation between inflammatory mediators in malaria infection, in this case through a synergistic mechanism that may contribute to the fever response, as IL-6 can provide a positive feedback for PGE2 production (Coceani and Akarsu 1998).

Since PGE2 can induce elevation in cAMP levels (Harris et al. 2002) which in turn can induce the production of IL-6 (Chen et al. 2006) (Chio et al. 2004), we investigated whether this pathway is induced in DCs. It is also known that malaria enhances cAMP production by immature erythrocytes *in vitro* (Hertelendy et al. 1979) and that IL-6 is produced by DCs *in vitro* in response to *Plasmodium* (Seixas et al. 2001). We now describe how PGE2 stimulates IL-6 production by a cAMP/PKA/p38-MAPK pathway. PGE2 may contribute to the regulation of inflammatory responses to malaria parasites not only through inhibition of TNF-α (Keller et al. 2006), but also through the induction of IL-6, since this cytokine contributes to the resolution of inflammation and to B cell proliferation (Jones 2005). DC-derived IL-6 may have local effects on stimulation of splenic B cells, which are required for Th1 to Th2 transition in malaria and also for avoiding persistence of *Plasmodium* (Grun and Weidanz 1981) (Taylor-Robinson and Phillips 1996).

IL-6 also regulates Th2 polarization, Treg inhibition, DC maturation, T cell and neutrophil apoptosis, and is considered an important regulator of the transition from innate to acquired immunity (Jones 2005). IL-6 is elevated in the serum of *P. falciparum*-infected individuals and is usually correlated with disease severity (Prakash et al. 2006) (Lyke et

al. 2004); however, the specific role of this cytokine in the immune response to malaria remains unknown. DCs secretion of IL-6 in the spleen may specifically influence B and T cell functions during malaria infection.

Cyclooxygenase activity has been reported to mediate production of IL-6 by macrophages in response to peptidoglycan (PGN), a well characterized ligand of TLR2 (Chen et al. 2006). This pathway seems to be parallel to the one we have identified for DC in response to *P. yoelii*, since they are both mediated by PGE2 activation of cAMP, PKA and p38MAPK, with only a slower kinetics triggered by PGN in macrophages. It is likely that *Plasmodium* GPI and/or hemozoin/DNA complexes contribute to IL-6 secretion in our system, as they induce activation of Toll-like receptors 2 (or 4) and 9, respectively, which triggers IL-6 production (Parroche et al. 2007) (Krishnegowda et al. 2005)

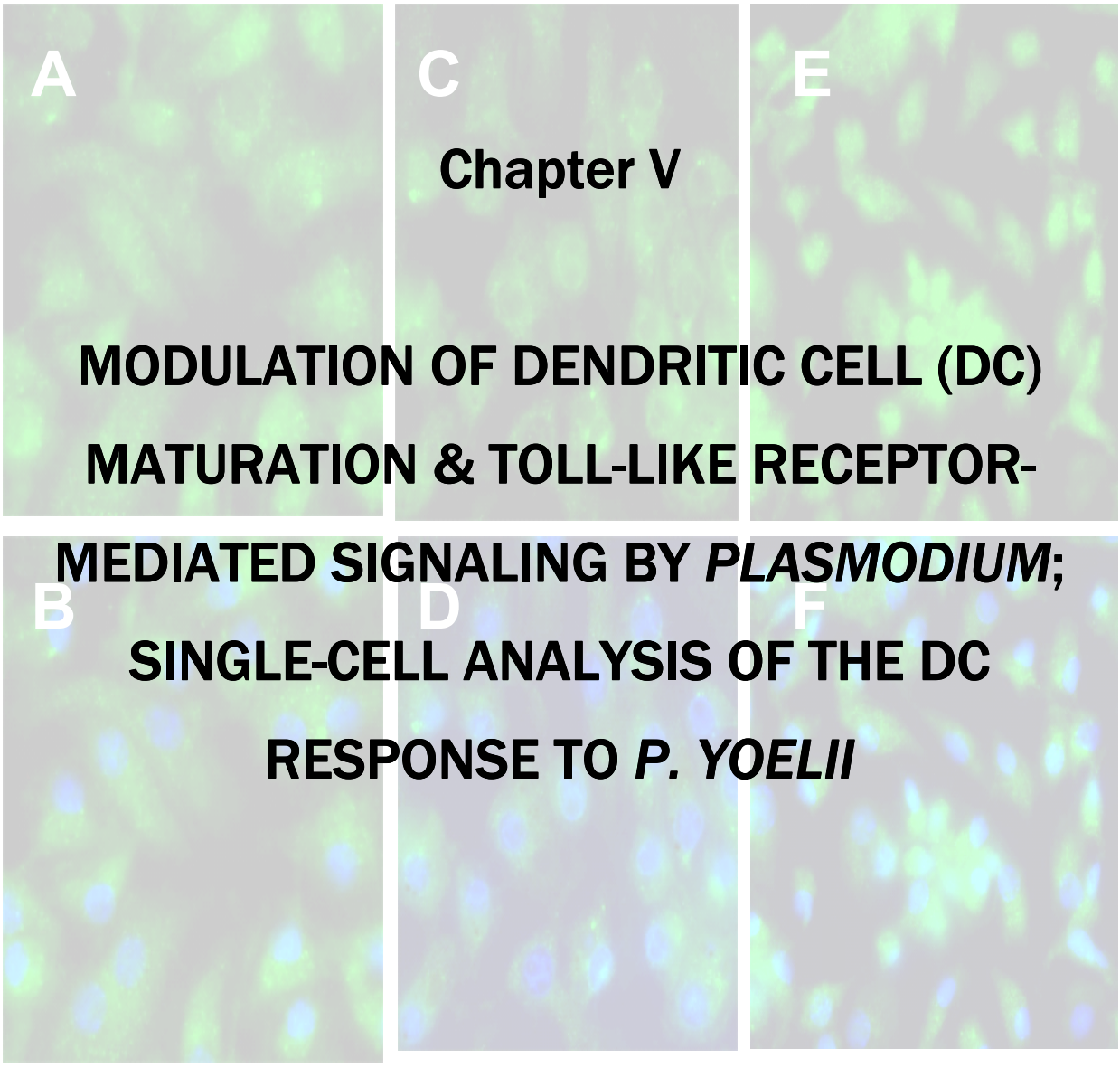
One of the purposes of this work was to identify signaling pathways involved in gene regulation in DCs by the malaria parasite – which is the case of cAMP – since this subject remains largely unexplored. The rapid increase in cAMP triggered by the parasite in DCs probably has a broad influence in the response of these cells to *Plasmodium*, given the large number of genes regulated by the two families of cAMP-dependent transcription factors: the CREB family (Mayr and Montminy 2001) and the C/EBP family (Wilson and Roesler 2002). In fact, PGE2-induced cAMP regulates migration of DCs (Legler et al. 2006) (Luft et al. 2002), DCs-mediated T cell polarization (Kubo et al. 2004) and also cytokine secretion (Kambayashi et al. 2001). Whether these effects are mediated by PGE2-induced cAMP during malaria infection remains to be characterized.

In terms of medical intervention, salicylate-derived antipyretics have been extensively used as a way of inhibiting COX activity and control fever and inflammation in malaria; however this strategy has resulted in negative effects on the infected individuals' pathologies, situation known as "salicylate poisoning" (English et al. 1996). Also in

mouse models, the administration of selective COX inhibitors is known to accelerate CM onset and increase mortality (Xiao et al. 1999) (Ball et al. 2004). Those observations have been recently proposed to be a consequence of the negative effect of PGE2 on TNF-α production (Keller et al. 2006); an inhibition of PGE2 production would likely result in an increase of circulating TNF-α, which is a strong promoter of adhesion molecule expression and many of malaria's pathologies (Schofield and Grau 2005).

We now provide evidence suggesting that PGE2 inhibition could reduce IL-6 levels in infected individuals. We were unable to detect differences in *ex vivo* DC production of IL-6, or in IL-6 mRNA levels of spleen DCs, between indomethacin- and DMSO-treated mice (data not shown). However, we still should not exclude the possibility that PGE2 inhibition can regulate IL-6 levels *in vivo*. What we could confirm is that indomethacin treatment decreases TNF-α, in our case at the mRNA level (data not shown), while others have observed the same at the protein level (Keller et al. 2006).

If regulation of IL-6 by PGE2 is confirmed *in vivo* it would provide an additional mechanism whereby cyclooxygenase inhibition leads to increased disease severity, since IL-6 is now considered as a molecule that promotes transition from innate to adaptive immunity given its roles as a neutrophil apoptotic signal, or inducer of IL-1 and TNF-α antagonists (Jones 2005); a reduced level of IL-6 could also contribute to an excess of regulatory T cells (Pasare and Medzhitov 2003) and a slower transition to Th2 responses (Jones 2005), two events that might have detrimental effects in parasite control (Taylor-Robinson and Phillips 1996) (Walther et al. 2005).



Chapter V

MODULATION OF DENDRITIC CELL (DC) MATURATION & TOLL-LIKE RECEPTOR-MEDIATED SIGNALING BY *PLASMODIUM*; SINGLE-CELL ANALYSIS OF THE DC RESPONSE TO *P. YOELII*

5.1 Do *P.yoelii*- infected erythrocytes (IEs) induce Toll-like receptor (TLR)-dependent signaling and dendritic cell (DC) maturation?

5.1.1 Dendritic cell responses to *P.yoelii*- IEs

The question of whether dendritic cells (DCs) mature or not in response to *Plasmodium*-infected erythrocytes (IEs) has been a subject of controversy between researchers, as pointed out in Chapter I.4. This discussion is of special relevance for our understanding of the role of DCs in the modulation of T cell responses against malaria blood stages infection; this is the only stage generating adaptive immunity that controls parasite growth in human infections (Struik and Riley 2004). Despite the fact that liver stage immunity induced by experimental vaccins can be fully protective (Nussenzweig et al. 1967), little protective immunity is found against liver stages in endemic areas (Struik and Riley 2004).

Three laboratories have shown that myeloid DCs do not mature in response to either *P. falciparum*- (Urban et al. 1999), *P.yoelii*- (Ocaña-Morgner et al. 2003), or *P. chaubaudi*- (Millington et al. 2006) infected erythrocytes (IEs), and are inhibited in their response to LPS stimulation of TLR4 when pre-incubated with IEs. In contrast, others have shown direct DC maturation in response to *P. chabaudi*- (Seixas et al. 2001) and *P. falciparum*--IEs (Pichyangkul et al. 2004) (plasmacytoid DCs in this case), or else during *P. yoelii* (Perry et al. 2004) or *P. chabaudi* infections (Leisewitz et al. 2004).

We have reported that mouse DCs respond to *P. yoelii*-IEs (Ocaña-Morgner et al. 2003) in a similar way as the one described for the response of human DCs to *P. falciparum*-

IEs. In Chapter III we described a transcriptomic analysis of spleen DCs during *P.yoelii* infection supporting the idea that DCs respond in a diverse way from "classic" maturation, as defined by the response to bacteria or viruses (Huang et al. 2001). We propose that DC response to *Plasmodium* is referred to as "divergent" maturation.

Given the discrepancies of observations between different laboratories, we decided to investigate whether maturation – determined by the increase in expression of co-stimulatory molecules – in response to *P. yoelii*-IEs had not been previously observed in the laboratory due to any bias of the experimental conditions.

To investigate whether infected *P.yoelii*-infected erythrocytes can activate DCs, we stimulated bone marrow-derived DC with different ratios of purified IEs: DC for 24h. No up-regulation of surface expression of the co-stimulatory molecules CD40, CD80 or CD86 was detected at any of the ratios tested (1 to 50 IEs:DC), (fig. 5.1A). We also performed shorter incubations of DCs with IEs, from 1h to 6h, upon which IEs were washed away, and DCs incubated in fresh medium for another 18h. Still, no induction of co-stimulatory molecules was detected (not shown) suggesting that long-term incubation of DCs with parasites is not responsible for the lack of DC maturation.

To induce the release of parasite molecules from the *P. yoelii*-IEs that are described to induce DC maturation, such as DNA/hemozoin complexes (Parroche et al. 2007) and glycophosphatidilinositol (GPI) anchors (Krishnegowda et al. 2005), we prepared freeze-thaw lysates of either *P. yoelii*-IEs or else non-infected erythrocytes (NEs). A marginal increase of CD86 expression was detected when DCs were stimulated with lysates of both infected and non-infected erythrocytes (fig. 5.1B), indicating that such an effect is not *Plasmodium*-specific. To control that both IEs and lysates were not toxic to DCs, we determined the number of non-viable cells in the co-cultures using a specific dye (7-amino-actinomycin D); no significant differences were found between treatments with IEs, IE lysates or DC alone (not shown).

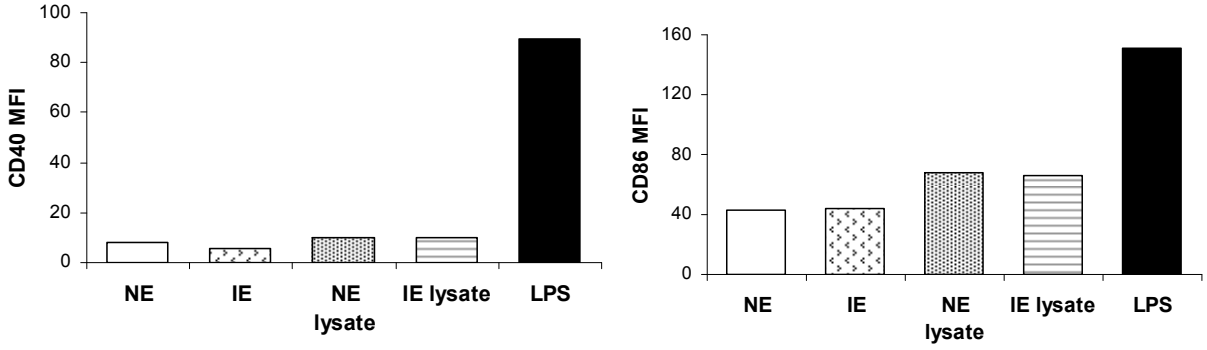


Figure 5.1 – Dendritic cells do not mature in response to *P. yoelii*. CD40 (A) and CD86 (B) expression in DCs incubated with *P.yoelii*-infected or non-infected erythrocytes (IE, NE, both 30/DC), the respective lysates, and LPS as maturation control.

GPI and DNA from *P. falciparum* induce secretion of IL-12, TNF-α and IL-6 from either DCs or macrophages (Parroche et al. 2007) (Krishnegowda et al. 2005).

We next investigated which cytokines are released by DCs in response to *P. yoelii*-infected erythrocytes. We found IL-6 production to be induced by overnight incubation of DC with Py-IEs in a dose-dependent manner, as described in a previous chapter (figure IV.x). However, IL-12 and TNF-α were not detectable after 18h incubation of DCs with 30 IEs:DC or else with IE lysates (not shown), similarly to studies with *P. falciparum* (Urban et al. 2001).

These results confirm our previous observations on the lack of DC maturation and cytokine production (with the exception of IL-6) in response to *P. yoelii*- IEs (Ocaña-Morgner et al. 2003). Also during *P. yoelii* infection we do not detect DC maturation, as described in Chapter III (fig. 3.7). Altogether, the data points to a defect of *P. yoelii* in triggering DC maturation, either through TLR-dependent pathways or others.

5.1.2 Is TLR-dependent signaling activated by *Plasmodium*?

Using diverse methodologies and criteria, we confirmed that DCs do not present the usual maturation characteristics in response to *P.yoelii*-infected erythrocytes (Py-IEs), including up-regulation of co-stimulatory molecules and production of IL-12. These processes are usually dependent on binding of molecular patterns of pathogens to Toll-like receptors (TLRs), but also other less studied families of receptors (Janeway and Medzhitov 2002), as described in chapter I.3

To investigate if the lack of DC maturation results from the absence of TLR activation by *Plasmodium* parasites, we collaborated with the laboratory of Dr. Gollenbock at the University of Massachusetts, where human embryonic kidney (HEK293) cells permanently transfected with TLRs are used for studies of TLR-mediated signaling. We used three cell lines transfected with the TLRs that have been described to be activated by molecules from *P. falciparum*: TLR2, TLR4 and TLR9. Each TLR-transfected cell line was incubated with various ratios of *P. yoelii*-infected erythrocytes/HEK cell (1 to 100 Py-IEs:HEK cell). TLR-dependent transcriptional activity of NF-κB was similar to background levels after incubation with any of the ratios of Py-IEs tested (fig. 5.2), suggesting that *P. yoelii* does not induce activation of NF-κB in response to either TLR2-, TLR4- or TLR9-depedent signaling. Transfected cells could activate NF-κB-dependent transcription in response to each of the respective TLR ligands (fig. 5.2).

These experiments suggest that whole *P. yoelii*-infected erythrocytes do not trigger TLR-dependent gene expression, despite the presence of molecules that have been described to activate the same receptors in *P. falciparum*-parasitized erythrocytes.

We will describe further ahead possible mechanisms for the lack of TLR-mediated responses to *P. yoelii* that might account for the inhibition of responses of DCs but also TLR- transfected cell lines.

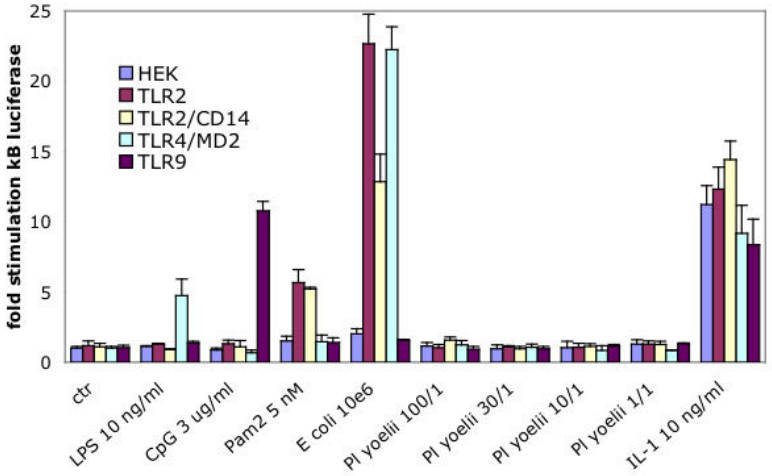


Figure 5.2 – Toll-like receptor-dependent gene expression is not triggered by P. yoelii in TLR-expressing cell lines. HEK cells transfected with either TLR2, TLR2+CD14, TLR4+MD2 or TLR9 were stimulated with *P. yoelii*-infected erythrocytes (from 1 to 100/HEK cell), and also with controls of non-specific TLR activation (*E. coli*, IL-1) as well as ligands for each TLR (TLR2-Pam2, TLR4-LPS, TLR9-CpG-DNA).

Another indirect way to study if TLR-dependent signaling is induced by *P. yoelii* is to use DCs with defects in signaling mediators such as MyD88, a molecule essential for some of the TLR-dependent pathways such as TLR9, while it is not essential for others, like TLR2 and TLR4 (Akira and Takeda 2004).

We have described in Chapter IV the pathway leading to the production of IL-6 by DCs in response to *P. yoelii*. However, we have not determined whether TLR activation mediates the production of either PGE2 or IL-6. To test if IL-6 production is triggered by TLR-dependent signaling pathways that rely on MyD88, we stimulated DCs generated from bone marrow precursors of MyD88-/- C57BL/6 mice with non-infected (NE) or *P. yoelii*-infected erythrocytes (Py-IEs), and compared to the response of C57BL/6 wild-

type (WT) DCs. IL-6 was secreted by MyD88-/- DCs in response to Py-IEs, although at lower levels than those from WT-DCs (fig. 5.3). These results suggest that *P.yoelii* is able to activate a MyD88- independent pathway for the secretion of this cytokine, at the same time that MyD88-dependent pathways may also contribute for expression of IL-6. The MyD88-dependet pathway is very likely to be dependent on *Plasmodium* DNA-mediated activation of TLR9 (Parroche et al. 2007).

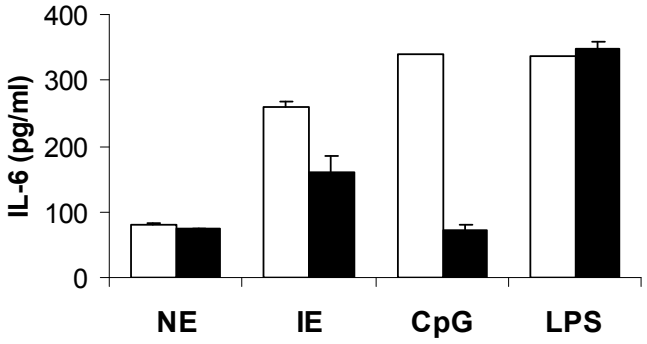


Figure 5.3 – IL-6 production in response to P. yoelii does not require MyD88. Production of IL-6 by both WT (white bars) and MyD88-/- C57BL/6 bone marrow-derived DCs (black bars) in response to *P.yoelii*-infected or non-infected erythrocytes (IE, NE, both 30/DC) was measured in cell culture medium after 18h. CpG (TLR9 activator) and LPS (TLR4 activator) were used as controls of MyD88-dependent and independent pathways, respectively.

5.2 Characterization of *Plasmodium*-mediated blockage of DC maturation in response to TLR stimulation

5.2.1 *P. yoelii* inhibits responses to all TLRs – expression of co-stimulatory molecules

P. yoelii-infected erythrocytes (Py-IEs), as well as *P. yoelii* infection, do not induce DC maturation, while Toll-like Receptor (TLR)-dependent gene transcription in transfected cells is not triggered by Py-IEs. We next decided to study whether infected erythrocytes

(IE) interfere with the capacity of DCs to mature in response to a secondary TLR activation.

We have reported that incubation of DC with *P. yoelii*-IE inhibits their capacity to mature in response to LPS stimulation (Ocaña-Morgner et al. 2003). This effect was found to be maximal after 16h to 24h of pre-incubation of DCs with Py-IEs. To investigate if the inhibition observed for LPS-dependent maturation is a general suppression of TLR-mediated responses, DCs were pre-incubated with *P.yoelii*-infected erythrocytes (IEs) for 24h before stimulation with ligands of TLR2 (zymosan 50μg/ml), TLR3 (polyI:C 25μg/ml), TLR5 (flagelin 100ng/ml), TLR7 (loxoribine 500μM) and TLR9 (CpG-ODN 1μg/ml), as well as TLR4 (LPS 1μg/ml). For all TLRs studied, the up-regulation of co-stimulatory molecules by DCs induced by the TLR ligands is severely inhibited by pre-incubation with Py-IEs at a ratio of 30IE:DC, while lower ratios resulted in a partial, or else no inhibition. Figure 5.4 shows results for DC membrane expression CD86, among the CD11c+, 7AAD- population. Similar results were obtained for CD40 and CD80.

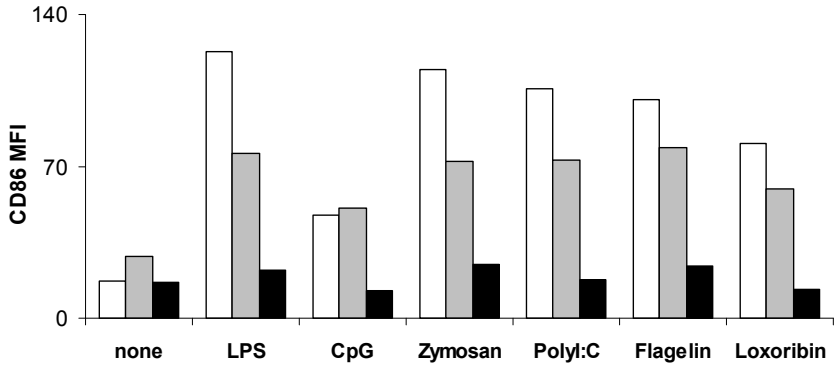


Figure 5.4 – TLR-mediated DC maturation is inhibited in the presence of *P. yoelii*. DCs were pre-incubated with either 30 non-infected erythrocytes/DC (white bars), 10 *P. yoelii*- infected erythrocytes (IE)/DC (gray bars) or 30 IE:DC (black bars) for 18 hours before addition of the following TLR ligands for another 24 h: LPS 1μg/ml (TLR4), CpG 1μg/ml (TLR9), Zymosan 50μg/ml (TLR2/TLR6), Poly I:C 25μg/ml (TLR3), Flagelin 100ng/ml (TLR5), Loxoribin 500μM (TLR7). At the end of stimulation, DCs are harvested and stained for membrane expression of CD86 (gated on CD11c+).

5.2.2 *P. yoelii* inhibits responses to all TLRs – IL-12 secretion

Since *P. yoelii*-infected erythrocytes (Py-IEs) inhibit the up-regulation of co-stimulatory molecules induced by TLR ligands, we decided to investigate if this modulation also applies to cytokine expression. The production of IL-12p70, the bio-active heterodimer, is of special relevance since it is crucial for the induction of T-cell responses of the Th1 type, as well as memory T cells (Trinchieri 2003).

The secretion of IL-12p70 by DCs was induced by all TLR ligands tested, except for PolyI:C (TLR3 activator). In all the cases where IL-12 was induced, pre-incubation with Py-IEs profoundly inhibited the production of this cytokine. This was the case for ratios between 10 and 30 IEs:DC (figure 5.5), the same way as for the expression of co-stimulatory molecules.

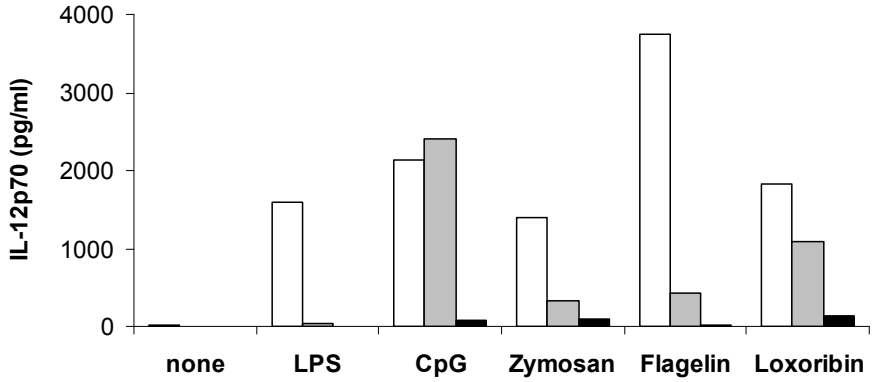


Figure 5.5 – TLR-mediated cytokine production is inhibited in the presence of *P. yoelii*. DCs were pre-incubated with either 30 non-infected erythrocytes:DC (white bars), 10 *P. yoelii*- infected erythrocytes(IE): DC (gray bars) or 30 IE: DC (black bars) for 18 hours before addition of the following TLR ligands for another 24 h: LPS 1μg/ml (TLR4), CpG 1μg/ml (TLR9), Zymosan 50μg/ml (TLR2/TLR6), Poly I:C 25μg/ml (TLR3), Flagelin 100ng/ml (TLR5), Loxoribin 500μM (TLR7). At the end of stimulation, cell culture medium is collected and used for ELISA quantification of IL-12p70.

The kinetics of inhibition of IL-12 production are similar, or even faster, to that observed for inhibition of CD86 expression, since release of IL-12 is severely impaired when DCs are pre-incubated with Py-IEs for 6h, but there is also a significant inibition in the absence of pre-incubation (time 0, simultaneous addition of Py-IEs and TLR stimulation) (figure 5.6). UEs can also induce a reduction in the IL-12 released by DCs, but to a lesser extent than a similar dose of IEs.

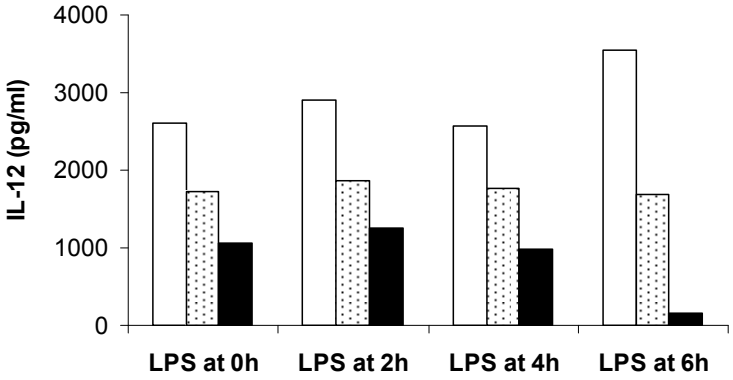


Figure 5.6 – Inhibition of TLR-mediated cytokine production by *P. yoelii* does not require pre-incubation. DCs were pre-incubated alone (white bars), with non-infected erythrocytes (30NE:DC, dotted bars), or *P. yoelii*- infected erythrocytes (30IE:DC, black bars), for different times from 0h to 6h, before addition of LPS for another 24h. IL-12p70 in the cell culture media was quantified by ELISA.

The above data on IL-12 production, and also the expression of co-stimulatory molecules, suggest that *P. yoelii*-IEs can severely impair the ability of DCs to respond to stimulation through any of the TLRs. Such an effect is maximum between 6h and 24h after the start of incubation, and requires a high-enough dose of parasitized erythrocytes, either 10 or 30IEs:DC. Such a broad effect on the ability of DCs to activate TLR-dependent gene expression is relevant for the understanding of the responses of malaria-infected individuals to concurrent infections, and also to vaccination, since immune responses in both cases are generally dependent on activation of TLR-dependent signaling pathways.

5.3 *P. yoelii* inhibits NF-κB transcriptional activity

We have described a general inhibition of TLR-dependent gene expression in DCs upon *in vitro* challenge with *P.yoelii*-infected erythrocytes (IEs). TLR-dependent signaling pathways rely to a large extent on NF-κB transcription factors for activation of gene expression (Akira and Takeda 2004), which is mediated by heterodimers of members of the NF-κB family: RelA/p65, RelB, c-Rel, nf-κb1/p50 and nf-κb2/p52 (Hoffmann et al. 2006).

We decided to investigate if NF-κB expression, or else its activation, which requires translocation from the cytoplasm into the nucleus, is impaired by *P.yoelii*-IEs.

We analyzed the localization of RelA/p65 subunits by immuno-fluorescence (IF) in DCs pre-incubated with either non-infected (NE) or *P.yoelii*-IEs (Py-IEs). We observed that incubation with a high dose of Py-IEs does not induce translocation of RelA/p65 into the nucleus of DCs (fig. 5.7). Oppositely to *P. yoelii*, LPS incubation induces RelA nuclear translocation (fig. 5.7). This data suggests that *P. yoelii* does not induce the activation of at least one member of the NF-κB family, which is characteristic of TLR activation.

To investigate if decreased NF-κB expression may result in a repression of NF-κB-dependent gene expression the latter hypothesis, a separate analysis of cytoplasmic and nuclear extracts of DCs is required, in order to quantify the nuclear translocation of NF-κB proteins. After several attempts, we were not able to detect NF-κB transcription factors by western blot with an antibody of high specificity.

It remains to be explored if any of the transcription factors of the NF-κB family have reduced expression in the presence of *P. yoelii*. However, microarray data presented in Chapter III indicates that the *RelB* gene has reduced expression in spleen DCs during *P. yoelii* infection at the transcript level (see Appendix I). Besides studies of global

expression, we also need to clarify if reduced NF-κB expression will associate or not with reduced NF-κB transcriptional activity, and if this is inhibited in response to secondary stimulation in DCs, as already shown in TLR-transfected cells (figure 5.2).

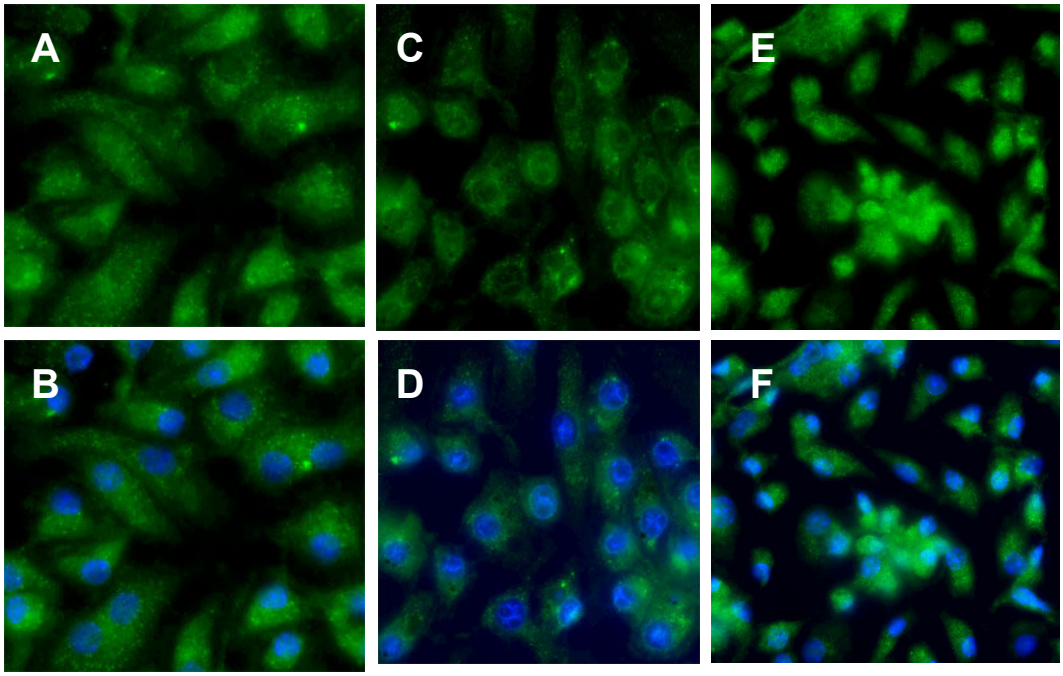


Figure 5.7 – *P. yoelii* inhibits RelA transcriptional activity. Immunofluorescence with anti-RelA for DCs incubated with non-infected erythrocytes (A, B), *P.yoelii*-infected erythrocytes (C, D) or LPS (E, F). Panels B, D and F show overlay of anti-RelA (green) with DAPI (blue) for nuclei staining.

We hypothesize that, during blood-stage malaria infection, spleen DCs will present low levels of nuclear NF-κB transcription factors and therefore will not be able to up-regulate a large part of the genes associated with the 'standard' maturation response, such as co-stimulatory molecules and IL-12, which require NF-κB translocation. Still, this hypothesis requires additional studies using various methodologies.

If an inhibition of NF-κB expression and/or nuclear translocation is confirmed, DCs can still contribute to the induction of inflammatory mediators such as PGE2 or IL-6 using

alternative signaling pathways that may not require NF-κB transcription factors, as those described in Chapter IV.

5.4. Visualizing phagocytosis of *P. yoelii*- IEs and its regulation of DC maturation

The role of opsonization and phagocytosis of *Plasmodium*-infected erythrocytes (IEs) in parasite clearance and immunity to malaria has been the subject of several studies, as discussed in Chapter I. So far, reports have focused on IE phagocytosis by macrophages and monocytes. DCs of the spleen are also likely to phagocytose IEs, with possibly relevant consequences for the regulation of immune responses (Engwerda et al. 2005). Even if the role of DCs phagocytosis in parasite clearance might be considered negligible because of the low numbers of DCs compared to macrophages, the possible consequences for the modulation of DC functions and therefore adaptive immunity by the parasite make these events very relevant.

It has been proposed that DC maturation is modulated by *P. falciparum*- IEs through receptor-mediated interactions involving CD36 on DCs and PfEMP1 on IEs (Urban et al. 2001); in murine malaria, the modulation of DC maturation is also present, but the strains of malaria that infect mice do not express any *var* genes or proteins related to PfEMP1. Therefore, the nature of the molecular interactions responsible for the modulation of DCs is not known.

In order to study the role of the phagocytosis of *P.yoelii*- IEs in the modulation of DC functions by this parasite, we devised a method for the labeling of erythrocytes, either infected or not, which allows for identification of cells that phagocytose parasites. That method is described in detail in Chapter II. Briefly, non-infected or else *P.yoelii*-infected

erythrocytes are incubated with carboxyfluorescein diacetate, succinimidyl ester (CSFE), which passively diffuses into cells; once in the cytoplasm, it generates well retained fluorescent conjugates. After careful wash, labeled erythrocytes are used for *in vitro* co-culture with DCs. A similar method was simultaneously developed in the Stevenson Laboratory (Ing et al. 2006).

We first investigated the extent to which homogenous cultures of bone marrow-derived DCs would uptake erythrocytes. As expected, the uptake of infected erythrocytes (IEs) was considerably higher than that of non-infected (58±6% vs 25±5%, Table 5.I); surprisingly, phagocytosis of non-infected erythrocytes (UEs) was rather frequent, even considering that a high dose of erythrocytes (30NEs:DC) was used.

Using antibodies to detect membrane expression of co-stimulatory molecules (CD40, CD86), we identified mature and immature DCs in cultures of cells pre-incubated with erythrocytes for 18h and then stimulated with LPS for another 24h. A clear association of DC maturation measured as surface staining of CD40 and CD86 with lack of erythrocyte phagocytosis and therefore lack of CFSE staining was observed (45-50% CD86+ in CFSE- DCs vs. 1%CD86+ among CFSE+DCs, Table 5.I). These results suggest that:

i) ca. 50% of DCs used for *in vitro* experiments respond to LPS stimulation;

ii) infected erythrocytes are phagocytosed more efficiently than non-infected;

iii) phagocytosis of erythrocytes, both *P. yoelii*-infected and non-infected, results in a virtually complete inhibition of DC maturation.

Uptake of both non-infected and *P. yoelii*-infected erythrocytes (IEs) associates with inhibition of maturation; the fact that phagocytosis of *P. yoelii*-IEs is more extensive explains the discrepancy between the frequencies of mature DCs in the whole population (37.8% with NEs, 19.5% with IEs).

	Frequency of Phagocytosis/ % of CFSE+ DCs	Mature DCs among CFSE - population	Mature DCs among CFSE + population	**Overall Maturation Frequency**
Non-infected erythrocytes	25%±4.2%	50%±5.1%	1%±1%	**37.8%**
P.yoelii-infected erythrocytes	58%±6.4%	45%±5%	0%±0%	**19.5%**

Table 5.I – Uptake of erythrocytes, either *P. yoelii*- infected or non-infected, inhibits DC maturation. Frequencies of phagocytosis and maturation of DCs incubated with P. *yoelii*-infected (IEs) or non-infected erythrocytes (NEs). Bone marrow-derived DCs were incubated for 18h with CFSE-labelled *P. yoelii*-IEs or (cont. from table 5.I) UEs (30:DC), and then stimulated for an additional 24h with LPS, or PBS as control. Maturation frequencies were determined by expression of CD86.

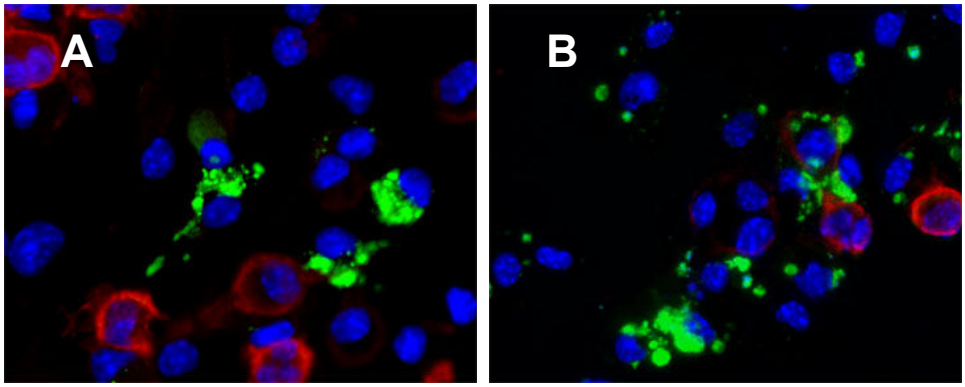


Figure 5.8 – Uptake of erythrocytes, either *P. yoelii*- infected or non-infected, inhibits DC maturation. Representative images of DCs co-cultured with CFSE-labeled erythrocytes (A – non-infected, B – *P. yoelii*-infected), and stained for CD86 membrane expression (red). DAPI was used for nuclei staining (blue).

Our studies using labelled erythrocytes confirm the previous results on the inhibition of DC maturation and IL-12 production using flow cytometry and ELISA, which give an average result of the whole DC population; in all studies, the inhibition induced by *P. yoelii*-IEs was much more pronounced with IEs, but specially for IL-12 production there was a considerable effect of NEs, even if lower than that of *P. yoelii*- IEs (fig. 5.6). The results from single cell analysis reveal that DCs can be inhibited similarly by infected and non-infected erythrocytes, suggesting that the previously observed effects of NEs result

from similar interactions to those that happen in the presence of *P. yoelii* but to a smaller extent, due to a reduced phagocytosis rate.

5.5. Discussion

We have described a general repression of TLR-dependent signaling and gene expression in DCs and TLR-transfected cells induced by *P. yoelii*- infected erythrocytes (IEs). These effects can be mimicked by non-infected erythrocytes (NEs).

We have extended previous observations on the inhibition of DC maturation in response to secondary stimulation of TLR4 with LPS (Urban et al. 1999) (Ocaña-Morgner et al. 2003) to stimulation of TLR2, TLR3, TLR5, TLR7 and TLR9. We observed an inhibition of expression of co-stimulatory molecules and IL-12, a cytokine relevant in the induction of Th1 responses (Trinchieri 2003). If confirmed *in vivo,* such a wide inhibition of TLR-dependent gene expression would be a likely mechanism by which *Plasmodium* promotes a general immune suppression of the host, given the central role of TLRs in shaping adaptive immunity; such general suppression includes reduced responses to malaria, co-infections and vaccination, as pointed in Chapter I.

In the laboratory we have confirmed that DCs do not mature during *P. yoelii* infection (fig. 3.7). Other laboratories have shown the same looking at late infection, and suggested that the inhibition is the result of a mechanism similar to endotoxin tolerance; such mechanism would require the activation of TLR signaling at an earlier stage of infection and lower parasitemia (Perry et al. 2005). However, as we thoroughly investigated, no DC maturation or TLR activation is observed either *in vitro* in response to *P. yoelii*-IEs (fig. 5.1), or else during *P. yoelii* infection as early as day 5 post-infection (fig. 3.7). We therefore propose that the *P. yoelii*-mediated inhibition of DC maturation is

distinct from an endotoxin tolerance-like mechanism since it does not involve TLR-dependent maturation in any phase of infection or *in vitro* stimulation.

The question that necessarily arises from our observations on TLR responses of DCs is: which level (or levels) of TLR-dependent signaling is (are) inhibited by *P. yoelii*?

The fact that the responses to a very large spectrum of TLRs are inhibited, including receptors with considerable differences in their signaling pathways, suggests that *Plasmodium* modulates a step that is shared by those pathways; it is therefore more likely to be closer to gene transcription than receptor binding or signaling events.

Co-stimulatory molecules and IL-12, whose expression is inhibited by *P. yoelii*, have in common the fact that they are mostly mediated by NF-κB-dependent transcription, like a large fraction of TLR-dependent gene expression (Kawai and Akira 2005). It is therefore natural to speculate that NF-κB transcription factors are prevented from activating gene expression in DCs by *Plasmodium*, either because they do not translocate to the nucleus, or because they are targeted for proteolysis and its overall expression is reduced.

The preliminary data suggesting that the activation of RelA subunits is inhibited when DCs are pre-incubated with *P.yoelii*-IEs indicates that DCs may have a reduced ability to trigger NF-κB-dependent gene expression in the presence of the parasite.This would apply to genes such as IL-6, IL-12 and TNF-α (Kawai and Akira 2005), which are all induced by purified *Plasmodium* DNA or GPI when these molecules are removed from the context of the whole infected erythrocyte (Parroche et al. 2007) (Krishnegowda et al. 2005). The fact that IL-6 is still secreted by DCs in response *to P. yoelii*-IEs (fig.4.2) even with lower NF-κB expression and/or activity can be explained by the induction of cyclic AMP-dependent transcription factors (CREB and C/EBP) by the PGE2/cAMP/PKA/p38 pathway described in Chapter IV. Whether this pathway relates to

a possible MyD88-independent signaling responsible for *il-6* expression (fig. 5.3) will be discussed in the next chapter.

The inhibition of RelA activation induced by *P. yoelii*, together with the quick production of PGE2 (Ocana-Morgner, in press), molecule that is able to induce a repressor of *il-12* transcription (Becker et al. 2001), provide two synergistic mechanisms by which the production of IL-12 is impaired in DCs incubated with *P. yoelii*-IEs, but also for the previously described IL-12 inhibition in mouse models (Xu et al. 2001) and infected individuals (Keller et al. 2006). The fact that this inhibition is maximal after 6h of pre-incubation suggests that transcription of a repressor of *il-12* gene is required but not necessary, since it is significant already at time 0. Whether a repressor of *il-12* is induced by PGE2 in the context of malaria remains to be investigated.

The association of erythrocyte phagocytosis with inhibition of DC maturation that was observed at the single-cell level is of notorious relevance for the understanding of the interactions between DCs and the infected erythrocyte.

We have described before how gene transcription is extensively regulated, and also how certain signaling mechanisms are activated in DCs during *P. yoelii* infection, or else *in vitro*. These events can have different outcomes depending on the expression of co-stimulatory molecules on the DC membrane, since this fact will determine if DCs will provide additional signals to the T cells besides the soluble ones, such as PGE2 and IL-6; those receptor-based interactions between DC and T cells are crucial to determine an optimal activation of T cells (Lutz and Schuler 2002).

We have described how DCs not only do not mature in response to *P. yoelii*, but furthermore are inhibited in their response to various ways of TLR stimulation; this was shown to be time and dose-dependent. Even if some degree of inhibition can be observed with high-enough doses of non-infected erythrocytes (NE), the inhibition

caused by *P. yoelii*-IEs is always much stronger than that of a similar dose of NEs. This was characterized using flow cytometry or ELISA to analyze average results for expression of co-stimulatory molecules, or else IL-12 production, in large populations of CD11c+ DCs. Later on, we devised a method using fluorescence microscopy to analyze separately the DCs which uptake erythrocytes, by means of CFSE labeling of both NEs and IEs. We observed a strong association of the presence of CFSE-labeled vesicles inside the DCs with the absence of co-stimulatory molecules, irrespectively of the nature of the erythrocytes present. This observation suggests that erythrocytes contain factor(s) that are strong inhibitors of DC maturation; this modulation of DC functions is amplified in *Plasmodium* infections due to an increased rate of erythrocyte phagocytosis.

Using the same methodology but a different *Plasmodium* parasite (*P. chabaudi*) and a different quantification method (flow cytometry), others have reached contrasting results (Ing et al. 2006). We believe the discrepancies can be explained by one of two factors: either *P. chabaudi* and *P. yoelii* have considerable differences in the way they modulate DCs, or else quantification analysis by flow cytometry mislead authors in the study of Ing *et al* due to cross-reaction between fluorescence channels, which is not the case in our fluorescence microscopy analysis.

We propose that the association we observe between phagocytosis of *P. yoelii* and inhibition of DC maturation is a consequence of the inherent tolerance of the mammalian immune system to self erythrocytes. DCs are known to be central players in the induction of tolerance and control of auto-immunity (Steinman et al. 2003); clearance of erythrocytes is one of the processes where auto-immune reactions must be prevented from being triggered by phagocytes that participate in that process (Oldenborg 2004). The fact that the infected erythrocyte increases the probability of phagocytosis, due to the modifications in the membrane of the erythrocyte, will increase the DC population that is in a tolerant or semi-mature state since expression of co-stimulatory molecules is

inhibited. Still, immune mediators like PGE2 or IL-6 can be released by DCs, and promote a wide array of immune processes; however, those inflammation mediators can also have deleterious effects on T cells, as we have observed for PGE2 in the case of malaria (Ocana-Morgner, in press).

The modifications in the IE membrane that promote phagocytosis are not unlike those that senescent erythrocytes undergo, as reviewed in (Bratosin et al. 1998) and (Arese et al. 2005). One example is the exposure of phosphatidylserine (PS), a molecule that binds to CD36 and other scavenger receptors (McGuinness et al. 2003), and is known to be induced by erythrocyte injury and to mediate erythrocyte clearance (Lang et al. 2005). PS has also been shown to be increased in *Plasmodium*-IEs (Eda and Sherman 2002). This molecule is a signal of membrane oxidation, either in senescent erythrocytes or else in apoptotic cells, which induce a tolerized state of DCs just like *Plasmodium*; such modulation is suggested to be mediated by CD36 (Urban et al. 2001). The infected erythrocyte is likely to oxidize faster and therefore expose higher amounts of PS, promoting its phagocytosis (Lang et al. 2005).

Additional mechanisms that promote phagocytosis of *Plasmodium*- IEs are related to the expression of *Plasmodium*-encoded proteins in the outer membrane of the IE. PfEMP1 protein attaches to receptors expressed in the DC or macrophage, including CD36, ICAM1 and VCAM1 (Sherman et al. 2003). PfEMP1 is likely to mediate the CD36-induced modulation of DC functions by *P. falciparum* (Urban et al. 2001). In murine malaria the ligands for CD36 have not been identified.

The absence of expression of co-stimulatory molecules is likely to be dependent from molecules within the host erythrocyte that will also be present in the infected one. Even if parasite-specific molecules, such as DNA and GPI, are able to induce expression of CD40, CD80 and CD86, the tolerance mechanisms triggered by molecules from the erythrocyte seem to overcome the former pathways, either TLR-dependent or others.

An example of the molecular mechanism that could be involved in preventing DC maturation is the signaling triggered by CD47, a receptor expressed by erythrocytes and considered a marker of "self" (Oldenborg et al. 2000). Among other functions (Oldenborg 2004), CD47 expression seems to be an important regulator of erythrocyte clearance (Okazawa et al. 2005) probably through its association with Band3, a determinant of erythrocyte senescence (Arese et al. 2005). CD47 binds SIRPα (signal regulatory protein α), which is expressed in macrophages and DCs and can induce negative signals for phagocytosis and macrophage activation through its two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (Oldenborg 2004). Whether SIRPα can regulate DC maturation is not studied.

The paradigm for the study of ITIMs negative signals in immune cells has been the NK cell (Ravetch and Lanier 2000), whose activation is promoted by activation motifs (ITAMs), but is inhibited by ITIMs. In macrophages, ITIMs preferentially induce negative signaling through SHP-1 (Src homology-2 (SH2) domain-containing protein tyrosine phosphatase 1) (Veillette et al. 1998). A clear role this phosphatase in regulation of DC functions has not been identified; however, lectin-like, ITIM-containing receptors such as DCAL-2 (Chen et al. 2006), DCIR (Bates et al. 1999) or LLIR (Huang et al. 2001) are known to bind SHP-1 and to have effects on DCs such as inhibition of TLR-induced IL-12 (Chen et al. 2006).

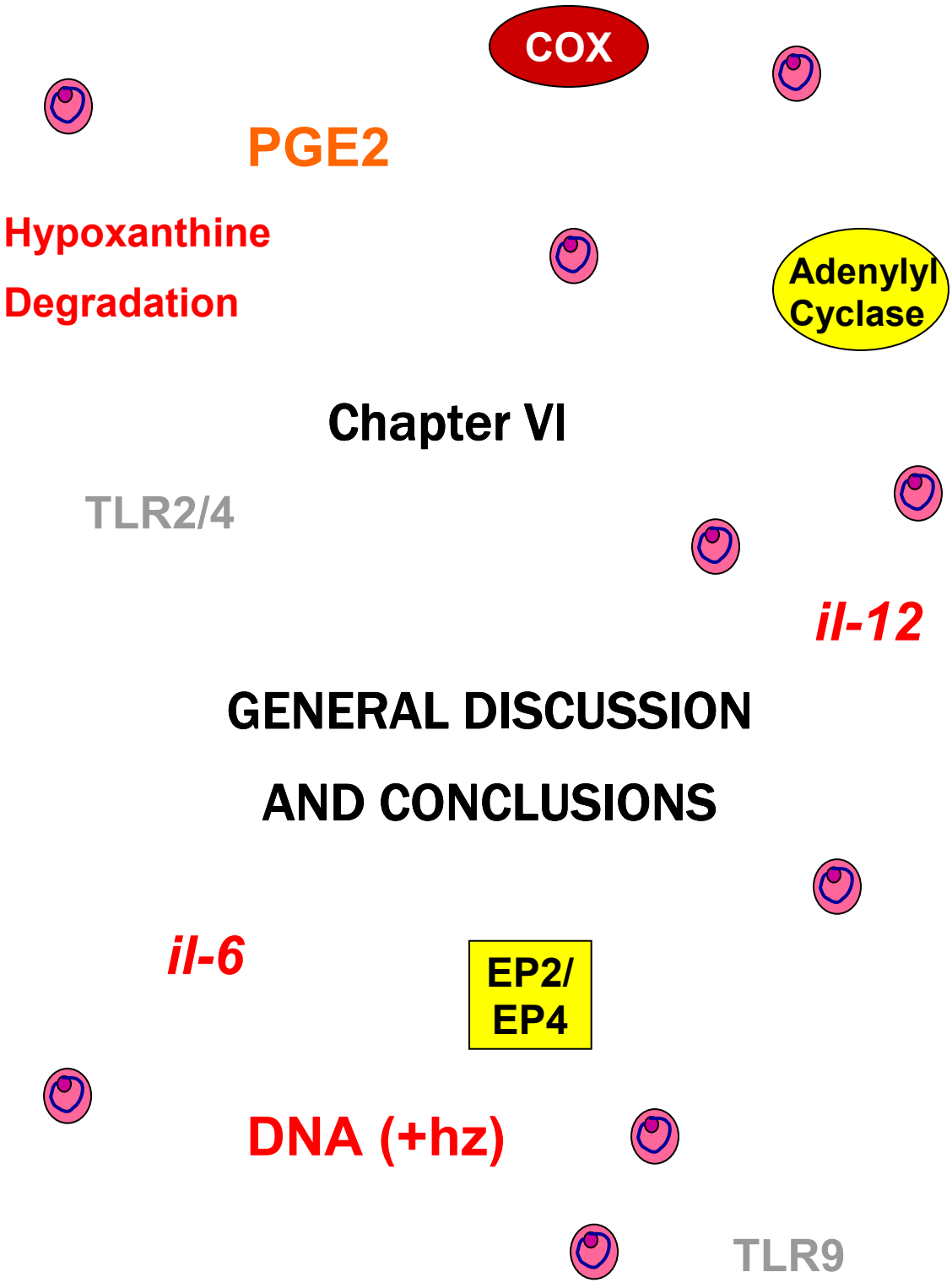
A role for CD47 interactions by DC receptors such as SHPS-1 has been identified in Langerhans Cells, including regulation of maturation (Fukunaga et al. 2006), but the signaling mediators were not identified. Still, these observations suggest that direct interactions of DC receptors with CD47 can regulate DC functions. Whether CD47 and SIRPα are involved in erythrocyte-mediated inhibition of DC maturation, especially in the context of malaria, is a subject that requires extensive investigation.

An alternative model for DC regulation that has been proposed for the case of malaria and apoptotic cells is the activation of CD36 (Urban et al. 2001); the signaling triggered by that receptor was not studied. Even if rodent parasites like *P. bergheii* bind extensively to CD36 (Franke-Fayard et al. 2005), a role for CD36 in DC regulation in those models is not clear, opposite to *P. falciparum,* where CD36 is required for cytokine production, at least in macrophages (Patel et al. 2007).

Contrary to the case of *P. falciparum* (Urban et al. 2001), inhibition of DC maturation by *P. yoelii* is not contact-dependent and does not require CD36 expression (Jamie Orengo, submitted for publication). However, in the case of direct interactions involving phagocytosis, it is possible that CD36 is engaged by infected erythrocytes, adding to the modulations induced by other pathways; a role for phosphatidylserine in this context is highly likely and requires urgent investigation.

A model for DC inhibition mediated by erythrocyte factors is supported by research that has identified non-infected erythrocytes as agents of inhibition of cellular responses in the context of human malaria (Struik et al. 2004).

The identification of the factors from the erythrocyte responsible for the modulation of DC maturation, and for the consequences on adaptive immunity, as well as the molecules from the DC that interact with them, is an important task for malaria immunologists in the near future.


COX
PGE2
Hypoxanthine
Degradation
Adenylyl
Cyclase
Chapter VI
TLR2/4
il-12
GENERAL DISCUSSION
AND CONCLUSIONS
il-6
EP2/
EP4
DNA (+hz)
TLR9

We have analyzed the overall transcriptomic regulation of dendritic cells (DCs) during infection with the rodent malaria parasite *P. yoelii*, and in more detail the signaling related to the expression of the *il-6* gene (Carapau et al. 2007). On the other hand, we studied the inhibition of Toll-like receptor (TLR)-dependent gene expression in the presence of *P. yoelii*, and how the phagocytosis of *P. yoelii*-infected erythrocytes (IEs) by DCs associates with the lack of DC maturation and of responses to stimulation by TLR-dependent pathways (Chapter V).

From the overall data obtained, three major questions arise about the different signaling pathways involved in the interactions of DCs and *P. yoelii*, and also the role of DCs in modulating immunity to *Plasmodium*; those questions will now be discussed in more detail.

6.1 Is cyclooxygenase (COX)-dependent increase in IL-6 production related to TLR-mediated signaling?

Analysis of IL-6 production by MyD88-/- DCs suggested that both MyD88-dependent and independent pathways are triggered in response to *P. yoelii*-IEs (fig. 5.3).

MyD88-dependent signaling can be activated by all of the TLRs that have been identified as receptors for *Plasmodium* molecules: TLR2 and TLR4 for GPI (Krishnegowda et al. 2005) and TLR9 for DNA+hemozoin (Parroche et al. 2007). However, MyD88-/- DCs will show reduced responses only to TLR9 activation since this receptor does not activate MyD88-independent pathways, oppositely to TLR2 or TLR4 (Akira and Takeda 2004). It is likely that the DNA-mediated engagement of TLR9 is responsible for the fraction of IL-6 release that requires MyD88; further studies are required to confirm this hypothesis. Also the possibility that the MyD88-independent induction of IL-6 is related to the activation of TLR2/TLR4 by *Plasmodium* GPI has not been addressed. Whether the

COX/cAMP-dependent pathway for expression of *il-6* (Chapter IV) constitutes a TLR2/TLR4-dependent, MyD88-independent pathway is another relevant question that should be investigated.

According to our data, *P. yoelii*- infected erythrocytes (IEs) do not induce expression of IL-12 or TNF-α, which are inducible by *Plasmodium* DNA or GPI by TLR-dependent mechanisms (Parroche et al. 2007). It seems likely that the inhibition of NF-κB by *P. yoelii*-IEs may be responsible for the inhibition of these two cytokines, as their expression is dependent on NF-κB activation (Akira and Takeda 2004) (Trinchieri 2003). IL-6 expression can also be regulated by NF-κB, but other transcription factors equally play a role in its expression such as CREB and C/EBP (Song and Kellum 2005); the latter have in common the characteristic of being cyclic AMP-inducible (Mayr and Montminy 2001) (Wilson and Roesler 2002). CREB and C/EBP are likely to be activated by *P. yoelii* –IEs through a cAMP/PKA/p38 pathway, resulting in expression of *il-6* (Carapau et al. 2007) regardless of the activation status of NF-κB transcription factors.
Besides the inhibition of NF-κB, the inhibition of *il-12* can also result from the pathway described for the activation of *il-6* since prostaglandin E2 (PGE2) is a strong inhibitor of IL-12 production (van der Pouw Kraan et al. 1995); recently, a PGE2-dependent repressor element in the il12p40 promoter was found (Becker et al. 2001).

As we have mentioned, it is possible that the cAMP-dependent production of IL-6 is related to TLR-dependent signaling triggered by *Plasmodium* DNA or GPI; this would require that TLR activation induces COX activity. TLRs are known to induce PGE2 release after the induction of *cox2* gene, as described in detail for bacterial peptidoglycan (PGN)-mediated activation of TLR2 (Chen et al. 2006); however, this

mechanism is not compatible with the rapid kinetics of PGE2 release in response to *P. yoelii*- IEs (within 30m; Carlos Ocana-Morgner, in press), which contrasts with the slower production of that prostaglandin (3-6h) in response to TLR activation.

As an alternative to a TLR-dependent mechanism, we can propose PGE2 release to be induced by lipid degradation within the erythrocyte membrane that is induced by the parasite, as described before (Schwarzer et al. 2003); these events include the accumulation of derivatives of arachidonic acid (AA), the precursor of prostanoids. Given the kinetics of PGE2 release in DC+ *P. yoelii*- IEs co-culture (Carlos Ocana-Morgner, in press), it is more likely that the process of *P. yoelii*-mediated production of PGE2 is mediated by the constitutively expressed COX1, instead of the inducible gene *cox2*. COX1 would act on the AA derivatives that are found in malaria-infected erythrocytes – which have been shown to have immune-modulatory properties (Schwarzer et al. 2003) – inducing the release of PGE2.

One metabolic pathway that is able to regulate PGE2 production in response to *Plasmodium* is the degradation of hypoxanthine, a product of nucleotide degradation. Studies in the laboratory show that infected erythrocytes accumulate high concentrations of hypoxanthine and that inhibition of hypoxanthine degradation using the xanthine oxidase (XO) inhibitor allopurinol abrogates production of PGE2 by DCs in response to *P. yoelii*, suggesting a role for either uric acid or reactive oxygen species (ROS), which are the products of the XO reactions, in inducing PGE2 (Jamie Orengo et al., submitted for publication). The molecular nature of the uric acid receptor is so far unknown (Shi et al. 2003), but the possibility of activating one of the TLRs has been ruled out (Chen et al. 2006). Therefore, the hypoxanthine degradation-mediated production of PGE2, and also IL-6, in the context of malaria does not involve TLRs, but instead the hypothetical uric acid receptor.

A PGE2-dependent pathway that does not require TLR activation explains why IL-6 is produced by DCs in response to *P. yoelii* even if these parasites do not trigger TLR-dependent gene expression in a transfected cell line system (fig. 5.2), do not induce DC maturation (fig. 5.1), and seem to inhibit nuclear translocation of NF-κB (fig. 5.7).

We propose that hypoxanthine degradation promotes COX activity, which is responsible for PGE2 release; cyclic AMP-dependent signaling is then triggered, leading to expression of *il-6* as well as other yet unidentified genes. Other mechanisms of PGE2 production that do no involve hypoxanthine might also take place in the context of malaria infection (as depicted in fig. 6.1) but with minor contributions given the effect of allopurinol on PGE2 production (Jamie Orengo et al., submitted for publication).

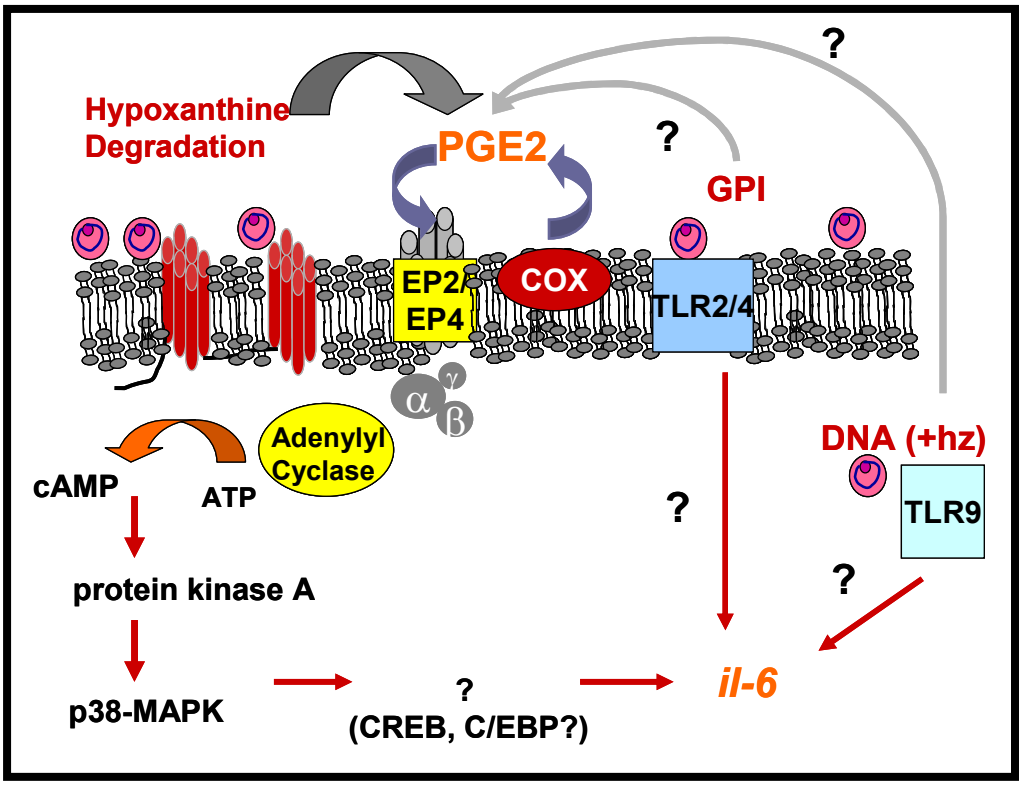


Figure 6.1 – Model for regulation of interleukin-6 gene expression in response to *Plasmodium*. Possible pathways promoting production by DCs of the immune mediators PGE2 and IL-6 in the context of malaria expression.

6.2 Is PGE2 production associated with erythrocyte phagocytosis and inhibition of DC maturation?

PGE2 has been detected to be produced in response to erythrocyte injury, and to be a central mediator of erythrocyte suicidal death, targeting themselves for phagocytosis (Lang 2005a) (Lang 2005b). These events involve the osmotic shock-mediated activation of the erythrocyte cyclooxygenases, PGE2-mediated activation of cationic channels, increases of Ca^{2+} intracellular concentrations, and finally the exposure of phosphatidilserine (PS) on the outer side of the erythrocyte membrane. The term *eryptosis* has been proposed to describe this process of self-induced clearance of erythrocytes, due to its similarities with some of the later stages of the apoptosis of nucleated cells, and (Lang 2005b).

Breakdown of PS asymmetry of the cell membrane, which has been associated with cytoadherence of *Plasmodium*-infected erythrocytes (IEs) (Eda and Sherman 2002), induces clearance of apoptotic nucleated cells, but also senescent erythrocytes, by macrophages and DCs through their scavenger receptors, such as CD36; these events induce a profound modulation of DC functions (Urban et al. 2001), as previously discussed.

We have shown that DCs respond to IEs by producing PGE2 and that this production depends on the cyclooxygenase (COX) activity of host cells (Ocana-Morgner, in press). Whether the COX activity required for PGE2 production is that of the DC cytoplasm, the erythrocyte cytoplasm or both is a question that requires further studies. Even if the activity is based in the cytoplasm of the erythrocyte, it is evident that PGE2 formation requires another cell type such as the DC, since control cultures of infected erythrocytes

(IEs) alone never have comparable levels of PGE2 to those of DC+IE co-culture (Ocana-Morgner et al, in press).

PGE2 or else cyclooxygenase activity have been shown to have the ability to modulate production of important cytokines such as TNF-α (Keller 2006) and IL-6 (Carapau 2007) in the context of malaria infection. If a role for DC- or DC+erythrocyte- based COX activity and PGE2 secretion in promoting erythrocyte phagocytosis, such data would make PGE2 a central regulator of innate as well as adaptive immunity to *Plasmodium*; such a role is highly likely given the induction of PS asymmetry by PGE2 and the contribution of PS asymmetry for cytoadherence.

In fact, increased phagocytosis of infected and non-infected erythrocytes in malaria-infected mice is observed during malaria infections (Omodeo-Sale et al. 2003). an observation that is compatible with this hypothesis. Whatever factors are found to regulate IE phagocytosis will also determine the inhibition of DC maturation, a process that has profound consequences on adaptive immunity.

6.3 Is inflammation compatible with cellular immunity?

The proposed roles of DCs in malaria vary from inducers of inflammatory cytokines (Seixas et al. 2001) and of T cell responses (Perry et al. 2004) to suppressors of both (Urban et al. 2001) (Ocaña-Morgner et al. 2003). More recently it was proposed that DCs switch from a pro-inflammatory, pro-cellular immunity phenotype at lower parasitemia to a suppressive phenotype at later infection or higher parasitemia (Perry et al. 2005), as discussed before.

As elegantly discussed by Haring *et al.* (Haring et al. 2006), pro-inflammatory cytokines such as IL-12 and IFN-γ are crucial mediators of cellular immunity, but not always in a

positive way, such as the inhibition of CD8+ memory T cell development when inflammation is prolonged over time. Another example of a pro-inflammatory cytokine that controls adaptive immunity is IL-6, as reviewed in (Jones 2005)).

Most pro-inflammatory cytokines are triggered during DC maturation by TLR-dependent signaling, usually through activation of NF-κB transcriptional activity, which also promotes expression of co-stimulatory molecules; this signal together with cytokine production promotes optimal T cell activation (Lutz and Schuler 2002).

Some pathogens such as the Toxoplasma parasite (McKee et al. 2004) are able to block DC maturation. From our preliminary data on TLR-dependent signaling and NF-κB expression, it seems that *Plasmodium* might have a similar mechanism to that of Toxoplasma. However, the inhibition of NF-κB seems to impair only the expression of co-stimulatory molecules and IL-12, while IL-6 is still secreted by a non-classic signaling pathway involving the second messenger cyclic AMP (Carapau et al. 2007). The data suggests that the host-parasite interactions in the case of *P. yoelii* promote cytokine production but inhibit the receptor-mediated signal for T cell activation provided by co-stimulatory molecules.

Even if IL-6 can have deleterious effects on T cells (Jones 2005), on the other hand this cytokine is a relevant inhibitor of regulatory T cells (Tregs) (Pasare and Medzhitov 2003), what might constitute a beneficial factor for the host since early production of Tregs seems to associate with uncontrolled parasitemia, both in rodent malaria (Hisaeda et al. 2004) and in human infection with *P. falciparum* (Walther et al. 2005). In the latter study it was shown a positive association between TGF-β and induction of Tregs; however, a role for IL-6 in down-regulating Tregs was not studied.

A role for DCs in promoting inflammation and fever through PGE2 and IL-6 is not incompatible with the role in suppressing cellular immunity that has been described

before (Ocaña-Morgner et al. 2003). The same molecules that promote inflammation can also inhibit CD8+ and CD4+ T cell responses, as it has been shown for the case of PGE2 (Ocana-Morgner, in press). Altogether, our data suggests that DCs play crucial roles in promoting inflammation and fever by the release of PGE2 and IL-6, while down-modulating activation of T cells, in part due to PGE2, but also by TGF-β (Ocana-Morgner, in press), together with the absence of co-stimulatory molecule expression.

6.4 Conclusions and Perspectives

In this thesis we have described the following roles of DCs in response to blood-stage *Plasmodium* infection in a murine model:

- extensive genetic remodeling in response to *P. yoelii* infection, resulting in the induction of cytokines such as IL-6, IL-10 and IFN-γ and the down-regulation of co-stimulatory molecules, besides other ca. 700 genes that are significantly modulated, including cell cycle regulators, defense response genes, signaling mediators and others;
- a significant part of the DC transcriptomic response to *P. yoelii* differs from the responses to bacterial, viral and parasitic pathogens, suggesting that DC/Plasmodium interactions are very uncommon among immune responses to microbes and require detailed characterization;
- DC secrete of the acute-phase protein and pyrogen IL-6 in response to a signaling mechanism that is dependent on cyclooxygenase activity and that is mediated by the second messenger cyclic AMP, suggesting a role for spleen DC in inducing inflammation through prostanoids (PGE2) and IL-6;

- DCs actively phagocytose *P. yoelii*-infected erythrocytes (Py-IEs), by mechanisms still not fully dissected; this process is associated with a blockage of DC maturation in response to IEs but also secondary stimulation, that seems to associate with a reduction in the transcriptional activity of the NF-κB family.

Together, these conclusions suggest a strong participation of DCs in promotion of inflammation by the production of IL-6, which will promote fever together with PGE2, another immune mediator also released by DC (Ocana-Morgner, in press). On the other hand, IL-6 is also a promoter of the resolution of inflammation; this fact together with the negative roles of PGE2 in T cell activation (Ocana-Morgner, in press) might contribute to an inhibition of cellular immunity to malaria.

The role of DCs might therefore be considered both beneficial and damaging for the host since the molecules released might inhibit an efficient cellular response to *Plasmodium*, but might also induce inflammation to a certain extent that is required to dampen the infection. Since PGE2 is able to inhibit TNF-α production (Keller et al. 2006), a PGE2/IL-6 combination might be able to control the extent to which inflammation is induced and reduce immune-based pathology.

The inhibition of cellular immunity by PGE2 but also by IL-6 – which acts as a pro-apoptotic factor in certain T cell populations (Jones 2005) – is favored in the absence of expression of co-stimulatory molecules on DCs, which is severely impacted by *P. yoelii*. Not only DCs are not able to induce CD40, CD80, CD83 and CD86 in response to infection (fig. 3.7), but these molecules are also inhibited even if a secondary stimulation induced by a TLR ligand is used. We report this inhibition to associate with Py-IE phagocytosis, and suggest to be mediated by a reduced transcriptional activity of Nf-κB

transcription factors. If Py-IE phagocytosis is confirmed to inhibit DC maturation during infection, this would be a central mechanism for the generalized immuno-suppression of individuals infected with *Plasmodium*, which has been studied, even if only superficially, for the last decades.

Many questions remain to be answered in respect to the roles of DCs during malaria infections, such as:

- Which are the factors of *Plasmodium* or the erythrocyte responsible for the induction of immune modulators such as PGE2 and IL-6? Do these factors bind to TLRs or other receptors?
- Which factors of *P. yoelii* -IEs promote its phagocytosis by DCs? Is PGE2 one of such factors?
- Which are the factors of *Plasmodium* or the erythrocyte responsible for the inhibition of DC maturation upon IE phagocytosis? Is inhibition of NF-κB activity one of the mechanisms for DC maturation? Which members of the NF-κB family are inhibited?
- Does IE phagocytosis induce a reduced expression and activity of NF-κB transcription factors?
- Is the inhibition of IL-12 in the presence of *P. yoelii* a result of reduced activity of NF-κB and/or is it mediated by a PGE2/cAMP-dependent mechanism?
- What other genes besides *il-6* are expressed in response to cAMP-dependent signaling?
- Does PGE2 or cAMP-dependent pathways regulate expression of co-stimulatory molecules or DC migration?

- Are cAMP- dependent pathways responsible for DC-mediated suppression of T cell activation?

The resolution of this and other questions by malaria researchers will provide us with relevant insights into the molecular mechanisms taking place during the early events in malaria infection; these events will determine the reaction of the different arms of the immune system – innate, adaptive, regulatory – to this parasitic infection.

Even if the characterization of innate responses to *Plasmodium* has seen a great expansion in recent years, many gaps remain in our understanding of the interactions between host cells and the malaria parasite, reality that has been confirmed with the studies presented in this Thesis – specially the extent of the gene regulations that remain to be characterized.

We show evidence of new roles for DCs in immunity to *Plasmodium*, but many questions still remain unanswered. We hope that the advances we report here will provide valuable information and will stimulate further studies in the participation of DCs in determining cytokine production, fever responses and adaptive immunity to malaria.

Plasmodium parasites are responsible for a heavy toll on human lives of the developing countries. We hope that new advances in the basic science of host/parasite interactions within the immune system, along with breakthroughs in drug discovery and vaccine development, will lead the way for a quick and dramatic reduction in the morbidity and mortality caused by malaria, and therefore the improvement of the health conditions of the poorest populations of the world.

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APPENDIX I

Genechip® data analysis: transcripts significantly regulated in spleen dendritic cells by *P. yoelii* infection

Appendix I -Table I

Genes up-regulated in spleen DCs by *P. yoelii* infection at both day 5 pi and day 10 pi

						Day 5 vs 0		Day 10 vs 0		Day 10 vs 5	
Probe_ID	p-value	Day 1	Day 5	Day 10	Gene Symbol	5vs0	p<0.01	10vs0	p<0.01	10vs5	p<0.01
1419060_at	0,000372	3,23	12,96	11,39	Gzmb	**9,73**	**U**	**8,16**	**U**	**-1,57**	**D**
1451632_a_at	0,008149	3,59	12,66	15,34	Igh-1a	**9,07**	**U**	**11,75**	**U**	**2,68**	**U**
1419684_at	0,000117	2,18	10,33	10,24	Ccl8	**8,15**	**U**	**8,06**	**U**	-0,09	
1450826_a_at	0,003868	4,38	12,25	12,03	Saa3	**7,87**	**U**	**7,65**	**U**	-0,22	
1424415_s_at	0,003325	3,39	9,68	9,05	Spon1	**6,69**	**U**	**6,02**	**U**	-0,67	
1422120_at	0,001067	3,15	9,43	11,02	Eaf2	**6,28**	**U**	**7,87**	**U**	**1,59**	**U**
1449305_at	0,004088	2,34	8,54	7,00	F10	**6,20**	**U**	**4,66**	**U**	**-1,54**	**D**
1422280_at	0,002123	2,48	8,62	6,63	Gzmk	**6,14**	**U**	**4,15**	**U**	**-1,98**	**D**
1423135_at	0,000876	2,50	8,53	6,30	Thy1	**6,03**	**U**	**3,80**	**U**	**-2,23**	**D**
1422062_at	0,007698	2,87	8,80	7,15	Msr1	**5,68**	**U**	**4,33**	**U**	**-1,35**	**D**
1422134_at	0,001739	2,01	7,31	8,38	Fosb	**5,30**	**U**	**6,37**	**U**	**1,07**	**U**
1421578_at	0,004799	4,11	9,36	8,66	Ccl4	**5,25**	**U**	**4,55**	**U**	-0,70	
1419561_at	0,008112	4,54	9,69	9,40	Ccl3	**5,14**	**U**	**4,86**	**U**	-0,28	
1426170_a_at	0,005987	3,59	8,66	5,12	Cd8b1	**5,07**	**U**	**1,53**	**U**	**-3,54**	**D**
1423100_at	0,000484	8,52	13,57	13,71	Fos	**5,05**	**U**	**5,19**	**U**	0,14	
1456212_x_at	0,007261	6,91	11,32	11,32	Socs3	**5,00**	**U**	**4,97**	**U**	-0,03	
1453677_a_at	0,004518	2,91	7,70	10,03	Derl3	**4,79**	**U**	**7,13**	**U**	**2,33**	**U**
1448191_at	0,00308	4,33	9,04	9,02	Plk1	**4,71**	**U**	**4,70**	**U**	-0,01	
1418021_at	0,000675	2,96	7,63	8,81	C4	**4,66**	**U**	**5,84**	**U**	**1,18**	**U**
1449171_at	0,004067	2,63	7,26	7,41	Ttk	**4,62**	**U**	**4,77**	**U**	0,15	
1448562_at	0,00431	2,41	7,00	3,69	Upp1	**4,58**	**U**	**1,28**	**U**	**-3,30**	**D**
1450871_a_at	0,00214	1,82	6,28	6,82	Bcat1	**4,46**	**U**	**5,00**	**U**	0,54	
1419943_s_at	0,003568	5,09	9,08	9,47	Ccnb1	**4,44**	**U**	**4,89**	**U**	0,46	
1437025_at	0,00324	3,48	7,62	7,81	Cd28	**4,24**	**U**	**4,44**	**U**	0,20	
1417185_at	0,001222	8,29	12,50	11,51	Ly6a	**4,22**	**U**	**3,22**	**U**	-0,99	
1417267_s_at	0,001899	2,77	6,96	8,98	Fkbp11	**4,19**	**U**	**6,21**	**U**	**2,02**	**U**
1450297_at	4,15E-05	2,26	6,38	3,42	Il6	**4,12**	**U**	**1,16**	**U**	**-2,97**	**D**
1419202_at	0,00331	4,37	8,41	6,35	Cst7	**4,04**	**U**	**1,97**	**U**	**-2,07**	**D**
1417813_at	0,006836	4,90	8,91	8,81	Ikbke	**4,01**	**U**	**3,91**	**U**	-0,10	
1439040_at	0,002502	3,22	7,06	6,67	Cenpe	**3,83**	**U**	**3,44**	**U**	-0,39	

1418186_at	0,000697	3,15	6,97	5,72	Gstt1	**3,82**	**U**	**2,58**	**U**	**-1,25**	**D**
1419042_at	0,002051	7,65	11,17	11,03	Iigp1	**3,82**	**U**	**3,50**	**U**	-0,32	
1417911_at	0,00116	5,91	9,66	10,00	Ccna2	**3,75**	**U**	**4,10**	**U**	0,35	
1424254_at	0,006125	6,88	10,57	9,67	Ifitm1	**3,68**	**U**	**2,78**	**U**	-0,90	
1427161_at	0,007091	4,90	8,56	8,42	Cenpf	**3,66**	**U**	**3,52**	**U**	-0,14	
1416961_at	0,008171	4,43	8,07	8,49	Bub1b	**3,64**	**U**	**4,06**	**U**	0,42	
1449877_s_at	0,000581	2,51	6,12	6,33	Kifc1	**3,61**	**U**	**3,82**	**U**	0,21	
1418919_at	0,005242	3,80	7,41	7,84	Sgol1	**3,60**	**U**	**4,04**	**U**	0,43	
1425156_at	0,003137	6,10	9,70	9,37	9830147J24Rik	**3,60**	**U**	**3,27**	**U**	-0,33	
1424046_at	0,008431	5,64	9,21	9,23	Bub1	**3,57**	**U**	**3,59**	**U**	0,02	
1435906_x_at	0,005705	7,79	11,29	10,62	Gbp2	**3,50**	**U**	**2,83**	**U**	-0,67	
1452181_at	0,008722	2,77	6,26	7,73	Ckap4	**3,49**	**U**	**4,96**	**U**	**1,47**	**U**
1448205_at	0,006806	6,20	9,67	10,27	Ccnb1-rs1 /// Ccnb1	**3,47**	**U**	**4,07**	**U**	0,61	
1425179_at	0,00313	3,41	6,84	6,66	Shmt1	**3,43**	**U**	**3,25**	**U**	-0,19	
1424292_at	0,001454	4,00	7,44	7,46	Depdc1a	**3,43**	**U**	**3,45**	**U**	0,02	
1418281_at	0,009101	5,10	8,53	8,41	Rad51	**3,43**	**U**	**3,31**	**U**	-0,12	
1416575_at	0,005558	3,21	6,63	6,89	Cdc45l	**3,42**	**U**	**3,69**	**U**	0,26	
1429171_a_at	0,005271	5,55	8,92	9,35	5730507H05Rik	**3,36**	**U**	**3,80**	**U**	0,44	
1415811_at	0,009253	4,34	7,68	7,28	Uhrf1	**3,34**	**U**	**2,94**	**U**	-0,40	
1419697_at	0,002575	4,19	7,52	6,92	Cxcl11	**3,33**	**U**	**2,73**	**U**	-0,60	
1424727_at	0,008322	6,45	9,71	9,16	Ccr5	**3,26**	**U**	**2,70**	**U**	-0,55	
1416299_at	0,004735	6,65	9,83	9,99	Shcbp1	**3,18**	**U**	**3,34**	**U**	0,16	
1420549_at	0,004903	7,55	10,73	10,41	Gbp1	**3,17**	**U**	**2,86**	**U**	-0,32	
1417587_at	0,006617	3,85	7,00	6,95	Timeless	**3,15**	**U**	**3,09**	**U**	-0,06	
1435054_at	0,002607	2,80	5,94	6,56	6820428D13	**3,13**	**U**	**3,76**	**U**	0,62	
1452257_at	0,00813	3,50	6,60	6,56	Bdh	**3,11**	**U**	**3,06**	**U**	-0,04	
1437621_x_at	0,009638	7,13	10,21	10,54	Phgdh /// LOC434252	**3,10**	**U**	**3,47**	**U**	0,36	
1425837_a_at	0,007919	4,25	7,33	7,59	Ccrn4l	**3,08**	**U**	**3,35**	**U**	0,26	
1430811_a_at	0,006939	5,67	8,73	9,14	Cdca1	**3,06**	**U**	**3,46**	**U**	0,40	
1449060_at	0,000983	2,82	5,65	5,82	Kif2c	**3,05**	**U**	**3,23**	**U**	0,18	
1448899_s_at	0,00147	3,76	6,81	7,10	Rad51ap1	**3,04**	**U**	**3,34**	**U**	0,30	
1423522_at	0,009475	4,88	7,89	7,33	Npm3	**3,01**	**U**	**2,45**	**U**	-0,56	
1419665_a_at	0,008709	5,59	8,59	7,78	Nupr1	**3,00**	**U**	**2,19**	**U**	-0,81	
1423920_at	0,005038	4,58	7,52	7,65	Brrn1	**2,94**	**U**	**3,07**	**U**	0,13	
1419513_a_at	0,004075	6,01	8,94	9,01	Ect2	**2,93**	**U**	**3,00**	**U**	0,07	
1416333_at	0,009204	5,59	8,51	7,37	Dok2	**2,92**	**U**	**1,78**	**U**	**-1,14**	**D**

1417823_at	0,006226	2,45	5,35	4,83	Gcat	**2,90**	**U**	**2,39**	**U**	-0,52	
1416802_a_at	0,00492	7,95	10,24	10,48	Cdca5	**2,90**	**U**	**3,08**	**U**	0,18	
1429295_s_at	0,004072	5,90	8,96	9,03	Trip13	**2,90**	**U**	**2,95**	**U**	0,05	
1435306_a_at	0,00452	6,01	8,40	8,49	Kif11	**2,89**	**U**	**3,23**	**U**	0,34	
1450496_a_at	0,007681	4,99	7,86	8,14	2810433K01Rik	**2,88**	**U**	**3,16**	**U**	0,28	
1420743_a_at	0,008041	4,78	7,05	8,16	Ppp3cc	**2,87**	**U**	**3,73**	**U**	0,87	
1435476_a_at	0,008233	7,96	10,60	10,66	Fcgr2b	**2,83**	**U**	**2,86**	**U**	0,03	
1423520_at	0,004918	5,21	8,03	7,88	Lmnb1	**2,82**	**U**	**2,67**	**U**	-0,15	
1448650_a_at	0,008851	4,84	7,65	7,97	Pole	**2,82**	**U**	**3,13**	**U**	0,31	
1449207_a_at	0,006628	4,49	7,30	7,76	Kif20a	**2,81**	**U**	**3,28**	**U**	0,46	
1424167_a_at	0,000996	3,79	6,57	7,00	Pmm1	**2,78**	**U**	**3,21**	**U**	0,43	
1423774_a_at	0,008498	5,62	8,51	8,98	Prc1	**2,76**	**U**	**3,15**	**U**	0,39	
1450862_at	0,004316	4,87	7,62	7,69	Rad54l	**2,75**	**U**	**2,81**	**U**	0,07	
1416757_at	0,006074	5,45	8,20	8,27	2310031L18Rik	**2,75**	**U**	**2,82**	**U**	0,08	
1437279_x_at	0,007362	5,94	8,46	9,89	Sdc1	**2,73**	**U**	**4,34**	**U**	**1,61**	**U**
1434437_x_at	0,003401	9,86	12,35	12,60	Rrm2	**2,73**	**U**	**3,00**	**U**	0,28	
1452242_at	0,003801	7,60	10,31	10,11	1200008O12Rik	**2,71**	**U**	**2,51**	**U**	-0,20	
1419172_at	0,007194	6,21	8,90	8,98	Dhfr	**2,69**	**U**	**2,77**	**U**	0,08	
1425225_at	0,009872	9,70	12,39	13,10	Fcgr3a	**2,69**	**U**	**3,40**	**U**	0,72	
1451128_s_at	0,009251	6,63	9,30	9,46	Kif22	**2,68**	**U**	**2,83**	**U**	0,15	
1450156_a_at	0,006203	4,09	6,77	7,63	Hmmr	**2,68**	**U**	**3,53**	**U**	0,86	
1426580_at	0,003971	5,38	7,73	7,77	Plk4	**2,66**	**U**	**2,51**	**U**	-0,14	
1450692_at	0,008128	3,94	6,60	7,00	Kif4	**2,65**	**U**	**3,06**	**U**	0,41	
1456006_at	0,00249	6,50	8,99	8,76	Bcl2l11	**2,63**	**U**	**2,35**	**U**	-0,27	
1422460_at	0,002505	7,95	10,57	10,82	Mad2l1	**2,62**	**U**	**2,87**	**U**	0,25	
1452754_at	0,002497	9,49	12,08	12,91	5730592L21Rik	**2,59**	**U**	**3,42**	**U**	0,83	
1418392_a_at	0,009132	8,14	10,71	10,28	Gbp4	**2,56**	**U**	**2,13**	**U**	-0,43	
1453107_s_at	0,006776	4,90	7,45	7,75	Foxm1 /// Pbp /// 49334	**2,56**	**U**	**2,85**	**U**	0,29	
1424128_x_at	0,006555	6,88	9,43	9,62	Aurkb	**2,55**	**U**	**2,74**	**U**	0,19	
1436990_s_at	0,005744	8,81	11,11	11,32	MGI:2143558	**2,51**	**U**	**2,73**	**U**	0,21	
1416497_at	0,008824	7,02	9,52	10,70	Cai	**2,51**	**U**	**3,69**	**U**	**1,18**	**U**
1454607_s_at	0,00819	7,33	9,56	9,37	Psat1	**2,50**	**U**	**2,43**	**U**	-0,07	
1422430_at	0,009871	6,69	9,17	9,14	Fignl1	**2,49**	**U**	**2,45**	**U**	-0,03	
1439377_x_at	0,002472	9,03	11,50	11,78	Cdc20	**2,47**	**U**	**2,75**	**U**	0,28	
1419153_at	0,002141	9,74	12,20	12,30	2810417H13Rik	**2,46**	**U**	**2,56**	**U**	0,09	
1416022_at	0,009636	8,50	11,17	10,32	Fabp5	**2,46**	**U**	**1,66**	**U**	-0,80	

1417065_at	0,00442	8,54	10,96	11,68	Egr1	**2,42**	**U**	**3,14**	**U**	0,72	
1423714_at	0,008611	6,37	8,77	9,08	Asf1b	**2,40**	**U**	**2,71**	**U**	0,31	
1417445_at	0,009764	4,28	6,68	6,86	Kntc2	**2,39**	**U**	**2,58**	**U**	0,18	
1448604_at	0,003611	5,80	8,19	8,12	Uck2	**2,39**	**U**	**2,32**	**U**	-0,07	
1452040_a_at	0,004377	6,54	8,93	9,56	Cdca3	**2,39**	**U**	**3,02**	**U**	0,63	
1421307_at	0,007881	3,75	6,28	6,12	Car13	**2,38**	**U**	**2,21**	**U**	-0,17	
1424278_a_at	0,00693	8,49	10,86	11,04	Birc5	**2,37**	**U**	**2,55**	**U**	0,17	
1424065_at	0,004107	8,92	11,28	12,68	Edem1	**2,36**	**U**	**3,76**	**U**	**1,40**	**U**
1454694_a_at	0,004829	9,11	11,47	11,42	Top2a	**2,36**	**U**	**2,30**	**U**	-0,06	
1450920_at	0,004817	7,70	10,05	10,25	Ccnb2	**2,35**	**U**	**2,55**	**U**	0,20	
1416939_at	0,001444	7,99	10,33	10,30	Pyp	**2,34**	**U**	**2,30**	**U**	-0,03	
1448314_at	0,00409	7,37	9,67	10,01	Cdc2a	**2,30**	**U**	**2,64**	**U**	0,34	
1450886_at	0,003406	5,99	8,29	8,24	Gsg2	**2,29**	**U**	**2,25**	**U**	-0,04	
1423006_at	0,004748	6,61	8,89	9,06	Pim1	**2,29**	**U**	**2,45**	**U**	0,17	
1417777_at	0,00732	6,17	8,45	9,03	Ltb4dh	**2,28**	**U**	**2,87**	**U**	0,59	
1417299_at	0,004805	3,53	5,81	6,02	Nek2	**2,28**	**U**	**2,49**	**U**	0,21	
1417450_a_at	0,005861	6,70	8,96	9,11	Tacc3	**2,26**	**U**	**2,41**	**U**	0,15	
1429655_at	0,00618	4,00	6,18	6,42	Nudcd1	**2,18**	**U**	**2,42**	**U**	0,24	
1428130_at	0,00469	8,49	10,27	11,05	Lman1	**2,16**	**U**	**3,37**	**U**	**1,21**	**U**
1424882_a_at	0,004283	2,97	5,11	5,63	2510015F01Rik	**2,14**	**U**	**2,66**	**U**	0,52	
1416558_at	0,001955	5,25	7,36	7,35	Melk	**2,12**	**U**	**2,11**	**U**	-0,01	
1416726_s_at	0,002665	9,61	11,69	11,72	Ube2s	**2,08**	**U**	**2,11**	**U**	0,03	
1452954_at	0,006912	8,92	11,00	11,27	Ube2c	**2,08**	**U**	**2,35**	**U**	0,27	
1450790_at	0,000712	3,37	5,45	7,11	Tgn	**2,08**	**U**	**3,74**	**U**	**1,67**	**U**
1419169_at	0,001827	7,59	9,65	9,56	Mapk6	**2,06**	**U**	**1,97**	**U**	-0,10	
1417725_a_at	0,002535	3,73	5,79	5,54	Sssca1	**2,06**	**U**	**1,81**	**U**	-0,25	
1455730_at	0,004084	5,11	7,17	7,35	Dlg7	**2,06**	**U**	**2,24**	**U**	0,18	
1448824_at	0,000858	7,22	9,26	10,48	Ube2j1	**2,04**	**U**	**3,26**	**U**	**1,22**	**U**
1427506_at	0,007573	3,82	5,84	5,91	Ppil5	**2,02**	**U**	**2,09**	**U**	0,07	
1450842_a_at	0,004024	8,05	10,07	10,32	Cenpa	**2,02**	**U**	**2,27**	**U**	0,25	
1424556_at	0,00605	1,90	3,92	6,36	Pycr1	**2,01**	**U**	**4,46**	**U**	**2,45**	**U**
1417141_at	0,001564	9,57	11,58	11,69	Igtp	**2,01**	**U**	**2,13**	**U**	0,12	
1417205_at	0,004018	5,96	7,96	8,53	Kdelr2	**2,00**	**U**	**2,57**	**U**	0,57	
1428492_at	0,00321	4,08	6,59	6,19	Glipr2	**1,97**	**U**	**1,74**	**U**	-0,23	
1449859_at	0,008708	3,64	5,59	5,52	Golt1b	**1,96**	**U**	**1,88**	**U**	-0,07	
1452254_at	0,000183	5,39	7,34	7,20	Mtmr9	**1,95**	**U**	**1,80**	**U**	-0,15	

1450459_at	0,005099	6,48	8,42	8,35	2010106G01Rik	**1,94**	**U**	**1,88**	**U**	-0,07	
1421546_a_at	0,009718	7,06	8,74	8,82	Racgap1	**1,94**	**U**	**2,08**	**U**	0,14	
1416069_at	0,004546	8,27	10,20	9,86	Pfkp	**1,93**	**U**	**1,59**	**U**	-0,34	
1426724_at	0,007409	5,81	7,68	7,07	Cnn3	**1,87**	**U**	**1,26**	**U**	-0,61	
1423914_at	0,000563	3,32	5,18	7,36	C630004H02Rik	**1,87**	**U**	**4,05**	**U**	**2,18**	**U**
1423291_s_at	0,005521	8,36	10,05	10,43	Hyou1	**1,86**	**U**	**2,15**	**U**	0,29	
1447999_x_at	0,000439	5,09	6,95	6,94	LOC380690 /// LOC436	**1,86**	**U**	**1,85**	**U**	-0,01	
1449107_at	0,004061	5,15	7,01	7,37	Nudt4	**1,86**	**U**	**2,21**	**U**	0,35	
1427938_at	0,002992	7,36	9,16	9,47	Mycbp	**1,80**	**U**	**2,11**	**U**	0,31	
1416234_at	0,009129	9,43	11,23	11,64	AA959742	**1,79**	**U**	**2,21**	**U**	0,41	
1417883_at	0,001576	6,65	8,43	8,35	Gstt2	**1,78**	**U**	**1,71**	**U**	-0,08	
1425750_a_at	0,003982	5,92	7,69	7,66	Jak3	**1,78**	**U**	**1,74**	**U**	-0,03	
1424394_at	0,00366	3,82	5,58	7,14	MGI:2149786	**1,75**	**U**	**3,32**	**U**	**1,56**	**U**
1431241_at	0,001649	5,01	6,75	6,30	Chchd3	**1,74**	**U**	**1,30**	**U**	-0,44	
1451509_at	0,004608	7,30	9,03	8,83	Taf9	**1,73**	**U**	**1,53**	**U**	-0,20	
1460251_at	0,001563	5,49	7,21	8,02	Fas	**1,72**	**U**	**2,53**	**U**	0,81	
1417125_at	0,006847	8,78	10,49	10,92	Ahcy	**1,71**	**U**	**2,14**	**U**	0,43	
1448844_at	0,001307	7,04	8,71	8,62	1810044O22Rik	**1,67**	**U**	**1,58**	**U**	-0,09	
1448651_at	0,003181	7,80	9,45	9,52	Nudt5	**1,65**	**U**	**1,72**	**U**	0,07	
1448441_at	0,007081	8,49	10,45	10,45	Cks1b	**1,63**	**U**	**1,59**	**U**	-0,05	
1424280_at	0,002771	7,61	9,24	9,58	Mospd1	**1,63**	**U**	**1,97**	**U**	0,34	
1416956_at	0,000908	6,64	8,27	8,42	Kcnab2	**1,63**	**U**	**1,79**	**U**	0,16	
1449221_a_at	0,004557	7,26	8,99	9,42	Rrbp1	**1,63**	**U**	**1,93**	**U**	0,30	
1423333_at	0,008246	5,51	7,12	8,21	1200007D18Rik	**1,61**	**U**	**2,69**	**U**	**1,09**	**U**
1417103_at	0,009952	8,45	10,05	9,59	Ddt	**1,60**	**U**	**1,14**	**U**	-0,46	
1421571_a_at	0,000866	10,11	11,71	12,35	Ly6c	**1,60**	**U**	**2,24**	**U**	0,64	
1429244_at	0,000102	5,72	7,32	7,97	2610524H06Rik	**1,60**	**U**	**2,25**	**U**	0,65	
1416746_at	0,002949	9,64	11,24	11,04	H2afx	**1,60**	**U**	**1,40**	**U**	-0,19	
1460742_at	0,003651	3,68	5,26	4,96	Alg3	**1,59**	**U**	**1,28**	**U**	-0,31	
1451259_at	0,000248	9,81	11,39	12,03	Smfn	**1,58**	**U**	**2,22**	**U**	0,64	
1421731_a_at	0,005758	8,28	9,86	10,17	Fen1	**1,58**	**U**	**1,90**	**U**	0,32	
1423726_at	0,004053	6,36	7,90	7,61	Vat1	**1,55**	**U**	**1,25**	**U**	-0,29	
1452577_at	0,003151	2,91	4,45	7,23	Igh-VJ558	**1,54**	**U**	**4,32**	**U**	**2,78**	**U**
1424705_at	0,002526	4,59	6,11	5,70	Rbmx2	**1,53**	**U**	**1,12**	**U**	-0,41	
1449007_at	0,0035	6,67	8,19	8,92	Btg3	**1,52**	**U**	**2,25**	**U**	0,74	
1417308_at	0,005576	10,31	11,81	11,54	Pkm2	**1,50**	**U**	**1,23**	**U**	-0,27	

1423650_at	0,006802	5,95	7,45	7,68	Rnf26	**1,50**	**U**	**1,73**	**U**	0,23	
1415860_at	0,008744	10,65	12,15	12,29	Kpna2	**1,50**	**U**	**1,64**	**U**	0,14	
1418856_a_at	0,004646	6,35	7,85	7,74	Fanca	**1,50**	**U**	**1,39**	**U**	-0,11	
1428047_s_at	0,004015	4,40	5,89	5,67	Zfa /// Zfx	**1,49**	**U**	**1,27**	**U**	-0,23	
1452927_x_at	0,003475	10,16	11,54	11,56	Tpi1	**1,49**	**U**	**1,50**	**U**	0,00	
1450416_at	0,001534	2,22	3,71	3,65	Cbx5	**1,48**	**U**	**1,43**	**U**	-0,06	
1448451_at	0,002197	8,95	10,42	10,07	Ak2	**1,47**	**U**	**1,12**	**U**	-0,35	
1427857_x_at	0,008862	6,66	7,83	10,51	Igk-V8	**1,46**	**U**	**4,03**	**U**	**2,56**	**U**
1450948_a_at	0,001797	6,69	8,13	8,19	Mrpl1	**1,44**	**U**	**1,51**	**U**	0,06	
1438847_at	0,000147	5,91	7,35	7,40	Mxd3	**1,44**	**U**	**1,50**	**U**	0,06	
1417773_at	0,003238	7,55	8,98	9,16	Nans	**1,43**	**U**	**1,61**	**U**	0,18	
1428025_s_at	0,003839	5,87	7,28	8,29	Pitpnc1	**1,41**	**U**	**2,43**	**U**	**1,02**	**U**
1452880_at	0,007042	5,70	7,09	7,23	Trip3	**1,39**	**U**	**1,53**	**U**	0,14	
1422768_at	0,000717	7,72	9,10	8,82	Syncrip	**1,38**	**U**	**1,09**	**U**	-0,28	
1418481_at	0,000999	6,18	7,55	7,52	Pkmyt1	**1,38**	**U**	**1,34**	**U**	-0,03	
1416184_s_at	0,003415	7,74	9,11	9,02	Hmga1	**1,37**	**U**	**1,27**	**U**	-0,10	
1450330_at	0,007909	2,34	3,71	5,90	Il10	**1,37**	**U**	**3,56**	**U**	**2,19**	**U**
1420013_s_at	0,002436	5,31	6,66	7,18	Lss	**1,35**	**U**	**1,87**	**U**	0,52	
1424538_at	0,001515	7,79	9,14	9,07	Ubl4	**1,35**	**U**	**1,28**	**U**	-0,06	
1424105_a_at	0,009956	8,45	9,78	9,98	Pttg1	**1,33**	**U**	**1,53**	**U**	0,20	
1453181_x_at	0,00598	8,12	9,45	9,27	Plscr1	**1,33**	**U**	**1,15**	**U**	-0,18	
1450694_at	0,000278	9,41	10,74	11,11	Fkbp2	**1,33**	**U**	**1,71**	**U**	0,37	
1434020_at	0,008762	8,68	10,05	9,97	Pdap1	**1,32**	**U**	**1,31**	**U**	-0,02	
1428112_at	0,006221	11,64	12,93	13,32	Armet	**1,30**	**U**	**1,68**	**U**	0,39	
1423299_at	0,008063	8,79	10,08	10,32	Txnl1	**1,28**	**U**	**1,53**	**U**	0,24	
1439030_at	0,007075	7,40	8,67	8,87	Gmppb	**1,28**	**U**	**1,47**	**U**	0,20	
1426923_at	0,008894	8,82	10,10	9,91	Hrb	**1,28**	**U**	**1,09**	**U**	-0,19	
1439436_x_at	0,007674	10,15	11,42	11,56	Incenp	**1,27**	**U**	**1,40**	**U**	0,13	
1436349_at	0,002317	10,01	11,27	11,32	2700094K13Rik	**1,26**	**U**	**1,30**	**U**	0,04	
1423784_at	0,007498	8,85	10,11	10,18	Gars	**1,26**	**U**	**1,33**	**U**	0,07	
1448574_at	0,001753	3,54	4,78	5,01	Nme6	**1,25**	**U**	**1,48**	**U**	0,23	
1424344_s_at	0,008773	7,22	8,47	8,49	Eif1a	**1,25**	**U**	**1,27**	**U**	0,02	
1449718_s_at	0,006738	4,52	5,76	5,99	4930453N24Rik	**1,25**	**U**	**1,47**	**U**	0,22	
1460705_at	0,001446	5,98	7,21	7,71	Rps6kb1	**1,23**	**U**	**1,74**	**U**	0,51	
1451092_a_at	0,001256	6,66	7,88	7,67	Rangap1	**1,22**	**U**	**1,01**	**U**	-0,21	
1418656_at	0,006414	9,48	10,70	10,63	Lsm5	**1,22**	**U**	**1,15**	**U**	-0,07	

1416470_a_at	0,009936	9,93	11,15	11,43	Rpn1	**1,22**	**U**	**1,50**	**U**	0,28	
1427061_at	0,003784	7,21	8,43	8,37	Rbbp8	**1,21**	**U**	**1,16**	**U**	-0,06	
1423722_at	0,004184	9,89	11,08	11,45	4930579A11Rik	**1,19**	**U**	**1,56**	**U**	0,37	
1415673_at	0,009968	7,69	8,88	8,99	Psph	**1,19**	**U**	**1,30**	**U**	0,11	
1460221_at	0,005843	10,91	12,09	12,11	MGI:1929282	**1,18**	**U**	**1,20**	**U**	0,02	
1450722_at	0,008006	7,78	8,95	9,15	Nup50	**1,17**	**U**	**1,37**	**U**	0,20	
1450697_at	0,005237	7,01	8,18	8,60	Slc30a7	**1,17**	**U**	**1,59**	**U**	0,43	
1448462_at	0,007446	7,59	8,83	8,71	Tdg	**1,16**	**U**	**1,18**	**U**	0,02	
1428113_at	0,003085	7,05	8,21	8,21	4930403J22Rik	**1,16**	**U**	**1,16**	**U**	0,00	
1451065_a_at	0,009715	10,59	11,81	11,71	Ddx39	**1,16**	**U**	**1,08**	**U**	-0,07	
1417075_at	0,002958	7,99	9,14	9,09	2010309E21Rik	**1,15**	**U**	**1,11**	**U**	-0,05	
1449089_at	0,007104	8,15	9,30	9,41	Nrip1	**1,14**	**U**	**1,25**	**U**	0,11	
1448284_a_at	0,008816	10,09	11,14	11,29	Ndufc1	**1,14**	**U**	**1,22**	**U**	0,08	
1434882_at	0,005539	9,33	10,46	11,07	Mtdh	**1,14**	**U**	**1,74**	**U**	0,61	
1419736_a_at	0,004687	9,88	11,02	11,08	Eif1ay	**1,14**	**U**	**1,20**	**U**	0,06	
1417058_a_at	0,009994	9,65	10,77	11,48	Krtcap2	**1,12**	**U**	**1,84**	**U**	0,71	
1451346_at	0,006317	7,56	8,68	8,62	Mtap	**1,12**	**U**	**1,06**	**U**	-0,06	
1415909_at	0,00757	8,28	9,40	9,29	Stip1	**1,12**	**U**	**1,01**	**U**	-0,11	
1438640_x_at	0,00592	10,72	11,86	11,92	Pgk1	**1,11**	**U**	**1,13**	**U**	0,02	
1427404_x_at	0,007854	11,20	12,31	12,22	Eno1	**1,11**	**U**	**1,02**	**U**	-0,09	
1421073_a_at	0,004882	4,56	5,66	5,79	Ptger4	**1,10**	**U**	**1,23**	**U**	0,13	
1423525_at	0,005659	3,71	4,81	4,75	Mastl	**1,10**	**U**	**1,04**	**U**	-0,06	
1433604_x_at	0,006892	10,06	11,23	11,32	Aldoa	**1,10**	**U**	**1,16**	**U**	0,06	
1416696_at	0,008645	7,01	8,11	8,22	D17Wsu104e	**1,09**	**U**	**1,21**	**U**	0,12	
1423134_at	0,000225	9,52	10,60	10,80	BC003324	**1,09**	**U**	**1,29**	**U**	0,20	
1416093_a_at	0,006327	8,79	9,87	10,09	Mrpl20	**1,08**	**U**	**1,31**	**U**	0,23	
1424429_s_at	0,008163	3,56	4,63	4,74	AI225782	**1,07**	**U**	**1,18**	**U**	0,11	
1449267_at	0,009267	6,74	7,81	8,07	3110023E09Rik	**1,07**	**U**	**1,33**	**U**	0,26	
1433480_at	0,00169	9,48	10,52	10,70	2900010J23Rik	**1,05**	**U**	**1,23**	**U**	0,18	
1438292_x_at	0,000936	9,62	10,65	11,95	Adk	**1,03**	**U**	**2,32**	**U**	**1,30**	**U**
1419690_at	0,000914	5,77	6,80	7,19	2610002M06Rik	**1,03**	**U**	**1,42**	**U**	0,40	
1430326_s_at	0,000519	9,52	10,54	10,70	1500040F11Rik	**1,02**	**U**	**1,18**	**U**	0,16	

Genes up-regulated in spleen DCs by *P. yoelii* infection at day 5 pi but not at day 10 pi

						Day 5 vs 0		Day 10 vs 0		Day 10 vs 5	
Probe_ID	p-value	Day 1	Day 5	Day 10	Gene Symbol	5vs0	p<0.01	10vs0	p<0.01	10vs5	p<0.01
1425832_a_at	0,006893	2,85	6,96	3,33	Cxcr6	**4,18**	**U**	0,67		**-3,51**	**D**
1417290_at	0,006802	3,12	5,89	2,99	Lrg1	**2,77**	**U**	-0,14		**-2,91**	**D**
1422934_x_at	0,009696	5,73	8,26	5,33	Defcr-rs7	**2,53**	**U**	-0,40		**-2,93**	**D**
1421826_at	0,002053	2,03	4,55	2,06	Dll4	**2,51**	**U**	0,02		**-2,49**	**D**
1419530_at	0,006295	6,63	8,43	6,21	Il12b	**1,79**	**U**	-0,42		**-2,22**	**D**
1449481_at	0,004454	2,24	3,85	2,34	Slc25a13	**1,62**	**U**	0,11		**-1,51**	**D**
1450456_at	0,005577	6,20	7,78	7,16	Il21r	**1,58**	**U**	0,96		-0,62	
1452414_s_at	0,004713	6,50	8,05	7,41	D19Ertd678e	**1,55**	**U**	0,91		-0,64	
1450843_a_at	0,002274	3,63	5,20	3,22	Serpinh1	**1,41**	**U**	-0,19		**-1,59**	**D**
1418024_at	0,00747	8,80	10,12	9,70	Narg1	**1,32**	**U**	0,90		-0,42	
1448794_s_at	0,0096	8,83	10,10	9,29	Dnajc2	**1,29**	**U**	0,64		-0,64	
1423801_a_at	0,008489	8,61	9,84	9,51	Aprt	**1,22**	**U**	0,90		-0,33	
1448543_at	0,000695	8,48	9,70	9,25	2310042G06Rik	**1,22**	**U**	0,77		-0,45	
1448947_at	0,00787	6,89	8,08	7,75	0610039J04Rik	**1,20**	**U**	0,86		-0,34	
1417090_at	0,003348	7,72	8,88	8,19	Rcn1	**1,17**	**U**	0,47		-0,70	
1424436_at	0,003805	7,39	8,55	8,24	Gart	**1,15**	**U**	0,85		-0,31	
1426264_at	0,00564	6,83	7,98	7,72	Dlat	**1,15**	**U**	0,89		-0,26	
1452012_a_at	0,002986	5,30	6,44	6,00	Exosc1	**1,14**	**U**	0,70		-0,44	
1452704_at	0,002955	7,74	8,86	8,61	1200015F23Rik	**1,11**	**U**	0,87		-0,24	
1426256_at	0,000891	8,69	9,79	9,64	Timm17a	**1,10**	**U**	0,95		-0,15	
1418633_at	0,004836	3,09	4,19	3,66	Notch1	**1,10**	**U**	0,57		-0,53	
1456071_a_at	0,00816	12,24	13,25	12,94	Cycs	**1,09**	**U**	0,77		-0,33	
1451483_s_at	0,005715	3,02	4,07	3,55	1700054N08Rik	**1,05**	**U**	0,53		-0,52	
1424577_at	0,008889	5,59	6,62	6,18	BC008103	**1,03**	**U**	0,59		-0,44	
1425878_at	0,006398	2,43	3,46	2,63	Cabp4	**1,03**	**U**	0,19		-0,83	
1419644_at	0,000377	4,60	5,63	5,44	Cstf2	**1,03**	**U**	0,84		-0,19	
1424343_a_at	0,006053	8,91	9,93	9,85	Eif1a	**1,03**	**U**	0,94		-0,08	
1419912_s_at	0,008266	7,94	8,96	8,94	Strap	**1,02**	**U**	1,00		-0,02	
1451319_at	0,008292	5,95	6,97	6,20	Senp1	**1,01**	**U**	0,24		-0,77	
1426922_s_at	0,000412	7,45	8,46	8,39	Hrb	**1,01**	**U**	0,94		-0,07	
1450914_at	0,002531	9,64	10,65	10,42	Ppp1r14b	**1,01**	**U**	0,78		-0,23	
1447919_x_at	0,004128	9,43	10,43	10,33	Ndufab1	**1,00**	**U**	0,91		-0,10	
1454817_at	0,005127	8,38	9,38	9,24	Wdr50	**1,00**	**U**	0,86		-0,14	
1427718_a_at	0,009611	7,28	8,28	7,95	Mdm2	**1,00**	**U**	0,67		-0,33	

Genes up-regulated in spleen DCs by *P. yoelii* infection at day 10 pi but not at day 5 pi

						Day 5 vs 0		Day 10 vs 0		Day 10 vs 5	
Probe_ID	p-value	Day 1	Day 5	Day 10	Gene Symbol	5vs0	p<0.01	10vs0	p<0.01	10vs5	p<0.01
1419764_at	0,009566	2,25	2,25	6,75	Chi3l3	0,01		**4,51**	**U**	**4,50**	**U**
1426178_at	0,00307	4,00	3,97	7,81	Igk-V8	-0,03		**3,81**	**U**	**3,85**	**U**
1424186_at	0,000715	2,33	2,34	6,08	2610001E17Rik	0,01		**3,74**	**U**	**3,73**	**U**
1419560_at	0,008464	1,88	2,25	5,47	Lipc	0,37		**3,58**	**U**	**3,22**	**U**
1417644_at	0,000773	2,21	2,22	5,42	Sspn	0,02		**3,21**	**U**	**3,19**	**U**
1416166_a_at	0,003621	9,07	10,06	11,37	Prdx4	0,99		**2,30**	**U**	**1,30**	**U**
1426442_at	0,006912	3,30	3,69	5,35	Gpm6a	0,40		**2,06**	**U**	**1,66**	**U**
1415932_x_at	0,00921	2,64	2,64	4,51	Atp9a	0,12		**1,82**	**U**	**1,71**	**U**
1428882_at	0,006791	4,18	4,98	5,96	2510001I10Rik	0,79		**1,78**	**U**	0,98	
1453573_at	0,00507	3,89	3,79	5,67	Hist1h3d	-0,11		**1,78**	**U**	**1,88**	**U**
1417059_at	0,003483	8,81	9,78	10,48	Krtcap2	0,98		**1,68**	**U**	0,70	
1449084_s_at	0,003839	3,41	3,20	5,05	Sh3d19	-0,21		**1,64**	**U**	**1,85**	**U**
1448355_at	4,12E-06	2,43	2,67	4,02	Prss16	0,25		**1,60**	**U**	**1,35**	**U**
1422147_a_at	0,005382	5,08	5,44	6,61	Pla2g6	0,37		**1,54**	**U**	**1,17**	**U**
1437841_x_at	0,005415	4,32	4,53	5,85	AI481750	0,21		**1,53**	**U**	**1,32**	**U**
1451412_a_at	0,003599	5,38	5,81	6,88	0610009H04Rik	0,43		**1,50**	**U**	**1,07**	**U**
1448769_at	0,009764	9,40	10,39	10,89	Slc35b1	0,99		**1,49**	**U**	0,50	
1435731_x_at	0,008713	7,05	7,73	8,50	Stag1	0,68		**1,45**	**U**	0,77	
1418531_at	0,003972	2,25	2,35	3,70	Oosp1	0,10		**1,45**	**U**	**1,35**	**U**
1424806_s_at	0,007494	7,59	8,18	9,04	1110039B18Rik	0,59		**1,45**	**U**	0,87	
1424471_at	0,005516	1,95	1,95	3,04	Rapgef3	-0,01		**1,44**	**U**	**1,46**	**U**
1448520_at	0,002337	2,96	3,27	4,32	Dclre1b	0,32		**1,36**	**U**	**1,05**	**U**
1439444_x_at	0,005682	9,69	10,50	11,04	1110014C03Rik	0,81		**1,35**	**U**	0,55	
1428004_at	0,001935	8,16	9,02	9,49	3300001G02Rik	0,86		**1,33**	**U**	0,47	
1436824_x_at	0,006546	9,58	10,52	10,90	Rnf26	0,94		**1,32**	**U**	0,39	
1450057_at	0,006962	5,27	6,09	6,59	2610042O14Rik	0,82		**1,31**	**U**	0,49	
1451449_at	0,007307	9,12	9,33	10,43	4933407N01Rik	0,21		**1,30**	**U**	**1,09**	**U**
1416863_at	0,004871	3,32	4,04	4,62	Abhd8	0,72		**1,30**	**U**	0,59	
1451973_at	0,003686	8,17	8,75	9,47	Ube1dc1	0,58		**1,30**	**U**	0,73	
1424373_at	0,00284	6,95	7,42	8,25	Armcx3	0,47		**1,30**	**U**	0,83	
1431422_a_at	0,000441	2,21	2,31	3,50	Dusp14	0,10		**1,29**	**U**	**1,19**	**U**

1435733_x_at	0,00389	7,62	8,57	8,90	1500026D16Rik	0,95	**1,28**	**U**	0,34	
1425927_a_at	0,000772	3,21	3,22	4,46	Atf5	0,01	**1,26**	**U**	**1,24**	**U**
1424566_s_at	0,004541	2,27	2,84	3,52	Polr3d	0,57	**1,25**	**U**	0,68	
1423242_at	0,000863	7,14	8,00	8,30	Mrps36	0,86	**1,16**	**U**	0,30	
1418137_at	0,002089	9,73	10,41	10,88	Mrp63	0,69	**1,15**	**U**	0,47	
1433627_at	0,005648	7,93	8,92	9,07	Sec23ip	0,98	**1,13**	**U**	0,15	
1423254_x_at	0,008237	10,68	11,65	11,80	Rps27l	0,97	**1,12**	**U**	0,15	
1417578_a_at	0,007576	7,16	7,73	8,34	Gmppa	0,56	**1,11**	**U**	0,55	
1450911_at	0,008912	11,75	12,33	12,85	Ppib	0,58	**1,10**	**U**	0,52	
1453750_x_at	0,003803	3,95	4,18	5,05	Pitpnc1	0,23	**1,10**	**U**	0,87	
1437102_at	0,000974	7,04	7,91	8,14	Ythdf1	0,86	**1,09**	**U**	0,23	
1416189_a_at	0,005319	11,82	12,55	12,91	Sec61a1	0,73	**1,09**	**U**	0,35	
1435803_a_at	0,003832	9,33	10,21	10,41	Eif4e2	0,89	**1,09**	**U**	0,20	
1448682_at	0,00882	10,89	11,83	12,01	Dnclc1	0,93	**1,08**	**U**	0,15	
1454963_at	0,008358	8,15	9,10	9,22	E430028B21Rik	0,95	**1,07**	**U**	0,12	
1428316_a_at	0,00953	9,20	9,82	10,25	Fundc2	0,62	**1,05**	**U**	0,43	
1455824_x_at	0,009655	11,40	12,01	12,45	Itm1	0,61	**1,05**	**U**	0,43	
1424222_s_at	0,006919	9,46	10,39	10,50	Rad23b	0,93	**1,04**	**U**	0,11	
1423120_at	0,007805	8,98	9,94	10,02	Ide	0,96	**1,04**	**U**	0,08	
1434299_x_at	0,001522	7,89	8,46	8,92	Rabl4	0,57	**1,03**	**U**	0,46	

Appendix I - Table II

Genes down-regulated in spleen DCs by *P. yoelii* infection at both day 5 pi and day 10 pi

						Day 5 vs 0		Day 10 vs 0		Day 10 vs 5	
Probe_ID	p-value	Day 1	Day 5	Day 10	Gene Symbol	5vs0	p<0.01	10vs0	p<0.01	10vs5	p<0.01
1427168_a_at	0,000168019	10,38	2,32	2,32	Col14a1	**-8,06**	**D**	**-8,06**	**D**	0,00	
1449153_at	2,84897E-05	9,47	2,67	2,52	Mmp12	**-6,80**	**D**	**-6,95**	**D**	-0,15	
1426172_a_at	0,003302319	10,10	4,01	3,09	Cd209a	**-6,10**	**D**	**-7,02**	**D**	-0,92	
1419696_at	0,000157623	8,90	3,51	2,11	Cd4	**-5,39**	**D**	**-6,79**	**D**	**-1,40**	**D**
1417600_at	0,000551951	9,68	4,34	4,46	Slc15a2	**-5,35**	**D**	**-5,22**	**D**	0,12	
1419144_at	0,002273179	11,45	6,23	6,63	Cd163	**-5,22**	**D**	**-4,83**	**D**	0,39	
1427427_at	0,003380937	10,69	6,18	3,62	Ryr3	**-5,05**	**D**	**-6,52**	**D**	**-1,47**	**D**
1416997_a_at	8,76017E-06	7,07	2,34	2,35	Hap1	**-4,73**	**D**	**-4,72**	**D**	0,00	
1419082_at	1,3782E-05	6,96	2,29	2,31	Serpinb2	**-4,68**	**D**	**-4,65**	**D**	0,03	
1449308_at	0,006107162	10,26	5,59	6,06	C6	**-4,67**	**D**	**-4,19**	**D**	0,47	
1423556_at	0,000818839	7,19	2,56	2,57	Akr1b7	**-4,64**	**D**	**-4,63**	**D**	0,01	
1449065_at	0,003967675	8,54	4,18	3,49	Cte1	**-4,36**	**D**	**-5,05**	**D**	-0,68	
1451527_at	0,005877907	6,59	2,30	2,26	Pcolce2	**-4,28**	**D**	**-4,32**	**D**	-0,04	
1439381_x_at	0,007501432	11,18	7,63	7,14	Marveld1	**-4,28**	**D**	**-4,62**	**D**	-0,34	
1419906_at	0,000962413	6,43	2,17	2,17	Hpgd	**-4,27**	**D**	**-4,26**	**D**	0,01	
1448249_at	0,000308944	7,50	2,80	2,88	Gpd1	**-4,19**	**D**	**-4,13**	**D**	0,06	
1451888_a_at	2,65029E-05	6,61	2,45	2,45	Odz4	**-4,17**	**D**	**-4,17**	**D**	0,00	
1430388_a_at	0,002873391	9,36	5,34	4,53	Sulf2	**-4,02**	**D**	**-4,83**	**D**	-0,81	
1432492_a_at	0,001305443	6,41	2,41	2,30	Haao	**-4,00**	**D**	**-4,11**	**D**	-0,11	
1417311_at	0,003909863	10,23	6,30	6,53	Crip2	**-3,93**	**D**	**-3,70**	**D**	0,23	
1422837_at	0,00300323	7,32	3,41	2,86	Scel	**-3,91**	**D**	**-4,46**	**D**	-0,56	
1437188_at	0,003126204	6,35	2,64	2,62	Gabbr1	**-3,90**	**D**	**-3,91**	**D**	-0,02	
1419423_at	0,005768851	10,72	6,84	7,15	Stab2	**-3,88**	**D**	**-3,57**	**D**	0,32	
1425546_a_at	0,008580727	12,98	9,11	9,73	Trf	**-3,86**	**D**	**-3,24**	**D**	0,62	
1416930_at	0,000195605	6,16	2,33	2,22	Ly6d	**-3,82**	**D**	**-3,94**	**D**	-0,12	
1448330_at	0,009084878	7,75	3,93	4,27	Gstm1	**-3,82**	**D**	**-3,49**	**D**	0,33	
1423378_at	0,002916855	8,55	4,74	4,64	Adam23	**-3,81**	**D**	**-3,91**	**D**	-0,10	
1420582_at	0,004134273	6,03	2,29	1,92	Cd209e	**-3,74**	**D**	**-4,11**	**D**	-0,38	
1423285_at	0,001918983	6,50	2,86	2,58	Coch	**-3,64**	**D**	**-3,91**	**D**	-0,28	
1453571_at	0,00026438	5,93	2,29	2,18	Depdc6	**-3,64**	**D**	**-3,75**	**D**	-0,11	

1426183_a_at	0,002339603	7,29	3,68	2,96	Cd209d	**-3,62**	**D**	**-4,34**	**D**	-0,72	
1448816_at	0,001267853	5,69	2,12	2,13	Ptgis	**-3,58**	**D**	**-3,56**	**D**	0,02	
1423693_at	0,009919983	10,86	7,32	8,25	Ela1	**-3,54**	**D**	**-2,62**	**D**	0,93	
1449405_at	0,002548999	6,33	3,16	3,42	Tns	**-3,51**	**D**	**-3,05**	**D**	0,46	
1418804_at	0,002365068	7,79	4,36	3,40	Sucnr1	**-3,43**	**D**	**-4,40**	**D**	-0,96	
1416638_at	0,000954103	6,75	3,33	3,41	Sall2	**-3,42**	**D**	**-3,35**	**D**	0,08	
1419584_at	0,002632117	5,92	2,56	2,54	AI428795	**-3,36**	**D**	**-3,38**	**D**	-0,02	
1434325_x_at	0,002300293	9,64	5,23	5,88	Prkar1b	**-3,36**	**D**	**-3,02**	**D**	0,34	
1417481_at	0,002121919	9,32	6,00	5,33	Ramp1	**-3,33**	**D**	**-3,99**	**D**	-0,67	
1416055_at	1,15421E-05	6,37	3,07	3,06	Amy2	**-3,30**	**D**	**-3,31**	**D**	-0,01	
1449992_at	0,008404137	5,72	2,94	3,27	MGI:2149952	**-3,25**	**D**	**-2,87**	**D**	0,38	
1422952_at	0,000700325	7,42	4,20	4,56	MGI:2388100	**-3,22**	**D**	**-2,87**	**D**	0,35	
1449952_s_at	0,00033757	7,90	4,74	3,51	Tmprss8	**-3,17**	**D**	**-4,39**	**D**	**-1,23**	**D**
1434109_at	0,002759688	6,18	3,01	3,14	Sh3bgrl2	**-3,16**	**D**	**-3,03**	**D**	0,13	
1417704_a_at	0,002538956	8,55	5,42	5,32	Arhgap6	**-3,12**	**D**	**-3,23**	**D**	-0,11	
1433558_at	0,00421687	7,98	4,87	4,91	Dab2ip	**-3,11**	**D**	**-3,07**	**D**	0,04	
1422438_at	0,001722963	8,81	5,72	7,18	Ephx1	**-3,08**	**D**	**-1,63**	**D**	**1,45**	**U**
1419811_at	0,009082816	6,19	3,11	4,18	D16Wsu65e	**-3,08**	**D**	**-2,01**	**D**	**1,06**	**U**
1418288_at	0,00523419	8,79	5,41	6,04	Lpin1	**-3,04**	**D**	**-2,56**	**D**	0,48	
1419219_at	0,003825353	7,46	4,44	4,74	Cyp4f18	**-3,01**	**D**	**-2,71**	**D**	0,30	
1451804_a_at	0,007911937	6,25	3,29	2,64	Lrrc16	**-2,95**	**D**	**-3,60**	**D**	-0,65	
1417676_a_at	0,005766781	9,38	6,79	5,80	Ptpro	**-2,94**	**D**	**-3,76**	**D**	-0,83	
1423286_at	0,009753393	4,84	1,90	1,92	Cbln1	**-2,93**	**D**	**-2,92**	**D**	0,01	
1426157_a_at	0,003633926	5,13	2,24	2,27	Cd209b	**-2,89**	**D**	**-2,87**	**D**	0,02	
1437687_x_at	0,000955128	7,17	4,28	4,55	Fkbp9	**-2,89**	**D**	**-2,62**	**D**	0,26	
1424358_at	0,001380356	9,16	6,29	4,02	Ube2e2	**-2,87**	**D**	**-5,14**	**D**	**-2,27**	**D**
1421960_at	0,00145639	8,07	4,60	5,31	Adcy3	**-2,85**	**D**	**-2,25**	**D**	0,61	
1456370_s_at	0,005784078	5,12	2,32	2,36	0610037L13Rik	**-2,80**	**D**	**-2,77**	**D**	0,04	
1417626_at	0,005848479	7,01	4,21	4,43	Usmg4	**-2,80**	**D**	**-2,59**	**D**	0,22	
1416697_at	0,009340161	11,45	9,65	8,65	Dpp4	**-2,80**	**D**	**-3,54**	**D**	-0,74	
1417590_at	0,004828162	11,37	8,57	8,42	Cyp27a1	**-2,79**	**D**	**-2,95**	**D**	-0,15	
1416700_at	0,009025022	7,83	5,39	3,91	Rhoe	**-2,75**	**D**	**-3,90**	**D**	**-1,15**	**D**
1422592_at	0,004232769	9,19	6,50	5,43	Catnd2	**-2,69**	**D**	**-3,76**	**D**	**-1,07**	**D**
1415996_at	0,002546659	12,16	9,48	10,33	Txnip	**-2,68**	**D**	**-1,83**	**D**	0,85	
1432329_a_at	0,00328853	5,32	2,41	2,36	Matk	**-2,67**	**D**	**-2,68**	**D**	-0,01	
1423756_s_at	0,00245463	5,41	2,78	1,98	Igfbp4	**-2,64**	**D**	**-3,43**	**D**	-0,79	

1428573_at	0,004502219	8,49	5,86	5,83	Chn2	**-2,63**	**D**	**-2,66**	**D**	-0,03	
1449411_at	0,003532467	5,45	2,82	2,91	Dscam	**-2,63**	**D**	**-2,54**	**D**	0,09	
1450476_at	0,001541679	9,36	6,75	6,73	Cnr2	**-2,61**	**D**	**-2,63**	**D**	-0,02	
1425087_at	0,001938094	6,12	3,51	3,49	2310003F16Rik	**-2,60**	**D**	**-2,63**	**D**	-0,03	
1429247_at	0,001175566	7,12	4,52	4,34	Anxa6	**-2,59**	**D**	**-2,77**	**D**	-0,18	
1431726_a_at	0,009833466	7,56	4,97	4,52	5530601I19Rik	**-2,59**	**D**	**-3,04**	**D**	-0,45	
1451486_at	0,00418022	11,54	8,96	8,45	1200006F02Rik	**-2,58**	**D**	**-3,09**	**D**	-0,51	
1432466_a_at	0,00902992	12,69	10,11	11,08	Apoe	**-2,58**	**D**	**-1,60**	**D**	0,98	
1452654_at	0,009089958	7,50	4,92	6,24	Zdhhc2	**-2,58**	**D**	**-1,26**	**D**	**1,32**	**U**
1449062_at	0,002145788	7,71	5,17	5,44	Khk	**-2,54**	**D**	**-2,27**	**D**	0,27	
1427341_at	0,000756213	7,43	4,91	4,65	E130103I17Rik	**-2,53**	**D**	**-2,78**	**D**	-0,26	
1417623_at	0,004016156	8,25	6,08	6,83	Slc12a2	**-2,51**	**D**	**-1,83**	**D**	0,68	
1425371_at	0,001708222	7,13	4,64	4,69	Polb	**-2,50**	**D**	**-2,44**	**D**	0,06	
1449013_at	0,001492214	7,00	4,51	4,88	Eef2k	**-2,49**	**D**	**-2,12**	**D**	0,37	
1454677_at	0,009716152	10,16	7,37	7,97	Timp2	**-2,46**	**D**	**-1,98**	**D**	0,48	
1416130_at	0,001898618	6,59	3,96	3,98	Prnp	**-2,46**	**D**	**-2,39**	**D**	0,07	
1455195_at	0,005230385	6,67	4,21	5,41	Rps24	**-2,45**	**D**	**-1,25**	**D**	**1,20**	**U**
1448005_at	0,005292501	12,20	9,75	9,71	Sash1	**-2,45**	**D**	**-2,49**	**D**	-0,04	
1452732_at	0,006764686	5,57	3,15	2,42	2300003P22Rik	**-2,42**	**D**	**-3,15**	**D**	-0,72	
1436910_at	0,008136059	7,10	4,68	4,52	A330066M24Rik	**-2,42**	**D**	**-2,59**	**D**	-0,16	
1420395_a_at	0,001456737	5,33	2,94	2,96	Kif9	**-2,39**	**D**	**-2,38**	**D**	0,01	
1425942_a_at	0,009991561	6,18	3,77	2,86	Gpm6b	**-2,33**	**D**	**-3,42**	**D**	**-1,09**	**D**
1454628_at	0,007541508	8,50	6,18	7,05	A930037G23Rik	**-2,32**	**D**	**-1,45**	**D**	0,86	
1418061_at	1,23247E-05	4,12	1,82	1,83	Ltbp2	**-2,31**	**D**	**-2,29**	**D**	0,01	
1448990_a_at	0,005134354	4,67	2,37	2,39	Myo1b	**-2,30**	**D**	**-2,28**	**D**	0,03	
1426340_at	0,007309281	8,28	5,99	6,11	Slc1a3	**-2,29**	**D**	**-2,17**	**D**	0,12	
1431929_a_at	0,001202696	6,98	5,29	5,29	Stx17	**-2,28**	**D**	**-2,41**	**D**	-0,13	
1421587_at	0,000928057	6,31	4,05	3,97	Klk27	**-2,26**	**D**	**-2,35**	**D**	-0,08	
1426743_at	0,001160324	9,53	7,28	8,16	MGI:2384914	**-2,26**	**D**	**-1,37**	**D**	0,88	
1451775_s_at	0,009791433	5,07	2,89	2,83	Il13ra1	**-2,25**	**D**	**-2,58**	**D**	-0,34	
1451584_at	0,003903094	10,13	7,90	6,31	Havcr2	**-2,23**	**D**	**-3,82**	**D**	**-1,59**	**D**
1427484_at	0,003848706	4,78	2,55	2,53	Eml5	**-2,23**	**D**	**-2,25**	**D**	-0,02	
1434442_at	0,003793529	5,76	3,53	2,84	D5Ertd593e	**-2,23**	**D**	**-2,92**	**D**	-0,69	
1453474_at	0,006938785	7,39	5,17	4,62	1300007F04Rik	**-2,22**	**D**	**-2,77**	**D**	-0,55	
1423059_at	0,003004869	9,58	7,36	7,04	Ptk2	**-2,22**	**D**	**-2,54**	**D**	-0,32	
1448999_at	0,000592773	7,29	5,08	5,25	Trappc5	**-2,21**	**D**	**-2,04**	**D**	0,17	

1432108_at	0,002084872	5,39	3,19	3,33	Rnf134	**-2,20**	**D**	**-2,06**	**D**	0,14	
1429761_at	0,008213995	8,16	5,99	4,37	Rtn1	**-2,17**	**D**	**-3,79**	**D**	**-1,62**	**D**
1426100_a_at	0,006873703	10,13	7,96	7,97	Tk2	**-2,16**	**D**	**-2,16**	**D**	0,00	
1450627_at	0,002171503	8,51	6,35	5,93	Ank	**-2,16**	**D**	**-2,58**	**D**	-0,42	
1422011_s_at	0,001373426	6,57	4,41	4,35	Xlr /// 3830403N18Rik	**-2,16**	**D**	**-2,21**	**D**	-0,06	
1435812_at	0,009851037	4,23	2,10	2,15	Unc50	**-2,13**	**D**	**-2,08**	**D**	0,05	
1417951_at	0,006108105	10,26	8,15	8,09	Eno3	**-2,11**	**D**	**-2,17**	**D**	-0,06	
1450062_a_at	0,004450078	7,91	5,80	5,81	Maged1	**-2,11**	**D**	**-2,10**	**D**	0,01	
1437394_at	0,003026724	4,38	2,31	2,48	Centg2	**-2,07**	**D**	**-1,90**	**D**	0,17	
1434796_at	0,007427753	7,67	5,59	6,00	Vamp4	**-2,07**	**D**	**-1,67**	**D**	0,40	
1423122_at	0,004597544	10,26	8,20	7,11	Avpi1	**-2,07**	**D**	**-3,16**	**D**	**-1,09**	**D**
1456430_at	0,003045324	6,32	4,26	4,27	D3Ertd789e	**-2,06**	**D**	**-2,05**	**D**	0,02	
1439069_a_at	0,006533035	10,73	8,90	9,24	4933439C20Rik	**-2,04**	**D**	**-1,62**	**D**	0,42	
1451809_s_at	0,001320958	6,94	4,90	5,09	Rwdd3	**-2,04**	**D**	**-1,85**	**D**	0,19	
1419297_at	0,000108441	11,09	9,06	8,17	H2-Oa	**-2,03**	**D**	**-2,92**	**D**	-0,89	
1452514_a_at	0,003883696	11,10	9,07	8,03	Kit	**-2,02**	**D**	**-3,06**	**D**	**-1,04**	**D**
1418195_at	0,000992838	6,92	4,90	4,95	Galnt10	**-2,02**	**D**	**-1,97**	**D**	0,06	
1455747_at	0,000340024	4,02	2,00	2,27	Ggtla1	**-2,02**	**D**	**-1,76**	**D**	0,27	
1420796_at	3,39395E-05	4,30	2,28	2,29	Ahrr	**-2,02**	**D**	**-2,01**	**D**	0,01	
1435357_at	0,003435221	9,67	7,65	6,59	D4Wsu53e	**-2,02**	**D**	**-3,08**	**D**	**-1,06**	**D**
1426915_at	0,000487951	6,21	4,20	3,43	Dapk1	**-2,01**	**D**	**-2,78**	**D**	-0,77	
1417505_s_at	0,005284153	4,67	2,67	2,59	Il11ra1 /// Il11ra2	**-2,00**	**D**	**-2,08**	**D**	-0,08	
1421384_at	0,009264033	6,62	4,64	3,22	Lyst	**-1,99**	**D**	**-3,40**	**D**	**-1,42**	**D**
1418435_at	0,009833769	10,22	8,14	8,75	Mkrn1	**-1,97**	**D**	**-1,53**	**D**	0,44	
1428297_at	0,009886207	6,19	4,22	4,01	Map4k2	**-1,97**	**D**	**-2,18**	**D**	-0,21	
1417707_at	0,006738022	10,70	8,73	8,84	B230342M21Rik	**-1,97**	**D**	**-1,86**	**D**	0,11	
1437180_at	0,003173729	7,34	5,38	5,25	6530403A03Rik	**-1,96**	**D**	**-2,09**	**D**	-0,13	
1419104_at	0,001776312	5,11	3,17	2,27	Abhd6	**-1,94**	**D**	**-2,84**	**D**	-0,89	
1430173_x_at	0,001490205	5,17	3,24	2,79	Cyp4f16	**-1,93**	**D**	**-2,38**	**D**	-0,45	
1451361_a_at	0,008664716	9,52	7,60	8,15	BC027342	**-1,92**	**D**	**-1,37**	**D**	0,55	
1417218_at	0,004042257	8,36	6,45	5,97	2810048G17Rik	**-1,91**	**D**	**-2,39**	**D**	-0,48	
1449452_a_at	0,001454315	8,52	6,62	2,45	Gp2	**-1,90**	**D**	**-6,07**	**D**	**-4,17**	**D**
1448686_at	0,003975186	8,05	6,16	6,55	Il16	**-1,88**	**D**	**-1,49**	**D**	0,39	
1425742_a_at	0,007133661	10,23	8,41	7,87	Tgfb1i4	**-1,87**	**D**	**-2,24**	**D**	-0,37	
1426514_at	0,006997614	7,05	5,19	5,19	4631426J05Rik	**-1,87**	**D**	**-1,86**	**D**	0,00	
1418553_at	0,007064653	6,79	4,92	4,74	Arhgef18	**-1,86**	**D**	**-2,05**	**D**	-0,19	

1428669_at	0,003984252	6,11	4,25	3,86	Bmyc	**-1,85**	**D**	**-2,25**	**D**	-0,40	
1423164_at	0,005329048	6,46	4,61	4,70	Tm7sf1	**-1,85**	**D**	**-1,77**	**D**	0,08	
1460324_at	0,007149789	6,72	4,87	4,31	Dnmt3a	**-1,85**	**D**	**-2,41**	**D**	-0,56	
1416111_at	0,00174001	11,84	10,00	9,86	Cd83	**-1,84**	**D**	**-1,99**	**D**	-0,14	
1451127_at	0,004232141	9,35	7,52	7,85	AW146242	**-1,84**	**D**	**-1,54**	**D**	0,30	
1438467_at	9,63033E-05	3,76	1,92	1,94	Mgl2	**-1,84**	**D**	**-1,82**	**D**	0,02	
1422064_a_at	0,001107281	5,25	3,43	4,04	Zbtb20	**-1,82**	**D**	**-1,21**	**D**	0,61	
1422751_at	0,003496652	7,16	5,34	4,67	Tle1	**-1,82**	**D**	**-2,49**	**D**	-0,67	
1416401_at	0,004112382	12,94	11,12	11,68	Kai1	**-1,82**	**D**	**-1,26**	**D**	0,56	
1448398_s_at	0,000277042	8,98	7,21	6,72	Rpl22	**-1,77**	**D**	**-2,26**	**D**	-0,49	
1418927_a_at	0,002539663	6,65	4,90	4,66	Habp4	**-1,75**	**D**	**-1,99**	**D**	-0,23	
1426992_at	0,006530512	9,62	7,86	8,11	Xpr1	**-1,75**	**D**	**-1,50**	**D**	0,25	
1426124_a_at	0,007362387	12,14	10,39	10,94	Clk1	**-1,74**	**D**	**-1,20**	**D**	0,54	
1418419_at	0,003439852	7,31	5,58	5,43	4632423N09Rik	**-1,73**	**D**	**-1,88**	**D**	-0,14	
1438211_s_at	0,007036273	7,96	5,68	6,22	Dbp	**-1,72**	**D**	**-1,48**	**D**	0,23	
1428122_s_at	0,005549255	7,91	6,20	6,34	2610528K11Rik	**-1,71**	**D**	**-1,57**	**D**	0,13	
1419135_at	0,003062747	12,78	11,08	10,23	Ltb	**-1,71**	**D**	**-2,56**	**D**	-0,85	
1451421_a_at	0,009574204	10,51	8,81	7,94	MGI:1913299	**-1,70**	**D**	**-2,57**	**D**	-0,87	
1454987_a_at	0,003574087	8,95	7,26	7,73	H2-Ke6	**-1,69**	**D**	**-1,22**	**D**	0,47	
1421340_at	0,000602229	7,03	5,35	4,83	Map3k5	**-1,69**	**D**	**-2,20**	**D**	-0,51	
1437901_a_at	7,28358E-05	9,37	7,69	7,91	Vps41	**-1,69**	**D**	**-1,47**	**D**	0,22	
1418238_at	0,000179639	8,33	6,65	6,34	Ivd	**-1,68**	**D**	**-1,99**	**D**	-0,31	
1426258_at	0,000274561	10,89	9,22	8,90	Sorl1	**-1,67**	**D**	**-1,99**	**D**	-0,32	
1436879_x_at	0,001348421	5,52	3,85	2,48	Afp	**-1,66**	**D**	**-3,04**	**D**	**-1,37**	**D**
1416207_at	0,007294289	9,85	8,20	8,40	Taz	**-1,65**	**D**	**-1,45**	**D**	0,20	
1419212_at	0,000901581	9,36	7,73	6,63	Icoslg	**-1,62**	**D**	**-2,73**	**D**	**-1,10**	**D**
1435896_at	0,002919949	6,13	4,53	4,52	Sfxn2	**-1,60**	**D**	**-1,61**	**D**	-0,01	
1438860_a_at	0,003649854	7,58	5,66	4,85	1110028E10Rik	**-1,58**	**D**	**-2,04**	**D**	-0,46	
1424226_at	0,001551408	6,99	5,41	4,49	9030617O03Rik	**-1,58**	**D**	**-2,49**	**D**	-0,92	
1423508_at	0,000395316	7,79	6,22	6,34	Myst4	**-1,57**	**D**	**-1,45**	**D**	0,12	
1419759_at	0,0039568	5,84	4,27	3,13	Abcb1a	**-1,57**	**D**	**-2,71**	**D**	**-1,14**	**D**
1423213_at	0,007830995	10,94	9,28	8,52	Plxnc1	**-1,57**	**D**	**-2,25**	**D**	-0,68	
1436970_a_at	0,002907786	4,52	2,97	3,06	Pdgfrb	**-1,56**	**D**	**-1,46**	**D**	0,10	
1424338_at	0,006333979	3,74	2,19	2,19	Slc6a13	**-1,55**	**D**	**-1,54**	**D**	0,00	
1416703_at	0,006193894	11,89	10,34	10,17	Mapk14	**-1,55**	**D**	**-1,72**	**D**	-0,18	
1451474_a_at	0,004215305	9,08	7,54	7,25	Parp8	**-1,55**	**D**	**-1,83**	**D**	-0,29	

1421861_at	0,000917712	7,66	6,13	6,44	Clstn1	**-1,53**	**D**	**-1,23**	**D**	0,31	
1425346_at	0,001616853	7,08	5,56	5,60	Zfp318	**-1,53**	**D**	**-1,48**	**D**	0,05	
1425115_at	0,002811428	4,18	2,66	2,74	Rbbp6	**-1,53**	**D**	**-1,44**	**D**	0,08	
1423212_at	0,000707612	7,84	6,33	5,87	Phc1	**-1,51**	**D**	**-1,97**	**D**	-0,46	
1449023_a_at	0,005246474	8,88	7,38	7,59	Ezh1	**-1,50**	**D**	**-1,30**	**D**	0,20	
1454884_at	0,007706125	9,29	7,79	6,30	Btbd4	**-1,50**	**D**	**-2,99**	**D**	**-1,49**	**D**
1418523_at	0,008980979	6,70	5,22	5,24	Arih2	**-1,48**	**D**	**-1,46**	**D**	0,02	
1452398_at	0,008073215	4,38	2,91	2,66	Plce1	**-1,48**	**D**	**-1,73**	**D**	-0,25	
1424054_at	0,000284628	6,07	4,59	4,63	Btbd2	**-1,47**	**D**	**-1,44**	**D**	0,04	
1419381_at	0,000824429	4,98	3,50	3,48	Terf2ip	**-1,47**	**D**	**-1,50**	**D**	-0,03	
1450464_at	0,005852925	6,74	5,27	5,15	E4f1	**-1,47**	**D**	**-1,59**	**D**	-0,12	
1429473_at	5,03923E-05	8,70	7,24	6,87	1200003I07Rik	**-1,46**	**D**	**-1,83**	**D**	-0,37	
1449878_a_at	0,004455883	9,37	7,91	8,15	Slc12a6	**-1,46**	**D**	**-1,22**	**D**	0,24	
1438933_x_at	0,001966986	8,31	6,86	6,89	Rasgrp2 /// LOC381240	**-1,44**	**D**	**-1,41**	**D**	0,03	
1419047_at	6,57479E-05	7,54	6,11	6,16	Pcnx	**-1,43**	**D**	**-1,38**	**D**	0,05	
1427252_at	0,000143343	4,16	2,75	2,79	Dmrtb1	**-1,41**	**D**	**-1,37**	**D**	0,04	
1420630_at	0,006233888	9,11	7,70	7,59	8430419L09Rik	**-1,41**	**D**	**-1,52**	**D**	-0,12	
1453013_at	0,00050227	9,27	7,86	7,82	1110034O07Rik	**-1,41**	**D**	**-1,45**	**D**	-0,04	
1449433_at	0,002871707	4,41	3,00	2,56	P2rx5	**-1,41**	**D**	**-1,85**	**D**	-0,44	
1421550_a_at	0,000782134	3,85	2,45	2,06	Trim34	**-1,40**	**D**	**-1,79**	**D**	-0,39	
1452625_at	0,00473404	8,33	6,94	6,96	Kctd2	**-1,39**	**D**	**-1,37**	**D**	0,03	
1454699_at	0,008005761	9,65	8,27	8,59	Sesn1	**-1,38**	**D**	**-1,06**	**D**	0,31	
1423630_at	0,002024447	3,95	2,57	2,50	Cygb	**-1,38**	**D**	**-1,45**	**D**	-0,07	
1418865_at	0,003000714	8,06	6,69	6,26	Zfp385	**-1,37**	**D**	**-1,80**	**D**	-0,43	
1426834_s_at	0,000684256	11,97	10,65	10,09	D930015E06Rik	**-1,32**	**D**	**-1,88**	**D**	-0,56	
1448361_at	0,004468719	9,39	8,08	7,89	Ttc3	**-1,31**	**D**	**-1,50**	**D**	-0,19	
1415834_at	0,007833188	10,70	9,39	8,50	Dusp6	**-1,31**	**D**	**-2,20**	**D**	-0,89	
1419248_at	0,001436363	13,22	11,91	11,56	Rgs2	**-1,31**	**D**	**-1,66**	**D**	-0,35	
1452333_at	0,003576874	3,92	2,62	2,65	Smarca2	**-1,31**	**D**	**-1,27**	**D**	0,04	
1433723_s_at	0,002286218	9,79	8,49	8,73	Serf2	**-1,30**	**D**	**-1,07**	**D**	0,24	
1449131_s_at	0,004231295	11,27	9,95	8,84	Cd1d1	**-1,30**	**D**	**-2,36**	**D**	**-1,07**	**D**
1430527_a_at	0,000747407	10,19	8,90	8,65	Rnf167	**-1,29**	**D**	**-1,54**	**D**	-0,24	
1433475_a_at	0,004486314	9,69	8,40	8,37	C78339	**-1,28**	**D**	**-1,32**	**D**	-0,03	
1421937_at	0,004943844	9,37	8,10	7,76	Dapp1	**-1,27**	**D**	**-1,61**	**D**	-0,34	
1449459_s_at	0,009268549	8,09	6,82	6,93	Asb13	**-1,27**	**D**	**-1,16**	**D**	0,11	
1460363_at	0,000284703	8,12	6,85	6,44	Tnrc6c	**-1,26**	**D**	**-1,68**	**D**	-0,42	

1423592_at	0,002485461	6,16	4,90	4,48	Rock2	**-1,26**	**D**	**-1,69**	**D**	-0,42	
1418723_at	0,004107283	4,18	2,93	2,93	Edg7	**-1,26**	**D**	**-1,25**	**D**	0,00	
1427032_at	0,001443058	11,29	10,03	10,22	Herc4	**-1,26**	**D**	**-1,06**	**D**	0,19	
1455315_at	0,003535724	6,26	5,01	4,92	2410019G02Rik	**-1,25**	**D**	**-1,34**	**D**	-0,09	
1426914_at	0,000496515	4,36	3,11	3,08	Marveld2	**-1,24**	**D**	**-1,27**	**D**	-0,03	
1425602_a_at	0,004129711	7,96	6,72	6,90	Rabep2	**-1,24**	**D**	**-1,06**	**D**	0,19	
1438610_a_at	0,005459139	9,46	8,23	8,27	Cryz	**-1,23**	**D**	**-1,19**	**D**	0,04	
1452187_at	0,001092454	7,41	6,19	6,26	Rbm5	**-1,23**	**D**	**-1,15**	**D**	0,07	
1455134_at	0,003413091	6,25	5,03	4,57	D730040F13Rik	**-1,22**	**D**	**-1,68**	**D**	-0,46	
1451130_at	0,004769076	10,88	9,66	9,76	2010315L10Rik	**-1,22**	**D**	**-1,12**	**D**	0,09	
1420398_at	0,004812015	9,83	8,62	7,11	Rgs18	**-1,21**	**D**	**-2,72**	**D**	**-1,51**	**D**
1434633_at	0,008501864	8,40	7,20	6,78	AW558298	**-1,21**	**D**	**-1,63**	**D**	-0,42	
1425235_s_at	0,009339064	3,35	2,15	2,16	1700051I12Rik	**-1,21**	**D**	**-1,20**	**D**	0,01	
1438651_a_at	0,002460808	6,14	4,93	2,10	Agtrl1	**-1,20**	**D**	**-4,03**	**D**	**-2,83**	**D**
1451132_at	0,008537905	10,06	8,86	8,49	Pbxip1	**-1,20**	**D**	**-1,57**	**D**	-0,37	
1418148_at	0,003358953	6,29	5,09	4,91	Abhd1	**-1,19**	**D**	**-1,38**	**D**	-0,19	
1448385_at	0,006416	10,43	9,24	9,20	Slc15a4	**-1,19**	**D**	**-1,23**	**D**	-0,04	
1434511_at	0,005553277	9,46	8,29	8,41	Phkb	**-1,18**	**D**	**-1,05**	**D**	0,12	
1422303_a_at	0,001535124	6,49	5,32	3,79	Tnfrsf18	**-1,17**	**D**	**-2,70**	**D**	**-1,53**	**D**
1434620_s_at	0,006125955	10,07	8,90	9,04	2610024E20Rik	**-1,17**	**D**	**-1,03**	**D**	0,14	
1434937_at	0,006594151	12,04	10,88	10,44	Phr1	**-1,16**	**D**	**-1,60**	**D**	-0,43	
1418291_at	0,004421497	6,46	5,30	5,27	Zfp87	**-1,16**	**D**	**-1,19**	**D**	-0,03	
1416727_a_at	0,004664849	12,73	11,58	11,55	Cyb5	**-1,16**	**D**	**-1,18**	**D**	-0,02	
1448323_a_at	0,007186238	3,82	2,66	2,68	Bgn	**-1,15**	**D**	**-1,14**	**D**	0,01	
1426769_s_at	0,006961749	8,36	7,21	7,30	Maml1	**-1,15**	**D**	**-1,06**	**D**	0,09	
1438058_s_at	0,00165788	7,09	5,94	5,51	Ptov1	**-1,15**	**D**	**-1,59**	**D**	-0,43	
1460647_a_at	0,00567316	5,97	4,82	4,60	Nr2f6	**-1,15**	**D**	**-1,37**	**D**	-0,22	
1415691_at	0,009447952	8,77	7,62	7,47	Dlgh1	**-1,15**	**D**	**-1,30**	**D**	-0,15	
1416880_at	0,001658926	12,47	11,33	11,37	Mcl1	**-1,15**	**D**	**-1,10**	**D**	0,04	
1452846_at	0,004377517	6,05	4,91	4,83	Ppfia4	**-1,14**	**D**	**-1,22**	**D**	-0,08	
1423843_at	0,00837375	5,78	4,64	4,62	BC027309	**-1,14**	**D**	**-1,15**	**D**	-0,01	
1432264_x_at	0,000686276	11,87	10,73	10,84	Cox7a2l	**-1,14**	**D**	**-1,03**	**D**	0,11	
1428871_at	0,005646457	9,00	7,92	7,99	4121402D02Rik	**-1,14**	**D**	**-1,16**	**D**	-0,01	
1419676_at	0,001033335	3,27	2,13	2,07	Mx2	**-1,14**	**D**	**-1,20**	**D**	-0,06	
1427113_s_at	0,002434435	7,25	6,12	5,91	2700049H19Rik	**-1,14**	**D**	**-1,34**	**D**	-0,21	
1424781_at	0,004691324	10,02	8,90	8,60	D10Ucla1	**-1,12**	**D**	**-1,42**	**D**	-0,30	

1423478_at	0,009090781	10,86	9,75	9,51	Prkcb1	**-1,12**	**D**	**-1,32**	**D**	-0,20	
1454635_at	0,001273661	7,68	6,56	5,96	Fbxl3	**-1,11**	**D**	**-1,72**	**D**	-0,61	
1417856_at	0,009892234	10,12	9,01	7,79	Relb	**-1,11**	**D**	**-2,33**	**D**	**-1,22**	**D**
1415856_at	0,008875217	7,82	6,72	4,56	Emb	**-1,11**	**D**	**-3,27**	**D**	**-2,16**	**D**
1455291_s_at	0,003976827	9,88	8,87	8,53	Znrf2	**-1,10**	**D**	**-1,42**	**D**	-0,32	
1428280_at	0,009496118	9,25	8,16	8,24	Fip1l1	**-1,10**	**D**	**-1,01**	**D**	0,08	
1427376_a_at	0,009624116	4,25	3,16	3,11	Map4k5	**-1,09**	**D**	**-1,14**	**D**	-0,05	
1425508_s_at	0,007400871	7,96	6,87	6,91	Arfrp1	**-1,09**	**D**	**-1,06**	**D**	0,04	
1452288_at	0,003302956	7,28	6,19	6,23	BB128963	**-1,09**	**D**	**-1,05**	**D**	0,05	
1451115_at	0,007465634	7,05	5,97	5,78	Pias3	**-1,08**	**D**	**-1,27**	**D**	-0,19	
1450513_at	0,000150046	3,02	1,94	1,93	Cd33	**-1,08**	**D**	**-1,09**	**D**	-0,01	
1423899_at	0,000791811	5,95	4,88	4,69	Trip12	**-1,07**	**D**	**-1,25**	**D**	-0,19	
1451075_s_at	0,00799941	11,13	10,07	10,03	Ctdsp2	**-1,06**	**D**	**-1,10**	**D**	-0,04	
1455286_at	0,000878899	10,00	8,94	8,82	Btbd1	**-1,06**	**D**	**-1,18**	**D**	-0,12	
1426894_s_at	0,007056595	7,28	6,22	5,29	C230093N12Rik	**-1,06**	**D**	**-1,99**	**D**	-0,93	
1425311_at	1,70598E-05	3,95	2,89	2,91	4930432F04Rik	**-1,06**	**D**	**-1,04**	**D**	0,02	
1416522_a_at	0,001557726	11,02	9,97	9,90	Grcc10	**-1,05**	**D**	**-1,11**	**D**	-0,06	
1423673_at	0,004771371	5,92	4,87	4,77	BC058638	**-1,05**	**D**	**-1,16**	**D**	-0,11	
1432474_a_at	0,001101896	4,63	3,58	3,60	Krtcap3	**-1,05**	**D**	**-1,03**	**D**	0,02	
1424478_at	0,007877284	4,14	3,09	2,95	Bbs2	**-1,04**	**D**	**-1,19**	**D**	-0,14	
1448275_at	0,003856774	6,48	5,45	4,98	Tmem19	**-1,03**	**D**	**-1,50**	**D**	-0,47	
1418996_a_at	0,004384757	8,64	7,61	7,57	4930469P12Rik	**-1,02**	**D**	**-1,07**	**D**	-0,04	
1424431_at	0,001230168	10,29	9,27	8,86	MGI:1926002	**-1,02**	**D**	**-1,43**	**D**	-0,41	
1418402_at	0,009723563	9,94	8,92	8,34	Adam19	**-1,02**	**D**	**-1,60**	**D**	-0,58	
1460571_at	0,005127908	6,79	5,77	5,66	Dicer1	**-1,02**	**D**	**-1,13**	**D**	-0,11	
1421878_at	0,00724327	7,99	6,99	6,96	Mapk9	**-1,00**	**D**	**-1,03**	**D**	-0,03	

Genes down-regulated in spleen DCs by *P. yoelii* infection at day 5 pi but not at day 10 pi

						Day 5 vs 0		Day 10 vs 0		Day 10 vs 5	
Probe_ID	p-value	Day 1	Day 5	Day 10	Gene Symbol	5vs0	p<0.01	10vs0	p<0.01	10vs5	p<0.01
1423902_s_at	0,00553771	7,20	5,29	6,52	Arhgef12	**-1,91**	**D**	-0,68		**1,23**	**U**
1456393_at	0,002692519	11,59	10,04	10,69	2310002J21Rik	**-1,55**	**D**	-0,90		0,65	
1452694_at	0,000159511	9,52	8,20	8,59	Ihpk1	**-1,32**	**D**	-0,93		0,38	
1436038_a_at	0,000351685	8,84	7,54	7,94	Dscr5	**-1,30**	**D**	-0,90		0,40	
1427075_s_at	0,009215595	9,07	7,80	8,08	5330414D10Rik	**-1,26**	**D**	-0,98		0,28	
1435866_s_at	0,004400236	7,20	5,96	6,57	Hist3h2a	**-1,24**	**D**	-0,63		0,61	
1435631_x_at	0,000216662	8,28	7,05	7,69	Sec15l1	**-1,23**	**D**	-0,60		0,64	
1418394_a_at	0,005853222	11,51	10,29	10,65	Cd97	**-1,22**	**D**	-0,86		0,36	
1416768_at	0,004123575	10,98	9,78	10,01	1110003E01Rik	**-1,21**	**D**	-0,98		0,23	
1426810_at	0,000199408	8,99	7,84	8,15	Jmjd1a	**-1,16**	**D**	-0,84		0,32	
1460189_at	0,004974183	9,06	7,97	8,21	Wdr23	**-1,13**	**D**	-0,87		0,25	
1423739_x_at	0,004353293	10,23	9,12	9,54	Aplp2	**-1,11**	**D**	-0,70		0,41	
1427174_at	0,004887878	8,11	7,02	7,17	LOC433533	**-1,09**	**D**	-0,94		0,15	
1452271_at	0,006783041	5,99	4,90	5,11	Xpr1	**-1,08**	**D**	-0,88		0,20	
1435018_at	0,008271571	7,71	6,62	6,87	5930434B04Rik	**-1,08**	**D**	-0,84		0,24	
1449441_a_at	0,003492695	8,47	7,39	7,89	Wbp1	**-1,08**	**D**	-0,57		0,51	
1432263_a_at	0,004764443	11,27	10,20	10,42	Cox7a2l	**-1,07**	**D**	-0,85		0,22	
1424862_s_at	0,00937081	6,55	5,48	5,67	Zzank1	**-1,07**	**D**	-0,88		0,19	
1424319_at	0,001321374	6,89	5,82	5,98	Oraov1	**-1,07**	**D**	-0,90		0,16	
1416429_a_at	0,003446559	11,67	10,60	10,84	Cat	**-1,06**	**D**	-0,83		0,24	
1419635_at	2,99638E-05	6,89	5,84	6,08	4833420G17Rik	**-1,04**	**D**	-0,81		0,24	
1417061_at	0,009799759	13,79	12,75	12,99	Slc40a1	**-1,04**	**D**	-0,80		0,24	
1452835_a_at	0,008942735	5,30	4,27	5,38	Polrmt	**-1,03**	**D**	0,08		**1,11**	**U**
1424023_at	0,00527071	7,26	6,23	6,27	BC005752	**-1,03**	**D**	-0,98		0,05	
1456243_x_at	0,006677152	13,04	12,02	12,05	Mcl1	**-1,03**	**D**	-1,00		0,03	
1423817_s_at	0,006626367	10,62	9,61	9,95	2010315L10Rik	**-1,00**	**D**	-0,67		0,34	

Genes down-regulated in spleen DCs by *P. yoelii* infection at day 10 pi but not at day 5 pi

						Day 5 vs 0		Day 10 vs 0		Day 10 vs 5	
Probe_ID	p-value	Day 1	Day 5	Day 10	Gene Symbol	5vs0	p<0.01	10vs0	p<0.01	10vs5	p<0.01
1416779_at	0,006728558	6,27	5,69	2,47	Sdpr	-0,57		**-3,79**	**D**	**-3,22**	**D**
1448392_at	0,005363138	5,86	6,72	2,87	Sparc	0,86		**-2,98**	**D**	**-3,85**	**D**
1419331_at	0,00894844	6,01	5,49	3,29	Cdh17	-0,53		**-2,73**	**D**	**-2,20**	**D**
1419192_at	0,001854037	10,76	9,81	8,38	Il4i1	-0,95		**-2,38**	**D**	**-1,43**	**D**
1425386_at	0,009680758	5,92	6,04	3,66	4833422F24Rik	0,12		**-2,26**	**D**	**-2,38**	**D**
1420823_at	0,008044589	6,83	6,71	4,72	Sema4d	-0,12		**-2,11**	**D**	**-1,99**	**D**
1448957_at	0,007983247	8,66	7,90	6,70	Rbpsuh	-0,76		**-1,96**	**D**	**-1,20**	**D**
1421930_at	0,007255491	6,67	6,80	3,99	Icos	0,49		**-1,94**	**D**	**-2,44**	**D**
1418998_at	0,006571343	10,25	9,31	8,33	Kmo	-0,94		**-1,92**	**D**	-0,98	
1452481_at	0,001231346	5,37	4,43	3,51	Plcb2	-0,94		**-1,86**	**D**	-0,92	
1440874_at	0,007793887	5,95	5,01	4,13	Slco5a1	-0,94		**-1,82**	**D**	-0,88	
1416236_a_at	0,009054213	7,28	6,74	5,48	Eva1	-0,54		**-1,79**	**D**	**-1,26**	**D**
1421375_a_at	0,005914159	11,59	11,42	9,84	S100a6	-0,17		**-1,75**	**D**	**-1,58**	**D**
1418057_at	0,004171746	7,21	6,51	5,47	Tiam1	-0,70		**-1,74**	**D**	**-1,04**	**D**
1417473_a_at	0,000311338	6,86	6,05	5,20	6330579B17Rik	-0,81		**-1,67**	**D**	-0,86	
1421210_at	0,001572049	8,78	8,04	7,14	C2ta	-0,75		**-1,64**	**D**	-0,89	
1454632_at	0,002342921	9,78	8,99	8,18	6330442E10Rik	-0,79		**-1,60**	**D**	-0,82	
1423466_at	0,002028029	9,81	9,56	8,30	Ccr7	-0,25		**-1,51**	**D**	**-1,26**	**D**
1426994_at	0,00248067	7,81	7,24	6,31	Phlpp	-0,57		**-1,50**	**D**	-0,93	
1426708_at	0,00449262	8,97	8,17	7,49	Antxr2	-0,80		**-1,48**	**D**	-0,68	
1428819_at	0,003482162	7,75	6,75	6,27	Mapre1	-1,00		**-1,48**	**D**	-0,48	
1426448_at	0,002640905	7,07	6,53	5,59	Pja1	-0,54		**-1,47**	**D**	-0,93	
1451393_at	0,007782853	5,49	4,58	4,09	Pex26	-0,90		**-1,40**	**D**	-0,50	
1418587_at	0,001242961	8,19	7,47	6,80	Traf3	-0,72		**-1,39**	**D**	-0,67	
1434510_at	0,003606587	6,25	5,90	4,85	Papss2	-0,35		**-1,39**	**D**	**-1,04**	**D**
1450626_at	0,001968568	8,56	7,88	7,20	Manba	-0,68		**-1,37**	**D**	-0,69	
1459900_at	0,003972977	5,71	5,14	4,35	C79468	-0,57		**-1,36**	**D**	-0,79	
1436991_x_at	0,009027038	12,41	11,71	11,23	Gsn	-0,79		**-1,35**	**D**	-0,56	
1450859_s_at	0,006649947	9,10	8,26	7,78	Ube2d3	-0,84		**-1,32**	**D**	-0,49	
1431843_a_at	0,001714432	9,11	8,56	7,78	Nfkbie	-0,55		**-1,32**	**D**	-0,77	
1434184_s_at	0,000249965	8,27	7,81	6,95	9430080K19Rik	-0,46		**-1,32**	**D**	-0,86	
1424368_s_at	0,008360773	11,09	10,27	9,79	Ubqln1	-0,83		**-1,30**	**D**	-0,48	

1425532_a_at	0,007441589	9,06	8,47	7,76	Bin1	-0,60	**-1,30**	**D**	-0,71
1416992_at	0,003210381	8,35	7,59	7,08	Mfng	-0,76	**-1,28**	**D**	-0,52
1424660_s_at	0,009014906	7,67	6,74	6,39	4632407F12Rik	-0,92	**-1,27**	**D**	-0,35
1424665_at	0,007286432	5,02	4,17	3,75	5430405G24Rik	-0,85	**-1,27**	**D**	-0,41
1433568_at	0,004030058	8,76	7,98	7,50	Papd4	-0,77	**-1,26**	**D**	-0,48
1427929_a_at	0,001925626	9,55	8,92	8,29	Pdxk	-0,62	**-1,25**	**D**	-0,63
1420535_a_at	0,007110713	8,30	7,72	7,05	6330412F12Rik	-0,58	**-1,25**	**D**	-0,67
1448345_at	0,002921544	8,82	8,04	7,58	Tomm34	-0,78	**-1,24**	**D**	-0,45
1427705_a_at	0,007116749	11,10	10,18	9,87	Nfkb1	-0,92	**-1,23**	**D**	-0,31
1422595_s_at	0,002093574	7,96	7,22	6,73	5730470L24Rik	-0,74	**-1,22**	**D**	-0,48
1452021_a_at	0,009431724	6,80	6,13	5,59	Hes6	-0,67	**-1,21**	**D**	-0,54
1423319_at	0,008908836	9,66	8,75	8,48	Hhex	-0,90	**-1,17**	**D**	-0,27
1460351_at	0,000506257	12,28	12,02	11,11	S100a11	-0,27	**-1,17**	**D**	-0,90
1450879_at	0,006676308	7,71	6,72	6,54	Atp9b	-0,99	**-1,17**	**D**	-0,18
1450649_at	0,003921243	12,61	11,77	11,44	Gng10	-0,83	**-1,16**	**D**	-0,33
1423297_at	0,007364945	7,71	6,94	6,56	Add3	-0,77	**-1,15**	**D**	-0,39
1418375_at	0,009431791	7,72	6,78	6,58	D10Wsu93e	-0,94	**-1,14**	**D**	-0,20
1448340_at	0,00474675	6,56	5,78	5,43	D9Wsu20e	-0,79	**-1,14**	**D**	-0,35
1424508_at	0,002934354	9,90	8,90	8,79	Ttc5	-1,00	**-1,11**	**D**	-0,11
1424145_at	0,00614656	5,38	4,80	4,29	Prr3	-0,58	**-1,09**	**D**	-0,51
1422516_a_at	0,006410463	7,94	7,02	6,85	Fibp	-0,92	**-1,09**	**D**	-0,17
1423928_at	0,008293006	9,05	8,15	7,97	Phgdhl1	-0,91	**-1,08**	**D**	-0,17
1436315_at	0,008307196	9,84	9,04	8,77	Myst3	-0,80	**-1,07**	**D**	-0,26
1423168_at	0,007960712	9,78	8,96	8,72	Prei3	-0,83	**-1,07**	**D**	-0,24
1419554_at	0,002207377	12,02	11,10	10,96	Cd47	-0,92	**-1,06**	**D**	-0,15
1451985_at	0,008645279	10,49	9,85	9,43	Lrrk1	-0,64	**-1,06**	**D**	-0,42
1428128_at	0,004314719	9,93	8,97	8,87	4921506J03Rik	-0,96	**-1,06**	**D**	-0,10
1415886_at	0,004452414	7,54	6,65	6,48	Sh2d3c	-0,89	**-1,06**	**D**	-0,17
1416709_a_at	0,003715773	8,89	8,17	7,85	AW552001	-0,73	**-1,04**	**D**	-0,31
1438559_x_at	0,00773065	12,09	11,39	11,06	1110028E10Rik	-0,69	**-1,03**	**D**	-0,34
1424645_at	0,009698957	8,41	7,73	7,38	Tnrc6c	-0,68	**-1,03**	**D**	-0,35
1426207_at	0,008541454	10,91	10,20	9,89	Ikbkb	-0,72	**-1,02**	**D**	-0,31
1428131_a_at	0,009966409	10,83	10,21	9,82	Cdc42se1	-0,63	**-1,01**	**D**	-0,39
1451574_at	0,005178203	7,07	6,22	6,06	Bcl9	-0,85	**-1,01**	**D**	-0,16
1451098_at	0,009989616	9,24	8,60	8,23	Pcoln3	-0,64	**-1,01**	**D**	-0,37
1427925_at	0,001435157	6,85	5,86	5,84	Stx17	-1,00	**-1,01**	**D**	-0,01
1426465_at	0,00118274	8,57	7,83	7,56	Dlgap4	-0,73	**-1,01**	**D**	-0,27

APPENDIX II

Genechip® data analysis: annotation of *P. yoelii*-modulated genes according to Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG)

Appendix II, Table I

Transcripts modulated in spleen DCs by *P. yoelii* according to Gene Ontology biological process.

Process	**Number of differentially regulated transcripts (induced, repressed)**
Cell cycle	45 (37, 8)
Defense response	45 (28, 16)
Phosphorylation	36 (20, 16)
Protein phosphorylation	36 (20, 16)
Regulation of transcription	32 (14, 18)
Transcription, DNA-dependent	32 (14, 18)
Protein catabolism	25 (11, 14)
Proteolysis and peptidolysis	23 (10, 13)
Response to pest/pathogen/parasite	22 (15, 7)
Apoptosis	17 (8, 9)
Ion transport	17 (5, 12)
Protein biosynthesis	16 (11, 5)
G-protein coupled receptor protein signaling pathway	16 (3, 13)
Cytoplasm organization and biogenesis	15 (6, 9)
Intracellular transport	14 (10, 4)
Hexose metabolism	13 (9, 4)
Response to chemical substance	13 (6, 7)
DNA repair	12 (9, 3)
Cytokinesis	11 (11, 0)
main pathways of carbohydrate metabolism	11 (8, 3)
Amino acid metabolism	10 (10, 0)
Glycolysis	10 (8, 2)
Inflammatory response	10 (8, 2)
Monosaccharide catabolism	10 (8, 2)
Protein transport	10 (7, 3)
Vesicle-mediated transport	10 (7, 3)

Appendix II, Table II

Transcripts modulated in spleen DCs by *P. yoelii* according to GO molecular function.

Pathway	**Number of differentially regulated transcripts (induced, repressed)**
ATP binding	56 (34, 22)
Protein kinase activity	34 (19, 15)
Serine-type endopeptidase	11 (5, 6)
rhodopsin-like receptor activity	9 (3, 6)
GTP binding	8 (6, 2)
phosphoric monoester hydrolase activity	6 (4, 2)
serine-type endopeptidase inhibitor activity	6 (3, 3)
Symporter activity	6 (1, 5)
Acyltransferase activity	5 (3, 2)
ATPase activity	5 (3, 2)
Ubiquitin protein ligase activity	5 (2, 3)
Cation channel activity	4 (1, 3)
Chemokine activity	4 (4, 0)
Metaloendopeptidase activity	4 (1, 3)
Receptor signaling protein serine/threonine kinase activity	4 (1, 3)

Appendix II, Table III

Transcripts modulated in spleen DCs by *P. yoelii* according to GO cellular component.

Pathway	**Number of differentially regulated transcripts (induced, repressed)**
Microtubule cytoskeleton	12 (10, 2)
Transcription factor complex	7 (4, 3)
Mitochondrial membrane	6 (5, 1)
Mitochondrial matrix	5 (4, 1)
Microsome	4 (1, 3)
Organellar ribosome	4 (3, 1)
Mitochondrial electron transport	3 (3, 0)
Actin cytoskeleton	3 (1, 2)
Cytosolic ribosome	3 (1, 2)

Appendix II, Table IV

Transcripts modulated in spleen DCs by *P. yoelii* according to KEGG (Kyoto Encyclopedia of Genes and Genomes) metabolic pathways.

Pathway	**Number of differentially regulated transcripts (induced, repressed)**
Glycolysis / Gluconeogenesis	9 (8, 1)
Purine metabolism	9 (8, 1)
Pyrimidine metabolism	7 (5, 2)
Apoptosis	6 (3, 3)
Carbon fixation	5 (5, 0)
Alzheimer's disease	5 (2, 3)
Fructose and mannose metabolism	5 (3, 2)
Glycerolipid metabolism	5 (3, 2)
Glycine, serine and threonine metabolism	4 (4, 0)
Ribosome	4 (2, 2)
One carbon pool byfolate	3 (3, 0)
Oxidative phosphorylation	3 (3, 0)
Complement and coagulation cascades	3 (2, 1)
Glutathione metabolism	3 (2, 1)
Phosphatidylinositol signaling	3 (1, 2)

APPENDIX III

Comparison of data obtained from Genechip® analysis of transcriptomic regulation of spleen dendritic cells by *P. yoelii* with data from the literature

Appendix III, Table I

Regulation of genes belonging to the common maturation program of DCs during *P. yoelii* infection.

Gene	Gene ID (mouse)	Probeset	p	log2 (d0)	log2 (d5)	log2 (d10)	Malaria regulation
Innate immunity							
tnfα	21926	1419607_at	.8300	7.12	7.37	7.39	**nc**
il-1β	16176	1449399_a_at	.6506	10.64	10.59	10.20	**nc**
il-6	16193	1450297_at	.00004	2.26	6.38	3.42	**+++ (**)**
cxcl2/gro2/mip2	20310	1449984_at	.0776	2.24	7.52	7.23	**ns**
ccl19/mip3b/elc	24047	1449277_at	.3223	1.97	1.96	1.98	**nc**
H2-Bf/Cfb/bf	14962	1417314_at	.0279	5.70	10.36	9.16	**+++ (*)**
Ptgir	19222	1427313_at	.0644	3.45	2.91	2,96	**nc**
cox2/ptgs2	19225	1417262_at	.0287	3.10	8.21	6.09	**+++ (*)**
Adaptive immunity							
cd86/b7.2	12524	1420404_at	.0324	11.13	9.96	9.28	**- (*)**
cxcl9/mig	17329	1418652_at	.0333	10.98	13.06	12.12	**++ (*)**
cxcl10/inp10	15945	1418930_at	.0186	7.42	10.35	9.16	**++ (*)**
cxcl11/itac	56066	1419697_at	.0026	4.19	7.52	6.92	**+++ (**)**
slamf1	27218	1425569_a_at	.0484	2.33	3.87	3.29	**+ (*)**
icam1	25464	1424067_at	.8640	10.95	10.98	10.76	**nc**
Psmb10/lmp10	19171	1448632_at	.1251	10.19	10.94	10.35	**nc**
Pbef	59027	1417190_at	.1040	8.00	9.34	8.82	**ns**
Immunity Receptors							
cd83	12522	1416111_at	.0017	11.84	10.00	9.86	**- (**)**
Il15ra #	16169	1448681_at	.0171	4.06	6.34	5.83	**++ (*)**
Il7r	16197	1448576_at	.0252	4.35	3.51	2.87	**- (*)**
Il4ra	16190	1421034_a_at	.1218	7.56	8.54	8.31	**nc**
tnfrsf9/4-1bb	21942	1460469_at	.0357	4.75	6.76	6.30	**++ (*)**
Immune transcription							
nfkb1/p50	18033	1427705_a_at	.0071	11.10	10.18	9.87	**- (**)**
nfkb2/p52	18034	1425902_a_at	.0213	9.32	8.77	8.06	**- (*)**
rel-a/p65	19697	1419536_a_at	.0188	9.98	9.47	9.44	**nc**

Immune transcription							
stat3	20848	1460700_at	.1348	9.72	10.27	9.92	**nc**
Irf4	16364	1421173_at	.3496	8.35	8.08	8.96	**nc**
isgf3g	16391	1421322_a_at	.0150	9.90	9.00	8.74	**- (*)**
Immune 'Inhibitors'							
ccl2/mcp1	20296	1420380_at	.5367	3.71	4.74	4.21	**ns**
Indo	15930	1420437_at	.0457	2.07	3.69	2.92	**+ (*)**
Apoptosis activators							
casp4/casp11	12363	1449591_at	.0362	5.15	7.19	6.21	**++ (*)**
trail/tnfsf10	22035	1420412_at	.4114	2.21	2.24	2.93	**nc**
Fas/tnfrsf6/cd95	14102	1460251_at	.0016	5.49	7.21	8.02	**++ (**)**
bak1	12018	1418991_at	.0890	8.18	9.08	8.56	**nc**
Apoptosis inhibitors							
cflar/flip	12633	1425687_at	.6842	6.94	6.99	6.70	**nc**
bag1	12017	1416394_at	.0611	10.03	10.45	10.38	**nc**
birc3/ciap2	11796	1425223_at	.0266	5.38	3.95	4.08	**- (*)**
bcl2a1a	12044	1419004_s_at	.1062	12.91	12.53	11.95	**nc**
mapt/tau	17762	1424719_a_at	.2524	2.45	2.46	2.50	**nc**
Cell Stress							
mt1	17748	1422557_s_at	.0605	8.62	9.04	10.11	**ns**
mt2a	17750	1428942_at	.0684	5.11	8.20	9.88	**ns**
map3k4	26407	1421450_a_at	.3811	7.77	7.48	7.45	**nc**
hspa1a	193740	1452388_at	.1185	3.27	8.91	7.76	**ns**
ninj1	18081	1448417_at	.1798	7.45	6.52	5.90	**ns**
sod2	20656	1448610_a_at	.0238	9.80	11.93	11.34	**++ (*)**
cbr1	12408	1460196_at	.6983	6.42	6.11	6.30	**nc**
Others							
Pgam1	18648	1426554_a_at	.0155	11.12	12.08	12.05	**nc**
inhba/activin ba	16323	1422053_at	.3407	1.97	1.97	1.99	**nc**
mmp19	58223	1421977_at	.2001	3.15	4.25	3.78	**ns**
extl2	58193	1422539_at	.8723	4.64	4.50	4.70	**nc**

Appendix III, Table I – Genes identified by Huang et al. (Huang *et al.*, 2001) as the common maturation core were compared to the regulation of respective *Mus Musculus* homologs by *P. yoelii*. + stands for a 2 to 4-fold increase, ++ for 4 to 8-fold; +++ for over 8-fold. nc = no change in expression. ns = regulation is not significant between pairs of

replica arrays. *p* is the probability of differential regulation between 2 samples for each gene for Multiclass testing. Asterisks indicate level of significance (* is 0.01<*p*<0.05; ** is *p*<0.01). The following genes were not compared since they do not have a homolog in *Mus Musculus* or else were not present in the MOE430A array: i*l8/cxcl8, cxcl1/gro1, cxcl3/gro3/mip2b, ccl4l2/mip1b, ccl20/mip3a/larc, ccl17/tarc, ccl22/mdc*. # after the gene name indicates genes with more than one probe with p<0.05, in which case the expression values for the probe with lower p value are presented.

Appendix III - Table II

Regulation of genes belonging to the common response of DCs to infections during *P. yoelii* infection

Gene ID	Probeset	Gene	Description	p	log2(D0)	log2(D5)	log2(D10)	malaria regulation
50797	1456175_a_at	Copb2	coatomer protein complex, subunit beta 2 (be	0,087	10,68	11,06	11,19	no change
66913	1417204_at	Kdelr2	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulu	0,024	9,88	10,59	11,15	**up + (*)**
17527	1420387_at	Mpv17	Mpv17 transgene, kidney disease mutant	0,699	7,06	7,12	7,03	no change
52538	1428146_s_at	Acaa2	acetyl-Coenzyme A acyltransferase 2 (mitoch	0,595	8,25	8,33	8,55	no change
15488	1455777_x_at	Hsd17b4	hydroxysteroid (17-beta) dehydrogenase 4	0,140	10,99	10,45	10,46	no change
20322	1438183_x_at	Sdh1/sord	sorbitol dehydrogenase 1	0,000	2,68	1,97	2,24	no change
99586	1427946_s_at	Dpyd	dihydropyrimidine dehydrogenase	0,371	1,94	1,95	1,96	no change
12359	1416429_a_at	Cat	catalase	0,003	11,67	10,60	10,84	**down - (**)**
20963	1418261_at	Syk	spleen tyrosine kinase	0,629	10,93	11,07	11,12	no change
19414	1415850_at	Rasa3	RAS p21 protein activator 3	0,891	9,94	9,87	9,99	no change
19286	1450660_at	Pts	6-pyruvoyl-tetrahydropterin synthase	0,038	9,33	8,42	8,52	no change
97212	1452173_at	Hadha	hydroxyacyl-Coenzyme A dehydrogenase/3-k	0,309	8,98	9,33	9,33	no change
73690	1424927_at	Glipr1	GLI pathogenesis-related 1 (glioma)	0,433	11,30	11,05	11,47	no change
56440	1416260_a_at	Snx1	sorting nexin 1	0,634	9,47	9,67	9,62	no change
108014	1417727_at	Sfrs9	splicing factor, arginine/serine rich 9	0,339	10,65	10,78	10,86	no change
14688	1417432_a_at	Gnb1	guanine nucleotide binding protein, beta 1	0,089	11,04	11,43	11,36	no change
15979	1448167_at	Ifngr1	interferon gamma receptor 1	0,014	10,36	8,90	8,76	**down - (*)**
22596	1451968_at	Xrcc5	X-ray repair complementing defective repair ir	0,442	2,98	3,14	3,97	no change
66589	1415755_a_at	Ube2v1	ubiquitin-conjugating enzyme E2 variant 1	0,884	8,85	8,97	8,91	no change
67680	1418005_at	Sdhb	succinate dehydrogenase complex, subunit B	0,143	10,73	11,39	11,41	no change
51792	1415819_a_at	Ppp2r1a	protein phosphatase 2 (formerly 2A), regulato	0,673	9,99	10,07	9,95	no change
16889	1423140_at	Lip1	lysosomal acid lipase 1	0,447	10,39	9,93	9,48	no change
13032	1416382_at	Ctsc	cathepsin C	0,031	12,21	13,72	13,63	**up + (*)**
12493	1425736_at	Cd37	CD37 antigen	0,061	8,49	5,81	5,94	**down/ns**
18458	1453840_at	Pabpc1	poly A binding protein, cytoplasmic 1	0,263	7,19	7,67	7,85	no change
14683	1450186_s_at	Gnas	GNAS (guanine nucleotide binding protein, alp	0,006	13,07	12,69	13,25	no change
18807	1416013_at	Pld3	phospholipase D3	0,098	11,60	10,09	10,53	**down/ns**
18826	1415983_at	Lcp1	lymphocyte cytosolic protein 1	0,002	14,13	14,25	13,70	no change
20130	1418448_at	Rras	Harvey rat sarcoma oncogene, subgroup R	0,538	9,33	8,79	8,95	no change
109711	1428585_at	Actn1	actinin, alpha 1	0,096	11,69	10,97	10,77	no change
12846	1418701_at	Comt	catechol-O-methyltransferase	0,231	6,72	6,49	7,24	no change

56420	1460288_a_at	Ppp4c	protein phosphatase 4, catalytic subunit	0,526	10,67	10,71	10,61	no change
110208	1438627_x_at	Pgd	Phosphogluconate dehydrogenase	0,072	10,31	10,74	10,66	no change
54709	1416233_at	Eif3s2	eukaryotic translation initiation factor 3, subun	0,284	10,72	11,15	11,19	no change
12847	1415706_at	Copa	coatomer protein complex subunit alpha	0,387	11,22	11,44	11,43	no change
104318	1437690_x_at	Csnk1d	Casein kinase 1, delta	0,035	10,61	10,05	9,49	**down - (*)**
109905	1448020_at	Rap1a	RAS-related protein-1a	0,034	9,86	9,57	9,31	no change
110829	1418232_s_at	Lims1	LIM and senescent cell antigen-like domains 1	0,029	9,88	9,67	9,33	no change
56451	1415891_at	Suclg1	succinate-CoA ligase, GDP-forming, alpha su	0,957	9,84	9,94	9,90	no change
68089	1423589_at	Arpc4	actin related protein 2/3 complex, subunit 4	0,177	10,51	10,83	10,15	no change
68137	1460211_a_at	Kdelr1	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulu	0,156	10,13	10,74	10,96	no change
16783	1415880_a_at	Lamp1	lysosomal membrane glycoprotein 1	0,433	12,85	12,57	12,58	no change
67388	1416699_at	1110008F1	RIKEN cDNA 1110008F13 gene	0,564	11,07	10,94	10,52	no change
53872	1433901_at	Gpiap1	GPI-anchored membrane protein 1	0,053	8,51	9,11	9,04	no change
19084	1452032_at	Prkar1a	protein kinase, cAMP dependent regulatory, ty	0,028	11,55	11,35	11,03	no change
68059	1423295_at	Tm9sf2	transmembrane 9 superfamily member 2	0,017	10,13	9,26	9,10	**down - (*)**
19324	1416082_at	Rab1	RAB1, member RAS oncogene family	0,134	11,47	12,13	12,20	no change
20955	1426269_at	Sybl1	synaptobrevin like 1	0,019	8,49	9,20	9,44	no change
19354	1417620_at	Rac2	RAS-related C3 botulinum substrate 2	0,054	10,70	11,23	11,42	no change
14569	1421742_at	Gdi2	guanosine diphosphate (GDP) dissociation inh	0,345	2,53	2,62	2,60	no change
19349	1415734_at	Rab7	RAB7, member RAS oncogene family	0,214	10,91	11,11	10,80	no change
233489	1451316_a_at	Picalm	phosphatidylinositol binding clathrin assembly	0,117	11,51	12,06	12,10	no change
17184	1438368_a_at	Matr3	matrin 3	0,342	12,62	12,31	12,23	no change
93687	1424827_a_at	Csnk1a1	casein kinase 1, alpha 1	0,237	11,91	12,13	12,06	no change
12995	1419035_s_at	Csnk2a1	casein kinase II, alpha 1 polypeptide	0,020	2,35	4,24	3,96	**up + (*)**
107513	1417764_at	Ssr1	signal sequence receptor, alpha	0,072	9,49	9,76	10,29	no change
72265	1423732_at	Tram1	translocating chain-associating membrane pro	0,114	11,32	11,55	11,92	no change
11845	1418822_a_at	Arf6	ADP-ribosylation factor 6	0,341	13,06	13,13	12,79	no change
22154	1416256_a_at	Tubb5	tubulin, beta 5	0,030	10,80	11,62	11,78	no change
26395	1416351_at	Map2k1	mitogen activated protein kinase kinase 1	0,030	10,06	9,72	9,42	no change
18521	1421743_a_at	Pcbp2	poly(rC) binding protein 2	0,071	12,22	12,48	12,62	no change
56086	1421819_a_at	Set	SET translocation	0,382	11,11	11,55	11,24	no change
170930	1415782_at	Sumo2	SMT3 suppressor of mif two 3 homolog 2 (yea	0,020	11,84	12,37	12,33	no change
29875	1417380_at	Iqgap1	IQ motif containing GTPase activating protein	0,384	12,47	12,41	11,99	no change
110172	1448769_at	Slc35b1	solute carrier family 35, member B1	0,010	9,40	10,39	10,89	**up + (**)**
71602	1449753_at	Myo1e	myosin IE	0,210	2,51	2,51	2,58	no change
20393	1416041_at	Sgk	serum/glucocorticoid regulated kinase	0,086	11,95	11,24	10,54	**down/ns**

26943	1417816_s_at	Tde1/serin	tumor differentially expressed 1	0,084	8,40	7,89	8,41	no change
56449	1451012_a_at	Csda	cold shock domain protein A	0,121	8,76	9,96	9,99	**up/ns**
27054	1416059_at	Sec23b	SEC23B (S. cerevisiae)	0,014	8,61	9,36	9,52	no change
266692	1433439_at	Cpne1	copine I	0,064	9,93	9,68	9,47	no change
22260	1416353_at	Nr1h2	nuclear receptor subfamily 1, group H, membe	0,084	8,47	8,24	8,17	no change
20652	1417697_at	Soat1	sterol O-acyltransferase 1	0,440	7,22	7,21	6,50	no change
16164	1427165_at	Il13ra1	interleukin 13 receptor, alpha 1	0,001	7,46	5,15	4,54	**down - - (**)**
68011	1448357_at	Snrpg	small nuclear ribonucleoprotein polypeptide G	0,212	10,99	11,44	11,66	no change
16592	1416021_a_at	Fabp5	fatty acid binding protein 5, epidermal	0,002	10,56	12,81	12,06	**up ++ (**)**
13167	1438093_x_at	Dbi	diazepam binding inhibitor	0,003	8,65	9,45	9,26	no change
67923	1427914_a_at	Tceb1	transcription elongation factor B (SIII), polypep	0,031	11,19	11,88	11,78	no change
12340	1439455_x_at	Capza1	capping protein (actin filament) muscle Z-line,	0,004	12,83	13,32	13,12	no change
16412	1426919_at	Itgb1	integrin beta 1 (fibronectin receptor beta)	0,243	3,05	3,10	3,17	no change
12261	1448274_at	C1qbp	complement component 1, q subcomponent b	0,059	9,98	10,98	10,94	**up/ns**
12330	1415692_s_at	Canx	calnexin	0,240	12,54	12,55	12,39	no change
22027	1415889_a_at	Tra1/hsp90	tumor rejection antigen gp96	0,016	13,25	14,00	14,22	no change
53381	1416166_a_at	Prdx4	peroxiredoxin 4	0,004	9,07	10,06	11,37	**up ++ (**)**
11957	1416143_at	Atp5j	ATP synthase, H+ transporting, mitochondrial	0,078	10,48	11,19	11,39	no change
228033	1454661_at	Atp5g3	ATP synthase, H+ transporting, mitochondrial	0,020	12,70	13,10	13,12	no change
12858	1439267_x_at	Cox5a	cytochrome c oxidase, subunit Va	0,187	13,25	13,41	13,33	no change
66576	1452133_at	Uqcrh	ubiquinol-cytochrome c reductase hinge prote	0,443	11,69	11,62	11,63	no change
12864	1415970_at	Cox6c	cytochrome c oxidase, subunit VIc	0,456	11,66	11,85	11,83	no change
12867	1448112_at	Cox7c	cytochrome c oxidase, subunit VIIc	0,079	10,23	10,43	10,53	no change
17992	1424085_at	Ndufa4	NADH dehydrogenase (ubiquinone) 1 alpha s	0,013	10,01	10,48	10,66	no change
12861	1417417_a_at	Cox6a1	cytochrome c oxidase, subunit VI a, polypepti	0,016	13,63	13,91	13,61	no change
67530	1455997_a_at	Uqcrb	ubiquinol-cytochrome c reductase binding pro	0,000	12,81	13,37	13,35	no change
12462	1459987_s_at	Cct3	chaperonin subunit 3 (gamma)	0,028	10,27	11,12	11,00	no change
12464	1415868_at	Cct4	chaperonin subunit 4 (delta)	0,260	2,21	2,22	2,24	no change
12465	1417258_at	Cct5	chaperonin subunit 5 (epsilon)	0,254	12,05	12,49	12,38	no change
13001	1416728_at	Csnk2b	casein kinase II, beta subunit	0,167	10,15	10,79	10,74	no change
11950	1433562_s_at	Atp5f1	ATP synthase, H+ transporting, mitochondrial	0,003	13,06	13,52	13,35	no change
11740	1438545_at	Slc25a5	solute carrier family 25 (mitochondrial carrier,	0,079	8,52	9,06	9,28	no change
12215	1456616_a_at	Bsg	basigin	0,028	9,90	10,17	10,82	no change
13135	1418528_a_at	Dad1	defender against cell death 1	0,009	10,49	11,12	11,27	no change
11966	1415814_at	Atp6v1b2	ATPase, H+ transporting, V1 subunit B, isofor	0,363	10,57	10,59	10,18	no change
20375	1418747_at	Sfpi1	SFFV proviral integration 1	0,218	11,53	11,18	10,77	no change

11804	1423739_x_at	Aplp2	amyloid beta (A4) precursor-like protein 2	0,004	10,23	9,12	9,54	**down - (**)**
11502	1416094_at	Adam9	a disintegrin and metalloproteinase domain 9	0,182	9,52	10,33	10,53	**up/ns**
72900	1428179_at	Ndufv2	NADH dehydrogenase (ubiquinone) flavoprote	0,021	11,13	11,77	11,75	no change
53610	1448103_s_at	Nono	non-POU-domain-containing, octamer binding	0,002	11,67	11,81	11,54	no change
18453	1437465_a_at	P4hb	prolyl 4-hydroxylase, beta polypeptide	0,067	11,14	11,54	11,76	no change
26554	1450756_s_at	Cul3	cullin 3	0,081	5,83	6,06	5,91	no change
19359	1424222_s_at	Rad23b	RAD23b homolog (S. cerevisiae)	0,007	9,46	10,39	10,50	**up + (**)**
56550	1416476_a_at	Ube2d2	ubiquitin-conjugating enzyme E2D 2	0,230	12,77	12,60	12,56	no change
68441	1428905_at	Rraga	Ras-related GTP binding A	0,051	7,20	7,89	8,00	no change
93765	1422559_at	Ube2n	ubiquitin-conjugating enzyme E2N	0,003	10,47	11,21	10,67	no change
11702	1448484_at	Amd1	S-adenosylmethionine decarboxylase 1	0,659	10,62	10,52	10,40	no change
98758	1456664_x_at	Hnrpf /// LC	heterogeneous nuclear ribonucleoprotein F ///	0,333	13,15	12,93	12,89	no change
19357	1416161_at	Rad21	RAD21 homolog (S. pombe)	0,019	11,68	12,30	12,31	no change
20912	1435058_x_at	Stxbp3a	syntaxin binding protein 3	0,033	8,84	8,38	8,32	no change
22384	1451521_x_at	Wbscr1	Williams-Beuren syndrome chromosome regi	0,019	10,88	11,22	10,96	no change
18655	1439435_x_at	Pgk1	phosphoglycerate kinase 1	0,004	10,11	11,20	11,18	**up + (**)**
213827	1451089_a_at	Arcn1	archain 1	0,099	10,35	10,77	10,94	no change
70349	1423301_at	Copb1	coatomer protein complex, subunit beta 1	0,374	9,90	10,08	10,35	no change
22209	1448772_at	Ube2a	ubiquitin-conjugating enzyme E2A, RAD6 hon	0,120	10,27	10,86	10,81	no change
76936	1426698_a_at	Hnrpm	heterogeneous nuclear ribonucleoprotein M	0,257	10,37	10,67	10,32	no change
66483	1448697_s_at	Rpl36al	ribosomal protein L36a-like	0,130	13,10	13,28	13,42	no change
53599	1431527_at	Cd164	CD164 antigen	0,170	2,19	2,20	2,23	no change
216558	1434486_x_at	Ugp2	UDP-glucose pyrophosphorylase 2	0,217	10,88	10,50	10,31	no change
15312	1438940_x_at	Hmgn1	high mobility group nucleosomal binding dom	0,397	13,39	13,36	13,57	no change
18148	1415839_a_at	Npm1	nucleophosmin 1	0,177	12,81	13,27	13,27	no change
15254	1424017_a_at	Hint1	histidine triad nucleotide binding protein 1	0,035	13,37	13,60	13,53	no change
18674	1416300_a_at	Slc25a3	solute carrier family 25 (mitochondrial carrier,	0,714	12,28	12,35	12,27	no change
22608	1418624_at	Nsep1/Ybx	nuclease sensitive element binding protein 1	0,003	12,11	12,89	12,90	no change
16828	1419737_a_at	Ldh1/ldha	lactate dehydrogenase 1, A chain	0,017	11,35	12,29	12,10	no change
20813	1418567_a_at	Srp14	signal recognition particle 14	0,216	10,20	10,35	10,19	no change
11973	1449711_at	Atp6v1e1	ATPase, H+ transporting, V1 subunit E isoforr	0,615	2,73	2,66	2,69	no change
56455	1417339_a_at	Dnclc1/Dyr	dynein, cytoplasmic, light chain 1	0,005	12,71	13,64	13,75	**up + (**)**
23983	1448642_at	Pcbp1	poly(rC) binding protein 1	0,083	12,29	12,59	12,65	no change
22333	1415998_at	Vdac1	voltage-dependent anion channel 1	0,007	9,24	10,13	9,89	no change
110213	1451586_at	Tegt	testis enhanced gene transcript	0,950	11,42	11,36	11,42	no change
53378	1423332_at	Sdcbp	syndecan binding protein	0,335	12,07	12,40	12,17	no change

11938	1416551_at	Atp2a2	ATPase, Ca++ transporting, cardiac muscle, s	0,361	7,13	7,56	7,55	no change
235072	1437995_x_at	sept7	septin 7, cell division cycle 10 homolog	0,126	11,33	11,53	11,48	no change
12462	1448122_at	Cct3/Tcp1	chaperonin subunit 3 (gamma), t-complex pro	0,082	11,07	11,84	11,84	no change
13207	1454793_x_at	Ddx5	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	0,001	13,36	12,93	12,95	no change
26440	1415695_at	Psma1	proteasome (prosome, macropain) subunit, al	0,188	11,71	12,34	12,13	no change
19167	1448442_a_at	Psma3	proteasome (prosome, macropain) subunit, al	0,144	12,36	12,85	12,58	no change
11843	1423052_at	Arf4	ADP-ribosylation factor 4	0,039	10,63	11,16	11,26	no change
66882	1423041_a_at	Bzw1	basic leucine zipper and W2 domains 1	0,002	9,88	10,32	10,31	no change
16952	1448213_at	Anxa1	annexin A1	0,065	10,88	10,30	10,44	no change
19744	1416636_at	Rheb	RAS-homolog enriched in brain	0,160	10,71	10,95	10,90	no change
11747	1425567_a_at	Anxa5	annexin A5	0,326	11,96	11,69	11,36	no change
326618	1433883_at	Tpm4	tropomyosin 4	0,051	11,05	11,40	11,06	no change
22631	1416102_at	Ywhaz	tyrosine 3-monooxygenase/tryptophan 5-mon	0,027	10,93	11,38	11,25	no change
19248	1450478_a_at	Ptpn12	protein tyrosine phosphatase, non-receptor ty	0,117	6,81	7,44	7,08	no change
12385	1437275_at	Catna1	Catenin alpha 1	0,051	2,44	2,45	2,52	no change
27057	1420808_at	Ncoa4	nuclear receptor coactivator 4	0,231	11,64	11,12	11,25	no change
11750	1416138_at	Anxa7	annexin A7	0,739	10,17	10,27	10,33	no change
22630	1420829_a_at	Ywhaq	tyrosine 3-monooxygenase/tryptophan 5-mon	0,510	9,88	10,04	9,99	no change
15902	1435176_a_at	Id2	inhibitor of DNA binding 2	0,618	14,12	14,32	14,06	no change
22201	1448116_at	Ube1x	ubiquitin-activating enzyme E1, Chr X	0,509	10,42	10,68	10,77	no change
18753	1422847_a_at	Prkcd	protein kinase C, delta	0,358	10,09	9,86	9,77	no change
18746	1417308_at	Pkm2	pyruvate kinase, muscle	0,006	10,31	11,81	11,54	**up + (**)**
20195	1460351_at	S100a11	S100 calcium binding protein A11 (calizzarin)	0,001	12,28	12,02	11,11	**down - (**)**
114584	1416656_at	Clic1	chloride intracellular channel 1	0,633	11,87	11,86	11,65	no change
11933	1423126_at	Atp1b3	ATPase, Na+/K+ transporting, beta 3 polypep	0,249	11,24	11,72	11,88	no change
13690	1415863_at	Eif4g2	eukaryotic translation initiation factor 4, gamm	0,226	12,54	12,95	12,91	no change
20194	1416762_at	S100a10	S100 calcium binding protein A10 (calpactin)	0,372	9,62	10,01	9,12	no change
66212	1417083_at	Sec61b	Sec61 beta subunit	0,031	11,97	12,42	12,62	no change
26442	1454940_at	Psma5	proteasome (prosome, macropain) subunit, al	0,122	2,72	2,73	2,78	no change
15381	1460240_a_at	Hnrpc	heterogeneous nuclear ribonucleoprotein C	0,088	12,09	12,47	12,49	no change
22042	1452661_at	Tfrc	transferrin receptor	0,136	11,29	10,28	11,77	**up/ns**
15081	1455725_a_at	H3f3b	H3 histone, family 3B	0,067	13,30	13,38	13,66	no change
56417	1425405_a_at	Adar	adenosine deaminase, RNA-specific	0,319	7,87	7,70	7,25	no change
19186	1417056_at	Psme1	proteasome (prosome, macropain) 28 subunit	0,128	12,26	12,49	12,03	no change
15502	1416288_at	Dnaja1	DnaJ (Hsp40) homolog, subfamily A, member	0,415	10,75	11,28	11,09	no change
26443	1437144_x_at	Psma6	proteasome (prosome, macropain) subunit, al	0,047	12,21	12,76	12,58	no change

22166	1456734_at	Txn1	thioredoxin 1	0,021	2,87	3,05	3,13	no change
20220	1434646_s_at	Sap18	Sin3-associated polypeptide 18	0,017	10,33	11,02	11,10	no change
19166	1448206_at	Psma2	proteasome (prosome, macropain) subunit, al	0,182	11,48	12,09	11,95	no change
217684	1454606_at	4933426M	RIKEN cDNA 4933426M11 gene	0,33029	8,48	8,90	8,57	no change
18024	1416543_at	Nfe2l2	nuclear factor, erythroid derived 2, like 2	0,79148	9,65	9,80	9,69	no change
22234	1421268_at	Ugcg	UDP-glucose ceramide glucosyltransferase	0,51328	6,75	7,43	7,36	no change
16004	1424112_at	Igf2r	insulin-like growth factor 2 receptor	0,06403	5,47	7,47	6,87	**up/ns**
76497	1417060_at	Ppp1r11	protein phosphatase 1, regulatory (inhibitor) s	0,13902	9,19	8,55	8,27	no change
11886	1416735_at	Asah1	N-acylsphingosine amidohydrolase 1	0,6544	10,88	10,51	10,49	no change
22195	1448879_at	Ube2l3	ubiquitin-conjugating enzyme E2L 3	0,05734	10,66	11,46	11,41	no change
22627	1426384_a_at	Ywhae	tyrosine 3-monooxygenase/tryptophan 5-mon	0,00908	12,21	12,56	12,27	no change

Appendix III, Table II - Genes identified by Jenner & Young (Jenner & Young, 2005) to be regulated simultaneously in human DCs in response to different infections and the regulation of respective *Mus Musculus* homologs by *P. yoelii*
+' stands for a 2 to 4-fold increase, '++' for 4 to 8-fold, '+++' for over 8-fold
nc' means no change in expression. 'ns' means regulation is not significant between pairs of replica arrays.
p is the probability of differential regulation between 2 samples for each gene for Multiclass testing.
Asterisks indicate level of significance (* is $0.01<p<0.05$; ** is $p<0.01$)
The following genes do not show up since they do not have a homolog in Mus Musculus or else were not present in the MOE430A array:
prdx3, rnase6, C21ORF106, grp58, znf267, psg11, pik4cb, rsu1, cox7b, gtf2a2, cd58

Appendix III, Table III

Regulation of genes belonging to the common response of DCs to intracellular parasites during *P. yoelii* infection.

Gene	Gene ID (mouse)	Probeset	p	log2 (d0)	log2 (d5)	log2 (d10)	Malaria regulation
traf1	22029	1423602_at	.0185	9.73	9.01	7.94	**- (*)**
nf-kb1/p50	18033	1427705_a_at	.0071	11.1	10.18	9.87	**- (**)**
cd83	12522	1416111_at	.0017	11.84	10	9.86	**- (**)**
cflar	12633	1449317_at	.0197	6.81	8.47	8.18	**+ (*)**
nr4a3	18124	1421080_at	.2515	1.92	1.92	1.93	**nc**
rel/c-rel	19696	1420710_at	.0105	4.92	6.01	6.08	**+ (*)**
casp7	12369	1448659_at	.1556	8.64	9.41	9.20	**nc**
ezh2	14056	1416544_at	.0245	8.93	10.25	10.18	**+ (*)**
marcks	17118	1456028_x_at	.1009	13.64	13.08	12.93	**nc**
tnfaip2	21928	1416273_at	.6850	6.27	7.05	6.95	**nc**
nfkbia/ik-a	18035	1448306_at	.2492	11.84	11.54	11.36	**nc**
tnfaip3	21929	1450829_at	.7680	6.28	6.65	6.45	**nc**
cd40/tnfrsf5	21939	1460415_a_at	.0031	8.90	8.99	7.95	**- (**)**
hist2h2aa1	15267	1418367_x_at	.0059	7.82	7.02	8.13	**+ (**)**
Il15ra #	16169	1448681_at	.0171	4.06	6.34	5.83	**++ (*)**
relB	19698	1417856_at	.0099	10.12	9.01	7.79	**-- (**)**
Irf1	16362	1448436_a_at	.2509	10.79	11.09	10.70	**nc**
tnfrsf4/ox40	22163	1420351_at	.4527	4.70	4.24	4.24	**nc**
bcl2a1a	12044	1419004_s_at	.1062	12.91	12.53	11.95	**nc**
nfkb2/p52	18034	1425902_a_at	.0213	9.32	8.77	8.06	**- (*)**
rel-a/p65	19697	1419536_a_at	.0188	9.98	9.47	9.44	**nc**
tradd	71609	1429117_at	.3289	7.34	7.58	7.22	**nc**
stat1	20846	1450033_a_at	.0944	11.12	12.30	11.88	**up/ns**
jak1	16451	1433803_at	.3155	8.90	8.84	8.76	**nc**
nmb	68039	1419405_at	.6200	2.35	2.36	2.37	**nc**
btg1	12226	1437455_a_at	.0052	13.26	12.49	12.42	**nc**
ptpn2	19255	1417140_a_at	8.8E-5	10.02	10.49	10.18	**nc**
wnt5a	22418	1436791_at	.2425	2.35	2.36	2.39	**nc**

dusp1	19252	1448830_at	.0194	9.56	12.19	12.35	**++ (*)**
maoa	17161	1428667_at	.2615	2.30	2.32	2.38	**nc**
gadd45b	17873	1450971_at	.0416	8.72	10.01	9.52	**+ (*)**
ptpn1	19246	1417068_a_at	.0671	11.49	12.41	12.32	**nc**
fscn1/snl	14086	1416514_a_at	.0138	13.32	13.88	12.50	**- (*)**
gypc	71683	1423878_at	.5643	8.67	8.35	8.75	**nc**
mox2	17470	1448788_at	.0109	6.52	7.09	6.60	**nc**
cst7	13011	1419202_at	.0033	4.37	8.41	6.35	**++++ (**)**
ccr7	12775	1423466_at	.0020	9.81	9.56	8.30	**- (**)**
lamp3	239739	1417057_a_at	.2745	9.72	10.50	10.23	**nc**
gp1ba	14723	1422316_at	.3610	2.05	2.05	2.07	**nc**
pbef1	59027	1417190_at	.1040	8.00	9.34	8.82	**up/ns**
sod2	20656	1448610_a_at	.0238	9.80	11.93	11.34	**++ (*)**
ebi2	321019	1449642_at	.6841	2.72	2.69	2.70	**nc**
cxcr4	12767	1448710_at	.2037	9.29	9.58	9.89	**nc**

Appendix III, Table III – Genes identified by Chaussabel et al. (Chaussabel *et al.*, 2003) to be regulated simultaneously in human DCs in response to three different intracellular parasites (*Leishmania major, Leishmania donovani*, and *Toxoplasma gondii*) and the regulation of respective *Mus Musculus* homologs by *P. yoelii*. + stands for a 2 to 4-fold increase, ++ for 4 to 8-fold; +++ for over 8-fold. nc = no change in expression. ns = regulation is not significant between pairs of replica arrays. *p* is the probability of differential regulation between 2 samples for each gene for Multiclass testing. Asterisks indicate level of significance (* is $0.01<p<0.05$; ** is $p<0.01$). The following genes do not show up since they do not have a homolog in *Mus Musculus* or else were not present in the MOE430A array: *tnrc3/maml3, dusp4, dusp5, ccl17, ccl20*.

APPENDIX IV

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Transcriptome profile of dendritic cells during malaria: cAMP regulation of IL-6

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Summary

Dendritic cells (DCs) have been proposed as mediators of immunity against malaria parasites, as well as a target for inhibition of cellular responses. Here we describe the transcriptomic analysis of spleen DCs in response to *Plasmodium* infection in a rodent model. We identified a high number of unique transcripts modulated in DCs upon infection. Many cellular functions suffer extensive genomic regulation including the cell cycle, the glycolysis and purine metabolism pathways and also defence responses. Only a small fraction of the regulated genes are coincident with the response induced by other pathogens, suggesting that *Plasmodium* induces a unique genetic re-programming of DCs. We confirmed regulation of a number of cytokines at the mRNA level including IL-6, IL-10 and IFN-γ. We further dissected a signalling pathway regulating *Plasmodium*-induced expression of IL-6 by DCs, which is mediated by release of PGE2, increases in intracellular cAMP and activation of PKA and p38-MAPK.

Introduction

Malaria is the leading cause of parasite-associated mortality worldwide. It is responsible for 800 000 deaths per year, according to a recent review of diverse estimates (Rowe *et al*., 2006), as well as a loss in 2% of GDP each year in Sub-Saharan Africa (Sachs and Malaney, 2002). Complications of the disease, including severe anaemia, respiratory distress, cerebral malaria and others, occur only after parasites invade erythocytes, where they multiply through asexual replication (Mackintosh *et al*., 2004).

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Blood stage malaria can be controlled by transfer of antibodies from infected individuals (Cohen *et al*., 1961). However, immunity to malaria which is able to control parasite levels takes several years to develop in exposed individuals from endemic areas (Wipasa *et al*., 2002).

Mounting adaptive immune responses requires dendritic cell (DC)-mediated priming of naïve T cells, upon DC maturation. This process is characterized by a reduction in the phagocytic activity of DCs, enhanced antigen presentation, increased expression of co-stimulatory molecules and cytokine production (Guermonprez *et al*., 2002). Recent research has shown that DC maturation is not uniform between different pathogens, resulting in different T cell polarization phenotypes (Kapsenberg, 2003). Still, a common 'core' of genes which is triggered in different conditions of DC maturation has been characterized using microarray technology (Huang *et al*., 2001; Jenner and Young, 2005). This common gene cluster is found to be largely modulated by different intracellular parasites, although each of these pathogens also specifically modulates additional transcripts (Chaussabel *et al*., 2003).

The responses of DCs to blood stage malaria have been a subject for controversy (Stevenson and Urban, 2006; Urban and Todryk, 2006). DCs have been found to mature *in vitro* in response to *Plasmodium chabaudi*-infected erythrocytes (Seixas *et al*., 2001), and also during *Plasmodium yoelii* infection (Perry *et al*., 2004). However, in different reports, both *P. falciparum*- and *P. yoelii*-infected erythrocytes were shown to fail to induce either human or mouse DC maturation, and furthermore inhibit maturation in response to secondary LPS stimulation (Urban *et al*., 1999; Ocaña-Morgner *et al*., 2003). More recently, it was proposed that DCs are activated at low loads of parasite in early infection, and then become refractory to stimulation, inducing malaria-specific IL-10-producing T cells (Perry *et al*., 2005). It has also been proposed that the cause of the *Plasmodium*-induced inhibition of DC maturation is haemozoin, a pigment of polymerized haem that is accumulated in infected erythrocytes upon *Plasmodium* infection (Keller *et al*., 2006a; Millington *et al*., 2006).

To shed light into the different observations on DC responses to *Plasmodium* parasites, we pursued a whole genome transcriptomic analysis of the response of myeloid DCs from spleen to a mouse malaria parasite (*P. yoelii*) at two different time points post infection (p.i.): a

medium-load time (day 5) and a high-load time (day 10, peak parasitaemia). We show that spleen DCs suffer extensive genetic regulation upon *P. yoelii* infection, comprising 694 unique transcripts. A large fraction of these is similarly regulated at both time points studied, and some of them have functions potentially related to parasite control. A comparison of *Plasmodium*-regulated genes with a set of genes previously identified as the common DC transcriptomic response to pathogens (Huang *et al.*, 2001) revealed a coincidence of 43% of genes regulated in the same way by *P. yoelii* as by other microbes. Some genes regulated coincide with the 'standard' DC maturation response, while a majority of them do not, suggesting that *P. yoelii* infection induces a 'divergent' maturation response at least at the gene expression level. The genes modulated by the parasite include several components of a signalling pathway leading to IL-6 secretion. We have characterized this signalling pathway in DCs, which is mediated by PGE2, increases in intracellular cAMP, PKA and p38-MAPK.

Results

Plasmodium yoelii *regulates 694 genes in spleen DCs, with many genes common between two different time points and loads of infection*

Plasmodium blood-stage parasites have been shown to either induce or else inhibit DC maturation, according to different reports that look at the expression of a restricted group of proteins associated with DC maturation (Stevenson and Urban, 2006; Urban and Todryk, 2006). To extend such studies to the whole genome, we used the GeneChip® technology from Affymetrix to analyse transcriptomic responses of DCs extracted from spleens of non-infected BALB/C mice (from hereon designated as 'day 0'), or else from *P. yoelii*-infected mice at two different time points p.i.: day 5 (8.1 ± 0.2% parasitaemia) and day 10 (28.3 ± 2.6%) p.i. For each condition, two RNA samples from independent experiments were obtained, adding up to six independent hybridizations onto the Mouse Expression Array MOE430.

There was a high reproducibility between replica samples as measured by the high Pearson correlation coefficient between data from the same time point (0.992–0.995) compared with different time points: day 0 versus day 5 p.i. (0.970–0.972), day 0 versus day 10 p.i. (0.959–0.970) and day 5 versus day 10 p.i. (0.984–0.990). These relationships are easily visualized by cluster analysis, which highlights the extensive similarity between day 5 p.i. and day 10 p.i. compared with samples from uninfected mice (Fig. 1). The same similarities between replicate arrays and between samples from infected mice can also be observed by scatter plots of

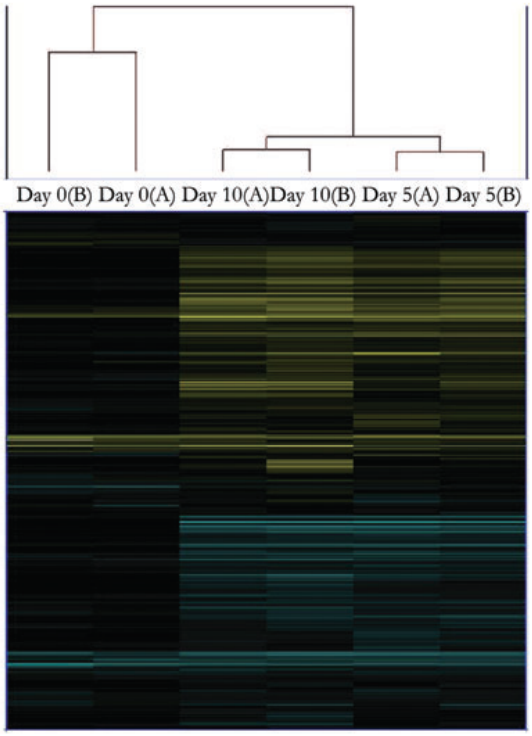

Fig. 1. Cluster analysis of genes detected by microarray analysis of spleen DCs at different times of *P. yoelii* infection. Six mRNA samples from CD11c+ splenocytes obtained from BALB/C mice at different times of infection were hybridized onto MOE430A GeneChip (Affymetrix). A cluster analysis (Pearson correlation, average linkage method) of the 6.646 genes detected as 'Present' in at least one of the samples, and with maximum–minimum signal values in the six samples above 1 (twofold change) is shown. Yellow and green indicate induced and repressed genes respectively.

expression values for all probesets in any given pair of samples (Fig. S1 in *Supplementary material*).

Using significance analysis of multiclass testing type using a cut-off of $P < 0.01$, we found that *P. yoelii* regulates a high number of unique genes or ESTs (694) at the RNA level at least twofold, considering both times p.i., and both induced and repressed transcripts (Table 1). The total number of genes was obtained after redundant probesets were excluded. The distribution of those 694 regulated transcripts was found to have an extensive overlap of 514 transcripts in the genes regulated at day 5 and day 10 (90% and 81% of total regulated transcripts at day 5 and day 10 respectively). This overlap was also found when induced and repressed genes were analysed separately (Fig. 2A).

We segregated the 694 differentially regulated transcripts between those that are: (i) induced upon infection (327 genes, 47.1%) and (ii) repressed upon infection (367

Table 1. Total number of MOE430 GeneChip unique genes/ESTs differentially regulated by *P. yoelii* in spleen DCs.

	Unique genes/ESTs		
Samples compared	Induced	Repressed	Total
D5/D0 (A)	276	298	574
D10/D0 (B)	293	341	634
Common A, B	242	272	514
D10/D5	51	56	107

Total numbers of genes induced or repressed are presented for all comparisons of any two experimental conditions: between non-infected and day 5 p.i. (D5/D0), non-infected and day 10 p.i. (D10/D0), and also between days 5 and 10 p.i. (D10/D5).

genes, 52.9%). No genes were found to have opposite regulation at day 5 and day 10 p.i. when each one is compared with DCs from non-infected mice. Examples of individual genes following different expression profiles (Fig. 2B) and complete lists of the transcripts in each group (Tables S1 and S2 in *Supplementary material*) are shown. The data suggest that the universe of genes regulated in DC of the spleen during *P. yoelii* infection is very similar at these two different times of infection, which correspond to significantly different levels of parasitaemia. Still, a limited number of genes were found

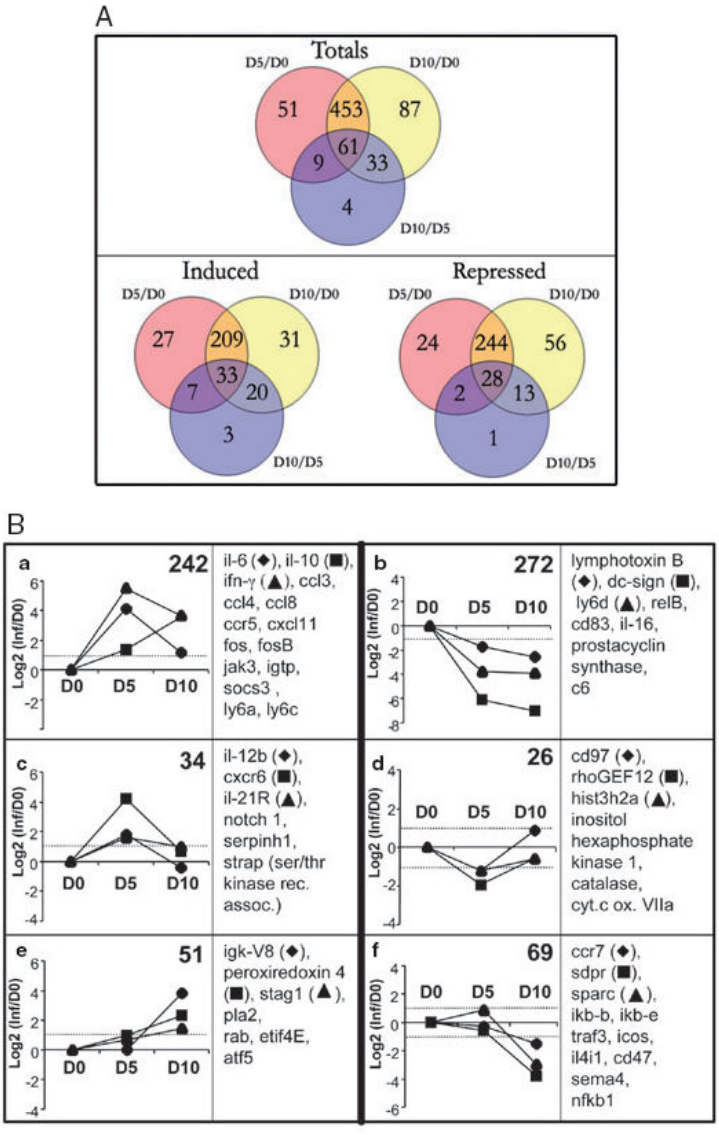

Fig. 2. Total numbers and examples of unique genes regulated at the transcription level in spleen DCs by *P. yoelii*-infected erythrocytes.
A. Venn diagrams show the numbers of total transcripts with differential regulation over twofold ($P < 0.01$). Expression levels for each pair of replica arrays: uninfected (D0), day 5 (D5) and day 10 (D10) were compared using multiclass significance analysis.
B. Genes with differential expression (over twofold, $P < 0.01$) between different times of infection were segregated according to expression profiles: induced (a, c and e) or repressed (b, d and f); either in both days (a and b), only on day 5 (c and d) or only on day 10 (e and f). Numbers in the upper right corner represent the total number of genes in each profile. Examples of genes in each profile are provided in the right panels. The variation in the expression levels of three genes of each group is represented in the graphs.

Table 2. Immunity-related genes that are induced upon *P. yoelii* infection.

Class	Gene	Gene ID	Log2 (D5/D0)	Log2 (D10/D0)	Log2 (D10/D5)	DC maturation
Chemokines and chemokine receptors	*ccl8/mcp-2*	20 307	+8.15	+8.06	ns	Induced
	ccl4/mip1-β	20 303	+5.25	+4.55	ns	Induced
	ccl3/mip1-α	20 302	+5.14	+4.86	ns	Induced
	cxcl11/i-tac	56 066	+3.33	+2.73	ns	Induced
	cxcr6	80 901	+4.18[a]	ns	−3.52	Not regulated
	ccr5	12 774	+3.26	+2.70	ns	Not regulated
Pattern recognition	*msr/sr-A*	20 288	+5.68	+4.33	−1.35	Not regulated
Transcription factors	*fos*	14 281	+5.05	+5.19	ns	Not regulated
	fos b	14 282	+5.30	+6.37	+1.07	Not regulated
Subset marker	*cd8 b1*	12 526	+5.07	+1.53	−3.54	Not regulated
Cytokines and receptors	*il6*	16 193	+4.12	+1.16	−2.97	Induced
	il10	16 153	+1.37	+3.56	+2.19	Induced
	il12b	16 160	+1.79	ns	−2.22	Induced
	il21r	60 504	+1.58	ns	ns	Not regulated
IFN response	*ifng*[b]	15 978	+5.50	+3.65	−1.85	Induced
	socs3	12 702	+4.23[a]	+4.29[a]	ns	Not regulated
	jak3	16 453	+1.78	+1.74	ns	Not regulated
	igtp	16 145	+2.01	+2.13	ns	Not regulated
Prostaglandin signalling	*ep4*	19 219	+1.10	+1.23	ns	Not regulated
Cytotoxicity and complement	*gzmb/ctla-1*	14 939	+9.73	+8.16	−1.57	Not regulated
	gzmk	14 945	+6.14	+4.15	−1.98	Not regulated
	c4/c4b/FDC	12 268	+4.66	+5.84	+1.18	Not regulated
Humoral immunity	*fcgr2b/CD32*	14 130	+2.83[a]	+2.86[a]	ns	Not regulated
	fcgr3a	246 256	+2.69	+3.40	ns	Not regulated
	igh-1a	380 793	+9.07	+11.75	+2.68	Not regulated
	igh-VJ558	16 061	+1.54	+4.32	+2.78	Not regulated
	Igk-v8	384 422	+1.46[a]	+4.03[a]	+2.57	Not regulated
Antigen presentation	*ly6a/tap*	110 454	+4.22	+3.22	ns	Induced
	ly6c	17 067	+1.60	+2.24	ns	Not regulated

a. Log2 values which are averages of two or more probes for the same gene.
b. Genes with $0.05 > P > 0.01$.
Genes listed are induced over twofold ($P < 0.01$) in at least one of the times of *P. yoelii* infection, when compared with expression in DCs from non-infected mice. DC maturation indicates whether a gene is induced, repressed or not regulated in the study of Huang *et al.* (2001).
ns, not significant.

to be regulated specifically at one or the other time of infection, possibly reflecting changes in the response of DCs over time of infection.

Plasmodium yoelii *preferentially regulates DC genes involved in cell cycle, defence responses, the glycolysis pathway and purine metabolism*

Using the Gene Ontology systems of classification and the Kyoto Encyclopedia of Genes and Genomes (KEGG), we analysed gene annotation for the total unique genes or ESTs that were regulated in the comparison between any two conditions (Tables S3–6 in *Supplementary material*). We found that the main cellular functions under parasite regulation are: 'cell cycle' (45 genes), 'defence responses' (44), 'transcription regulators' (32) and 'apoptosis' (18) (Table S3 in *Supplementary material*). The class of 'defence response' includes both induced (28) and repressed (16) genes. Some examples of induced genes are chemokines (*ccl8*, *ccl4*, *ccl3*, *cxcl11*) and their receptors (*ccr5*, *cxcr6*), cytokines such as *il-6*, *il-10*, *il-12p40* and *ifn-g*, and also members of the immunoglobulin superfamily (Table 2). These data suggest that DCs are actively engaged in interactions with the immune system that might contribute to parasite killing. However, transcripts such as *cd80*, *cd83*, *cd1d1* and *icos*, which can interact directly with co-receptors in NK and T cells, are repressed by *P. yoelii*, suggesting an impaired capacity of DCs to communicate with other cells of both the innate and adaptive arms of the immune system. Other *P. yoelii*-repressed transcripts include *nfkb1*, a gene that is usually induced upon DC maturation (Huang *et al.*, 2001) (Table 3).

Besides the classification according to biological processes, Gene Ontology also annotates genes according to their molecular functions (Table S5 in *Supplementary material*). The genes regulated by *P. yoelii* in DCs preferentially fall within the following functions: ATP binding (56), kinase activity (34), serine-type endopeptidase (11), rhodopsin-like receptor activity (8) and GTP binding (8). The data for the latter two categories, together with the high number of genes with kinase activity, suggest that *P. yoelii*

Table 3. Immunity-related genes that are repressed upon *P. yoelii* infection.

Class	Gene	Gene ID	Log2 (D5/D0)	Log2 (D10/D0)	Log2 (D10/D5)	DC maturation
Co-stimulation	*cd80*[b]	12 519	−2.25	−3.04	ns	Induced
	cd83	12 522	−1.84	−1.99	ns	Induced
	cd86[b]	12 524	−1.17	−1.85	ns	Induced
	icosl	50 723	−1.62	−2.73	−1.1	Not regulated
Antigen presentation	*cd1d1*	12 479	−1.30	−2.36	−1.06	Not regulated
	ly6d	17 068	−3.82	−3.94	ns	Not regulated
	h2-Oa/HLA-DOA	15 001	−2.03	−2.92	ns	Not regulated
	c2ta	12 265	ns	−1.64	ns	Not regulated
Nf-kb-dependent transcription	*ikb-b/nfkbib*	18 036	ns	−1.02	ns	Not regulated
	ikb-e/nfkbie	18 037	ns	−1.32	ns	Not regulated
	relB/nfkb1	19 698	−1.11	−2.33	−1.22	Induced
	traf3	22 031	ns	−1.39	ns	Not regulated
Cytokines and cytokine signalling	*ltb/tnfsf3*	16 994	−1.71	−2.56	ns	Not regulated
	il16	16 170	−1.88	−1.49	ns	Not regulated
	il4i1	14 204	ns	−2.38	−1.43	Not regulated
	il11ra1	16 157	−2	−2.08	ns	Not regulated
	il13ra1	16 164	−2.25[a]	−2.58[a]	ns	Not regulated
	ltbp2	16 997	−2.31	−2.29	ns	Not regulated
	tgfb1i4/tsc22d1	21 807	−1.87[a]	−2.25[a]	ns	Not regulated
Chemokine receptors	*ccr7*	12 775	ns	−1.51	−1.26	Induced
Pattern recognition	*cd209a (DC-SIGN)*	170 786	−6.10	−7.02	ns	Not regulated
	cd209b (SIGNR1)	69 165	−2.89	−2.87	ns	Not regulated
	cd209d (SIGNR3)	170 779	−3.62	−4.34	ns	Not regulated
Complement	*C6*	12 274	−4.62	−4.19	ns	Not regulated
Prostaglandin metabolism	*ptgis*	19 223	−3.58	−3.56	ns	Not regulated
Other receptors	*cd163*	93 671	−5.22	−4.83	ns	Not regulated
	cd33	12 489	−1.08	−1.09	ns	Repressed

a. Log2 values which are averages of two or more probes for the same gene.
b. Genes with $0.05 > P > 0.01$.
Genes listed are repressed over twofold ($P < 0.01$) in at least one of the times of *P. yoelii* infection, when compared with expression in DCs from non-infected. DC maturation indicates whether a gene is induced, repressed or not regulated in the study of Huang *et al.* (2001).
ns, not significant.

modulates the activity of signalling pathways that are dependent on G protein-coupled receptors and also involve kinases.

Plasmodium yoelii preferentially regulates the following metabolic pathways: glycolysis and gluconeogenesis, purine and pyrimidine metabolism, and protein biosynthesis and catabolism (Table S6 in *Supplementary material*).

Altogether, the data on functional annotation of *P. yoelii*-regulated transcripts of DCs show that the parasite exerts pressure on many cell functions including diverse metabolic pathways, but also the survival of DCs (cell cycle and apoptosis), the ability of DCs to regulate their gene expression (mediators of signalling pathways and gene transcription), as well as its function as part of the immune response.

Plasmodium yoelii-*induced transcriptomic regulation of DC compared with other pathogens*

To have a better understanding of how the global transcriptomic regulation of DC by *Plasmodium* overlaps with the response to other pathogens, and how similar it is to a regular DC maturation process, we compared a list of genes that are simultaneously regulated by three diverse pathogens (a bacterium, a virus and a fungus) in human DCs (Huang *et al*., 2001) to the genes regulated in *P. yoelii* infection. There are essential differences in the experimental systems used for this comparison, as Huang *et al.* use human DCs incubated *in vitro* with the different pathogens. However, another comparison that includes microarrays performed *in vivo* and *in vitro* found a defined common host transcriptional response (Jenner and Young, 2005).

To avoid errors resulting from comparing studies that use different statistical methods, we ranked the all the genes analysed by their expression change in infected versus uninfected mice, from maximum increase to maximum decrease, regardless of their statistical significance. Genes with more than twofold regulation are considered upregulated and are found within the first 1453 of all the microarray probes. From 49 human genes with homologues in the mouse, 21 (43%) were also induced by *P. yoelii* in at least one of the infection days (Table S7 in *Supplementary material*). The majority of the common core of genes induced by different pathogens in DCs is either not significantly modulated by *P. yoelii* (20) or else repressed by the parasite (8).

In an extensive comparison of transcriptomic analysis of host immune cells' responses to different pathogens, a set of 197 genes was found to be exclusively regulated in DCs (Jenner and Young, 2005). Comparison of this group of genes with their mouse homologues regulated by *P. yoelii* revealed that only 16% of genes belonging to the common DC response to pathogens are regulated by the malaria parasite (Table S8 in *Supplementary material*).

Transcriptomic analysis of DC responses to three different intracellular parasites (*Toxoplasma gondii*, *Leishmania major* and *Leishmania donovani*) identified a cluster of genes that are simultaneously induced by all three (Chaussabel *et al*., 2003). From the 41 genes that have mouse homologues, we found that *P. yoelii* induces 24% of them (10), while the majority (76%, 31) are either repressed or not significantly regulated (Table S9 in *Supplementary material*). Despite the different experimental approaches used in the microarrays used for these comparisons, these data suggest that the transcriptomic response of DCs to *P. yoelii* is largely different not only from non-parasitic pathogens, but also from other intracellular parasites such as *Leishmania* and *Toxoplasma*.

Plasmodium-*mediated regulation of cytokine production by DC – confirmation using quantitative real-time polymerase chain reaction*

Transcriptomic analysis shows that DC expression of cytokine genes such as *il-6*, *il-10* and *ifn-γ* is induced by *P. yoelii* infection. To confirm modulation of these cytokines at the mRNA level, we performed quantitative real-time polymerase chain reaction (PCR) analysis (Fig. 3). We also included in this analysis the cyclooxygenase 2 (*cox2/pgts2*) gene, which is responsible for the inflammation-inducible production of PGE2 (Harris *et al*., 2002), an important mediator of immune functions, and the regulatory subunit of PKA (*prkar1*) as an example of a gene that is repressed during *P. yoelii* infection. As *Plasmodium* regulates a high number of genes that are not described in the common DC response to different pathogens, we confirmed the regulation of three immune-related genes (*fos*, *msr* and *c4b*) that are specifically regulated by *Plasmodium* (Fig. 3).

Our detailed analysis of the expression of these genes throughout infection confirms the results obtained with the microarray analysis. Quantitative PCR analysis also reveals that the increase in expression of some immune-related genes can be detected as soon as 3 days after infection and is still significant at late times (day 22) when parasitaemia is undetectable in the blood. The response at later time points appears to be similar in trend but lower in magnitude compared with responses before or during the peak of parasitaemia.

Expression of co-stimulatory molecules on the surface of DCs during P. yoelii *infection*

Transcriptomic analysis indicates that the genes for the co-stimulatory molecules CD80, CD83 and CD86 are downregulated in both days of infection analysed (Table 3). CD40 was not significantly regulated in infected mice when compared with uninfected. The level of surface expression of these molecules is commonly used to determine the maturation state of DCs. To confirm that these molecules are not induced by *P. yoelii* infection, we analysed their surface expression on DCs from spleens of uninfected or *P. yoelii*-infected mice at days 5 and 10. We found that the surface expression of all four co-stimulatory molecules analysed was not increased in DCs upon infection (Fig. 4).

Plasmodium yoelii-*infected erythrocytes induce cAMP increases in DCs*

Activation of specific signalling pathways can be detected by microarray analysis, as it frequently results in feedback regulatory mechanisms that modulate the expression of the genes involved in each particular pathway. Our analysis indicates that several genes involved in prostaglandin synthesis and signalling are regulated in DCs: *cox2/ptgs2* (cyclooxygenase 2), *ep4* (a PGE2 receptor) and *ptgis* (prostacyclin synthase), suggesting that *P. yoelii* may be activating this signalling pathway. Prostaglandin receptors are coupled to G proteins, which can trigger production of cAMP, a second messenger with multiple effects on gene regulation (Harris *et al*., 2002). We also found a significant number of GTP-binding proteins (8) that show regulation of mRNA levels by *P. yoelii* (Table S4 in *Supplementary material*). We have previously described the quick release of PGE2 by DCs in response to *P. yoelii*-infected erythrocytes (C. Ocana-Morgner, submitted). As PGE2 induces increases in intracellular cAMP levels, we investigated if *Plasmodium*-induced PGE2 activates the production of this second messenger. We found that incubation of bone marrow-derived DCs with *P. yoelii*-infected erythrocytes specifically induces increases in intracellular cAMP that are not observed after incubation with uninfected erythrocytes (Fig. 5A). cAMP levels were also observed to increase with the time and numbers of infected erythrocytes added to DCs (Fig. 5B and C).

To study whether the cAMP increase is mediated by PGE2, we analysed the effect of indomethacin, a cyclooxygenase inhibitor (Harris *et al*., 2002), on *P. yoelii*-induced cAMP production by DCs. Pre-incubation of DCs with indomethacin resulted in a significant inhibition of *P. yoelii*-mediated increase in cAMP (Fig. 5C), suggesting that PGE2 contributes to the increase in this second messenger in response to the parasite.

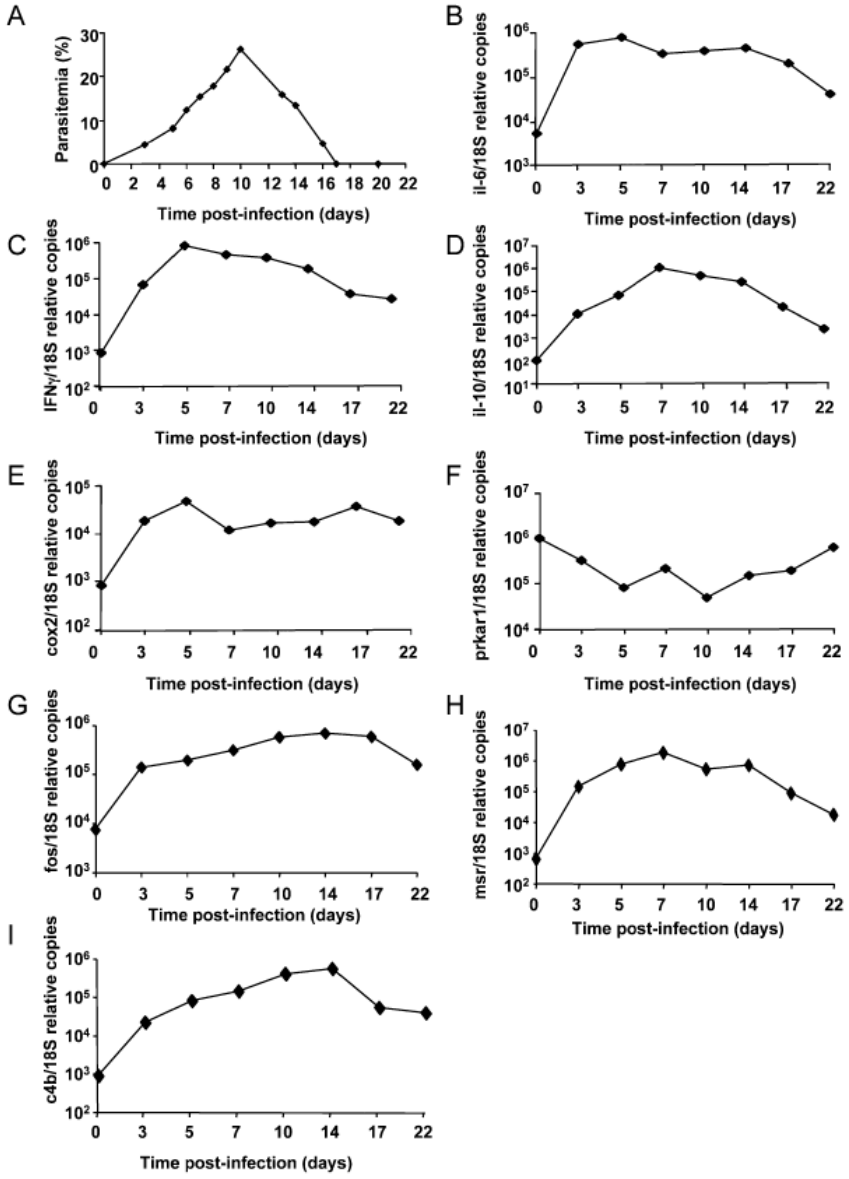

Fig. 3. Real-time PCR analysis of expression in spleen DCs of selected genes over time of *P. yoelii* blood-stage infection. A group of transcripts which showed differential regulation in microarray analysis was selected for real-time PCR analysis in spleen DCs collected at different times of *P. yoelii* infection. (A) Evolution of *P. yoelii* parasitaemia over time. Genes selected for quantitative mRNA analysis: *il-6* (B), *ifn-γ* (C), *il-10* (D), *cox2/pgts2* (E), *prkar1* (F), *fos* (G), *msr* (H), *c4b* (I). All genes were normalized to the expression of mouse 18S ribosomal RNA. Results show average of duplicated samples. Variation between samples was always lower than 10%.

These results suggest that *P. yoelii*-infected erythrocytes induce PGE2-mediated cAMP signalling in DCs. As cAMP regulates numerous signalling pathways in DCs, this finding may have broad implications for the understanding of DC/*Plasmodium* interactions.

Production of IL-6 by DCs in response to P. yoelii *is dose dependent and is mediated by PGE2*

IL-6 is an important mediator of the acute-phase response, with multiple effects on immune cells, such as

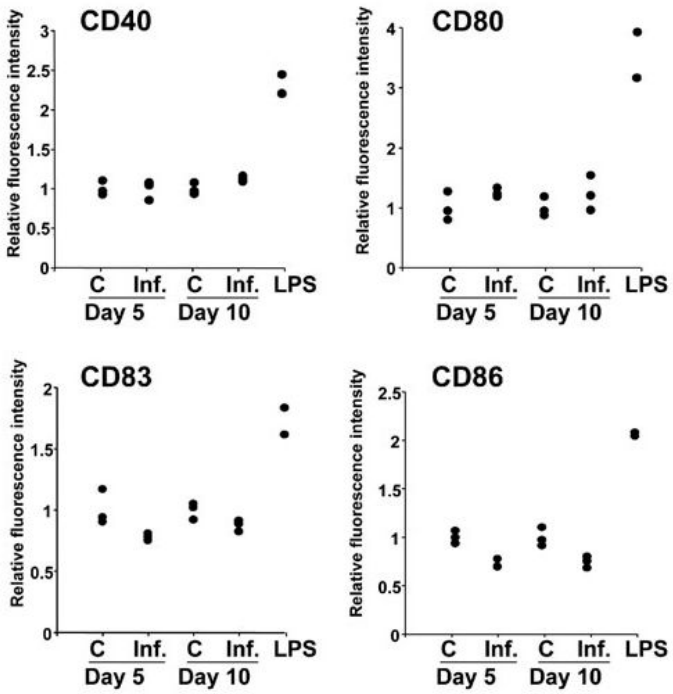

Fig. 4. Expression of co-stimulatory molecules on the surface of splenic DCs from *P. yoelii*-infected mice. CD11c+ DCs were obtained from the spleens of groups of three mice control (C) or infected with *P. yoelii* (Inf.) at either 5 or 10 days of infection. Co-stimulatory molecules were stained with fluorescently labelled specific antibodies and expression levels were analysed by FACS. Results are expressed as relative fluorescence intensity for the DCs of each mouse compared with the average of DCs from control uninfected mice. As positive control, two mice were injected with LPS 24 h before analysis.

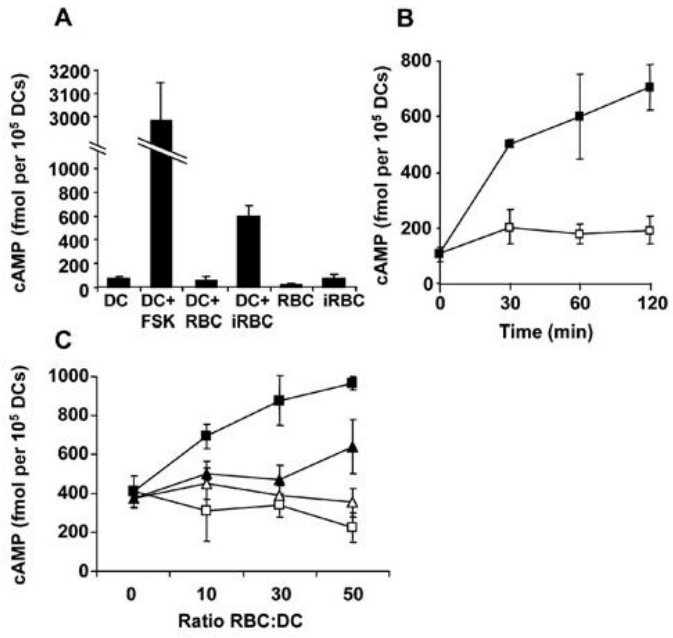

Fig. 5. *Plasmodium yoelii*-infected erythrocytes induce rapid accumulation of intracellular cAMP in DCs, which is inhibited by indomethacin. Intracellular cAMP was measured in DCs (A) incubated for 3 h alone or with the adenylate cyclase activator forskolin (FSK), uninfected erythrocytes (RBC), *P. yoelii*-infected erythrocytes (iRBC) or else uninfected or *P. yoelii*-infected erythrocytes incubated alone; (B) upon different times of incubation with either uninfected (white symbols) or *P. yoelii*-infected (black symbols) erythrocytes; (C) incubated for 3 h with different doses of uninfected (white symbols) or *P. yoelii*-infected (black symbols) erythrocytes, with the cyclooxygenase inhibitor indomethacin (5 μg ml^{-1}) (triangles) or DMSO as negative control (squares). Standard deviations of triplicated samples are shown. Results are representative of three independent experiments.

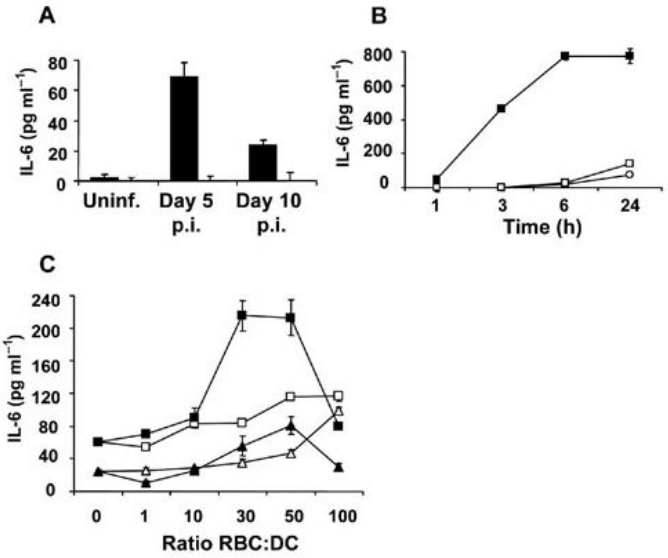

Fig. 6. *Plasmodium yoelii* induces IL-6 secretion by DCs. IL-6 secretion was measured in the culture medium of (A) CD11c+ (black bars) or CD11c– (white bars) splenocytes from *P. yoelii*-infected or uninfected mice after 18 h incubation; (B) bone marrow-derived DCs at different times of incubation alone (circles), with *P. yoelii*-infected (black squares) or uninfected (white squares) erythrocytes; (C) bone marrow-derived DCs incubated for 18 h with different doses of uninfected (white symbols) or *P. yoelii*-infected (black symbols) erythrocytes, with the cyclooxygenase inhibitor indomethacin (5 μg ml^{-1}) (triangles) or DMSO as negative control (squares). Standard deviations of triplicated samples are shown. Results are representative of at least two independent experiments.

resolution of inflammation, B cell stimulation, Th2 polarization of CD4+ T cells, and also preventing induction of regulatory T cells (Jones, 2005). We found that IL-6 mRNA is strongly induced in splenic CD11c+ cells during *P. yoelii* infection. To confirm that spleen DCs secrete this cytokine during malaria infection, we isolated both CD11c+ (DCs) and CD11c– splenocytes from *P. yoelii*-infected mice (days 5 and 10 p.i.) and analysed IL-6 released from these cells. DCs from both days of infection produced high levels of IL-6 compared with uninfected controls or with CD11c– spleen cells, which did not produce detectable levels of IL-6 (Fig. 6A).

We next characterized IL-6 secretion by DCs *in vitro*. IL-6 is released in response to *P. yoelii*-infected erythrocytes as early as 3 h after incubation with maximal secretion at 6 h (Fig. 6B). IL-6 production was also found to depend on the dose of infected erythrocytes (Fig. 6C). As PGE2 is quickly released in response to *P. yoelii* (C. Ocana-Morgner, submitted) and PGE2 increases IL-6 gene expression in DCs (Rubio *et al.*, 2005), we investigated the role of this immune mediator in *P. yoelii*-induced IL-6 release. We found that pre-incubation of DCs with indomethacin inhibited IL-6 production in response to infected erythrocytes and brought secretion down to background levels (Fig. 6C), suggesting that PGE2 mediates the enhanced IL-6 production of DCs in response to *P. yoelii*.

cAMP, PKA and p38-MAPK mediate P. yoelii*-induced secretion of IL-6 by DCs*

Our findings indicate that *P. yoelii*-induced PGE2 contributes to the observed increases in intracellular cAMP concentrations, as well as to the release of IL-6 by DCs. As cAMP can increase expression of IL-6 in different cell types through activation of either PKA-dependent (Chio *et al.*, 2004; Chen *et al.*, 2006) or PKA-independent pathways (Yin *et al.*, 2006), we first investigated if these pathways are also functional in DCs. Addition of synthetic PGE2 or 8-Br-cAMP, a cell-permeable analogue of cAMP, triggers secretion of IL-6 in DCs (Fig. 7A). Furthermore, PGE2-derived IL-6 could be inhibited by H89, a PKA inhibitor, suggesting that DCs can activate IL-6 production by a PGE2/cAMP/PKA pathway.

In other cell types, cAMP signal transduction pathways can also activate the exchange protein directly activated by cAMP (EPAC), which acts independently of PKA (Yin *et al.*, 2006). However, a specific activator of EPAC (8-CPT-2′OMe-cAMP) (Enserink *et al.*, 2002) did not induce IL-6 secretion in DCs, suggesting that this pathway is not involved in the production of IL-6 by DCs.

We next investigated if cAMP mediates the production of IL-6 in response to *P. yoelii*-infected erythrocytes. DCs were pre-incubated with a cAMP derivative (Rp-8Br-cAMPS), which acts as a competitive inhibitor of cAMP-dependent pathways. We found a significant inhibition of IL-6 secretion induced by *P. yoelii*-infected erythrocytes in the presence of Rp-8Br-cAMPS. Similar results were observed when DCs were pre-incubated with the PKA inhibitor H-89 (Fig. 7B).

Downstream effects of cAMP and PKA are frequently mediated by p38-MAPK, a subclass of mitogen-activated protein kinases, in response to different stimuli and in different cell types (Pomerance *et al.*, 2003; Delghandi *et al.*, 2005). p38-MAPK was also found to contribute to IL-6 expression in response to *Plasmodium*-derived GPI

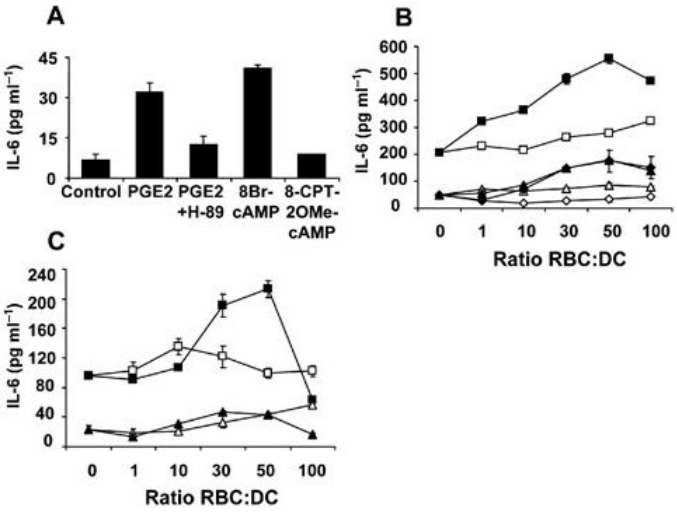

Fig. 7. cAMP, PKA and p38-MAPK mediate induction of IL-6 by *P. yoelii* in DCs. IL-6 was measured in the culture medium of (A) DCs incubated for 6 h alone, with PGE2 200 pg ml^{-1} (either in the absence or in the presence of the PKA inhibitor H89 10 μM), a permeable cAMP analogue (8-Br-cAMP, 500 μM) or a specific activator of EPAC (8-CPT-2OMecAMP 20 μM); (B and C) DCs incubated for 18 h with different doses of uninfected (white symbols) or *P. yoelii*-infected (black symbols) erythrocytes (B) in the presence of H89 10 μM (rhomboids), Rp-8Br-cAMPS 1 μM (triangles), or DMSO as negative control (squares); (C) in the presence of the p38-MAPK inhibitor SB203580 10 μM (triangles) or an inactive chemical analogue of the same inhibitor SB203474 10 μM as control (squares). Standard deviations of triplicated samples are shown. Results are representative of at least two independent experiments.

(Zhu *et al*., 2005). Pre-incubation of DCs with a specific p38-MAPK inhibitor (SB203580) completely abrogated *P. yoelii*-induced secretion of IL-6, while a chemical analogue of this inhibitor that does not interfere with p38-MAPK activity (SB203474) did not have any effect on IL-6 secretion (Fig. 7C).

Discussion

Global transcriptomic analysis of the transcriptomic modulation of DCs by *P. yoelii*-infected erythrocytes shows that blood-stage parasites exert an extensive regulation of gene expression of DCs during malaria infection. Gene regulation is very diverse, including different cell functions and metabolic pathways. Some of these have been previously identified through microarray analysis of different models of malaria infection using whole mouse spleen (Schaecher *et al*., 2005) or brain (Delahaye *et al*., 2006) and monkey mononuclear blood cells (Ylostalo *et al*., 2005), including the glycolysis pathway, kinase activities, ATP binding, transcription regulators, cell cycle and immunoglobulin genes. However, we found that genes with functions related to cell cycle regulation and defence responses have even a larger number of genes regulated in DCs compared with whole spleen (Schaecher *et al*., 2005), suggesting these two functions have a more extensive regulation in DCs than other cell types residing in the spleen.

Dendritic cells incubated with *P. yoelii*-infected erythrocytes have extended viability when compared with classic maturation stimuli (Ocaña-Morgner *et al*., 2003), which is likely to be related to the extensive regulation of genes with functions in cell cycle (45 transcripts) and also apoptosis (17 transcripts). Such gene regulation might be responsible for the increases in the numbers of DCs in both red pulp and marginal zones of the spleen in spleens of malaria-infected individuals compared with sepsis controls (Urban *et al*., 2005). In particular, the induction of *cyclins A2*, *B1* and *B2* suggests that DCs may have increased proliferation rates (Schwartz and Shah, 2005). On the other hand, the genetic regulation of apoptosis includes both the induction and repression of pro-apoptotic genes, such as *fas* (Dutta *et al*., 2006) and *ask1/mekk5* (Ichijo *et al*., 1997), respectively, as well as modulation of transcripts with both pro- and antiapototic activities (*relB*, *nfkb1/p50*, *c-fos* and *traf3*) (Liebermann *et al*., 1998; Dutta *et al*., 2006), suggesting a rather complex regulation of the survival pathways.

It is noteworthy that a large majority of genes (over 70%) that are significantly induced, or else repressed, after 5 days of infection have the same modulation after 10 days. However, we found a significant number of genes (180) regulated specifically at only one of the time points p.i., which may reflect the progression of the immune response from early to late infection.

A quantitative and more extensive kinetic analysis of specific cytokine genes revealed similar profiles of regulation over the course of infection, with some genes have maximal induction as soon as day 3 p.i. (*il-6*), others at day 5 p.i. (*ifn-γ*, *cox2*), while others show a gradual increase over time up to day 10 p.i. (*il-10*, *fos*, *msr*, *c4b*).

The transcriptomic profile is complex with some genes that are typically upregulated during DC maturation being also induced by *P. yoelii* (such as *il-6*, *cox2*, *ccl3*, *ccl4*, *cxcl9*, *cxcl10*, *cxcl11*), while others are not regulated (such as *tnfα*, *il1-β*, *ccl19*, *icam-1*) or even repressed (such as *cd86*, *cd83*, *nfkb1*, *nfkb2*). Conversely, a large number of genes modulated by *P. yoelii* are not typically associated

with DC maturation, including induced genes such as *fos*, *msr*, *c4b* (confirmed by real-time PCR), *ccr5*, *cxcr6*, *gzmb*, *gzmk*, *fcgr2b*, *fcgr3a*, and repressed genes such as *cd209a/dc-sign*, *c6*, *cd1d1* and *traf3*.

We confirmed induction of three transcripts with relevance in immune responses: *fos*, a transcription factor that has been associated with Th2-inducing stimuli (Agrawal *et al*., 2003); *msr/sr-a*, a scavenger and pattern recognition receptor (McGuinness *et al*., 2003); and *c4b*, a marker of follicular DCs (Taylor *et al*., 2002).

The upregulation of *fos* is of special interest as *fos* is induced by Toll-like receptor (TLR) agonists that modulate DCs into inducers of T cell responses of the Th2 type, which are gradually induced in late *Plasmodium* rodent infections (Agrawal *et al*., 2003).

The differences between *P. yoelii* and the common maturation response are also reflected when gene regulation is analysed by biological processes as defined by Gene Ontology. While defence response genes are extensively regulated by both *P. yoelii* and other microbes, other categories such as cell cycle, protein phosphorylation and protein catabolism are extensively modulated by *P. yoelii* but are not highly represented in the common maturation programme (Huang *et al*., 2001).

These data suggest that the DC responses to *P. yoelii* might have significant differences from the standard maturation programme. In particular, we found that the increase in co-stimulatory molecules, which is a hallmark of maturation, is not present in DCs from *P. yoelii*-infected mice. We propose that the response to *Plasmodium* is a 'divergent' response from what is known as the 'standard' maturation response. A *Plasmodium*-related parasite, *Toxoplasma* (McKee *et al*., 2004), and also helminth antigens (Kane *et al*., 2004) induce atypical responses in DCs suggesting that these pathogens also modulate the host immune response. However, the DC transcriptional responses to helminthes are very limited (Chaussabel *et al*., 2003; Kane *et al*., 2004), while *Plasmodium* induces extensive DC gene regulation. On the other hand, a comparison of *P. yoelii* and *Toxoplasma*-induced transcripts (Table S9 in *Supplementary material*) revealed a low degree of similarity.

Our finding that DC-derived PGE2 contributes to the production of IL-6 has consequences for the understanding of the mutual regulation between inflammatory mediators in malaria infection, in this case through a synergistic mechanism that may contribute to the fever response, as IL-6 can provide a positive feedback for PGE2 production (Coceani and Akarsu, 1998). Other studies have shown that PGE2 is elevated during malaria infections in monkeys (Yang *et al*., 1999), modulates cytokine levels during malaria, and inversely correlates with the incidence of malarial anaemia (Keller *et al*., 2006b) and severe disease in children (Perkins *et al*., 2001). As PGE2 can induce elevation in cAMP levels (Harris *et al*., 2002), which in turn can induce the production of IL-6 (Chio *et al*., 2004; Chen *et al*., 2006; Yin *et al*., 2006), we investigated whether this pathway is induced in DCs. It is also known that malaria enhances cAMP production by immature erythrocytes *in vitro* (Hertelendy *et al*., 1979) and that IL-6 is produced by DCs *in vitro* in response to *Plasmodium* (Seixas *et al*., 2001). We now describe how PGE2 stimulates IL-6 production by a cAMP/PKA/p38-MAPK pathway. PGE2 may contribute to the regulation of inflammatory responses to malaria parasites not only through inhibition of TNFα (Keller *et al*., 2006b), but also through the induction of IL-6, as this cytokine contributes to the resolution of inflammation and to B cell proliferation (Jones, 2005). DC-derived IL-6 may have local effects on stimulation of splenic B cells, which are required for Th1 to Th2 transition in malaria and also for avoiding persistence of *Plasmodium* (Grun and Weidanz, 1981; Taylor-Robinson and Phillips, 1996).

IL-6 also regulates Th2 polarization, Treg inhibition, DC maturation, T cell and neutrophil apoptosis, and is considered an important regulator of the transition from innate to acquired immunity (Jones, 2005). IL-6 is elevated in the serum of *P. falciparum*-infected individuals and is usually correlated with disease severity (Lyke *et al*., 2004; Prakash *et al*., 2006); however, the specific role of this cytokine in the immune response to malaria remains unknown. DCs secretion of IL-6 in the spleen may specifically influence B and T cell functions during malaria infection.

Cyclooxygenase activity has been reported to mediate production of IL-6 by macrophages in response to peptidoglycan (PGN), a well-characterized ligand of TLR-2 (Chen *et al*., 2006). This pathway seems to be parallel to the one we have identified for DC in response to *P. yoelii*, as they are both mediated by PGE2 activation of cAMP, PKA and p38MAPK, with only a slower kinetics triggered by PGN in macrophages. It is likely that *Plasmodium*-derived GPI and haemozoin contribute to IL-6 secretion in our system, as they induce activation of TLR-2 (or -4) and -9, respectively, which triggers IL-6 production (Coban *et al*., 2005; Krishnegowda *et al*., 2005).

One of the purposes of this work was to identify signalling pathways involved in gene regulation in DCs by the malaria parasite – which is the case of cAMP – as this subject remains largely unexplored. The rapid increase in cAMP triggered by the parasite in DCs probably has a broad influence in the response of these cells to *Plasmodium*, given the large number of genes regulated by the two families of cAMP-dependent transcription factors: the CREB family (Mayr and Montminy, 2001) and the C/EBP family (Wilson and Roesler, 2002). In fact, PGE2-induced cAMP influences migration of DCs (Luft *et al*., 2002; Legler *et al*., 2006), DCs-mediated T cell polarization

(Kubo *et al*., 2004) and also cytokine secretion (Kambayashi *et al*., 2001). Whether these effects are mediated by PGE2-induced cAMP during malaria infection remains to be characterized.

Experimental procedures

Mice and infections

BALB/C and Swiss Webster mice were purchased from Taconic or NIH and housed in the New York University Medical Center Animal Facility according to protocols approved by IACUC. *P. yoelii yoelii* 17XNL (non-lethal) strain was used for this study. BALB/C mice were infected with 5×10^6 *P. yoelii*-infected red blood cells (RBC) injected intraperitoneally. LPS (10 µg per mouse) was injected intraperitoneally.

Dendritic cell extraction

Total splenocyte suspensions from either non-infected or *P. yoelii*-infected mice were obtained after mechanical disruption of spleens. Cell suspensions were passed through a 100 µm pore nylon cell strainer and erythrocytes lysed with ammonia salt buffer. Myeloid spleen DCs were obtained by purification of CD11c+ cells from splenocyte preparations using anti-mouse CD11c-coupled magnetic beads (Miltenyi biotech) following manufacturer's instructions. The isolated cell population is > 85% CD11c+.

Culture of bone marrow-derived DCs

Bone marrow cells were cultured for differentiation into a population enriched in CD11c+ cells with a DC phenotype as described before (Bruna-Romero and Rodriguez, 2001). Briefly, cells from tibias and femurs of BALB/C mice were cultured for 8 days in DMEM medium supplemented with 10% FCS, 100 IU ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 2 mM glutamine, supplemented with 30% of conditioned medium from Ag8653 cells expressing recombinant GM-CSF (Stockinger *et al*., 1996). The non-adherent cells in these cultures are typically 85% CD11c+.

Isolation of erythrocytes and incubation with DCs

Blood was collected in heparin (10 U ml^{-1} final concentration) from Swiss Webster mice, either uninfected or infected with *P. yoelii yoelii* 17XNL. Blood was passed through 100 µm pore nylon cell strainer, diluted in PBS and centrifuged at 800 *g* for 3 min. Buffy coat was removed by pipetting. Purification of schizonts from infected erythrocyte suspension was performed on a gradient of 60% Accudenz® (Accurate Chemical & Scientific) solution in PBS by centrifugation at 200 *g* for 25 min at 20°C. The intermediate fraction containing schizont-infected erythrocytes was removed and washed with PBS. This fraction typically contains > 85% schizonts. DCs were pre-incubated for 30 min with the indicated drugs, or else DMSO, before addition of uninfected erythrocytes or schizonts at a ratio of 30 per DC, except when indicated otherwise. Inhibitors used were: 5 µg ml^{-1} indomethacin, 10 µM H89, 10 µM Rp-8Br-cAMPS, 10 µM SB203580, 10 µM SB203474 (inactive analogue of SB203580) (Calbiochem). The following cAMP analogues (Calbiochem) were also used: 500 µM 8-Br-cAMP, 20 µM 8-CPT-2OMecAMP (EPAC-specific activator). PGE2 was from Cayman Chemicals. LPS from *Escherichia coli* (Sigma) was used at 1 µg ml^{-1}.

RNA extraction and array hybridization

Total RNA from spleen CD11c+ cells (ranging from 2 to 5×10^6 cells) was obtained from cell lysates using the RNeasy mini kit (Qiagen). Each GeneChip experiment was performed with each one of the 6 independent RNA samples (two replicate samples for each of the three conditions). RNA was processed for use on Affymetrix (Santa Clara, CA, USA) GeneChip Mouse Expression 430A 2.0 Arrays, according to the manufacturer's One-Cycle Target Labelling Assay using Affymetrix reagents. Briefly, 2 µg of total RNA to which known concentrations of Poly A RNA controls were added (GeneChip Eukaryotic Poly A RNA Control Kit) was used in a reverse transcription reaction (One-Cycle DNA synthesis kit) to generate first-strand cDNA. After second-strand synthesis, double-stranded cDNA was used in an *in vitro* transcription (IVT) reaction to generate biotinylated cRNA (3′-Amplification Reagents for IVT-Labelling). Size distribution of the cRNA and fragmented cRNA was assessed using an Agilent 2100 Bioanalyser with a RNA 6000 Nano Assay.

Ten micrograms of fragmented cRNA was used in a 200 µl hybridization containing added hybridization controls. One hundred and thirty microlitres of mixture was hybridized on arrays for 16 h at 45°C. Arrays were scanned on an Affymetrix GeneChip scanner 3000. All quality parameters for the arrays were confirmed to be in the recommended range.

GeneChip® data analysis

Results of expression levels detected with the MOE430A GeneChip® in the form of .Cel files were loaded into ArrayAssist Software (Stratagene). A normalized master data table was created consisting of a matrix of expression (signal) values extracted from all the .Cel files using the sequence-enhanced Robust Multi-Array Average (GC-RMA) algorithm (Wu and Irizarry, 2004). Results were then analysed by multiclass testing method ($P < 0.01$) for comparison of average expression levels within each pair of replicate arrays, and by cluster analysis using Pearson Correlations. Gene Ontology and KEGG pathway terms were obtained using the DAVID Functional Annotation Tools (http://david.abcc.ncifcrf.gov/).

Real-time quantitative PCR

One microgram of each sample of total RNA was used for reverse transcription with MuLV reverse transcriptase (Applied Biosystems). cDNA (200 ng) from each sample was used for real-time PCR amplification of target cDNA sequences using the Rotor 3000 (Corbett Robotics). Primers were either custom designed or from Qiagen. Data analysis was performed using Rotor-Gene 6 (Corbett Research).

Monoclonal antibodies and flow cytometry

Splenocytes were stained with APC-anti-CD11c, 7AAD (for exclusion of non-viable cells) and FITC-anti-CD40, FITC-anti-CD80,

FITC-anti-CD86 (Pharmigen) or FITC-anti-CD83 (Biolegend). CD11c-positive, 7AAD-negative cells were gated and analysed for expression of the co-stimulatory molecules using FACSCalibur® (Becton Dickinson).

Quantification of IL-6 and cAMP by ELISA

Concentrations of IL-6 in the culture medium of DCs were determined using mouse 'BD Opteia' reagents (BD Biosciences). Intracellular cAMP in bone marrow-derived DCs was determined by competitive ELISA of cell lysates using the 'cAMP Direct Biotrack EIA' (GE Healthcare).

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Supplementary material

The following supplementary material is available for this article online:

Figure S1. Scatter plots of Expression Values for all Probesets in the MOE430A 2.0 Genechips ® for different pairs of samples, either duplicates or non-duplicates.

Table S1. Complete list of Transcripts induced by *P. yoelii* in Spleen DCs according to their expression profiles over the two times post-infection (pi) studied: induced at day 5 and day 10 pi; induced only at day 5 pi; induced only at day 10 pi.

Table S2. Complete list of Transcripts repressed by *P. yoelii* in Spleen DCs according to their expression profiles over the two times post-infection (pi) studied: repressed at day 5 and day 10 pi; repressed only at day 5 pi; repressed only at day 10 pi.

Table S3. Transcripts modulated by *P. yoelii* in Spleen DCs according to Gene Ontology Biological Processes.

Table S4. Transcripts modulated by *P. yoelii* in Spleen DCs according to Gene Ontology Molecular Functions.

Table S5. Transcripts modulated by *P. yoelii* in Spleen DCs according to Gene Ontology Cellular Components.

Table S6. Transcripts modulated by *P. yoelii* in Spleen DCs according to KEGG Metabolic Pathways.

Table S7. Regulation of genes belonging to the common maturation program of DCs (Huang *et al*. 2001) during *P. yoelii* infection in mice.

Table S8. Regulation of genes belonging to the common response of DCs to pathogens (Jenner and Young, 2005) during *P. yoelii* infection in mice

Table S9. Regulation of genes belonging to the common response of DCs to intracellular parasites (Chaussabel *et al.* 2003) during *P. yoelii* infection in mice.

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