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THE ROLE OF INTERFERON REGULATORY FACTOR 5 IN MYELOID CELLS DURING *LEISHMANIA DONOVANI* INFECTION

Par

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SUMMARY

Interferon Regulatory Factor 5 (IRF-5) is a transcription factor that has been reported to be an important regulator of the activation of Type I IFN genes and genes encoding for key pro-inflammatory cytokines (Takaoka *et al.*, 2005). Additionally, IRF-5 has been shown as a specific marker for inflammatory macrophages and to promote Th1 development (Krausgruber *et al.*, 2011). In previous studies, we have shown that in the absence of IRF-5, mice not only failed to develop Th1 responses against *Leishmania donovani*, but also had a severely reduced inflammatory infiltration in the spleen and the liver (Paun *et al.*, 2011). However, the expression of IRF-5 in IFNγ^{+} CD4^{+} T cells is not required for the expansion of Th1 cells. Indeed, IRF-5 expression in CD4^{+} T cells even resulted in cell death by upregulation of death receptor 5 (DR5) (Fabie *et al.*, 2018). This suggests that IRF-5 expression by other cell sources may be required to induce protective Th1 responses. A model of liver disease revealed that mice lacking IRF-5 in myeloid cells induce Th2 rather than Th1 cells (Alzaid *et al.*, 2016). Thus, we hypothesized that IRF-5 expression in myeloid cells strictly contributes to Th1 development during *L. donovani* infection.

In this study, we generated myeloid cell-specific *Irf5*$^{-/-}$ mice by breeding *Irf5* $^{flox/flox}$ with *LysM – Cre* mice to study the role of IRF-5 in myeloid cells during *L. donovani* infection. To our surprise, qPCR results revealed that LysM-Cre only promoted partial *Irf5* deletion in myeloid cells during the first two weeks of infection; after that, no deletion was observed. *L. donovani* infected *Irf5-LysM-Cre*$^{+}$ mice had similar parasite burdens as well as percentages of IFNγ-producing CD4^{+} T cells in the spleen and liver compared to control *Cre*$^{-}$ mice at any time point after infection with *L. donovani*. These mice also recruited a similar number of myeloid cells to the spleen compared to *Cre*$^{-}$ mice. In contrast, *Irf5-CMV-Cre*$^{+}$ mice, in which IRF-5 is deleted in all the cell types, showed a significant impairment of IFNγ-producing CD4^{+} T cells and a remarkable reduction of myeloid cell recruitment to the spleen. Additionally, these mice were more susceptible to *L. donovani* infection than *Cre*$^{-}$ mice. Our results suggested that the *LysM-Cre* mouse model is not appropriate to study gene deletion in myeloid cells in *L. donovani* infected mice, perhaps because it fails to work in monocytes, which massively infiltrate the spleen of *L. donovani* infected mice during the chronic stage of disease.

Using *Irf5-CD11c-Cre*$^{+}$ mice, which had a better deletion efficiency, we show that IRF-5 ablation in myeloid cells significantly decreased myeloid cell recruitment to the spleen during chronic VL; however, IFNγ-producing CD4^{+} T cells developed similarly to IRF-5-sudfficient mice. Taken together, our data shown that, IRF-5 expression in myeloid cells is required for the recruitment or differentiation of myeloid cells in the spleen but not for Th1 development during *L. donovani* infection. This also suggests that IRF-5 in other cell types such as B cells might play an important role in Th1 responses to *L. donovani*.

Résumé

L'interferon regulatory factor 5 (IRF-5) est un facteur de transcription qui est un important régulateur de l'activation des gènes de l'IFN de type I et des gènes codant pour des cytokines pro-inflammatoires clés (Takaoka *et al.*, 2005). De plus, IRF-5 a été montré comme étant un marqueur spécifique des macrophages inflammatoires et favorisant le développement de Th1 (Krausgruber *et al.*, 2011). Dans des études antérieures, nous avions montré qu'en l'absence d'IRF-5, les souris non seulement ne développaient pas de réponses Th1 contre *L. donovani*, mais avaient également une infiltration inflammatoire fortement réduite dans la rate et le foie (Paun *et al.*, 2011). Cependant, l'expression d'IRF-5 dans les lymphocytes T $CD4^+$ $IFN\gamma^+$ n'est pas requise pour l'expansion de la population Th1. En effet, l'expression de l'IRF-5 dans les lymphocytes T $CD4^+$ entraîne la mort cellulaire par une régulation à la hausse du récepteur de mort 5 (DR5) (Fabie *et al.*, 2018). Ceci suggère que l'expression d'IRF-5 par d'autres cellules peut être nécessaire pour induire des réponses Th1 protectrices. Un modèle de maladie hépatique a révélé que les souris dépourvues d'IRF-5 dans les cellules myéloïdes induisent une réponse Th2 plutôt qu'une réponse Th1 (Alzaid *et al.*, 2016). Ainsi, nous avons émis l'hypothèse que l'expression d'IRF-5 dans les cellules myéloïdes contribue au développement de Th1 au cours de l'infection à *L. donovani*.

Dans cette étude, nous avons généré des souris $Irf5^{-/-}$ spécifiques pour les cellules myéloïdes en croisant des souris $Irf5^{flox/flox}$ avec des souris *LysM–Cre* afin d'étudier le rôle de l'IRF-5 dans les cellules myéloïdes au cours d'une infection par *L. donovani*. À notre grande surprise, les résultats de la qPCR ont révélé que *LysM-Cre* n'induisait qu'une délétion partielle d'IRF-5 dans les cellules myéloïdes au cours des deux premières semaines de l'infection; après cela, aucune délétion n'a été observée. Les souris *Irf5-LysM-Cre*$^+$ infectées par *L. donovani* présentaient une charge parasitaire, ainsi que des pourcentages de lymphocytes T $CD4^+$ producteurs d'IFNγ similaires dans la rate et le foie, par rapport aux souris *Cre*$^-$ témoins à tout moment après l'infection par *L. donovani*. Ces souris ont également recruté un nombre similaire de cellules myéloïdes dans la rate par rapport aux souris *Cre*$^-$. En revanche, les souris témoins négatives *Irf5-CMV-Cre*$^+$, dans lesquelles IRF-5 est délété dans tous les types de cellules, ont présenté une altération significative des lymphocytes T $CD4^+$ producteurs d'IFNγ, une réduction remarquable dans le recrutement de cellules myéloïdes dans la rate et une plus grande susceptibilité à l'infection par *L. donovani* que les souris *Cre*$^-$. Nos résultats suggèrent que ce modèle murin n'est pas approprié pour étudier la délétion de gènes dans les cellules myéloïdes de souris infectées par *L. donovani*, peut-être parce qu'il ne fonctionne pas dans les monocytes, qui infiltrent massivement la rate de souris infectées par *L. donovani* au cours de la phase chronique de la maladie.

En utilisant des souris *Irf5-CD11c-Cre*$^+$, qui présentaient une meilleure efficacité de délétion, nous montrons que l'ablation d'IRF-5 dans les cellules myéloïdes réduit significativement le recrutement de cellules myéloïdes dans la rate pendant la LV chronique; cependant, les lymphocytes T $CD4^+$ producteurs d'IFNγ se sont développées de manière similaire aux souris exprimant IRF-5. Ensemble, nos données ont montré que l'expression d'IRF-5 dans les cellules myéloïdes était nécessaire pour le recrutement ou la différenciation des cellules myéloïdes dans la rate, mais pas pour le développement de la réponse Th1 au cours d'une infection par *L. donovani*. Cela suggère également qu'IRF-5 dans d'autres types de cellules tels que les lymphocytes B pourrait jouer un rôle important dans les réponses Th1 contre *L. donovani*.

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α : Alpha

β : Beta

γ : Gamma

°C : Degree Celsius

µg : Microgramm

µl : Microlitre

µm : Micron

APCs : Antigen presenting cells

APC : Allophycocyanin

BMDCs : Bone marrow-derived dendritic cells

BMDMs : Bonne morrow-derived macrophages

DCs : Dentritic Cells

GM-CSF : Granulocyte-macrophage colony-stimulating factor

FACS : Fluorescence-activated cell sorting

FBS : Fetal bovine serum

FITC : Fluorescein isothiocyanate

FMO : Fluorescence minus one

HIF : Hypoxia inducible factor

HPRT : Hypoxanthine-guanine phosphoribosyltransferase

HS : Horse Serum

IFN : Interferon

Ig : Immunoglobuline

IL : Interleukine

IMDM : Iscove's Modified Dulbecco's Medium

iNOS : Inducible Nitric Oxyde Synthase

iv : Intravenous

LDU : Leishman-Donovan Units

MACS : Magnetic-activated cell sorting

mg : Milligram

MHC : Major histocompatibility complex

mM : Millimolar

ml : Milliliter

ng : Nanogram

ns : Non significant

PALS : Periarteriolar Lymphoid Sheaths

PB : Pacific blue

PBS : Phosphate Buffered Saline

PCR : Polymerase Chain Reaction

PE : Phycoerythrine

PECy7 : Phycoerythrine cyanin 7

PerCP : Peridinin Chlorophyll Protein Complex

p.i : Post-infection

PSG : Penicillin-streptomycin-glutamin

rpm : Rotations per minute

RAG$^{-/-}$: Rature lymphocyte-deficient mouse

RPMI: Roswell Park Memorial Institute medium

ROS : Reactive Oxygen Species

RT-PCR : Real-time polymerase chain reaction

SEM : Standard Error of the Mean

SN : Supernatant

TCR : T Cell Receptor

TGF : Transforming growth factor

Th1 : Type 1 T helper cells

Th2 : Type 2 T helper cells

Th17 : Type 17 T helper cells

TLR : Toll-like receptor

TNF : Tumor necrosis factor

Tr1 : Type 1 T regulatory cells

VL : Visceral leishmaniasis

WT : Wild-type

CHAPTER 1. INTRODUCTION

1.1. Visceral Leishmaniasis

1.1.1. Leishmaniasis

The leishmaniasis are a set of vector-bone parasitic diseases, which are considered as a major public health problem worldwide. This disease affects an estimated 12 million people in 88 countries, has an approximate annual incidence of 700 000 to 1 million cases and still causes yearly 20,000–30,000 deaths (World Health Organization, 2018). Leishmaniasis belongs to the most neglected tropical and sub-tropical diseases, affecting populations in the poorest areas, who have difficulty to access diagnosis and effective treatment. Leishmanisis patient present a wide range of symptoms, from the most common form that is self-healing cutaneous to visceral form, the most severe manifestation of leishmaniasis, for which the mortality rate approaches 100 percent if left treated. The drugs used to treat leishmaniasis have many limitations including the long course of treatment and severe side-effects, and are associated with development of resistance. To date, no vaccine against *Leishmania* is available. Human leishmaniasis is caused by about 20 of the over 30 identified species of *Leishmania* (Rodrigues *et al.*, 2016). There are 3 major medical conditions of the disease:

- Cutaneous leishmaniasis (CL), the most common form, is characterized by a single or multiple transient skin lesions on exposed area of the body (papule or ulcer) that often heal spontaneously in immunocompetent hosts and leave a scar, although the parasites will persist in small quantities throughout life. Approximated 95% of CL cases occur in the Mediterranean basin, Americas, the Middle East and Central Asia (**Fig. 1A**).
- Mucotaneous leishmaniasis (ML) can cause partial or total destruction in the membrane of the mouth, the nose or on the genital mucosa. About 90% of mucocutaneous leishmaniasis cases occur in Bolivia, Brazil, Ethiopia and Peru.
- Visceral leishmaniasis (VL), the life-threating form, also known as kala-azar, is caused by parasites of *L. donovania*/*L. infantum* (also called *L. chagasi*) complex in the Old World and by *L. infantum* in the New World. Visceral leishmaniasis is clinically comparable in both the Old and New World and is characterized by weight loss, irregular bouts of fever, hypergammaglobulinemia, hepatosplenomegaly, and immunosuppression. The annual incidence of VL is estimated to be about 50 000 to 90 000 cases. Most of the cases of VL appear in East Africa, Brazil and South-East Asia (**Fig. 1B**) (World Health Organization, 2018).

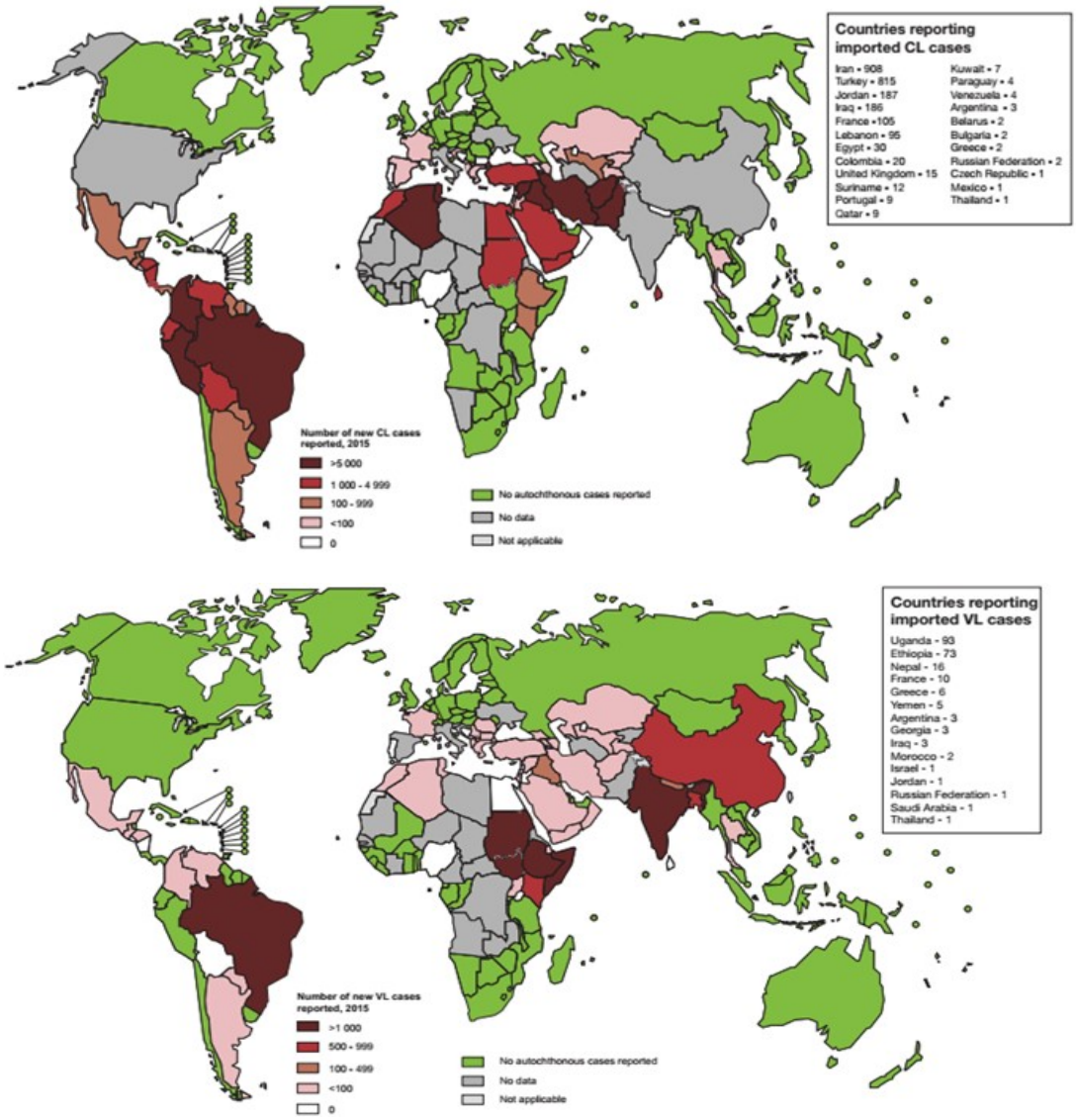


Figure 1.Status of endemicity of cutaneous (A) and visceral leishmaniasis (B) worldwide, 2015. Figure is from (World Health Organization, 2018)

The leishmaniases are mainly zoonoses that are naturally transmitted between animals and humans by the bite of infected sandflies. Domestic and wild animal can be reservoir hosts; wild animals carry the parasite but do not develop disease, whereas dogs can develop symptoms of leishmaniasis. Leishmaniasis can also be transmitted anthroponotically, with human populations being the main reservoir for infection. Anthropotic transmission of cutaneous and visceral leishmaniasis is significant in the Indian subcontinent, in East Africa and in urban regions.

1.1.2. Life cycle of *Leishmania*

Leishmania parasites are transmitted between hosts (human, dogs, rodents, etc.) by the bite of infected female phlebotomine sandflies. The vectors of leishmaniasis are species and

subspecies of *Phlebotomus* in the Old World and *Lutzomyia* in the New World. *Leishmania* have a dimorphic life cycle and undergo dramatic morphological transformation to adapt and survive in the hosts (**Fig. 2**). Within the digestive tract of the sandfly vector, *Leishmania* exist as extracellular, flagellated and motile promastigostes, while in the host they inhabit and replicate as non-flagelleted, non-motile intracellular amastigostes within the phagolysosomal vacuoles of phagocytes (Kaye & Scott, 2011). During the blood meal, parasites are regurgitated and delivered to the host as promastigostes in the saliva of sandflies, together with parasite-derived mucin-rich gel in the sandfly midgut. *Leishmania* parasites are then phagocytosed by one of the phagocytes in the local environment and subsequently establish residence inside macrophages. Inside the macrophages, the metacyclic promastigostes transform into, and replicate as, aflagellated amastigotes within phagosomal vascuoles. The constant replication of amastigotes inside infected phagocytic cells causes cell death by apoptosis. The released amastigotes are then taken up by the neighboring phagocytic cells. The transmission cycle is completed when another sandfly takes up infected macrophages with the blood meal. In the midgut of sandflies, the amastogotes are rapidly releases from the phagocytes and convert into promastigotes (Kaye & Scott, 2011).

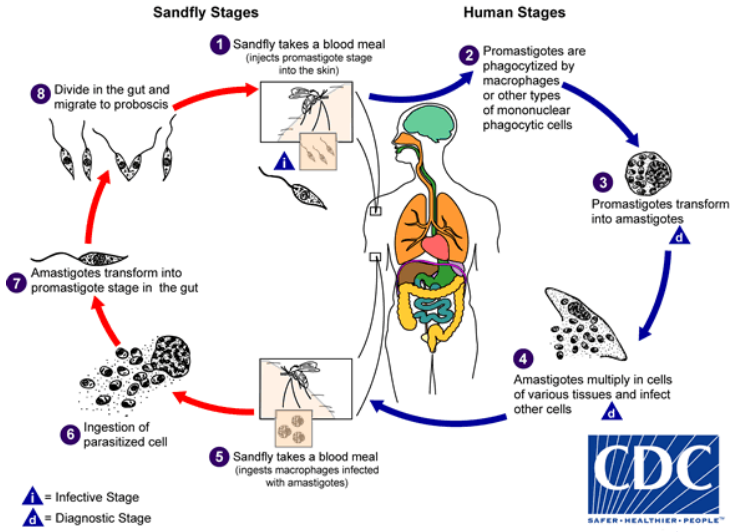


Figure 2.*Leishmania* life cycle.

Leishmania promastigotes develop in the midgut of female sand flies to the metacyclic infectious promastigote population. Upon a blood meal, parasites are entering the human host, reaching their host mononuclear phagocytes, mainly macrophages. In here, promastigotes develop into the disease propagating amastigote form, re-infecting new cells. Upon a following blood meal, amastigotes are ingested and redifferentiation in the sandfly to promastigotes occurs, closing the *Leishmania* life cycle.

1.1.3. Pathogenesis of VL

In humans, *L. donovani* and *L. infantum* establish infection in the mononuclear phagocyte system of visceral organs, mostly the liver, spleen and bone marrow. During the incubation

period that can last between 2 and 18 months, VL is manifested by irregular fevers, significant weight loss, enlargement of the spleen (splenomegaly) and sometimes liver (hepatomegaly). Moreover, when the infection occurs in the bone marrow, patients develop pancytopenia or deficiency in red blood cells, white cells and platelets production of the bone marrow (Barrett & Croft, 2012; Ready, 2014). The disease gradually brings the infected individuals into the immunosuppression state that makes them vulnerable to opportunistic infections. The disease is progressive and become systemic infection, potentially lethal if left treated, with a fatality rate of 75-95% (Ready, 2014).

Post-kala-azar dermal leishmaniasis (PKDL) or dermal leishmanoid, which develops in patients that recovered from VL, is characterized by dermal lesions that can be heavily parasitized. PKDL occurs mainly in *L. donovani*-infected patients in the Indian subcontinent and in Sudan (Zijlstra *et al.*, 2003). The lesions usually begin around the mouth and can spreads to other parts of the body.

1.1.4. Diagnosis and treatment of VL

The gold standard for *Leishmania* diagnosis is the presence of amastigotes in a tissue biopsy (Kumar & Nylen, 2012). Amastigotes in the samples can be detected using microscopic indentification as the first step. More recently, the development of PCR became the fist-line diagnostic method in hospital and research centers to detect the presence of *Leishmania,* because of its high sensitivity and easy manipulation. Other diagnostic tools such as serological test like ELISA, Western blot or direct agglutination and immunochromatography using the rk39 antigen are also used for detecting VL in combination with clinical symtoms (Barrett & Croft, 2012).

Fortunately, VL can be treatable in most cases. The most well-known drug against VL is pentavalent antomonials (SbV), which has been the standard medicine for 70 years. Although resistance to SbV is common in India, Pentostam and Glucantime remain the effective treatment in the rest of the world. Amphotericin B is usually used as the second-line of VL treatment and becomes the first-line in the areas with SbV resistance. Additionally, Miltefosine has also been used as the first oral treatment for VL (Barrett & Croft, 2012). However, all the current drugs used in VL treatment are associated with toxicity, severe side effects and high cost. To date, there is no effective anti-leishmanial vaccine. Thus, there is an urgency to develop an effective vaccine to reduce costs and toxicity of the treatment (Kumar & Nylen, 2012).

1.1.5. Experimental models of VL

1.1.5.1. Dog model

Wild canines and domestic dogs are the main natural hosts of visceral leishmaniasis caused by *L. infantum* infection. Since they are the main reservoir of these parasites, they can be used as a highly relevant model to study VL. Moreover, *L. donovani* also can establish infection in the dog, which displays pathologies similar to humans (Loria-Cervera & Andrade-Narvaez, 2014). Because of high cost and ethics, dogs are not typically used widely, only in special experiments. Small rodents are the preferred model (Kumar & Nylen, 2012).

1.1.5.2. Hamster model

Syrian golden hamsters (*Mesocricetus auratus*) are highly susceptible to both *L. donovani* as well as *L. infantum* (Keithly, 1976; Requena *et al.*, 2000). Hamsters are considered the best experimental model to study visceral leishmaniasis, since they can develop similar clinicopathologic features and immunopathologic mechanisms as humans. Indeed, in these animals the visceral parasite burdens progressively increase, eventually cause death of the host (Gifawesen & Farrell, 1989; Melby *et al.*, 2001; Requena *et al.*, 2000). As in humans, an up-regulated expression of Th1-associated cytokines (IFNγ, TNF and IL-12) and down regulation in Th2-like cytokine (IL-4) expressions were observed in the spleen of *L. donovani*- infected hamsters (Melby *et al.*, 2001). However, hamsters poorly produce IFNγ-inducible NOS2 enzyme, which is strictly involved in the generation of reactive nitrogen intermediates like NO, a well-known anti-leishmanial factor. This defect can explain the incapacity to control *Leishmania* infection in hamsters (Melby *et al.*, 2001; Wilson *et al.*, 2005). Moreover, a considerable amount of the macrophage deactivating cytokine IL-10 is progressively observed in visceral organs, which parallels the IL-10 expression observed in spleen and bone marrow of VL patients (Karp *et al.*, 1993). However, the use of hamsters as a model is still limited due to the lack of immunological tools such as antibodies, cytokine or cell markers.

1.1.5.3. Mouse model

To date, most of our current understanding about regulation of immunity during VL was achieved from the results of experiments using the mouse model. Experimental VL can be established initially by intravenous injection of *L. donovani* or *L. infantum* amastigotes with a dose of 10^7 or higher, while natural infection can be mimicked by a lower dose intradermal

injection (Ahmed *et al.*, 2003; Kaye *et al.*, 2004). Susceptibility or resistance to *L. donovani* infection is determined by genetic factors that expressed in both humans and mice. In mice, activation of macrophage antimicrobial mechanisms is controlled by the expression of *Slc11a1* gene (formerly *Nramp1*) which encode for phagosomal iron and manganese transporter (Stanley & Engwerda, 2007). Genetically resistant mouse strains (for example CBA) express a functional *Slc11a1* gene, which controls the early parasite growth through a mechanism distinct from adaptive immune responses and phagocyte oxidase activity or inducible nitric oxide synthase (Jabado *et al.*, 2000; White *et al.*, 2005). In contrast, susceptible mouse strains possess a nonfunctional *Slc11a1* gene by having a (Glysine → Aspartic)$_{169}$ amino acid mutation. Genetically vulnerable to *L. donovani* mouse strain include BALB/c and C57BL/6 are commonly used for experimental VL (Stanley & Engwerda, 2007). Although the parasite burden in the liver of *Slc11a1* mutant mice is higher 50-100 time than of functional *Slca1* mice during first 2 weeks of infection, most genetic susceptible mice are able to control parasite growth in the liver after this time (Kaye *et al.*, 2004). The resolution efficiency depends on MHC- II haplotypes (H-2 loci) (Kumar & Nylen, 2012).

In mice, the immune response to *L. donovani* or *L. infantum* can be distinct between organs (liver, spleen and bone marrow) (Kaye *et al.*, 2004). In the liver, parasites replicate rapidly in the first few weeks of infection following intravenous or intracardiac injection (Kumar & Nylen, 2012). Then, the development of cell-mediated immune responses and granulomas controls parasite growth. Over 2-3 months of infection, infected livers clear the parasite and are subsequently resistant to reinfection (Stanley & Engwerda, 2007). In contrast, the parasite burden in the spleen increases slowly but progressively over a longer period of time. Parasite persistence is associated with disruption of the splenic architecture and impairment of immune responses (Bankoti & Stager, 2012). Eventually, splenic parasite replication is also controlled and the parasite burden plateaus (Engwerda *et al.*, 2004a).

1.2. Regulation of immunity in the *L. donovani* experimental model

Experimental infection with *L. donovani* is characterized by distinct organ-dependent immune responses. The liver is a self-healing acute site with minimal tissue disruption and resistance to reinfection, while chronic infection is established in the spleen and the bone marrow with serious tissue damage. In general, IFNγ secretion by both $CD4^+$ and $CD8^+$ T cells is required to control parasite growth.

1.2.1. The self- healing infection in the liver

In the liver, resident Kupffer cells are the main target for *L. donovani* (McElrath *et al.*, 1988). In the first few weeks of infection, the anti-leishmanial capacity of Kupffer cells is impaired and the number of parasites rapidly increases (Rodrigues *et al.*, 2016). Parasite clearance in the liver depends on the formation of granulomas. This is initiated by the production of chemokines by infected Kupffer cells, followed by the recruitment of monocytes, neutrophils, $CD4^+$ and $CD8^+$ T cells, secretion of inflammatory cytokines and ultimately activation of anti-parasitic mechanisms in the infected Kupffer cells (Stanley & Engwerda, 2007). Following *L. donovani* infection, several chemokines are rapidly released including C-C motif ligand- 2, CCL2 (monocytes chemoattractant protein, MCP-1), CCL3 (macrophage inflammatory protein- 1α, MIP-1α) and C-X-C motif chemokine- 10, CXCL10 (IP-10) by infected Kupffer cells (Cotterell *et al.*, 1999). The chemokine productions are required to attract immune cells including neutrophils and monocytes, which play an essential role in the initiation and maintenance of the anti-leishmanial defense (Cervia *et al.*, 1993; Smelt *et al.*, 2000). Other cells of the innate immune system, particularly natural killer (NK) cells, NK T cells, and invariant NK (iNKT) cells also play an important role in initiating granuloma formation by their ability to produce a large amount of inflammatory cytokines such as IFNγ (Amprey *et al.*, 2004; Svensson *et al.*, 2005). Moreover, both iNKT cells and IFNγ are essential for CXCL10 production, which attracts $CD4^+$ and $CD8^+$ T cells to eventually establish granuloma maturation (Amprey *et al.*, 2004; Beattie *et al.*, 2010; Svensson *et al.*, 2005). After one week of infection, $CD4^+$ and $CD8^+$ T cells are increasingly recruited to the granuloma and subsequently become predominant immune cells (Stern *et al.*, 1988).

Following *L. donovani* infection, several cytokines play a critical role in granuloma formation and parasite clearance, including IL-12, IFNγ, TNF and lymphotoxin (LT) (Stanley & Engwerda, 2007). IFNγ production by Th1 responses, which is initiated by IL-12 production by dendritic cells (DCs), plays a critical role in the development of granulomas in the liver (Gorak *et al.*, 1998; Scharton-Kersten *et al.*, 1995). The importance of IFNγ has been demonstrated by using IFNγ-deficient mice (Taylor & Murray, 1997), anti-IFNγ antibody-treated mice (Squires *et al.*, 1989), or treatment with IFNγ (Taylor & Murray, 1998). In granulomas, IFNγ enhances anti-leishmanial mechanisms of infected Kupffer cells (Stanley & Engwerda, 2007). However, anti-leishmanial mechanisms can also be activated by TNF, which can moderately compensate for IFNγ absence (Taylor & Murray, 1997). TNF, which can be observed at both early and late stages of infection, plays a crucial role in immune cell recruitment and maturation of hepatic granulomas (Engwerda *et al.*, 1996; Rodrigues *et al.*, 2016). Following T cells activation, reactive oxygen intermediates (ROI) and reactive

nitrogen intermediates (RIN) are generated by infected hepatic macrophages (Stanley & Engwerda, 2007). In TNF-deficient mice, *L. donovani* amastigotes developed unrestrained due to lack of granuloma development. These mice eventually die because of severe hepatic necrosis after 2 months of infection (Murray *et al.*, 2000). Another TNF-related cytokine, LTα has been shown to induce leukocyte recruitment to infected Kupffer cells (Engwerda *et al.*, 2004b). However, unlike TNF, which is necessary for host long-term survival, LTα is required for controlling the parasite growth in the first 2 weeks of infection (Engwerda *et al.*, 2004b). Although both TNF and LTα contributes to cell migration into the sinusoidal area of infected liver, they have distinct roles, since TNF is required for leukocytes recruitment to the liver and LTα induces leukocyte recruitment from periportal areas by promoting VCAM-1 expression on sinusoid lining cells (Engwerda *et al.*, 2004b). Finally, IL-12 secreted from DCs is essential for the development of protective immunity to *L. donovani* and the induction of both IFNγ production and hepatic granuloma development *(Bankoti & Stager, 2012)*. Additionally, the treatment using exogenous IL-12 to infected mice significantly reduced parasite burden in the liver (Murray & Hariprashad, 1995).

1.2.2. The establishment of persistent infection in the spleen

The spleen is a highly organized lymphoid organ. This organ consists of a white and red pulp area, which are separated by a third compartment, the marginal zone. The white pulp of the spleen comprises lymphoid tissue, the bulk of which is arranged around a central arteriole to form the periarteriolar lymphoid sheaths (PALS), which are composed of T and B cells. The red pulp consists of venous sinuses and cellular cords, which contain, among others, resident macrophages, granulocytes, lymphocytes, platelets, and erythrocytes. The marginal zone (MZ) contains marginal zone B cells, macrophages and dendritic cells (Cesta, 2006). Due to the contact between the spleen and the circulation system, the spleen is responsible for initiating immune responses to systemic infection. Specially, the spleen is a crucial site to generate immune responses during the acute phase of experimental *L. donovani* infection as well as disease resolution in the liver. However, protective immune reactions are also possibly generated in other tissue such as bone marrow or hepatic lymph node (Stanley & Engwerda, 2007).

In contrast to the liver, the spleen is the site of persistent infection, associated with the failure to generate efficient immune responses and the disruption of the splenic microarchitecture (Ato *et al.*, 2002; Engwerda *et al.*, 2002). In the acute phase, parasite burden increases slowly due to the effective parasite control of immune responses (Kaye *et al.*, 2004; Stanley & Engwerda, 2007). Following *L. donovani* infection, amastigotes are rapidly taken up and

removed from the blood stream by marginal zone macrophages (MZM) and marginal zone metallophilic macrophages (MMM) (Gorak *et al.*, 1998). Parasites are sometime observed in the white pulp area, commonly PALS, which suggests they possibly reside in DCs (Gorak *et al.*, 1998). An early upregulation of IL-12 production by DCs was also observed following infection, which is reflected by the increase of IL-12p40 subunit (Gorak *et al.*, 1998). This IL-12p40 production is mainly localized to the PALS region, and partially in the MZ, demonstrating that following antigen acquisition and activation in the MZ, DCs migrate to the PALS, which is required for full activation of DCs (Ato *et al.*, 2006). The IL-12p40 secreted by DCs then induces the activation of $CD4^+$ and $CD8^+$ T cells (Stanley & Engwerda, 2007). Following IL-12 stimulation, naïve $CD4^+$ T cells polarize toward IFNγ-secreting $CD4^+$ T cells (Th1). IFNγ plays a crucial role in macrophage activation and the induction of intracellular anti-parasitic mechanisms (Taylor & Murray, 1997). DCs migration to the PALS area is regulated by CCR7 ligands CCL19 and CCL21 (Ato *et al.*, 2006). DCs and naïve $CD4^+$ T cells express high level of CCR7, which is essential for the migration to PALS according to CCL19 and CCL21 gradients secreted by $gp38^+$ stromal cells and the central arteriole (Stanley & Engwerda, 2007). In mice lacking these chemokine or following chemokine signaling blockade, DCs migration and activation are significantly impaired, resulting in a remarkable reduction of IL-12 production in these mice compared to wild-type mice. Eventually, chemokine-deficient mice are more susceptible to *L. donovani* than wild-type mice (Ato *et al.*, 2006). Thus, DCs migration from MZ to PALS is crucial for their full activation as well as induction of protective immunity to *L. donovani*.

After d14 of infection, disruption of splenic microarchitecture and severe splenomegaly occur. This is followed by impairment of immune responses and the establishment of persistent infection (Bankoti & Stager, 2012; Engwerda *et al.*, 2002). Although TNF is required for the maturation of granulomas in the liver, it contributes to the establishment of chronic infection in the spleen by promoting MZ disruption, decreasing DCs migration to the PALS, and subsequently impairing DCs activation (Engwerda *et al.*, 2002; Stanley & Engwerda, 2007). TNF, possibly secreted by macrophages, partially mediates MZ reorganization with selective depletion of MZM though a not yet clear mechanisms that may involve apoptosis (Engwerda *et al.*, 2002; Murray *et al.*, 2000). TNF is also responsible for the disorganization of the PALS and the loss of $gp38^+$ stromal cells (Ato *et al.*, 2002). Destruction of stromal cells associates with the loss of their chemokine productions, including CCL19 and CCL21 (Ato *et al.*, 2002). These chemokines are well-known chemoattractants of CCR7-expressing cells, which include mature DCs, naïve $CD4^+$ T cells and a subset of memory T cells (Cyster, 1999; Zlotnik & Yoshie, 2000). Additionally, elevated TNF in the spleen promotes the expression of IL-10, an immune suppressor that

directly induces CCR7 downregulation of DCs, inhibiting their migration to the PALS (Ato *et al.*, 2002). During chronic VL, DCs not only show impaired migratory capacity but also upregulate the inhibitory receptor B7-H1 (Joshi *et al.*, 2009). B7-H1-expressed by DCs binds to PD-1 on the T cell surface and inhibits their proliferation and cytokine production (Bankoti & Stager, 2012). Upregulation of B7-H1 in DCs partially contributes to the dysfunction and depletion of $CD8^+$ T cells during chronic VL (Joshi *et al.*, 2009). Moreover, disruption of the splenic structure is associated with the loss of the follicular DC (FDC) network, which is essential for the development of B cell germinal centers (GC). Thus, FDC destruction is associated with the loss of GC, which profoundly affects B cell functions and contributes to hypergammaglobulinaemia typically during chronic VL (Smelt *et al.*, 1997). Another major factor contributes to immune suppression in the spleen is IL-10. In VL patient, IL-10 was detected at high levels in the serum, splenic aspirates, bone marrow and lymph nodes (Rodrigues *et al.*, 2016). Additionally, IL-10 deficient mice are highly resistant to *L. donovani* infection (Murphy *et al.*, 2001) and IL-10 receptor blockade results in increased IFNγ expression (Murray *et al.*, 2003). During *L. donovani* infection, IL-10 can be expressed by various immune cell type including T cells, B cells, macrophages, NK cells, and DCs (Rodrigues *et al.*, 2016). However, $CD25^-FoxP3^-CD4^+$ T (Tr1) cells which also produce IFNγ are the main source of IL-10 production during chronic VL in mice and humans (Nylen *et al.*, 2007; Stager *et al.*, 2006). As an immunosuppressive cytokine, IL-10 inhibits immune responses by deactivating anti-leishmanial mechanisms and the expression of costimulatory receptors in macrophages, inhibiting IFNγ production in T cells, and impairing DC migration to the PALS (Rodrigues *et al.*, 2016; Stanley & Engwerda, 2007).

During the experimental model of VL, humoral immune responses also become dysfunctional and IgM, complement, and IL-10 secreted by B cells contribute to disease exacerbation (Bankoti *et al.*, 2012; Deak *et al.*, 2010) Interestingly, mice lacking B cells are highly resistance to *L. donovani* (Smelt *et al.*, 2000). Moreover, depletion of MZ B cells enhances both $CD4^+$ and $CD8^+$ T cell responses resulting in decreased parasite load (Bankoti *et al.*, 2012). In infected mice, B cells undergo polyclonal activation. This activation contributes to disease exacerbation and results in hypergammaglobulinemia (Bankoti *et al.*, 2012; Silva-Barrios *et al.*, 2016). However, circulating IgGs during infection are non-specific to *Leishmania* (Deak *et al.*, 2010). Recently, it was demonstrated that amastigotes activate B cells via endosomal Toll-like receptors (TLRs), thus leading to the production of several cytokines including predominantly IFN-I and IL-10. The combined action of these two molecules seems to be responsible for hypergammaglobulinemia (Silva-Barrios *et al.*, 2016).

In our lab, we also showed that, although IRF-5 contributes to protective immune responses in the liver during *L. donovani* infection (Paun *et al.*, 2011), this transcription factor seems to

be involved in the establishment of persistent infection in the spleen. The inflammatory environment in the spleen during the acute phase induces IRF-5 activation, which promotes HIF-1α expression in DCs. HIF-1α upregulation in DCs inhibits the expression of IL-12, while promoting IL-10 transcription, which prevents antigen-specific $CD8^+$ T cells expansion and their effector function, resulting in disease exacerbation (Hammami *et al.*, 2018; Hammami *et al.*, 2015). Over the course of infection, IRF-5 expression is significantly elevated in IFNγ-producing $CD4^+$ T cells. Interestingly, upregulation of IRF-5 in Th1 cells was induced by tissue disruption. Indeed, apoptotic material triggered the TLR7 signaling pathway (Fabie *et al.*, 2018), leading to the upregulation of IRF-5 and the induction of DR5, which resulted in apoptosis of protective $CD4^+$ T cells. Another transcription factor, HIF-1α, also contributes to immune suppression by enhancing inhibitory functions of myeloid cells (Hammami *et al.*, 2017). Thus, activation of IRF-5 and HIF-1α may contribute to the suppression of protective $CD4^+$ and $CD8^+$ T cell responses, which favors the establishment of parasite persistence.

1.2.3. Immune responses in the bone marrow

Similarly to the spleen, *L. donovani* amastigotes also establish persistent infection in the bone marrow (Cotterell *et al.*, 2000b). During *L. donovani* infection, the hematopoietic capacity is significantly enhanced in the bone marrow and spleen of infected mice and is associated with the establishment of persistent infection (Cotterell *et al.*, 2000b). A rapid but transient mobilization of myeloid progenitor cells into the bloodstream is induced by the parasite, accompanied with increased levels of GM-CSF and MIP-1α production. The dramatical multiplication of parasites in the spleen and bone marrow after two weeks of infection is associated with an increase in both frequency and hematopoietic activity of progenitor cells, including CFU-granulocyte and monocyte in these organs (Cotterell *et al.*, 2000b). Both *in vivo* and *in vitro* experiments revealed that *L. donovani* directly targets bone marrow stromal macrophages but not hematopoietic stem cells (HSC) or myeloid progenitor cells (Cotterell *et al.*, 2000a). Moreover, parasites were also found in myeloblast-like cells and monocytes in the bone marrow (Abidin *et al.*, 2017). Infection of stromal macrophages induces the production of GM-CSF and TNF, which increases their capacity to promote myelopoiesis (Cotterell *et al.*, 2000a). Moreover, *L. donovani*-induced myelopoiesis promotes the differentiation of non-classical myeloid progenitors with a regulatory phenotype, which expressed high levels of regulatory markers including Sca1, MHC-II, IL-10 and Galectin-3 (Abidin *et al.*, 2017). These data demonstrate that, *L. donovani* establishes persistent parasite infection in the bone marrow by inducing emergency hematopoiesis (Abidin *et al.*, 2017). Additionally, unlike the long term HSC (LT-HSC) from naïve mice, which mostly remain in a quiescent state, the majority LT-HSC from *L. donovani* infected

mice progress to cell-cycle, resulting in functional exhaustion (Pinto *et al.*, 2017). Critically, the active proliferation of LT-HSC from infected mice was promoted by IFNγ-expressing $CD4^+$ T cells with resident effector phenotype in the bone marrow. Interestingly, the differentiation of pathogenic $CD4^+$ T cells was induced by intrinsic TNF signaling, but not intrinsic IFNγ pathway (Pinto *et al.*, 2017).

1.3. The role of myeloid cells in *L. donovani* infection

1.3.1. Neutrophils

Neutrophils, the most abundant of granulocytes, are an essential factor of the innate immune response because of their contribution to the elimination of various pathogens. However, their specific role in the immune response to *L. donovani* has not been clearly elucidated. During *L. donovani* infection, neutrophils are continuously recruited to the spleen and liver (Hammami *et al.*, 2017). In the liver, neutrophils and monocytes are initially recruited to promote granuloma formation in a chemokine-dependent manner within the first few days of infection (Stanley & Engwerda, 2007). Neutrophils seem to contribute to protective immune responses to *L. donovani*. Indeed, selective depletion of neutrophils using NIMP-R14 monoclonal antibody resulted in significantly increased parasite burden in the spleen, liver and bone marrow (McFarlane *et al.*, 2008). Enhanced susceptibility to the parasite is linked to the prevention of hepatic granuloma development, splenomegaly, and lower iNOS expression in granulomas. In the spleen of neutrophil-depleted mice, high levels of IL-10 and IL-4 were observed. In these mice, protective immune responses were also lower than those in wild-type mice (McFarlane *et al.*, 2008). However, the mechanisms by which neutrophils control parasite growth is still unclear. However, our study revealed that during chronic VL, splenic neutrophils expressed similar markers to polymorphonucleated-myeloid-derived suppressor cells (PMN-MDSC) and could therefore be involved in inhibiting protective immune responses (Hammami *et al.*, 2017).

1.3.2. Monocytes/macrophages

Similarly to neutrophils, $Ly6C^{hi}$ inflammatory monocytes have been shown to increasingly migrate to the spleen and liver over the course of VL and contribute to parasite survival during *L. donovani* infection (Hammami *et al.*, 2017; Terrazas *et al.*, 2017). Recruited monocytes play a detrimental role in VL and contribute to immune suppressive responses (Hammami *et al.*, 2017; Terrazas *et al.*, 2017). The recruitment of inflammatory monocytes is regulated via STAT1 signaling pathway (Terrazas *et al.*, 2017). $Ly6C^{hi}$ monocyte migration

blockade leads to a remarkable reduction of the parasite burden in both spleen and liver and is associated with a lower frequency of IFNγ^{+} IL-10^{+} Tr1 cells (Terrazas *et al.*, 2017). Although monocytes from infected spleen and liver differentiate into macrophage lineage but not DCs, they have distinct phenotypes. Indeed, hepatic monocytes upregulate iNOS and TNF, which are essential for disease resolution, and splenic monocytes express high level of arginase that is associated with parasite survival (Hammami *et al.*, 2017; Terrazas *et al.*, 2017). Moreover, monocytes also acquire an MDSC-like phenotype in a HIF-1α- dependent manner in the spleen during chronic VL (Hammami *et al.*, 2017).

Under inflammatory or infectious conditions, blood monocytes are recruited to inflamed sites, where they then can differentiate into macrophages and/or dendritic cells (DCs). Two well-known phenotypes of macrophages have been described as classically activated macrophages or pro-inflammatory (M1) macrophages and alternatively activated macrophages or anti-inflammatory (M2) macrophages. M1 and M2 macrophages are modeled *in vitro* by IFNγ or lipopolysaccharide (LPS) and IL4 and/or IL-13, respectively (Lawrence & Natoli, 2011; Mosser & Edwards, 2008). M1 macrophages have functions of antigen presenting cells and are associated with anti-microbicidal activity because of their increased expression of IL-12, MHC-II and nitric oxide synthase 2 following IFNγ stimulation. In contrast, M2 macrophages are associated with parasitic infection, wound healing and lipid metabolism and are identified by markers such as FIZZ1, arginase 1, IL-10 and mannose receptor 1 (or CD2060) (Gordon & Martinez, 2010).

In the case of *L. donovani* infection, macrophages play a dual role: on one hand macrophages house *Leishmania,* which survives and replicates inside macrophages, suppressing their anti-leishmanial capacity (Arango Duque & Descoteaux, 2015); on the other hand, activated macrophages are the major cells involved in parasite elimination (Stanley & Engwerda, 2007). Macrophages destroy intracellular parasites by the synthesis of various mediators of the inflammatory reaction such as cytokines (TNF, IL-1, IL-6, IL-12), reactive oxygen and nitrogen species, which eventually lead to intracellular lysis (Stanley & Engwerda, 2007). In order to fully acquire their leishmanicidal effector functions, macrophages must be properly stimulated by signals present in the microenvironment. In fact, stimulation with cytokines associated with Th1 lymphocytes, especially IFNγ, enables them to acquire an effector function against intracellular pathogens. These activated macrophages are referred to M1 phenotype. Conversely, if they are stimulated by a Th2-associated cytokine, i.e. IL-4, IL-13, IL-33, TGF-β and IL-10, there is polarization towards an M2 phenotype or otherwise called alternative activation (Mantovani *et al.*, 2013). This phenotype is able to prevent protective immunity against *L. donovani* (Mukhopadhyay *et al.*, 2015). Recently, our results demonstrated that HIF-1α upregulation induces the M2-like

phenotype and inhibited anti-leishmanicidal functions of macrophages during *L. donovani* infection (Hammami *et al.*, 2017). The importance of this duality between phenotype M1/M2 during VL requires further clarification and therefore more investigation.

1.3.3. Dendritic cells

In case of experimental VL, DCs also play a dual role: they are not only required for the development of protective immune responses but are also involved in parasite survival (Kaye & Scott, 2011). Indeed, DCs are essential for the implementation of protective Th1 responses (Kaye *et al.*, 2004); their IL-12 production plays a crucial role for the development of IFNγ$^+$ CD4$^+$ T cells (Stager *et al.*, 2006). Moreover, splenic DCs also produce IL-23p19 subunit (Maroof & Kaye, 2008), which in combination with IL-12p40 forms active IL-23. IL-23 in combination with other cytokines present in the splenic microenvironment promotes the differentiation of Th17 CD4$^+$ T cells that appear to have a rather important role in the resolution of infection (Ghosh *et al.*, 2013).

In contrast, DCs can also play an inhibitory role. Beside macrophages, amastigotes can also be detected in DCs (Kaye & Scott, 2011). As described above, during VL, DCs migration and function are inhibited, which favors chronic infection. Specifically, *L. donovani* has been demonstrated to inhibit DCs maturation and prevent their expression of CCR7, which results in the impairment of their migration into PALS (Ato *et al.*, 2002). Furthermore, the combination of cytokine productions of infected DCs including IL-12, IL-27, and IL-10 promotes IFNγ/IL-10 co-producing Tr1 development that inhibit the leishmanicial capacities of macrophages (Owens *et al.*, 2012). Additionally, the inflammatory environment during VL inhibits the production of IL-12 and enhances the production of IL-10 by DCs, which limits the expansion of CD4$^+$ and CD8$^+$ T cells (Hammami *et al.*, 2018; Hammami *et al.*, 2015). Finally, DCs upregulate the inhibitory molecule B7-H1, which promotes CD8$^+$ T cells exhaustion (Joshi *et al.*, 2009).

Thus, the inflammatory responses are dramatically increased during *L. donovani* infection, and results in a significant recruitment of immune cells. The transcription factor IRF-5 has been demonstrated to induce the expression of several proinflammatory cytokines in responses to viral infection and PRRs activation in various innate immune cells. We have also shown that the IRF-5 largely regulate the inflammatory response during experimental *L. donovani* infection. Thus, in this study, we aimed to specifically identify the role of IRF-5 expression in myeloid cells during VL.

1.4. Interferon regulatory factor 5

1.4.1. The interferon regulatory factor family

The family of transcription factor interferon regulatory factor (IRF) consists of nine members (IRF-1-9), which were originally identified based on their involvement in type I interferon (IFNI) induction (Miyamoto *et al.*, 1988; Wathelet *et al.*, 1998). Since then, they have also been described as regulators of many biological processes related to various diseases, oncogenesis and hematopoiesis differentiation, such as innate and adaptive immune responses, cell cycle and apoptosis, and metabolism, (Barnes *et al.*, 2002a; Eguchi *et al.*, 2008; Taniguchi *et al.*, 2001; Yanai *et al.*, 2012; Zhao *et al.*, 2015). The IRFs contain multiple domain proteins that share significant similarity in the conserved N-terminal DNA-binding domain (DBD). This domain, which contains 5 repeated tryptophan residues, recognizes a similar DNA sequence to the IFN-stimulated response element motif (ISRE; G(A)AAANN GAAANN) (Takaoka *et al.*, 2008; Tanaka *et al.*, 1993). The flexible biological activities of IRFs are regulated by the IRF- associated domain (IAD) located at carboxyl-terminal. Beside IRFs' intrinsic transactivation potential, this domain is critically required for specific homomeric and heteromeric interaction of IRF family members or the interaction with other transcription factors or cofactors resulting in activation or repression of their target genes (Chen *et al.*, 2008; Ikushima *et al.*, 2013; Panne *et al.*, 2007; Takaoka *et al.*, 2008). The IAD consists of 2 types: (1) IAD1, which was initially identified in IRF-8 and is conserved in all IRF excluding IRF-1 and IRF-2 and (2) IAD 2, which is shared only between IRF-1 and IRF-2 (Ikushima *et al.*, 2013; Takaoka *et al.*, 2008).

Most members of the IRF family, such as IRF-1, IRF-2, IRF-4, IRF-5, IRF-7, and IRF-8, have been shown to be involved in shaping innate and adaptive immune responses mediated by pattern recognition receptors (PRRs) (Tamura *et al.*, 2008). Moreover, IRF-1, IRF-2, IRF-4, and IRF-8 have revealed their roles in the development and differentiation of various cell types of immune system. Major progress has also identified the essential role of IRF-5 and IRF-7 in autoimmunity and inflammation, as well as IRF-1, IRF-4, IRF-5 and IRF-8 in the control of cell growth, cell metabolism, apoptosis and oncogenesis (Marsili *et al.*, 2016; Tamura *et al.*, 2008).

1.4.2. Diverse functions of IRF-5

IRF-5 was originally demonstrated to be directly involved in inducing type I interferon production by innate immune cells in response to viral infections (Barnes *et al.*, 2001). Since then, many more functions have been ascribed to this transcription factor. For instance, it has been shown to be a mediator of tumor suppression (Hu *et al.*, 2005) and to be

implicated in cell cycle, apoptosis (Barnes *et al.*, 2003; Fabie *et al.*, 2018), fungal and parasitic infections (del Fresno *et al.*, 2013; Paun *et al.*, 2011), and in the regulation of pro-inflammatory cytokines (Krausgruber *et al.*, 2011; Takaoka *et al.*, 2005). IRF-5 has been reported to be expressed in various immune cell types, such as macrophages, neutrophils, conventional dendritic cells (cDC), plasmacytoid dendritic cells (pDC), B cells, and T cells (Fabie *et al.*, 2018; Heng & Painter, 2008; Ishikawa *et al.*, 2015).

The role of IRF-5 in the regulation of the inflammatory response is well documented. In cDCs, plasmacytoid DCs (pDCs) and macrophages, IRF-5 regulates the production of various pro-inflammatory cytokines, including tumor necrosis factor (TNF), IL-6, IL-12, and IL-23, following TLR7 and/or TLR9 stimulation (Takaoka *et al.*, 2005). Moreover, it was shown to be a specific marker for inflammatory (M1) macrophage polarization both *in vitro* and *in vivo* (Krausgruber *et al.*, 2011; Weiss *et al.*, 2013). The important role of IRF-5 in immune responses to microbial infection and inflammation has been revealed using IRF-5 deficient mice. These mice showed resistance to lethal endotoxin shock (Takaoka *et al.*, 2005) as well as susceptibility to *L. donovani* infection, which was associated with an impaired development of Th1 responses and severely decreased inflammatory infiltration (Paun *et al.*, 2011; Takaoka *et al.*, 2005). More recently, IRF-5 was reported to directly mediate apoptosis of $CD4^+$ T cells and hepatocytes in chronic diseases (Alzaid *et al.*, 2016; Fabie *et al.*, 2018). The mechanism of apoptosis induction requires the upregulation of DR5, Fas, tumor necrosis factor related apoptosis-inducing ligand (TRAIL) pathway or loss of mitochondrial membrane potential, all of which are regulated by IRF-5 (Alzaid *et al.*, 2016; Cevik *et al.*, 2017; Fabie *et al.*, 2018; Hu & Barnes, 2009).

IRF-5 has been also described as a tumor suppressor. IRF-5 is directly activated by p53 and translocates to the nucleus upon DNA damage, suggesting a role of this transcription factor in DNA-damage responses (Barnes et al., 2003). IRF-5 inhibits tumor cell growth by promoting pro-apoptotic gene expression, including *p21*, *Bak*, *caspase 8*, *DAP kinase 2*, and *Bax,* in a pathway distinct from p53 (Barnes *et al.*, 2003; Hu *et al.*, 2005). IRF-5 also inhibits the transformation potential of oncogenic viruses like Epstein Barr virus and Kaposi's sarcoma-associated herpes virus and then inhibits the tumor cell growth (Bi *et al.*, 2011b; Xu *et al.*, 2011). It also inhibits the replication of hepatitis C virus and suppresses the progression of hepatocellular carcinoma by dismissing the migration and invasion of hepatocytes (Cevik *et al.*, 2017). Moreover, IRF-5 expression cannot be detected in acute and chronic lymphocytic leukemia, gastrointestinal cancer and is associated with breast cancer development and metastatic spread, suggesting a putative role of IRF-5 as tumor suppressor (Barnes *et al.*, 2003; Bi *et al.*, 2011a; Yamashita *et al.*, 2010). In contrast, there

was a high expression level of IRF-5 in thyroid carcinomas, in which it promotes the proliferation and tumorigenesis potential of thyroid tumor cells (Massimino *et al.*, 2012).

In humans, IRF-5 polymorphisms are associated with increased risk of various autoimmune diseases, such as systemic lupus erythematosus (SLE) (Graham *et al.*, 2006), rheumatoid arthritis (RA) (Dieguez-Gonzalez *et al.*, 2008; Stahl *et al.*, 2010), systemic sclerosis (SSc) (Carmona *et al.*, 2013), multiple sclerosis (MS) (Kristjansdottir *et al.*, 2008), and inflammatory bowel disease (IBD) (Dideberg *et al.*, 2007). In SLE patients, a significant elevation of IRF-5 expression was observed in the blood as compared to healthy donors (Hellquist *et al.*, 2009). Furthermore, augmented IRF-5 expression is linked with the SLE risk haplotype and with elevated type I IFN production in SLE patients (Niewold *et al.*, 2008). The *Irf5*$^{-/-}$ SLE mouse model revealed the importance of IRF-5 in regulating type I IFN levels (Feng *et al.*, 2012), limiting Th2 responses (Feng *et al.*, 2012; Xu *et al.*, 2012), promoting inflammatory monocyte recruitment (Yang *et al.*, 2012), and altering IgG class switching (Feng *et al.*, 2012) and the development of plasma cells (Lien *et al.*, 2010). IRF-5 was also highly expressed in antigen-induced arthritis murine model; in this model IRF-5 regulates neutrophil recruitment to injury sites by controlling the expression and secretion of chemokines (Weiss *et al.*, 2015).

1.4.3. IRF-5 signaling pathway

The microbial sensing by innate immune system is initially mediated by pattern recognition receptors (PRRs); these include Toll-like receptor (TLRs), RIG-like receptors (RLRs), NOD-like receptors (NLD) and C-type lectin receptors (CLR) (Takeuchi & Akira, 2010). Upon recognition by these receptors of a variety of pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs), the intracellular signaling cascades are triggered, which results in the transcription of inflammatory mediators and ultimately the elimination of pathogens and infected cells (Takeuchi & Akira, 2010). IRF-5 has been shown to be involved in all of these four PRRs signaling pathways upon stimulation by pathogens as well as danger signals (**Fig. 3**) (Chang Foreman *et al.*, 2012; del Fresno *et al.*, 2013; Lazear *et al.*, 2013; Takaoka *et al.*, 2005; Zhao *et al.*, 2015). Chemically-induced DNA damage can also trigger the expression of IRF-5-dependent genes in tumor cell lines (Couzinet *et al.*, 2008; Hu & Barnes, 2009; Hu *et al.*, 2005).

IRF-5 is described as one of the molecules downstream of the Toll-like-receptors/Myeloid differentiation primary response 88 (TLRs/MyD88)-dependent signaling pathway, which results in the induction of the expression of type I interferon and various pro-inflammatory cytokines (Takaoka *et al.*, 2005). This pathway is initiated by triggering intracellular TLRs like

TLR7, TLR8, and TLR9 in innate immune cells (Schoenemeyer *et al.*, 2005; Takaoka *et al.*, 2005). Upon TLRs stimulation, IRF-5 directly interacts with the middle domain (TIR domain) of MyD88 and interleukin 1 receptor-associated kinase 1 (IRAK 1), and is activated by post-translational modification (PTM) such as phosphorylation by IκB kinase β (IKKβ) and polyubiquitination by TNF receptor associated factor 6 (TRAF6) (Balkhi *et al.*, 2008; Lopez-Pelaez *et al.*, 2014). Modified IRF-5 then undergoes dimerization and translocates to the nucleus to trigger cytokine gene transcription (Schoenemeyer *et al.*, 2005; Takaoka *et al.*, 2005). In T cells, IRF-5 can be activated upon triggering of TLR7 by apoptotic cell materials. This activation in turn promotes the expression of the death receptor 5 (DR5) and caspase 8 and ultimately leads to cell death (Fabie *et al.*, 2018). In other studies, NF-κB is shown to share the positive regulators including TRAF6 and IKKβ with IRF-5. Specifically, IRF-5 binds to NF- κB p65 (RElA) in murine macrophages and with p50 in human pDCs, co-regulating the inflammatory activities of these cells (Saliba *et al.*, 2014; Steinhagen *et al.*, 2013). In contrast, IRF-4 can compete and inhibit the activation and physical functions of IRF-5, because of its capacity to bind to the same central domain of MyD88 like IRF-5 (Negishi *et al.*, 2005). IRF-5 PTM is also inhibited by a member of the Src family, Lyn, by directly binding to IRF-5 and suppressing its transcriptional activity (Ban *et al.*, 2016).

RLRs comprise RIG-I, MDA5 and LGP2 and promote innate immune responses mainly against viral infections. The activity of the RLR family requires adaptor mitochondrial antiviral-signaling protein (MAVS) to regulate the transcription of proinflammatory cytokines and type I IFN. RIG-I can recognize various ssRNA viruses, such as influenza A and B virus, Newcastle disease virus (NDV), Sendai virus (SeV), West Nile virus (WNV) or vesicular stomatitis virus (VSV) (Dixit & Kagan, 2013). NDV and VSV have also been shown to induce IRF-5 activation (Barnes *et al.*, 2002b). In the absence of IRF-5, mice are more susceptible to VSV, NDV and herpes simplex virus type 1 (HSV-1) and express lower levels of serum type I IFN and IL-6 (Yanai *et al.*, 2007). Interestingly, MAVS-dependent induction of IFN-stimulated genes can be depended on IRF-5, which suggests that there may be a novel signaling pathway between IRF-5 and RLRs (Lazear *et al.*, 2013).

Among different NLRs, the nucleotide-binding oligomerization domain containing protein 2 (NOD2) receptor was shown to induce IRF-5 activation. Upon NOD2 stimulation via the ligands muramyl dipeptide or peptidoglycan, TANK-binding kinase-1 (TBK-1), TRAF6 and, downstream of NOD2, receptor interacting protein 2 (RIP2) directly phosphorylate IRF-5. This sufficiently activates IRF-5 to promote gene transcription, nuclear accumulation, and apoptosis (Chang Foreman *et al.*, 2012; Pandey *et al.*, 2009). A recent study also described the interaction between NOD and IRF-5, which resulted in severe pneumonia by boosting type I interferon in the mouse model of *Staphylococcus aureus* pneumonia (Parker *et al.*,

2014). IRF-5 is also activated by CLRs in response to fungal infection. IFN-β production of DCs was shown to depend on members of CLR family, the Dectin-1 and Dectin-2, which recognize the β-glucan complex of *C. albicans* cell wall. Dectin-1-regulated IFN-β expression is strongly depended on the tyrosine kinase Syk/ Card9 and IRF-5 (del Fresno *et al.*, 2013). However, the precise mechanism by which IRF-5 is activated by NOD2 and the relationship among IRF-5 with other NLRs or CLRs requires further investigations.

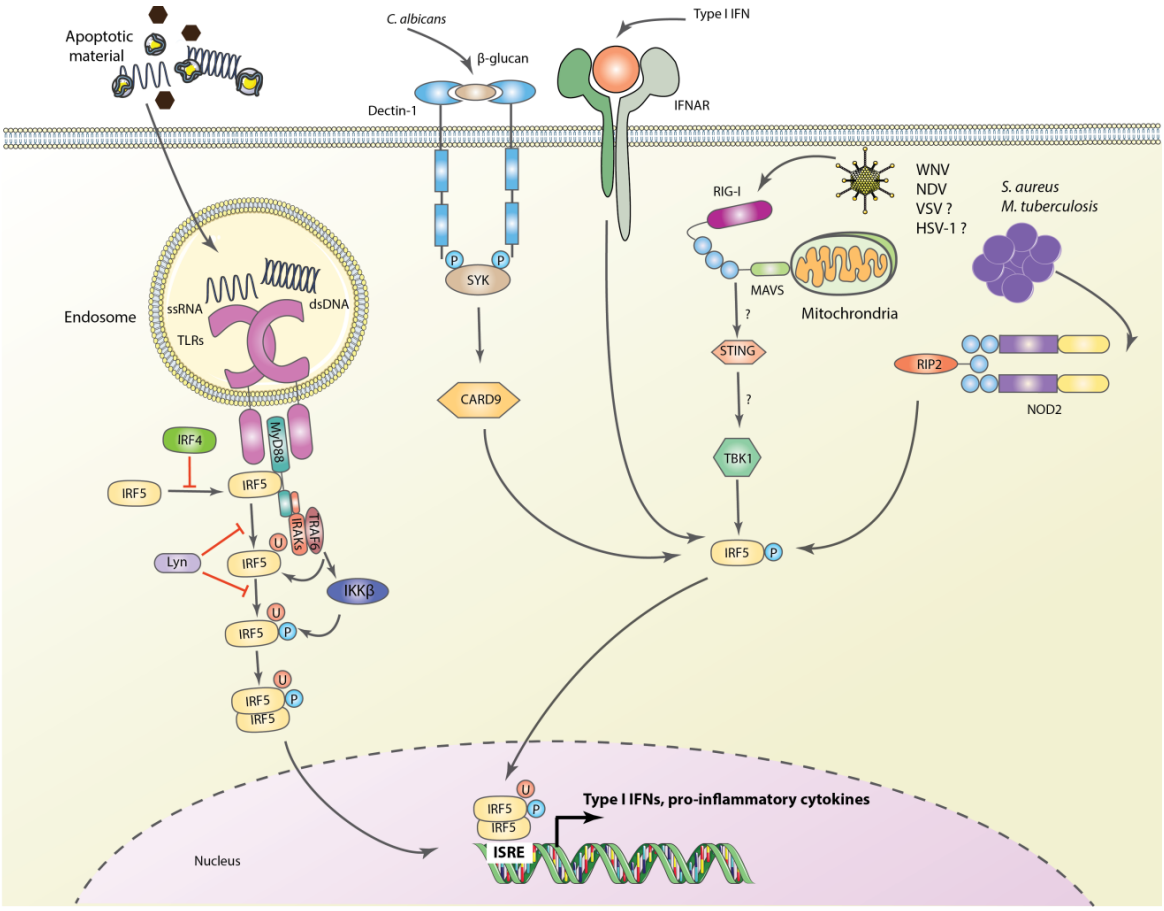


Figure 3.Pathways involved in IRF-5 activation.

Activation of IRF-5 is indicated by black arrows via endosomal TLR stimulation, DECTIN-1, RIG-I, NOD2 ligands or directly by type I IFN and VSV, HSV-1 binding. Following to the association of IRF-5 to the middle domain of MyD88 via TLR ligand binding, IRF-5 is ubiquitinated by TRAF6 and then phosphorylated by IKKβ. IRF-5 then forms a dimer, translocates to the nucleus, and activates the transcription of target genes. IRF-4 suppresses the biological function of IRF-5 by competing with its binding site on the MyD88 adaptor. IRF-5 ubiquitination and phosphorylation are inhibited by Lyn. The kinase cascade controlling IRF-5 activation downstream of other receptors is not yet known. Figure is adapted from (Ban *et al.*, 2018; Barnes *et al.*, 2002a; del Fresno *et al.*, 2013; Fabie *et al.*, 2018; Pandey *et al.*, 2009; Parker *et al.*, 2014).

Additionally, IRF-5 can also be activated by chemically- induced DNA damage, leading to apoptosis in tumor cell lines (Hu & Barnes, 2009; Hu *et al.*, 2005) and Fas and TRAIL-mediated cell death in hepatocytes and DCs (Couzinet *et al.*, 2008). IRF-5 is also described as a target of p53 upon DNA-damage stimulation (Bi *et al.*, 2014; Hu *et al.*, 2005). Type I IFN

has been shown to directly activate *Irf5* transcription (**Fig. 3**) (Schoenemeyer *et al.*, 2005). However, the kinases involved in these processes are still unknown.

1.5. The role of IRF-5 in immune cells

1.5.1. IRF-5 and neutrophils

IRF-5 has been shown to be constitutively expressed in blood neutrophils and to significantly increase in activated neutrophils in the synovial fluid of arthritic mice (Ericson *et al.*, 2014). In this study, they also identified the importance of IRF-5 for optimal secretion of several cytokines and chemokines, including IL-10, IP-10, MIP-1α, MIP-1β, and TNF (Ericson *et al.*, 2014). In acute inflammation models, like antigen-induced inflammatory arthritis and lung injury, IRF-5 regulates the recruitment of neutrophils to the inflammatory site (Weiss *et al.*, 2015). Interestingly, IRF-5 deficient mice developed a significant impairment in the number of neutrophils to the inflamed knee at the early stages of arthritis leading to a decrease in the Th1/Th17 and γδT IL-17^{+} cells in the joint at the later stages. The reduction in neutrophil numbers was also observed in lung injuries. However, IRF-5 does not alter the intrinsic capacity of neutrophils to migrate, but defects in recruitment results from deficient production of neutrophil chemoattractants such as chemokine (C-X-C motif) ligand 1 (CXCL1), which is produced by macrophages and dendritic cells (Weiss *et al.*, 2015).

1.5.2. IRF-5 in monocytes/macrophages

IRF-5 has been shown to control inflammatory M1 macrophage polarization both *in vitro* and *in vivo* (Chistiakov *et al.*, 2018; Krausgruber *et al.*, 2011; Weiss *et al.*, 2013). The development of macrophages from human monocytes and murine hematopoietic stem cells is characterized by a high level of IRF-5 expression. IRF-5 directly activates M1-associated cytokine including TNF, IL-6, IL-12 and IL-23 subunits secretion, while simultaneously suppressing the transcription of M2 marker protein like IL-10 and TGF-β (Dalmas *et al.*, 2015; Krausgruber *et al.*, 2011). Conversely, another member of the IRF family, IRF-4, which restricts IRF-5 function by competing the binding site in MyD88 adaptor, appears to control M2-associated markers' expression (Negishi *et al.*, 2005; Satoh *et al.*, 2010; Sun *et al.*, 2016). The mouse model of antigen-induced arthritis (AIA), IRF-5 is also upregulated in macrophages recruited at the inflamed site, suggesting that IRF-5 can be defined as a reliable marker of inflammatory macrophages (Weiss *et al.*, 2013; Weiss *et al.*, 2015). In the context of liver fibrosis either from nonalcoholic fatty liver disease or following hepatitis C virus infection, IRF-5 is significantly activated in Kupffer cells. High levels of IRF-5

expression are positively associated with clinical markers of liver damage, like plasma transaminase and bilirubin levels (Alzaid *et al.*, 2016). Besides controlling M1 macrophage polarization, IRF-5 in myeloid cells also affects T cell responses in the liver. Indeed, an increase in regulatory -like T cell differentiation and IL-10 production was observed in myeloid cell-specific IRF-5 deficient mice. Moreover, IRF-5 in myeloid cells promotes apoptosis of hepatocytes and liver macrophages through the Fas/Fas ligand pathway, whilst repressing the expression of anti-apoptotic genes *Bcl2* and *Bcl-X*$_L$ (Alzaid *et al.*, 2016). Also, the role of IRF-5 in the regulation of inflammation and metabolic outcomes in human and murine macrophages has been verified upon nucleotide- binding oligomerization domain containing 2 (NOD2) stimulation. Following this PRR stimulation, IRF-5 directly associated with RIP2, IRAK1 and TRAF6, then directly regulated the expression of glycolytic pathway genes, pro-inflammatory cytokines, HIF-1α as well as M1 polarization by activating Akt2 (Hedl *et al.*, 2016).

1.5.3. IRF-5 in dendritic cells

IRF-5 was shown to be constitutively expressed in both conventional dendritic cells (cDC) and plasmacytoid dendritic cells (pDC) (Heng & Painter, 2008). IRF-5 seems to contribute to the differentiation and the migration of pDCs. Indeed, *Irf5*$^{-/-}$ mice display a higher frequency of pDCs in the bone marrow, but have significantly reduced numbers of these cells in the spleen and peripheral blood; this defect in recruitment is also associated with a reduction in IFNα production in a DOCK2-dependent manner.(Purtha *et al.*, 2012). IRF-5 not only affects DC migration, but is also involved in cytokine production. Indeed, IRF-5 critical contributes to the induction of TNF, IL-6 and IL-12 in both cDCs and pDCs following stimulation of TLR agonists (Takaoka *et al.*, 2005) and viral infection in mice (Dai *et al.*, 2011). Moreover, IRF-5 and NF-κB have been shown to be co-regulators for IFN-β and IL-6 expression in human pDC upon TLR9 stimulation (Steinhagen *et al.*, 2013). During West-Nile virus infection, the co-operation of IRF-5 with IRF-3 and IRF-7 is responsible for type I IFN production in myeloid DCs (Lazear *et al.*, 2013). A study also reported a critical role of IRF-5 in immune responses against fungal infection by promoting IFNβ production through the Dectin-1 signaling pathway in β1,3-glucans- stimulated dendritic cells (del Fresno *et al.*, 2013). Moreover, it appears that the IRF-5-mediated IFNα production in pDCs is hormonally regulated in women (Griesbeck *et al.*, 2015). Another example of regulation of cytokine production by IRF-5 is seen in the lupus model, where follicular dendritic cells (FDCs) upon uptake of self-antigen secrete IFNα following stimulation of the TLR7/IRF-5 signaling pathway. IFNα secreted by FDCs induces the autoantibody production of B cells in germinal centers, which contributes to pathology progression (Das

et al., 2017). Moreover, FAS-induced apoptosis in DC upon TLR stimulation are promoted by IRF-5 (Couzinet *et al.*, 2008). And finally, in the experimental *L. donovani* model, IRF-5-mediated inflammation induces the expression of HIF-1α, which then inhibits DC function and $CD8^+$ T cell expansion, favoring the establishment of parasite persistence in the spleen (Hammami *et al.*, 2015).

1.5.4. IRF-5 and B cells

IRF-5 has a direct impact on the terminal B cell differentiation to plasma cells (Lien *et al.*, 2010; Yasuda *et al.*, 2014). IRF-5 has been shown to be required for B cell maturation and the expression of Blimp 1 that regulates plasma cell differentiation. IRF-5-deficient mice developed aged-related splenomegaly, which correlates with the expansion of the marginal zone. However, old *Irf5*$^{-/-}$ mice showed a dramatical impairment in Ig-producing plasma cells and in the level of natural antibodies and IgG, but not IgM in the serum. Moreover, IL-6 production by splenic B cells requires IRF-5 expression mediated by TLR7 and TLR9 (Lien *et al.*, 2010). In agreement with the study in murine B cells, IRF-5 is also involved in human B cell activation, proliferation, survival, and differentiation into plasma cells. Moreover, IRF-5 induces the expression of *Irf4*, *Erk1,* and *Myc* during the early stage of B cell activation and proliferation in response to TLR9/BCR-induced plasma cells differentiation (De *et al.*, 2017). The production of antinuclear autoantibodies such as IgG2 by B cells strictly requires IRF-5 presence in the case of murine systemic lupus erythematosus (SLE) (Savitsky *et al.*, 2010). Also, estrogen can stimulate the expression and translocation of IRF-5 in splenic B cells, which leads to class switching to IgG2a/c (Fang *et al.*, 2012; Shen *et al.*, 2010). Lastly, the expansion of age-associated B cell populations that accumulate in the early stages of autoimmune diseases is controlled by IRF-5 (Manni *et al.*, 2018).

1.5.5. IRF-5 and T cell functions

Although IRF-5 is constitutively expressed in various cells types such as monocytes, dendritic cells, macrophages, neutrophils and B cells, it is not found in naïve T cells (Fabie *et al.*, 2018; Heng & Painter, 2008). However, T cells have been shown to significantly upregulate IRF-5 transcription and translation during infection (Fabie *et al.*, 2018; Ishikawa *et al.*, 2015; Paun *et al.*, 2011). Most of the literature reports a role for IRF-5 in regulating T cell differentiation and activation by affecting the cytokine environment and macrophage or dendritic cell functions. For instance, IRF-5 mediates macrophage differentiation towards inflammatory M1 macrophage phenotypes and consequently promotes Th1 and Th17 responses (Krausgruber *et al.*, 2011; Xu *et al.*, 2012). However, in the context of

experimental visceral leishmaniasis, IRF-5 is upregulated and translocated to the nucleus in T cells, especially in $CD4^+$ T cells producing IFNγ. Interestingly, its activation is induced by apoptotic cell material via TLR7, but not by microbial sensing. IRF-5 expression activates apoptosis by upregulating DR5 and caspase 8 leading to $CD4^+$ T cells death. During human T cell leukemia type 1 (HTLV-1) infection, IRF-5 is constitutively expressed in infected T cells leading to activation of the TNF family cytokine translation. The expression of IRF-5 was specifically induced by viral oncoprotein Tax of HTLV-1, but not type I IFN (Ishikawa *et al.*, 2015). To date, the presence of IRF-5, as well as its role in $CD8^+$ T cells, has not yet been described.

1.6. Hypothesis

Recognition of pathogens by pattern recognition receptors triggers a signaling cascade that results in the upregulation of the transcription of genes involved in the inflammatory response. Two families of transcription factors are mainly involved in the transcriptional activation of the early inflammatory genes: the family of the NF-κB factors, and the family of the interferon regulatory factors (IRF). Interferon Regulatory Factor 5 (IRF-5), a member of the IRF family, is a transcription factor that has been demonstrated to be an important regulator of the activation of both Type I IFN genes and genes encoding for key pro-inflammatory cytokines such as TNF, IL-6, IL-12, and IL-23 (Takaoka *et al.*, 2005). IRF-5 has also been shown to be involved in cell cycle and apoptosis (Barnes *et al.*, 2003; Hu *et al.*, 2005). This transcription factor can be activated by TLR7 and TLR9 via the MyD88 signaling pathway and/or directly by Type I interferon and viral infections. IRF-5 expression has been reported in various immune cell types, including conventional dendritic cells (cDC) and plasmacytoid dendritic cells (pDCs), macrophages, B cells, neutrophils and, recently, activated $CD4^+$ T cells (Fabie *et al.*, 2018; Heng & Painter, 2008).

Visceral leishmaniasis (VL), commonly known as kala-azar, is a potentially lethal disease, characterized by long-term low grade fever, hepatosplenomegaly, pancytopenia, hypergammaglobulinemia, and immunosuppression (Kaye *et al.*, 2004; Kumar & Nylen, 2012)**.** *Leishmania donovani,* a species of intracellular protozoan parasites belonging to the genus *Leishmania*, is one of the etiological agents of VL (Kumar & Nylen, 2012). This intracellular protozoan parasite systemically infects and proliferates in macrophages and other phagocytic cells of internal organs, typically spleen, liver and bone marrow. Both $CD4^+$ and $CD8^+$ T cells producing IFN-γ are required to control infection. DCs-derived IL-12 is necessary to promote protective $CD4^+$ and $CD8^+$ T cell responses. Myeloid cells play an important role during experimental VL. Indeed, *L. donovani* strongly induces emergency

myelopoiesis in the bone marrow promoting the production and release of inflammatory monocytes with a regulatory phenotype. Monocytes and neutrophils are constantly recruited to the spleen, where they acquire myeloid-derived suppressor cell (MDSC) functions (Abidin *et al.*, 2017; Hammami *et al.*, 2017)**.** In a previous study, we reported the essential role of IRF-5 in inducing inflammation during *L. donovani* infection. We have found that in the absence of IRF-5, mice not only fail to develop Th1 responses, but also had a severely reduced inflammatory infiltration in the spleen and the liver (Paun *et al.*, 2011). This defect in Th1 responses did not depend on a $CD4^+$ intrinsic function of IRF-5, since T cell-specific *Irf5*$^{-/-}$ mice developed stronger Th1 responses than their wild type counterpart (Fabie *et al.*, 2018). Hence, we hypothesized that IRF-5 expression in myeloid cells is required to promote and sustain protective Th1 responses and initiate inflammation. This hypothesis is also based on the literature that shows that IRF-5 expression promotes inflammatory (M1) macrophages both *in vitro* and *in vivo*, which are required to support Th1 development (Alzaid *et al.*, 2016; Krausgruber *et al.*, 2011). Moreover, mice lacking IRF-5 in myeloid cells failed to develop IFNγ-secreting $CD4^+$ T cells in the context of chronic liver diseases (Alzaid *et al.*, 2016). Thus, the aim of my project will be to study the role of IRF-5 in myeloid cells using cell-specific knockout mice and the experimental model of visceral leishmaniasis. First, I will investigate the expression of IRF-5 in myeloid cells during acute *L. donovani* infection. Next, I will examine the role of IRF-5 in myeloid cells in the immune response against *L. donovani* infection, taking into account cell recruitment, development of protective and inhibitory $CD4^+$ T cell responses, and hepatic and splenic parasite burden. To test our hypothesis, we established two objectives:

1. To characterize IRF-5 expression in myeloid cells during experimental VL
2. To study the role of IRF-5 in myeloid cells during *Leishmania donovani* infection

CHAPTER 2. MATERIAL AND METHODS

2.1. Mice

B6.129S7-*Rag1*^{tm1Mom} mice were purchased from The Jackson Laboratory. Conditional IRF-5 knock-out mice in myeloid cells were generated by mating *Irf5*$^{flox/flox}$ mice with mice expressing the cre-recombinase under the control of the LysM or the CD11c promoter as described in section **3.2**. All mice were located under specific pathogen-free conditions at the INRS- Institut Armand Frappier animal facility and used at 6-10 weeks of age.

2.2. Parasites

2.2.1. Parasite purification

L. donovani amastigotes strain LV9 were kept by sequential passage in *Rag*$^{-/-}$ mice. The infected spleen of *Rag*$^{-/-}$ mice was collected and digested by 0.4 mg/ml filtered Collagenase D (Worthington, #43E14253) in 3 ml plain RPMI, then incubated on rotating wheel for 35 minutes at room temperature (RT). After the incubation, the digested spleen pieces were homogenized and passed through a 100 µm cell sieve on a 50 ml falcon by using a sterile plunger of a 5 ml syringe. Plain RPMI was added to reach up to 20 ml volume; tubes were spun down 10 min at 3100 rpm at RT to wash. The supernatant was removed and the pellet was resuspended in 20 ml of plain RPMI. 400 µl of 25mg/ml filtered saponin (Sigma, #84510) in plain RPMI was added, and then the mixture was rotated gently for 5 minutes at RT. The volume was topped up 50 ml by adding plain RPMI and washed three times at 3100 rpm at RT for 10 min. After discarding the supernatant, the pellet was resuspended in 1 ml of plain RPMI and then passed through a 1 ml syringe with a 26- gauge (G) needle to break up the clumps. Plain RPMI was added to make up the volume to 10 ml and then parasites were counted with a Thoma chamber. Mice were infected with $2x10^7$ amastigotes intravenously.

2.2.2. Parasite fixation

Parasites (purified as described above) were resuspended at a concentration of 10^9/ml. 4% paraformaldehyde (PFA) (Sigma, #158127) in 1x PBS was added with the equal volume of the parasite solution for a final PFA concentration of 2%. The solution was then incubated on ice for 30 minutes. Plain RPMI was added to the solution that was then spun down for 10 minutes at 3100 rpm at RT. The washing was repeated 3 times. The parasite pellet was

resuspended at a concentration of 2 x 10^8 or 5 x 10^8/ml in RPMI supplemented with 10% PSG + 10% FBS (complete RPMI), then transferred to an eppendorf for storage at -20°C.

2.2.3. Estimation of parasite burden: Leishman-Donovan Units (LDU)

To determine the parasite load, infected spleen and livers were cut and impressed on the slides, then fixed by methanol. The slides were air dried then stained for 20 minutes in a Giemsa solution (45ml H_2O supplemented with 5.55 ml Giemsa (Sigma-Aldrich, # 489000), 3.6 ml of Sorenson A (9.5g Na_2HPO_4/l H_2O) and 1.4 ml of Sorenson B (9.07g KH_2PO_4/l H_2O). The staining was stopped by removing the Giemsa solution. Stained slides were rinsed off with water and allowed to air dry. The dried slides were observed under a 100X objective microscope with oil immersion. Leishman-Donovan Units (LDU) were calculated by using the formula: LDU = number of amastigotes per 1000 host cell nuclei x organ weight (in grams) (Bradley & Kirkley, 1977).

2.3. T cell restimulation

2.3.1. Bone marrow-derived dendritic cell (BMDC) maturation

Femurs and tibias of a mouse were collected in sterile 1x PBS or plain RPMI. The bone marrow was harvested by flushing tibiae and femora using a 1 ml syringe with a 26G needle with 5 ml of warm 10% PSG IMDM. Aggregated cells were gently disrupted by pipetting up and down few times and separated in 6 sterile petri dishes. 10 ml of warm 10% PSG + 10% FBS + 10% of GM-CSF IMDM was then added to each petri dish and incubated at 37°C. 3 days later 10 ml of warm 10% PSG + 10% FBS + 10% of GM-CSF IMDM was added to each petri dish and the incubation was continued for 4 more days.

2.3.2. BMDC stimulation with fixed parasites

After 7-10 days in culture, non-adherent cells (mainly DCs) were harvested using a sterile Pasteur pipette and then centrifuged for 7 minutes at 1200 rpm. Cells were counted and resuspended at a concentration of 5x10^5/ml in warm complete RPMI. 5x10^6 fixed LV9 (MOI 1:10) was then added to the cell suspension. The mixture was plated in a 24 well-plate with one well for one mouse or one condition, the plate was cultured overnight at 37°C.

2.3.3. Antigen-specific splenic T cell restimualtion

BMDC pulsed with parasites from step **2.3.2** were taken out of the incubator. 500 µl of supernatant was removed from each well. A million cells of homogenized splenocytes from naïve or infected mice were transferred to a sterile FACS tube with cap. One tube was used for one mouse or one condition. Cells were washed by adding 1x PBS and centrifuged at 1200rpm at 4°C for 7 minutes. After discarding the supernatant, the cellular pellet was resuspended in 500 µl of warm complete RPMI. These total splenocytes were added to the BMDC, then incubated for 2h at 37°C. 5 µg/ml brefeldin A (BD Bioscience, #555029) was added per well and cells were incubated for 4 more hours at 37°C. At the end of the incubation, cells were transferred to FACS tubes for flow cytometry staining.

2.3.4. Isolation of mononucleated cells from the liver and antigen-specific restimulation of hepatic $CD4^+$ T cells

The liver was placed in a petri dish and cut into pieces with sterile scalpels that were then digested for 40-45 minutes at RT in 5 ml of 0.4 mg/mL filtered Collagenase D in plain RPMI. After digestion, liver were homogenized through a sieve to obtain single-cell suspension; then, the volume was adjusted to 50 ml and washed by centrifuging 5 minutes at 500 rpm at 4°C. The supernatant was then collected in a new 50ml tube. 1x PBS was added to a final volume of 50 ml, the tube was then spun 7 min at 1200 rpm, 4°C. The pellet was homogenized in 3 ml of Percoll (GE Healthcare, #17-0891-01) 40% in complete RPMI, the mixture then was delicately added on top of 3ml of Percoll 70% in complete RPMI to create the gradient in a 15 ml falcon. The tube was centrifuged for 15 minutes at 1700 rpm at RT without acceleration or break. After centrifugation, the debris on the top was discarded by slow aspiration; the cell layer in the middle was transferred to a new 15 ml tube and washed with 1x PBS. Cell pellet was resuspended in 500 µl of warm RPMI complete. 500 µl of supernatant was removed from each well of BMDC plate from **2.3.2.** Isolated hepatic mononucleated cells were then added to each well. 5 µg/ml brefeldin A was added per well and cells were incubated for 4 more hours. At the end of incubation, cells were transferred to FACS tubes for flow cytometry staining.

2.3.5. Restimulation of splenic T cells with phorbol myristate acetate (PMA)/ionomycin

About 1 million cells of homogenized splenocytes suspension is transferred into FACS tubes and washed with 1X PBS. The pellets are resuspended in 1 ml of warm complete RPMI

supplemented with 5 ng/ml Phorbol myristate acetate (PMA) (Sigma-Aldrich, # P8139), 500 ng/ml Ionomycin (Sigma-Aldrich, # I0634) and 5 µg/ml Brefeldin A, and then incubated for 4h at 37°C.

2.4. Myeloid cell isolation using magnetic-activated cell sorting (MACS) (positive selection)

The spleen from naïve or infected mice was cut into pieces and digested with 3 ml of 0.4 mg/mL filtered Collagenase D in a 15 ml falcon for 35 minutes at RT on a rotating wheel. The digested spleens were passed through a cell sieve with the plunger of a sterile 5ml syringe and then washed with 1x PBS. After removing the supernatant, the cellular pellet was transferred to a 15 ml falcon and washed with 10 ml of cold MACS buffer (1x PBS supplemented with 1% of FBS and 5mM EDTA). The cell pellet was resuspended in 90 µl of MACS buffer, labeled by adding 10 µl of CD11b beads (Miltenyi Biotech, # 130-049-601), and incubated for 15 minutes at 4°C. The cells then were washed with MACS buffer, while the MS column (Miltenyi Biotech, #130-042-201) was calibrated with 500 µl of MACS buffer. The labeled cells were resuspended in 500 µl of MACS buffer and added to the column. After washing the column 3 times with 500 µl MACS buffer, the magnetically labeled $CD11b^+$ cells were retained in the column and then eluted after removal of the column from the separator by 3 ml of MACS buffer.

2.5. Flow cytometry

The cytokine production of $CD4^+$ T cells in the spleen and liver was assessed by flow cytometry. Briefly, after incubation from 2.3.3; 2.3.4; and 2.3.5, cells were harvested into a FACS tube and washed with FACS buffer (1X PBS supplemented with 5mM EDTA, 0.01% sodium azide and 1% of horse serum; pH 7.4). Cells were then stained with BV421-conjugated anti-CD3 (BD Biosciences, #562600) and FITC-conjugated anti-CD4 (BD Pharmingen™, #553729). The staining was incubated for 20 minutes at 4°C in the dark. Cells were washed with FACS buffer and fixed with 100 µl PFA 4% in 20 minutes at 4°C. After washing with FACS buffer, cells were permeabilized with FACS buffer supplemented with 0.1% saponin (permeabilization buffer) and stained with the following antibodies: APC-conjugated anti-IFNγ (BD Pharmingen™, #554413) and PECy7-conjugated anti-TNF (BD Pharmingen™, #557644) for cells that were restimulated with BMDC previously pulsed with fixed amastigotes or PE-conjugated anti-IL-10 (BD Pharmingen™, 554467) for cells that were stimulated with PMA/ionomycin. Cells were washed with permeabilization buffer and acquired on a BD LSR Fortessa cell analyzer (Becton Dickinson).

For observing myeloid population, 10^6 isolated cells from 2.4 or 10^6 single-cell splenocytes were transferred into a FACS tube and washed with FACS buffer. Cells were then stained with PB-conjugated anti-CD11b (BD Horizon^{TM}, #562605), FITC-conjugated anti-MHC-II (BD PharmingenTM, #553623), APC-conjugated anti-CD11c (eBioscience, #17-0114-82), PerCP-conjugated anti-Ly6C (Bioscience, #1280028), PE-conjugated anti-Ly6G (Bioscience, #127608), and PECy7-conjugated anti- F4/80 (Bioscience, #123114). The staining was incubated for 20 minutes at 4^{o}C in the dark. Cells were washed with FACS buffer and fixed with PFA 4% for 20 minutes at 4^{o}C. After washing with FACS buffer, cells were acquired on a BD LSR Fortessa cell analyzer (Becton Dickinson). The results were analyzed using the FlowJo software (Tree Star).

2.6. Real-time PCR

RNA from isolated myeloid cells from naïve or infected mice at various time points was extracted using the RNeasy mini kit (QIAGEN) following the manufacturer's protocol. Reverse transcription was conducted using the iScript cDNA synthesis kit (Bio-Rad) as described by the manufacturer. Real-time PCR analysis was performed using iTaq Universal SYBR Green Supermix (Bio-Rad). The IRF-5 and HPRT genes were amplified using following primers: *Hprt*, 5′-GTT GGA TAC AGG CCA GAC TTT GTT G-3′ and 5′-GAT TCA ACC TTG CGC TCA TCT TAG GC-3′; *Irf5*, 5′-TAG AGG CTA CCC AGG AGC AA-3′ and 5′-GCC CAC TCC AGA ACA CCT TA-3′. All PCRs were carried out with the Stratagene mx3000p real-time PCR system. Data were normalized to HPRT and expressed as fold increase to naive controls.

2.7. Statistical analysis

Statistical analysis of the results was obtained by using paired Student's t-test or multi-way ANOVA. Statistical analysis was conducted using Graphpad Prism 6.0 software with $p < 0.05$ considered to be significantly different. All experiments were repeated at least twice.

CHAPTER 3. RESULTS

3.1. IRF-5 expression in myeloid cells during *L. donovani* infection

A previous study in the laboratory demonstrated that IRF-5 is required for promoting inflammation during *L. donovani* infection (Hammami *et al.*, 2015). However, it failed to determine the cellular source responsible for this phenomenon. To test our hypothesis that IRF-5 expression in myeloid cells drives the inflammatory response, we first assessed the kinetic of IRF-5 expression in myeloid cells during the early stages of *L. donovani* infection. C57BL/6 mice were infected with *L. donovani* amastigotes and euthanized at day 1, 3, 5, and 7 after infection. Spleens were then collected to isolate myeloid cells ($CD11b^+$ cells) by magnetic cell sorting. IRF-5 expression was determined by qPCR and compared with that in the non-myeloid cell population ($CD11b^-$ cells). In the CD11b+ fraction, IRF-5 mRNA was already upregulated at day 1 of infection and was expressed at higher levels compared with other cell types. However, IRF-5 expression in myeloid cells decreased at d3 and d5 p.i, to levels similar to those seen in $CD11b^-$ cells, to be upregulated again at d7 p.i (**Fig. 4**). These changes are associated with the cellularity of the spleen during the progression of the disease. After *L. donovani* infection, the spleen starts increasing in size and cell number until d3 p.i. After that, anti-inflammatory responses are initiated (Maroof *et al.*, 2009) and the spleen becomes smaller. From d7 p.i. on, inflammatory cells are slowly, but constantly recruited to the spleen, which gradually increases its cellularity until day 21. At this point, a massive infiltration of inflammatory cells, mainly inflammatory monocytes (Hammami *et al.*, 2017), occurs and the spleen enlarges massively.

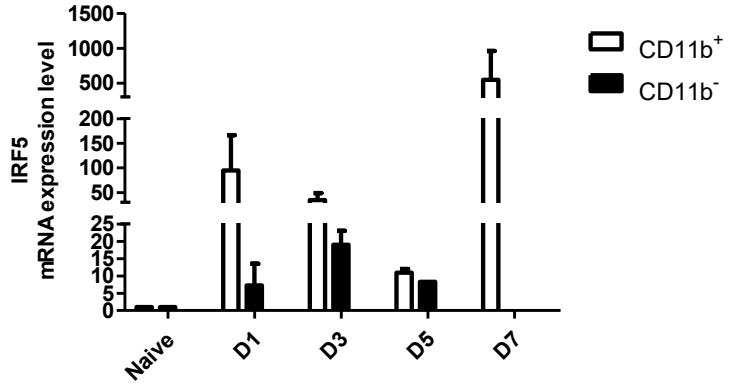


Figure 4.IRF-5 is upregulated in myeloid cells during *L. donovani* infection.

C57BL/6 mice were infected with *L. donovani* and euthanized at d1, 3, 5 and 7 p.i. Real time PCR analysis of myeloid cells isolated inform the spleen of naive and infected C567BL/6 mice measuring mRNA for IRF-5. All data is presented as the mean ± SEM, n=4 mice per group.

3.2. Construction of myeloid-specific knockout mice

To better identify the role of IRF-5 in myeloid cells, we generated myeloid-specific *Irf5*$^{-/-}$ mice using the cre-lox system, which is often used to generate tissue-specific knockout mice. These mice have a gene of interest that is "floxed" and thus inoperative in specific cell types or in a certain tissue, while other cell types and tissues exhibit an unchanged, functional gene expression. In order to generate mice lacking IRF-5 in myeloid cells, we crossed two strains of mice: *C57BL/6 Irf5*$^{flox/flox}$ and *C57BL/6 LysM-Cre*$^{+}$. *LysM-Cre*$^{+}$ mice express the cre recombinase in cells expressing lysozyme 2, namely most myeloid cells (**Fig. 5**). Approximately 50% of the progeny will be heterozygote for the *Irf5*^{flox} allele and the *Cre* transgene. After confirmation of the genotype by PCR, these mice were bread back with *C57BL/6 Irf5*$^{flox/flox}$ mice to obtain mice homozygous for the *Irf5*^{flox} allele. The second generation (F2) contains approximately 25% of mice homozygous for the *Irf5*^{flox} allele and heterozygous for the *Cre* transgene. These mice were defined as *C57BL/6 Irf5*$^{flox/flox}$ *LysM-Cre*$^{+}$ (*LysM-Cre*) mice and used for the experiments. Finally, their *Cre*$^{-}$ littermates, identified as *Irf5*$^{flox/flox}$, were used as controls. In some experiments, *C57BL/6 Irf5*$^{flox/flox}$ *CMV-Cre*$^{+}$ (*CMV-cre*) mice were also used. These mice completely lack IRF-5 in all the cell types.

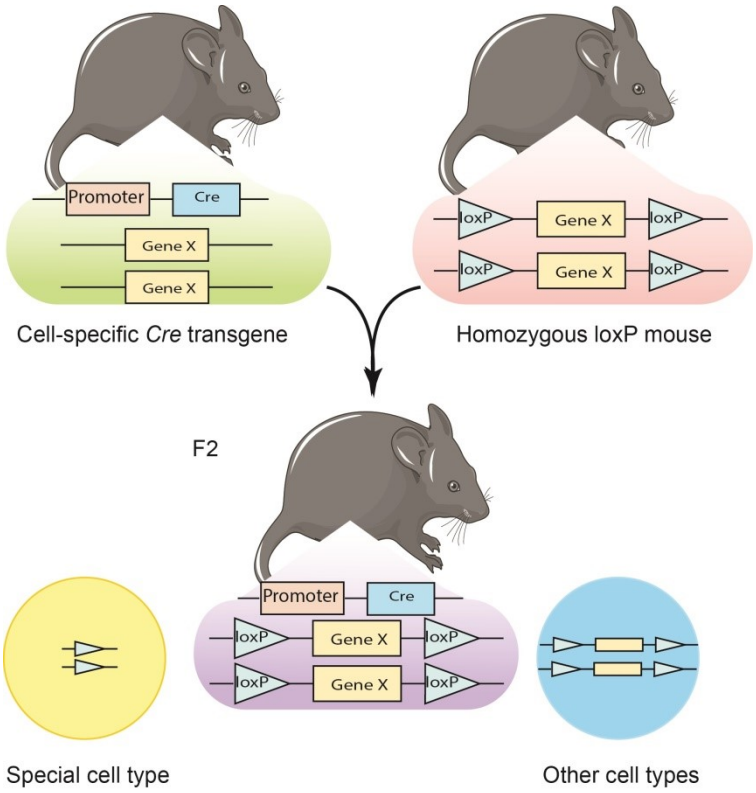


Figure 5.Breeding strategy for the generation of cell specific knockout mice.

Mice with the Cre protein expressed in a specific cell type (for example myeloid cells) are bred with mice that contain a target gene surrounded by *loxP* sites. When the mice are bred, the cells carrying *Cre* will lose the target gene, while in other cell types the target gene will not be deleted.

3.3. Lack of IRF-5 expression in myeloid cells does not affect the course of *L. donovani* infection

In a previous study, we showed that IRF-5 plays an essential role in the maintenance of the inflammatory cell infiltration induced by *L. donovani* infection. IRF-5 deficient mice failed to develop splenomegaly, which is a hallmark of *L. donovani* infection, but they have higher parasite burden than WT mice (Paun *et al.*, 2011). Since myeloid cells, especially monocytes, accumulate in the spleen of infected mice during VL progression (Hammami *et al.*, 2017), we wanted to see whether IRF-5 expressed by myeloid cells influences the course of *L. donovani* infection. To do that, we infected *C57BL/6 Irf5*$^{flox/flox}$ *LysM-Cre*$^{+}$ (here called *LysM-cre*) or their *Cre*$^{-}$ littermates (here called WT), and *C57BL/6 Irf5*$^{flox/flox}$ *CMV-Cre*$^{+}$ (*CMV-cre*) mice with *L. donovani* amastigotes. Hepato- and splenomegaly were evaluated at different time points of infection for the various mouse strains. As expected, we observed an increase in the weight of the spleen in WT mice compared with naïve mice throughout the time course of infection, particularly at day 28 p.i, when the spleen of WT mice were five times larger than the ones of naïve mice. In agreement with Paun et al., *CMV-cre* mice did not develop splenomegaly. Surprisingly, *LysM-cre* mice revealed a similar spleen weight as in WT mice (**Fig. 6A**). No significant difference in liver weights was observed among the three groups (**Fig. 6B**). Although *Irf5*$^{-/-}$ mice had smaller spleens, they were more susceptible to *L. donovani* by having higher parasite burdens in the spleen and liver than WT mice (Paun *et al.*, 2011). Hence, we next investigated whether *LysM-cre* mice were more susceptible to the parasite. In the spleen of WT mice, the parasite burden increased slowly in the first 2 weeks after infection; then the number of parasites increased dramatically in the later stages of infection (**Fig. 6C**). Although, the splenic parasite burden in *CMV-cre* mice was slightly higher, it was not significantly different to the WT group. Moreover, we observed similar parasite burdens in the spleen of *LysM-cre* and WT mice during the whole course of infection (**Fig. 6C**). In contrast, *CMV-cre* mice developed significantly higher parasite burden in the liver than WT mice, while a similar level of infection was observed in the liver of WT and *LyM-cre* mice (**Fig. 6D**). Taken together, IRF-5 expression in myeloid cells does not affect the course of *L. donovani* infection in the spleen and the liver.

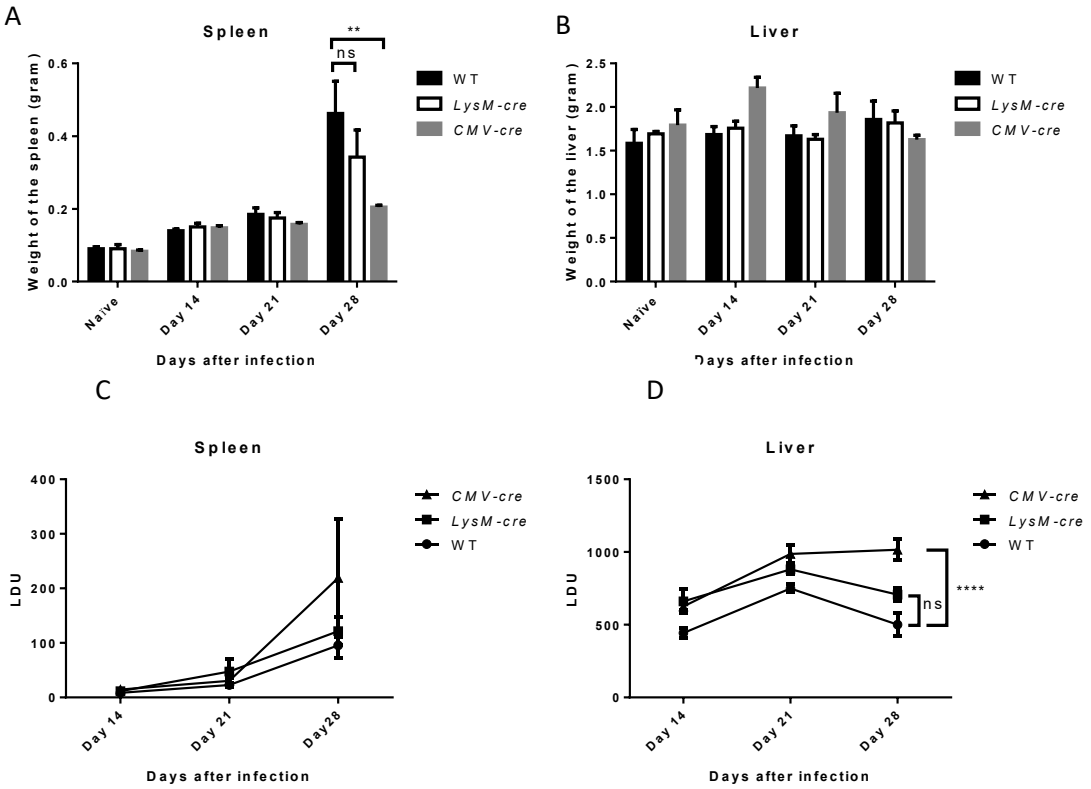


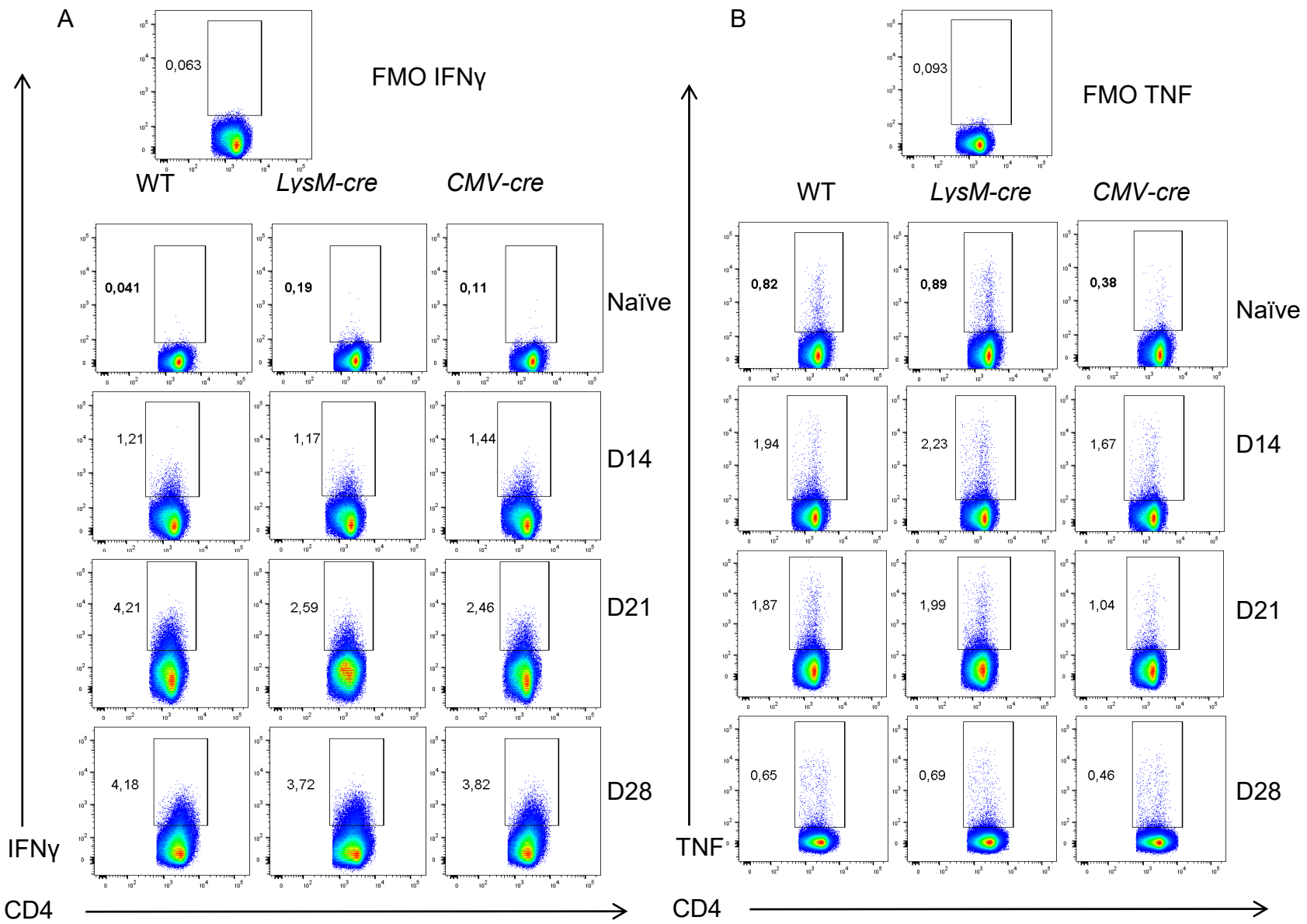
Figure 6.*LysM-cre* mice are not more susceptible to *L. donovani* infection than WT mice.

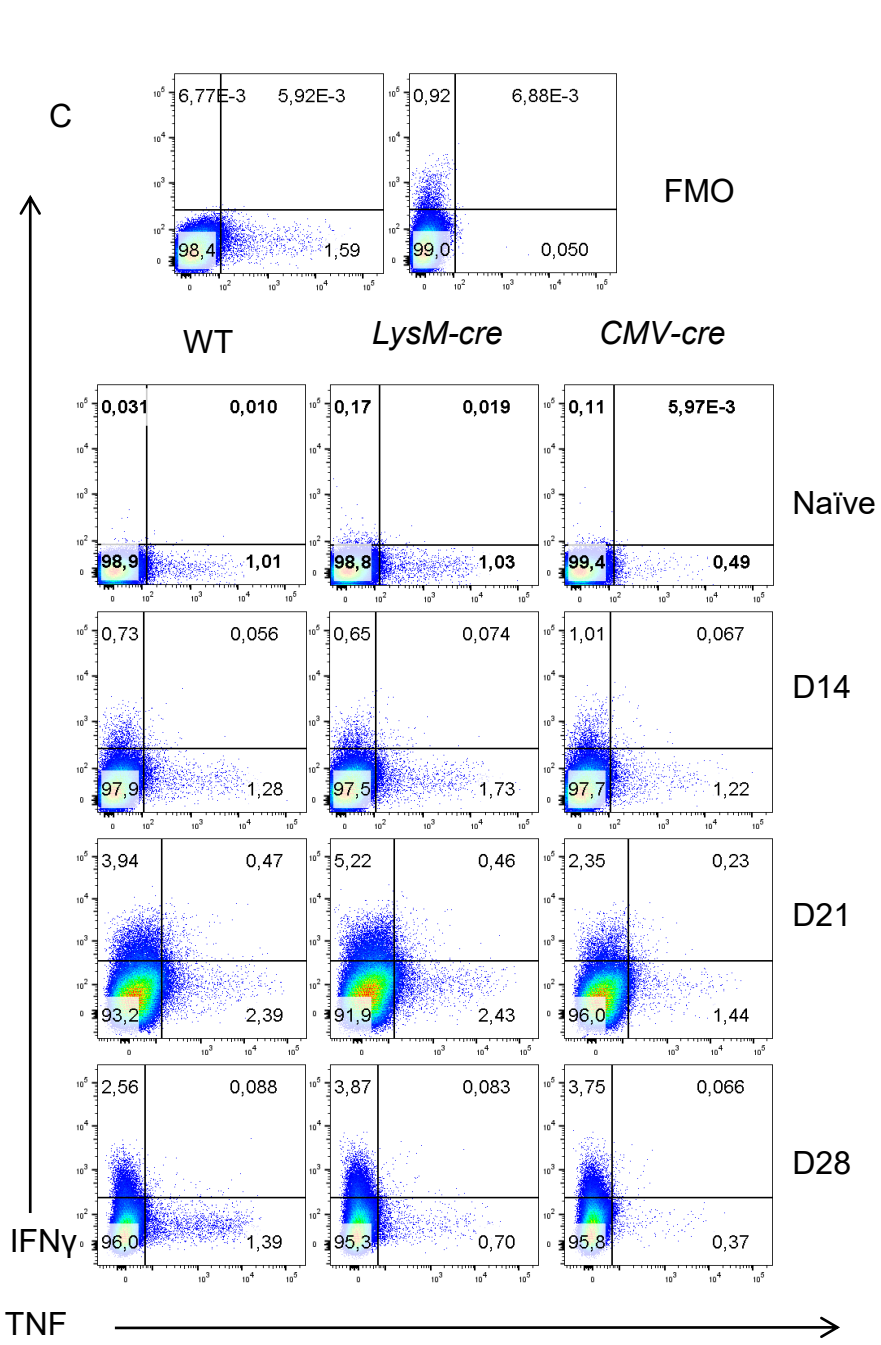
WT, *LysM-cre* and *CMV-cre* mice were infected with *L. donovani* and euthanized at d14, 21, and 28 p.i. (A) Spleen and (B) liver weights of WT, *LysM-cre* and *CMV-cre* naïve and infected mice. (C-D) Leishmania Donovan Units (LDU) were calculated from impression smears for the spleen (C) and the liver (D) of infected mice from the three groups. Error bars indicate mean ± SEM with 4 mice per group, ns denote not significant, ** denote p<0.01, **** denote p< 0.0001.

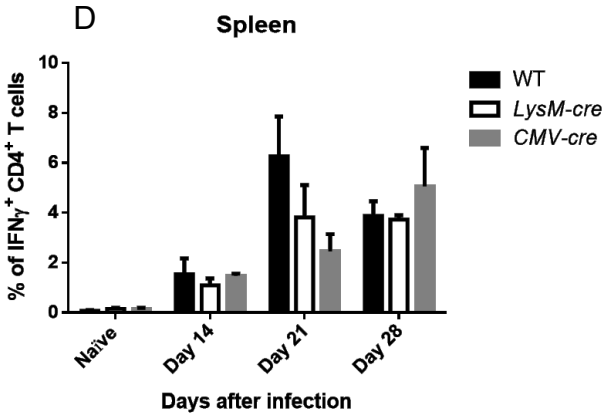
3.4. IRF-5 expression in myeloid cells is not required for T cell responses in the spleen following *L. donovani* infection

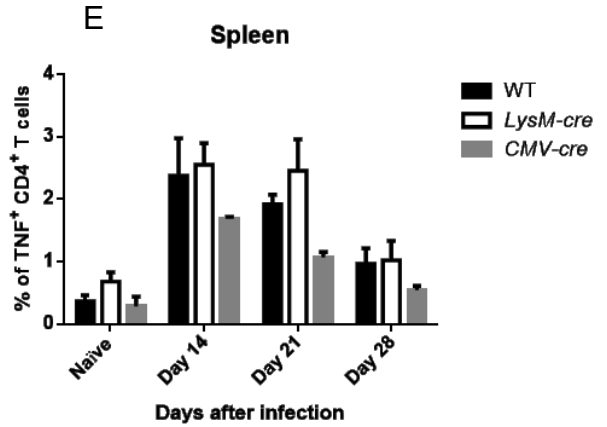
Myeloid lineage includes some cells that are professional antigen presenting cells (APC), such as macrophages and some types of dendritic cells. Since IRF-5 expression in macrophages was shown to be required to induce T helper 1 (Th1) responses (Krausgruber *et al.*, 2011), we assessed antigen-specific, IFNγ and TNF-producing $CD4^+$ T cell responses. These cells are known to activate macrophages and therefore help eliminate the parasite (Kaye & Scott, 2011). In order to investigate the IFNγ and TNF secretion, splenocytes from naïve and infected mice, isolated at different time points of infection, were stimulated with bone marrow-derived dendritic cells pulsed with fixed *L. donovani* parasites for 6h in presence of Brefeldin A, and analyzed by flow cytometry. In WT mice, splenic $IFN\gamma^+$ $CD4^+$ T-cells were already detected at d14, although at low frequency (approximately 1.5% of total $CD4^+$ T cells); these responses peaked at d21 p.i (about 6% of total $CD4^+$ T cells) and

decreased again to 3% at d28 p.i. *LysM-Cre* and *CMV-Cre* mice developed comparable IFNγ–producing $CD4^+$ T cells responses in the spleen than WT mice (**Fig. 7A and 7D**). TNF^+ $CD4^+$ T cells or IFNγ and TNF co-expressing $CD4^+$ T frequencies were also equivalent among the three groups of mice over the whole course of infection (**Fig. 7B, C, E and F**). Taken together, IRF-5 expression in myeloid cells does not affect Th1 responses in the spleen during *L. donovani* infection.


A
0,063
FMO IFNγ
WT
LysM-cre
CMV-cre
0,041
0,19
0,11
Naïve
1,21
1,17
1,44
D14
4,21
2,59
2,46
D21
4,18
3,72
3,82
D28
IFNγ
CD4
B
0,093
FMO TNF
WT
LysM-cre
CMV-cre
0,82
0,89
0,38
Naïve
1,94
2,23
1,67
D14
1,87
1,99
1,04
D21
0,65
0,69
0,46
D28
TNF
CD4


C
6,77E-3
5,92E-3
98,4
1,59
0,92
6,88E-3
99,0
0,050
FMO
WT
LysM-cre
CMV-cre
0,031
0,010
98,9
1,01
0,17
0,019
98,8
1,03
0,11
5,97E-3
99,4
0,49
Naïve
0,73
0,056
97,9
1,28
0,65
0,074
97,5
1,73
1,01
0,067
97,7
1,22
D14
3,94
0,47
93,2
2,39
5,22
0,46
91,9
2,43
2,35
0,23
96,0
1,44
D21
2,56
0,088
96,0
1,39
3,87
0,083
95,3
0,70
3,75
0,066
95,8
0,37
D28
IFNγ
TNF


D
Spleen
% of IFNγ+ CD4+ T cells
WT
LysM-cre
CMV-cre
Naïve
Day 14
Day 21
Day 28
Days after infection


E
Spleen
% of TNF+ CD4+ T cells
WT
LysM-cre
CMV-cre
Naïve
Day 14
Day 21
Day 28
Days after infection

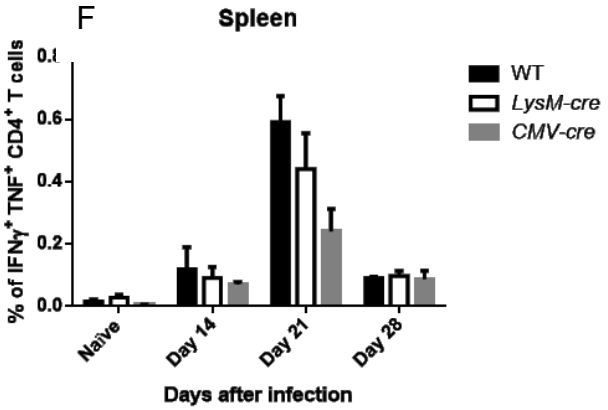

F
Spleen
% of IFNγ+ TNF+ CD4+ T cells
WT
LysM-cre
CMV-cre
Naïve
Day 14
Day 21
Day 28
Days after infection

Figure 7.*LysM-cre* mice develop similar Th1 responses in the spleen than WT mice following *L. donovani* infection.

WT, *LysM-cre* and *CMV-cre* mice were infected with *L. donovani* and euthanized at d14, 21, and 28 p.i. (A-C) Representative scatter plots showing splenic $IFN\gamma^+$(A), TNF^+ (B) and $IFN\gamma^+$ TNF^+ (C) $CD4^+$ T cells from WT, *LysM-cre* and *CMV-cre* naïve and *L. donovani* -infected mice at different time points of infection. The percentage of $CD4^+$ T cells producing IFNγ (D), TNF (E) and co-expressing IFNγ and TNF (F) following *L. donovani* infection in the spleen from three groups of mice. Data is shown as the mean ± SEM with 3 mice per group.

IL-10 is one of the major factors that contribute to the progression of VL by directly suppressing protective T cell responses and APC functions (Saraiva & O'Garra, 2010; Stager *et al.*, 2006). During chronic VL, IL-10 is mainly secreted by $CD25^-$ $FoxP3^-$ $CD4^+$ T cells, which also produce IFNγ (Stager *et al.*, 2006). This co-expressing population, named Tr-1, limits immunopathology but also favours parasite survival (Belkaid *et al.*, 2002; Montes de Oca *et al.*, 2016; Stager *et al.*, 2006). Thus, we wanted to investigate whether the absence of IRF-5 in myeloid cells would affect the expansion of Tr-1 cells. To do that, splenocytes of infected or naïve mice were stimulated with PMA/ ionomycin in presence of Brefeldin A for 4 hours, then $IFN\gamma^+$ $IL\text{-}10^+$ $CD4^+$ T co-producers were assessed by flow cytometry. We observed a significantly larger expansion of Tr-1 cells in *CMV-cre* mice compared with WT mice. However, *LysM-cre* mice showed similar Tr-1 frequencies than the WT group (**Fig 8**). Thus, conditional IRF-5 depletion in the myeloid cell population does not seem to affect the development of $IFN\gamma^+$ $IL\text{-}10^+$ $CD4^+$ T cells during VL.

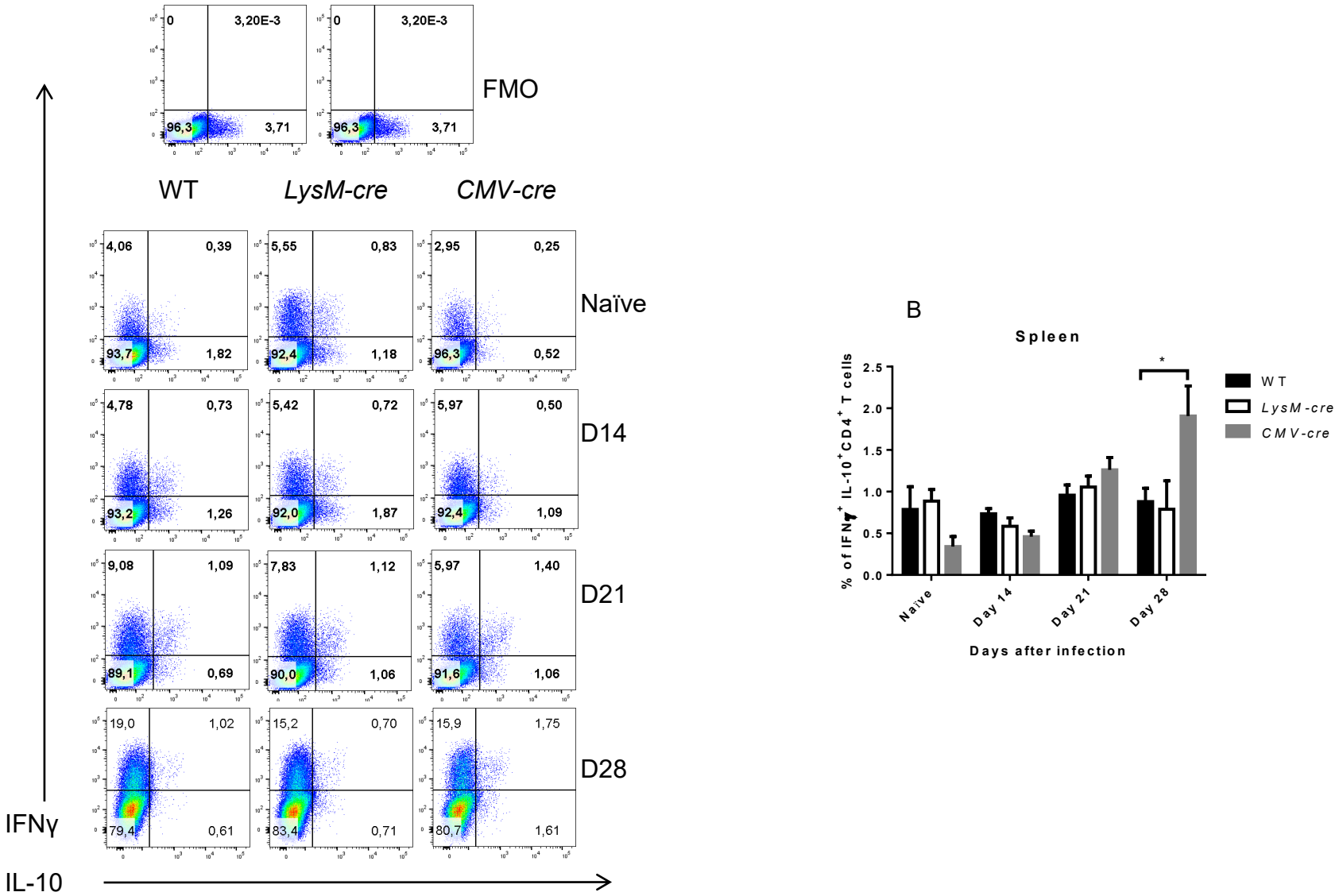


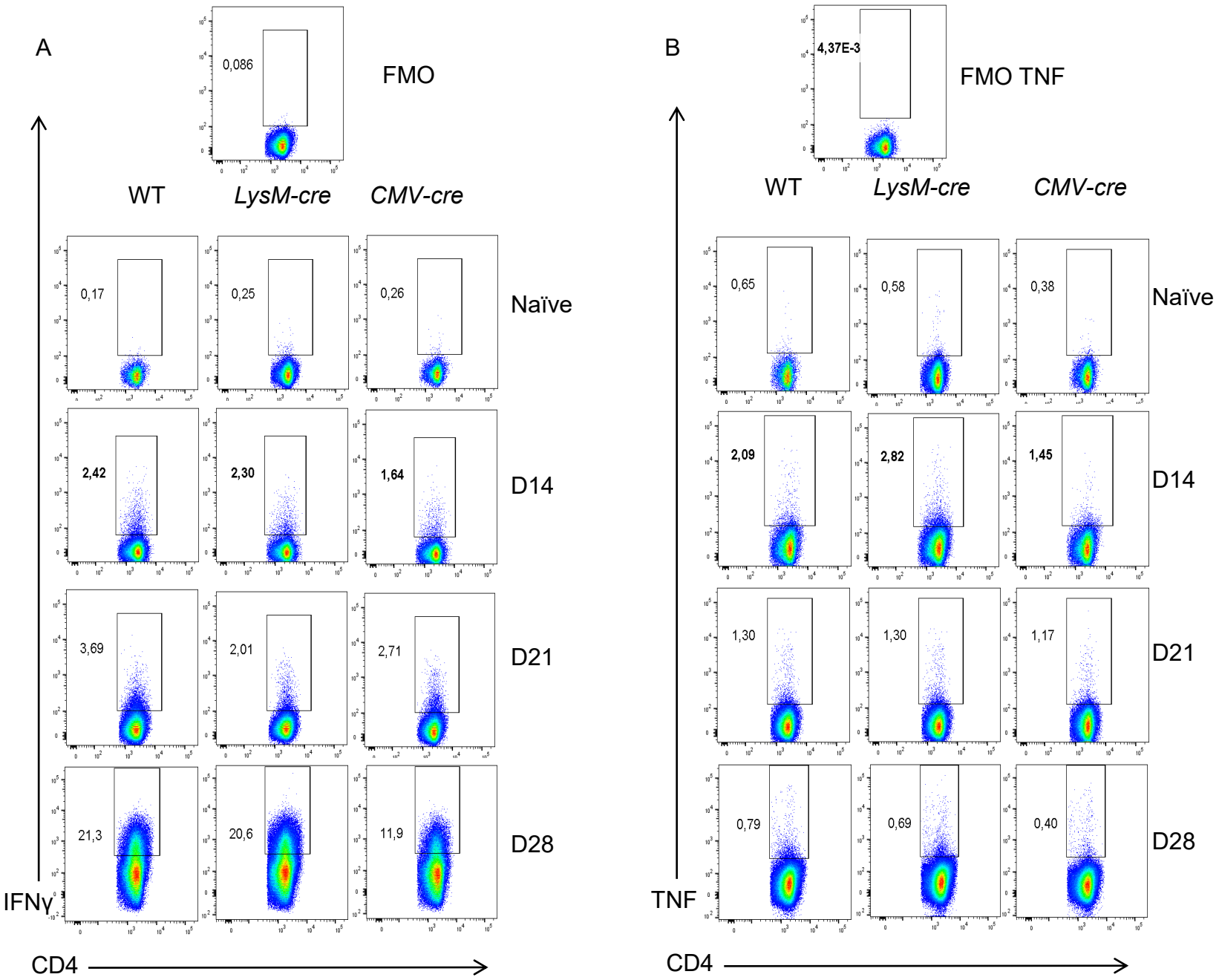
Figure 8.*LysM-cre* mice develop similar Tr1 responses than WT mice following *L. donovani* infection.

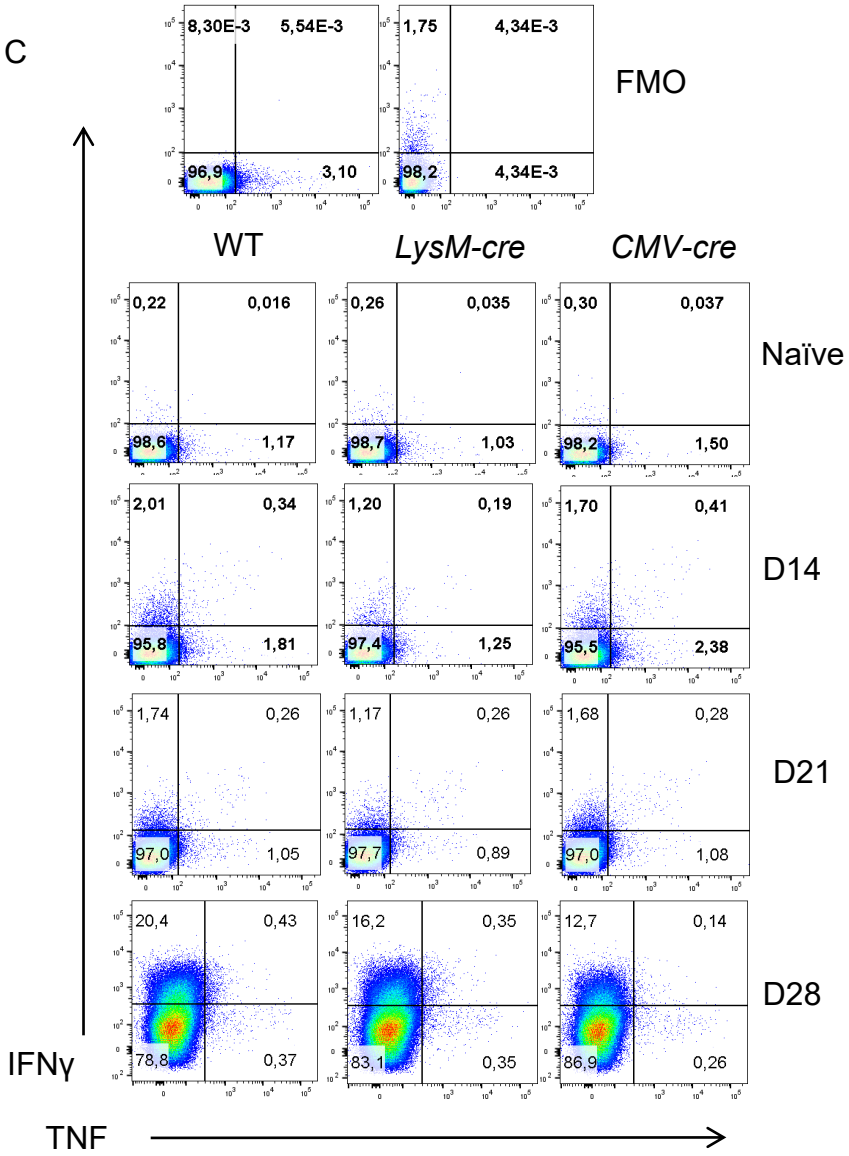
WT, *LysM-cre* and *CMV-cre* mice were infected with *L. donovani* and euthanized at d14, 21, and 28 p.i..Graphs show representative scatter plots (A) and percentages (B) of splenic IFNγ$^+$ IL-10$^+$ CD4$^+$ T cells from WT, *LysM-cre* and *CMV-cre* naïve mice and mice infected with *L. donovani* at different time points of infection. Data is shown as the mean ± SEM with 3-4 mice per group, * denote p<0.05.

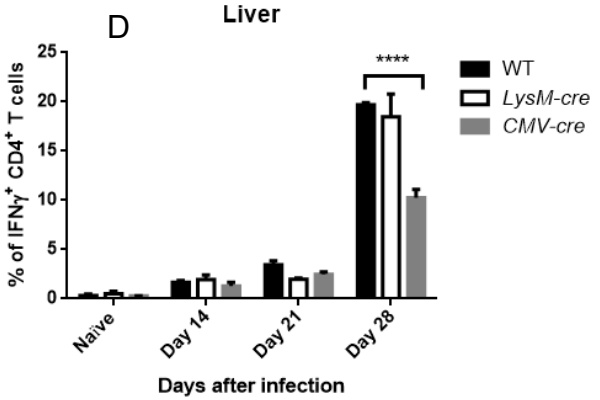
Taken together, T cell responses to *L. donovani* in the spleen do not require IRF-5 expression in $CD11b^+$ cells.

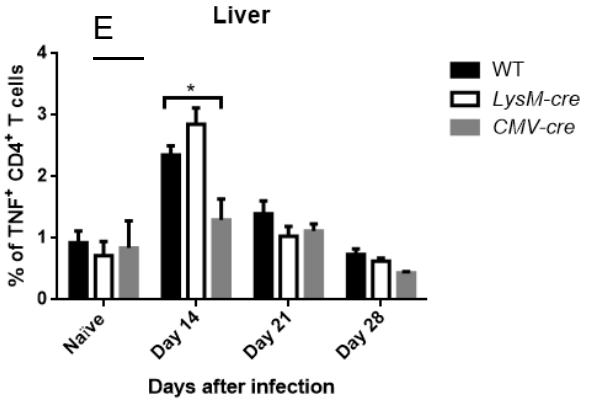
3.5. IRF-5 in myeloid cells is not required for Th1 responses in the liver following *L. donovani* infection

In the experimental model of VL, IFNγ production by $CD4^+$ T cells determines the resolution of the infection in the liver by contributing to the development of inflammatory granulomas and activating the parasite elimination mechanisms of infected Kupffer cells (Faleiro *et al.*, 2014; Gorak *et al.*, 1998). A previous study in the laboratory demonstrated that *Irf5*$^{-/-}$ mice failed to induce IFNγ-producing $CD4^+$ T cells in the liver during chronic VL (Paun *et al.*, 2011). Moreover, IRF-5 is known to play a critical role in M1 macrophages by promoting Th1 responses (Krausgruber *et al.*, 2011). Also, myeloid cells such as neutrophils and monocytes play an essential role in granuloma formation (Stanley & Engwerda, 2007); hence IRF-5 expression in these myeloid cells could potentially affect the generation of protective T cell responses to *L. donovani* in the liver. To examine this hypothesis, we assessed antigen-specific Th1 responses in the liver of *L. donovani*-infected mice. In agreement with previous observations (Paun *et al.*, 2011), the frequency of $IFN\gamma^+$ $CD4^+$ T constantly increased to peak at d28 p.i,, when nearly 20% of $CD4^+$ T cells in the liver produced IFNγ in WT mice. While the development of $IFN\gamma^+$ $CD4^+$ T cells was significantly impaired in *CMV-cre* mice at d28 p.i. *LysM-cre* mice were able to generate strong Th1 response, comparable with those observed in the WT group (**Fig. 9A and 9D**). Likewise, the frequency of TNF single-producing $CD4^+$ T cells was maximal at d14 p.i (approximately 2.3% of total $CD4^+$ T cells) in WT mice and gradually decreased over the course of infection. Again, *CMV-cre* $CD4^+$ T cells generated significantly fewer TNF single-producers compared with WT mice and no differences were detected between *LysM-cre* and WT mice (**Fig. 9B and 9E**). Similarly, we did not observe any difference in the frequency of IFNγ and TNF co-producers between the WT and the *Lysm-cre* group; however, *CMV-cre* mice displayed a significant reduction in this cell population at d28 p.i. (**Fig. 9C and 9F**). Thus, conditional ablation of IRF-5 in myeloid cells using the *LysM-cre* model does not affect the development of T cell responses to *L. donovani* in the liver.


A
0,086
FMO
WT
LysM-cre
CMV-cre
0,17
0,25
0,26
Naïve
2,42
2,30
1,64
D14
3,69
2,01
2,71
D21
21,3
20,6
11,9
D28
IFNγ
CD4
B
4,37E-3
FMO TNF
WT
LysM-cre
CMV-cre
0,65
0,58
0,38
Naïve
2,09
2,82
1,45
D14
1,30
1,30
1,17
D21
0,79
0,69
0,40
D28
TNF
CD4


C
FMO
8,30E-3
5,54E-3
96,9
3,10
1,75
4,34E-3
98,2
4,34E-3
WT
LysM-cre
CMV-cre
Naïve
D14
D21
D28
0,22
0,016
98,6
1,17
0,26
0,035
98,7
1,03
0,30
0,037
98,2
1,50
2,01
0,34
95,8
1,81
1,20
0,19
97,4
1,25
1,70
0,41
95,5
2,38
1,74
0,26
97,0
1,05
1,17
0,26
97,7
0,89
1,68
0,28
97,0
1,08
20,4
0,43
78,8
0,37
16,2
0,35
83,1
0,35
12,7
0,14
86,9
0,26
IFNγ
TNF


D
Liver
% of IFNγ+ CD4+ T cells
****
WT
LysM-cre
CMV-cre
Naïve
Day 14
Day 21
Day 28
Days after infection


E
Liver
% of TNF+ CD4+ T cells
*
WT
LysM-cre
CMV-cre
Naïve
Day 14
Day 21
Day 28
Days after infection

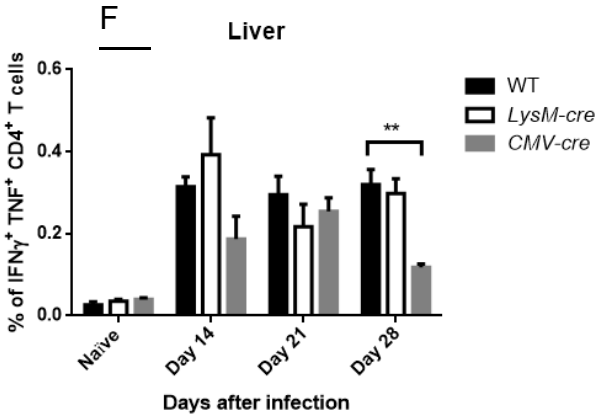

F
Liver
% of IFNγ+ TNF+ CD4+ T cells
**
WT
LysM-cre
CMV-cre
Naïve
Day 14
Day 21
Day 28
Days after infection

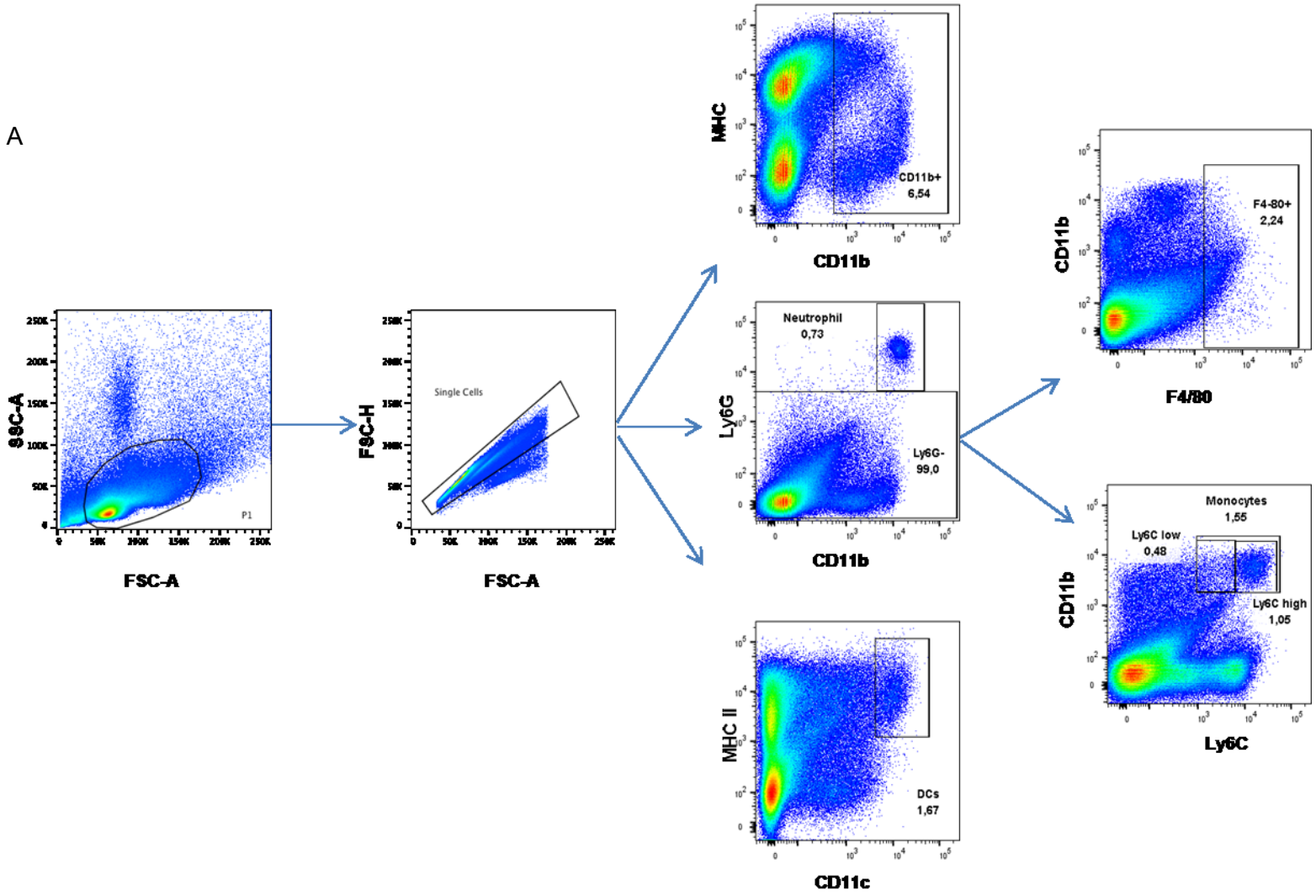
Figure 9.*LysM-cre* mice develop similar Th1 responses in the liver than WT mice following *L. donovani* infection.

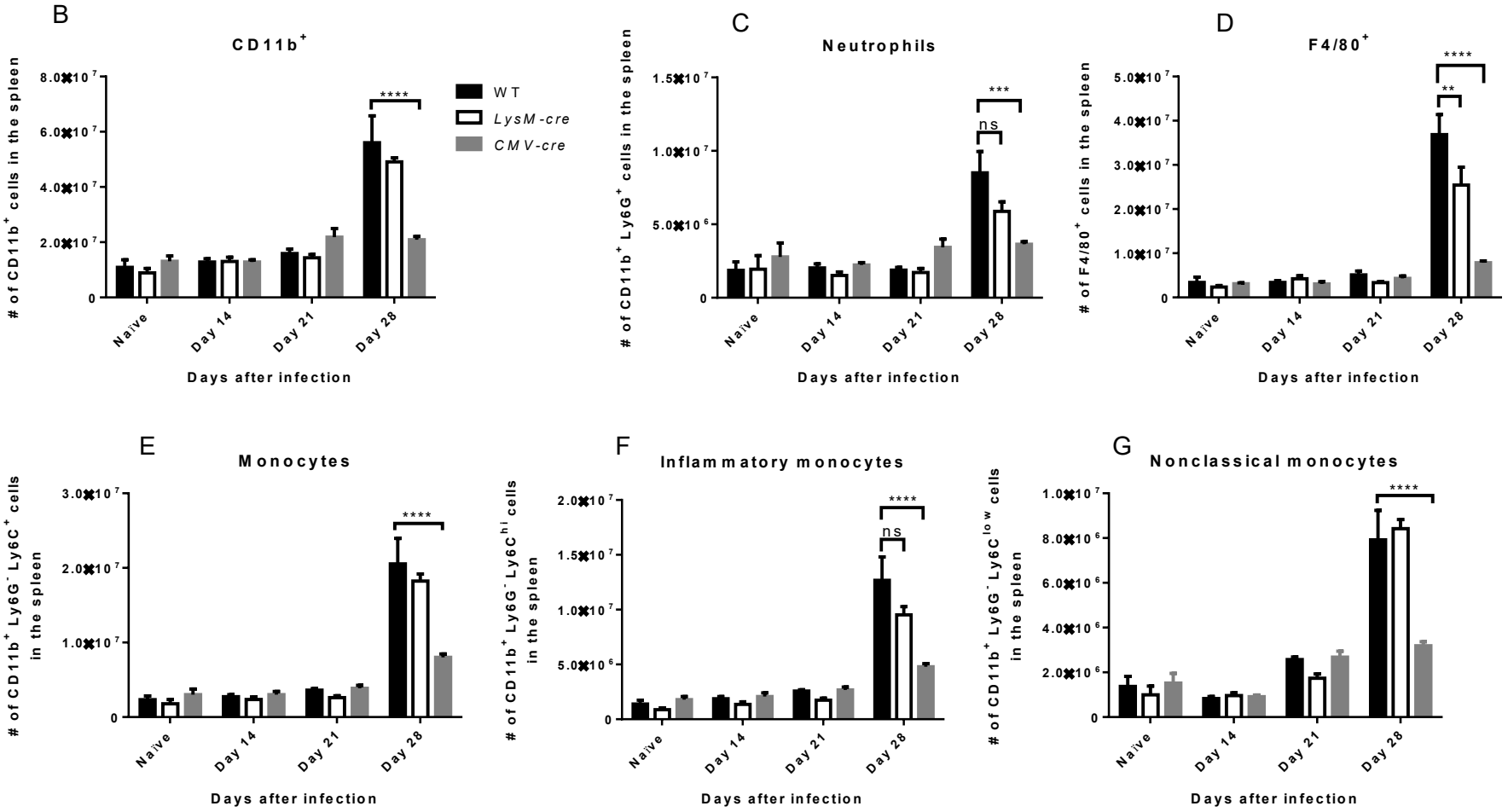
WT, *LysM-cre* and *CMV-cre* mice were infected with *L. donovani* and euthanized at d14, 21, and 28 p.i. (A-C) Representative scatter plots showing IFNγ$^+$(A), TNF$^+$ (B) and IFNγ$^+$ TNF$^+$ double producing (C) CD4$^+$ T cells in the liver of WT, *LysM-cre* and *CMV-cre* naïve and infected mice at different times p.i. (D-F) Graphs show the percentage of CD4$^+$ T cells producing IFNγ (D), TNF (E) and co-expressing IFNγ and TNF (F) following *L. donovani* infection in the liver from three groups of mice. Data is shown as the mean ± SEM with 3-4 mice per group, * denote p<0.05, ** denote p<0.01, **** denote p< 0.0001.

3.6. *LysM-cre* mice showed equivalent levels of myeloid cell recruitment to the spleen in response to *L. donovani* than WT mice

During VL, emergency hematopoiesis is skewed towards myelopoiesis with a heightened production of inflammatory monocytes (Ly6C^{hi} monocytes) (Abidin *et al.*, 2017). These monocytes already acquire a regulatory phenotype in the bone marrow and then develop into myeloid-derived suppressor cells (MDSC) in the spleen (Hammami *et al.*, 2017). We have previously shown that *Irf5*$^{-/-}$ mice failed to recruit pro-inflammatory cells and did not develop splenomegaly during chronic *L. donovani* infection (Paun *et al.*, 2011). Hence, we wanted to know whether IRF-5 expression in myeloid cells is required for the recruitment of these cells to the spleen. As expected, we observed a gradual increase in the number of myeloid cells present in the spleen over the course of infection, which went from around 10^7 in naïve mice to 6 x 10^7 cells at d28 p.i (**Fig. 10B**). All splenic myeloid cell populations increased accordingly over the time course of infection: neutrophils went from 2.5 x 10^6 to 8.5 x 10^6 at d28p.i. (**Fig. 10C**); F4/80$^+$ cells, which are mainly macrophages and other monocyte-derived cells, massively expanded from 0.5 x 10^7 to 3.75 x 10^7 (**Fig. 10D**); monocytes increased from 0.3 x 10^7 to 2 x 10^7 (**Fig. 10E-G**); and dendritic cells from 0.28 x 10^7 to 2.4 x 10^7 (**Fig. 10H**). In contrast and in agreement with our previous study (Paun *et al.*, 2011) myeloid cell recruitment to the spleen was significantly impaired in *L. donovani*-infected *CMV-cre* mice during chronic VL. Interestingly, infected *LysM-cre* mice displayed similar myeloid cell numbers than WT mice (**Fig. 10B-H**). To note, *CMV-cre* did not only fail to recruit an equivalent number of myeloid cells to the spleen but also develop a different composition of myeloid cells since they contain higher frequency of neutrophils (**Fig. 10I**) and a lower percentage of F4/80$^+$ cells than WT mice (**Fig. 10K**). However, *LysM-cre* mice did not show any difference in frequency of these cell compared with WT mice (**Fig. 10I-K**). Taken together, IRF-5 expressed by CD11b$^+$ cells does not seem to contribute to the recruitment or differentiation of myeloid cells to the spleen during chronic VL.

A


SSC-A
FSC-A
P1
FSC-H
FSC-A
Single Cells
MHC
CD11b
CD11b+
6,54
Ly6G
CD11b
Neutrophil
0,73
Ly6G-
99,0
MHC II
CD11c
DCs
1,67
CD11b
F4/80
F4-80+
2,24
CD11b
Ly6C
Monocytes
1,55
Ly6C low
0,48
Ly6C high
1,05


B
CD11b+
# of CD11b+ cells in the spleen
8.0✖10 7
6.0✖10 7
4.0✖10 7
2.0✖10 7
0
****
WT
LysM-cre
CMV-cre
Naïve
Day 14
Day 21
Day 28
Days after infection
C
Neutrophils
# of CD11b+ Ly6G+ cells in the spleen
1.5✖10 7
1.0✖10 7
5.0✖10 6
0
***
ns
Naïve
Day 14
Day 21
Day 28
Days after infection
D
F4/80+
# of F4/80+ cells in the spleen
5.0✖10 7
4.0✖10 7
3.0✖10 7
2.0✖10 7
1.0✖10 7
0
****
**
Naïve
Day 14
Day 21
Day 28
Days after infection
E
Monocytes
# of CD11b+ Ly6G- Ly6C+ cells in the spleen
3.0✖10 7
2.0✖10 7
1.0✖10 7
0
****
Naïve
Day 14
Day 21
Day 28
Days after infection
F
Inflammatory monocytes
# of CD11b+ Ly6G- Ly6Chi cells in the spleen
2.0✖10 7
1.5✖10 7
1.0✖10 7
5.0✖10 6
0
****
ns
Naïve
Day 14
Day 21
Day 28
Days after infection
G
Nonclassical monocytes
# of CD11b+ Ly6G- Ly6Clow cells in the spleen
1.0✖10 7
8.0✖10 6
6.0✖10 6
4.0✖10 6
2.0✖10 6
0
****
Naïve
Day 14
Day 21
Day 28
Days after infection

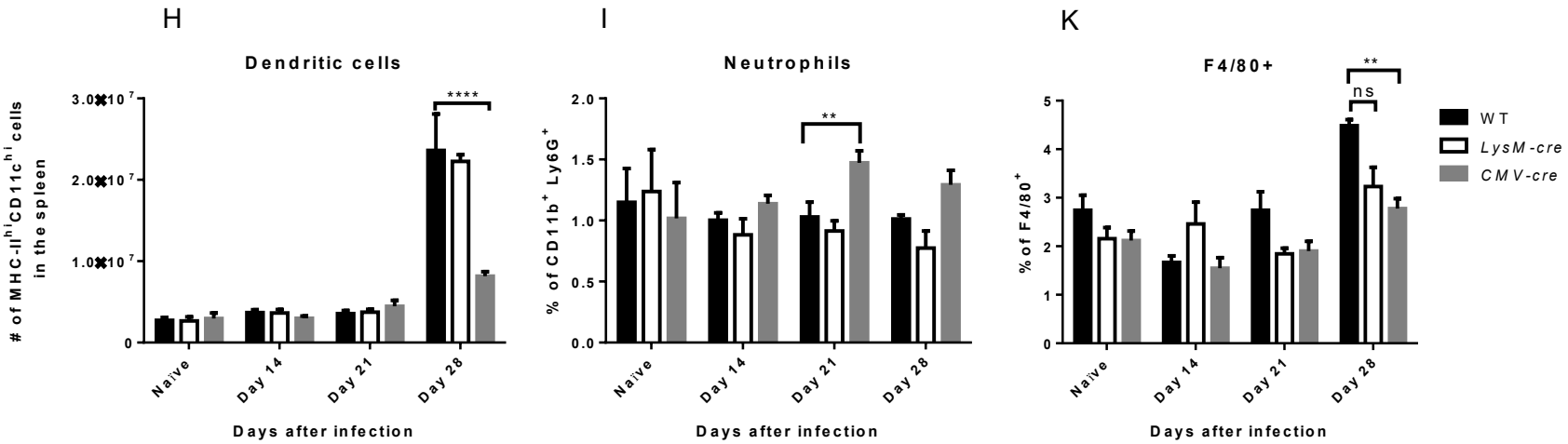


Figure 10.*LysM-cre* mice show similar numbers and frequency of myeloid cells in the spleen than WT mice.

WT, *LysM-cre* and *CMV-cre* mice were infected with *L. donovani* and sacrificed at d14, d21 and d28 p.i. (A) Representative FACS plots showing gating strategy for defining myeloid cell populations; neutrophils were excluded before analyzing monocytes and macrophages. (B-I) Absolute numbers of splenic $CD11b^+$ cells (B), $Ly6G^+$ neutrophils (C), $F4/80^+$ cells (D), total monocytes (E),$Ly6C^{high}$ monocytes (F), $Ly6C^{low}$ monocytes (G), and MHC-II^{high} $CD11c^{high}$ dendritic cells (H). (I-K) percentages of neutrophils (I) and $F4/80^+$ cells (K). Data is shown as the mean ± SEM with 3-4 mice per group, ns denote not significant, ** denote p<0.01, *** denote p<0.001, **** denote p< 0.0001

3.7. *LysM-Cre* failed to delete *Irf5* in splenic myeloid cells after d14 p.i

We were puzzled by the fact that all the parameters we assessed in *L. donovani*-infected *LysM-cre* mice were surprisingly similar to WT but not to *CMV-cre* mice. Our results also did not show any difference in macrophage and Th1 development as previous studies from other groups (Krausgruber *et al.*, 2011; Weiss *et al.*, 2013). We then suspected that the deletion efficiency of the cre recombinase was somehow diminished in splenic myeloid cells of *LysM-cre* mice. To answer this question, we infected WT and *LysM-cre* mice with *L. donovani* amastigotes and monitored *Irf5* expression by qPCR in splenic $CD11b^+$ cells isolated at various time points of infection. 10^6 cells were used to extract total RNA and conduct qPCR to observed IRF-5 deletion efficiency. As we suspected, we only saw a partial reduction in the expression of IRF-5 mRNA levels in splenic *LysM-cre* myeloid cells at d14p.i; in contrast, no deletion was observed in splenic *LysM-cre* myeloid cells isolated from naïve or infected mice at d21 and d28p.i (**Fig. 11**). To have a better proof, we also evaluated the IRF-5 expression at the protein level by using the Image Stream technology. However, we had difficulty in detecting the signal of IRF-5 in myeloid cells despite the fact that we were able to observe IRF-5 in other cell types, such as $CD4^+$ and $CD8^+$ T cells.

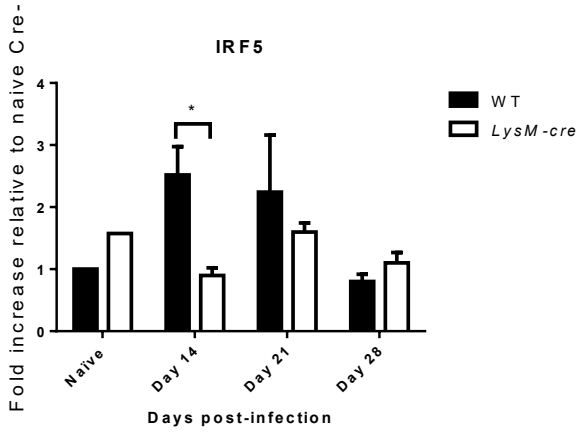


Figure 11: *LysM-Cre* failed to delete IRF-5 in splenic myeloid cells after d14 p.i.

WT and *LysM-cre* mice were infected with *L. donovani* and euthanized at d14, 21, and 28 p.i. Real-time PCR analysis of myeloid cells isolated inform the spleen of naive and infected mice measuring mRNA for IRF-5 and expressed as fold increase compared to naïve WT mice. Data represents mean ± SEM of 2 experiments, n=8, * denote $p<0.05$.

Our results are in agreement with a study (Abram *et al.*, 2014) reporting that *LysM-cre* had 37% deletion efficiency in splenic $CD11b^+$ cells. Interestingly, *LysM-cre* promoted significant deletion in neutrophils and tissue macrophages such as bronchoalveolar and peritoneal macrophages, but was not efficient in splenic red pulp and marginal zone macrophages.

LysM-cre also just showed 40% deletion in inflammatory and resident monocytes, and 10% in splenic dendritic cell populations. Hence, our results only demonstrate that IRF-5 expression in neutrophils does not play a role in the induction of Th-1 responses or in the migration of myeloid cells to the spleen. The role of IRF-5 in other splenic myeloid cells such as monocytes, macrophages or dendritic cells cannot be elucidated using *LysM-cre* mice.

3.8. *Irf5$^{flox/flox}$-Cd11c cre* mice show a lower level of myeloid cell recruitment, but similar Th1 responses

Because LysM-specific IRF-5-deficient mice are not a good model to study the role of IRF-5 in monocytes/macrophages in the spleen and the vast majority of splenic CD11b$^+$ cells during VL are CD11c$^+$ (Hammami *et al.*, 2017; Hammami *et al.*, 2015), we decided to use CD11c-specific IRF-5 deficient mice to investigate the role of IRF-5 in myeloid cells, particularly monocytes and monocyte-derived cells, during chronic VL. We infected WT *and Irf5$^{flox/flox}$-CD11c Cre$^+$* (*CD11c-cre*) with *L. donovani* amastigotes and monitored splenic myeloid cell populations and Th1 responses throughout the courses of infection. During the acute phase of infection (0-6 days), both groups of mice had similar numbers of CD11b$^+$ cells in the spleen. WT and *CD11c-cre* also showed similar numbers of CD11b$^+$ cells between d7 and 21 of *L. donovani* infection. However, at d28 p.i., CD11b$^+$ cells were massively recruited to the spleen of WT mice, but not to the spleen of *CD11c-cre* mice (**Fig. 12A**). To note, *CD11c-cre* mice also did not present any splenomegaly which is a visible characteristic of VL in WT mice (**Fig. 12B**). We next investigated whether the absence of IRF-5 in myeloid cells affected protective immune responses to *L. donovani*. Although fewer IFNγ$^+$ cells were detected in the spleen of *CD11c-cre* mice at d28 p.i. compared with WT mice, the difference was not significant. WT and *CD11c-cre* mice developed equivalent frequencies of IFNγ-producing CD4$^+$ T cells in liver during the whole course of infection (**Fig. 12C-D**). Taken together, IRF-5 expression in myeloid cells is required for myeloid cell recruitment or differentiation but not for the development of Th1 responses during VL.

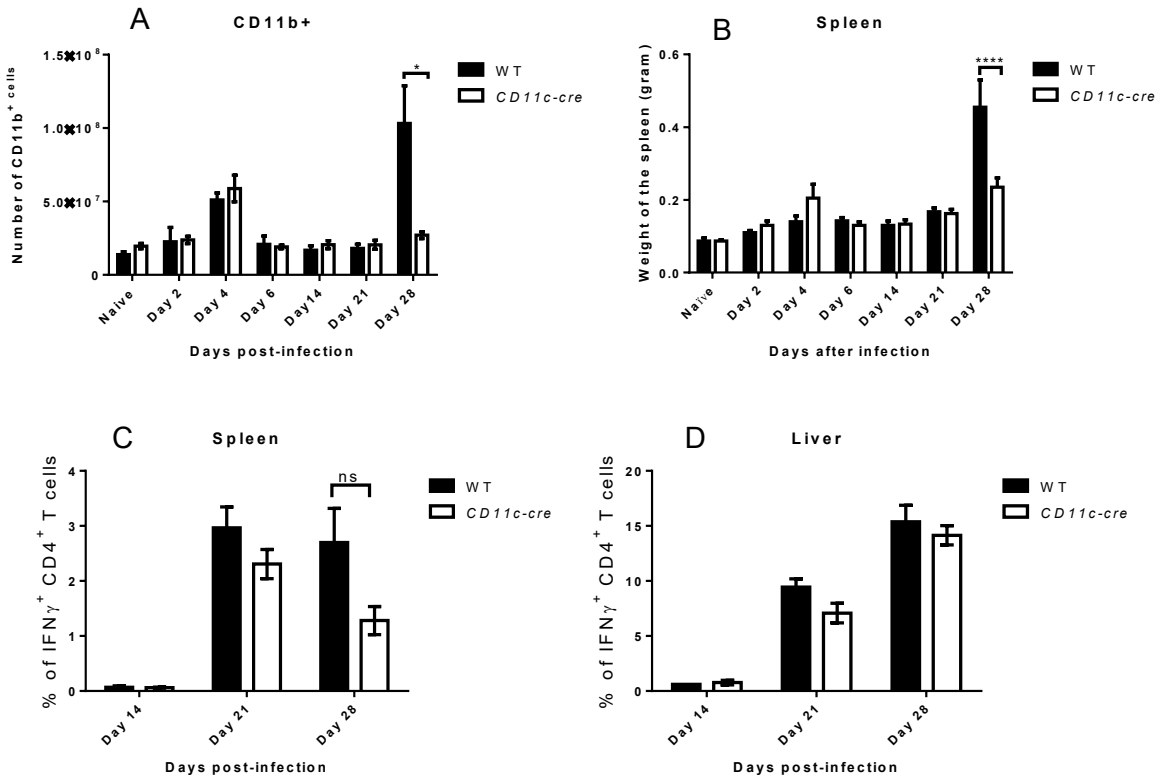


Figure 12.*CD11c-cre* mice show impaired numbers of splenic myeloid cells but equivalent frequency of Th1 responses to *L. donovani* infection than WT mice.

WT and *CD11c-cre* mice were infected with *L. donovani* and sacrificed at various time points of infection. (A) Number of myeloid cells in spleen and (B) spleen weights of WT and *CD11c-cre* naïve and infected mice. (C-D) Frequency of IFNγ$^+$ producing CD4$^+$ T cells was assessed by FASC in the spleen (C) and the liver (D) of infected mice from the two groups. Error bars indicate mean ± SEM with 4 mice per group, ns denote not significant, * denote p<0.05, **** denote p< 0.0001.

CHAPTER 4. DISCUSSION

L. donovani promotes emergency myelopoiesis in the bone marrow and induces a dramatic recruitment of myeloid cells to the spleen, which results in splenomegaly (Abidin *et al.*, 2017; Hammami *et al.*, 2017). Additionally, the inflammatory responses and the development of protective $CD4^+$ T cells during *L. donovani* infection are largely depended on IRF-5 expression (Paun *et al.*, 2011), but not by $CD4^+$ T cells (Fabie *et al.*, 2018). This suggests that Th1 development and initiation of pro-inflammatory responses during VL is possibly regulated by IRF-5 expression in antigen presenting cells. However, we could not prove our hypothesis using LysM-Cre–specific *Irf-5*$^{-/-}$ mice. Our results demonstrate that these mice are not an adequate model to study specific gene deletion in splenic myeloid cells during *L. donovani* infection. Hence, we used another promoter to specifically knock-out *Irf5* in myeloid cells, namely, the CD11c promoter. Interestingly, IRF-5 expression in myeloid cells regulated the migration or differentiation of myeloid cells in the spleen, but was not involved to the development and maintenance of Th1 cells.

LysM-cre conditional deletion has been successfully used to study the role of specific genes in several publications. LysM-cre effectively and specifically deletes genes in myeloid cells derived from the bone marrow (Imtiyaz *et al.*, 2010), macrophages in the lungs (McCubbrey *et al.*, 2017), Kupffer cells in the liver (Alzaid *et al.*, 2016), and skin or gut resident myeloid cells (Andrianne *et al.*, 2017; Malik *et al.*, 2018). In contrast, the deletion efficiency of LysM-cre in splenic myeloid populations was reported to be about 35%. Particularly, 75% deletion was achieved in neutrophils, less than 40% in both splenic MZM and red pulp macrophages, and approximately 10% in either cDCs or pDCs (Abram *et al.*, 2014). Interestingly, deletion in blood monocytes was also only about 40% or less. Furthermore, analysis of mice with an insertion of enhaced GFP (EGFP) into the LysM gene revealed that LysM is mostly highly expressed in granulocytes, especially neutrophils, followed by macrophages, and only occasionally in monocytes (Faust *et al.*, 2000). Following *L. donovani* infection, monocytes are the major cells that are progressively recruited to the spleen during chronic infection (Hammami *et al.*, 2017). Moreover, these cells can differentiate into macrophages and a major subset of DCs. Our results are in agreement with the literature. In our hands, the *LysM-cre* mouse model is not adequate to study gene functions in myeloid cells in the spleen, except perhaps in neutrophils. But in our model, IRF-5 expression in neutrophils is not required for inducing the inflammatory response following *L. donovani* infection.

The *CD11c-cre* mouse model has been shown to have good ablation efficiency in splenic myeloid cells, which highly express CD11c during chronic infection (Hammami *et al.*, 2017; Hammami *et al.*, 2018; Hammami *et al.*, 2015). Our results demonstrate that mice lacking

IRF-5 in CD11c^{+} cells display a massive impairment of myeloid cell recruitment to the spleen compared with WT mice. Moreover, in these mice *L. donovani* fail to induce splenomegaly, similarly to what was observed in total *Irf5*$^{-/-}$ mice (Paun *et al.*, 2011). We do not know what causes this defect in cell recruitment. Lower myeloid cell numbers in the spleen of CD11c-specific *Irf5*$^{-/-}$ mice could be resulting from an impairment of recruitment to the spleen (due to defective chemokine expression), reduced myelopoiesis in the bone marrow, or increased susceptible to cell death. The recruitment of cells to target organ are regulated by IL-23 or chemokines-produced by macrophages (Town *et al.*, 2009; Weiss *et al.*, 2015). However, *Irf5*$^{-/-}$ mice express higher level of IL-23 than WT mice (Paun *et al.*, 2011). Meanwhile, IRF-5-expressed macrophages are known to regulate the expression of CXCL1 and other chemokines, which are chemoattractants for myeloid cells to the inflamed site (Weiss *et al.*, 2015). Additionally, the differentiation of monocytes into CD64^{+} macrophages is regulated by IRF-5 (Weiss *et al.*, 2015). We can probably exclude the fact that IRF-5-deficient myeloid cells are more prone to apoptosis, because IRF-5 is actually a known mediator of cell death (Couzinet *et al.*, 2008; Fabie *et al.*, 2018; Hu & Barnes, 2009). Thus, the presence of lower inflammatory infiltration during chronic VL in the absence of IRF-5 in myeloid cells most likely results from impairment in recruitment or differentiation.

Our results also showed that IRF-5 expression in myeloid cells is not essential for the development of Th1 responses during VL. Our results are in disagreement with the literature that showed that the IRF-5-dependent polarization of M1 macrophages promoted to the development of Th1 (Krausgruber *et al.*, 2011). This could be because of the fact that these experiments were conducted *in vitro*. It is thus possible that *in vivo*, other transcription factors and pathways compensate for the lack of IRF-5. Along with IRF-5, other members of the IRF transcription factor family including IRF-3 and IRF-7 are known as master regulators of the expression of several pro-inflammatory cytokines and type I IFN (Ikushima *et al.*, 2013; Tamura *et al.*, 2008). A study demonstrated the combination of these three transcription factors induces type I IFN and IFN stimulated gene expression in response to West Nile virus infection (Lazear *et al.*, 2013). However, *Irf7*$^{-/-}$ mice showed a comparable frequency of IFNγ-producing CD4^{+} T cells than WT mice during VL (Paun *et al.*, 2011), which suggests that IRF-3 and IRF-5 functions may compensate each other during VL. Another factor that could make IRF-5 redundant is NF-κB. NF-κB is a major regulator of the transcription of pro-inflammatory cytokines such as TNF, IL-1, IL-6, IL-23 and various chemokines (Taniguchi & Karin, 2018). NF-κB can also be activated by TLRs stimulation via MyD88 signalling pathway. It appears that NF-κB and IRF-5 share many binding sites in the promoter of pro-inflammatory cytokines, which suggests a close relationship in collaborative co-activation and function at their target promoters (Moynagh, 2005). Specifically, NF-κB

p50 and IRF-5 are described as co-mediators for IFN-β and IL-6 expression upon TLR9 stimulation in human pDCs (Steinhagen *et al.*, 2013). Additionally, another subunit of NF-κB, ReIA, has been shown to interact with IRF-5 to induce transcriptional activation of inflammatory cytokines in human macrophages and dendritic cells (Krausgruber *et al.*, 2010; Saliba *et al.*, 2014). However, NF-κB can still be active even when IRF-5 is inhibited by chemical inhibition or Lyn kinase (Ban *et al.*, 2016; Cushing *et al.*, 2017). This suggests that a compensatory function of NF-κB could also occur in IRF-5-deficient myeloid cells during *L. donovani* infection.

In previous studies we showed that IRF-5 regulated the development of inflammation and Th1 responses during chronic VL (Paun *et al.*, 2011), but that this was not depend on an intrinsic IRF-5 effect in $CD4^+$ T cell (Fabie *et al.*, 2018). Our results reveal that the absence of IRF-5 in myeloid cells did not affect the differentiation of $IFN\gamma^+$ $CD4^+$ T cells. Either the lack of IRF-5 is compensated by other pathways of activation or IRF-5 expression in B cells could be involved. B cells have been demonstrated to be involved in sustaining inflammation during chronic VL via the upregulation of inflammatory cytokines and the induction of hypergammaglobulinemia (Silva-Barrios *et al.*, 2016). Additionally, IRF-5 is known to regulate the activation, proliferation, differentiation and antibody production of human naïve B cells (De *et al.*, 2017). Moreover, mice lacking IRF-5 showed a reduction in hypergammaglobulinemia and type I IFN levels, as well as increase in Th2 responses (Feng *et al.*, 2012). Thus, IRF-5 expression in B cells may favour the differentiation of Th1 responses. Further studies are required to investigate the role of IRF-5 in B cells during VL.

CAHPTER 5. CONCLUSIONS

In conclusion, we propose that the *LysM-Cre* mouse model is not adequate to study gene deletion in myeloid cells in the spleen. This model cannot be used to study gene deletions during VL, probably because the majority of cells recruited to the spleen during *L. donovani* infection are monocytes and *LysM-Cre* is not adequate to delete genes in monocytes. Using *Irf5*$^{flox/flox}$*-Cd11c* mice, we observe defective myeloid cell recruitment to the spleen at d28p.i.v. However, IRF-5 expression in myeloid cells is not required for the development of Th1 responses or for parasite control. These results suggest a possible role of IRF-5 in B cells in promoting inflammation and protective $CD4^+$ T cell responses against *L. donovani*.

CHAPTER 6. SYNOPSIS

6.1. Introduction

6.1.1. Leishmaniose

La leishmaniose est un ensemble de maladies parasitaires transmises par des vecteurs, considérée comme un problème de santé publique majeur dans le monde entier. Cette maladie affecte environ 12 million de personnes dans 88 pays, avec une incidence annuelle approximative de 700 000 à 1 million de cas et provoque de 20 000 à 30 000 décès chaque année (World Health Organization, 2018). La leishmaniose fait partie des maladies tropicales et subtropicales les plus négligées, affectant les populations des régions les plus pauvres, qui ont des difficultés à obtenir un diagnostic et un traitement efficace. Les patients atteints de leishmaniose présentent un large éventail de symptômes, allant de la forme la plus commune qui consiste en une forme cutanée à guérison spontanée à la forme viscérale, la manifestation la plus grave de la leishmaniose, dans laquelle le taux de mortalité avoisine les 100% si elle n'est pas traitée. Les médicaments utilisés pour traiter la leishmaniose présentent de nombreuses limitations, notamment une longue durée de traitement, des effets secondaires graves et sont associés à l'apparition de résistances. A ce jour, aucun vaccin n'est disponible. La leishmaniose humaine est causée par environ 20 des 30 espèces de Leishmania identifiées (Rodrigues et al., 2016). Il existe 3 conditions médicales principales de la maladie:

- La leishmaniose cutanée (CL), la forme la plus courante, se caractérise par une ou plusieurs lésions cutanées transitoires sur les parties exposées du corps (papule ou ulcère) qui guérissent souvent spontanément chez les hôtes immunocompétents et laissent une cicatrice, bien que les parasites persistent en petite quantité tout au long de la vie. Environ 95% des cas de CL sont observés dans le bassin méditerranéen, l'Amérique, le Moyen-Orient et l'Asie centrale.
- La leishmaniose cutanée muqueuse peut entraîner une destruction partielle ou totale de la muqueuse de la bouche, du nez ou de la gencive. Environ 90% des cas de leishmaniose muco-cutanée surviennent en Bolivie, au Brésil, en Éthiopie et au Pérou.
- La leishmaniose viscérale (LV), forme mortelle appelée aussi kala-azar, est causée par des parasites de type *L. donovania* / *L. infantum* (également appelé *L. chagasi*) dans le

vieux monde et par *L. infantum* dans le nouveau monde. La leishmaniose viscérale est cliniquement comparable dans l'Ancien et le Nouveau Monde et se caractérise par une perte de poids, des poussées de fièvre irrégulières, une hypergammaglobulinémie, une hépatosplénomégalie et une immunosuppression. L'incidence annuelle de la LV est estimée aux alentours de 50 000 à 90 000 cas. La plupart des cas de LV surviennent en Afrique de l'Est, au Brésil et en Asie du Sud-Est (World Health Organization, 2018).

Les leishmanioses sont principalement des zoonoses naturellement transmises entre animaux et humains par la piqûre de phlébotomes infectés. Les animaux domestiques et sauvages peuvent être des hôtes réservoirs; les animaux sauvages sont porteurs du parasite mais ne développent pas la maladie, alors que les chiens peuvent développer des symptômes de leishmaniose. La leishmaniose peut également être transmise de manière anthropique, les populations humaines constituant le principal réservoir d'infection. La transmission anthropique de la leishmaniose cutanée et viscérale est importante dans le sous-continent indien, en Afrique de l'Est et dans les régions urbaines.

6.1.2. Facteur régulateur de l'interféron 5

Il a été démontré, à l'origine, qu'IRF-5 est directement impliqué dans l'induction de la production d'interféron de type I par des cellules immunitaires innées en réponse à des infections virales (Barnes *et al.*, 2001). Depuis, de nombreuses autres fonctions ont été attribuées à ce facteur de transduction. Par exemple, il s'est avéré qu'il était un médiateur de la suppression tumorale (Hu *et al.*, 2005) et qu'il était impliqué dans le cycle cellulaire, l'apoptose (Barnes *et al.*, 2003; Fabie *et al.*, 2018), les infections fongiques et parasitaires (del Fresno *et al.*, 2013; Paun *et al.*, 2011) ainsi que dans la régulation de cytokines pro-inflammatoires (Krausgruber *et al.*, 2011; Takaoka *et al.*, 2005). Il a été rapporté qu'IRF-5 était exprimé dans divers types de cellules immunitaires, telles que les macrophages, les neutrophiles, les cellules dendritiques classiques (cDC), les cellules dendritiques plasmacytoïdes (pDC), les cellules B et les cellules T (Fabie *et al.*, 2018; Heng & Painter, 2008; Ishikawa *et al.*, 2015).

Le rôle d'IRF-5 dans la régulation de la réponse inflammatoire est bien documenté. Dans les cDC, les CD plasmacytoïdes (pDC) et les macrophages, IRF-5 régule la production de diverses cytokines pro-inflammatoires, y compris le tumor necrosis factor (TNF), IL-6, IL-12 et IL-23, suite à la stimulation des TLR7 et / ou 9 (Takaoka *et al.*, 2005). De plus, il s'est avéré être un marqueur spécifique de la polarisation des macrophages, en macrophages inflammatoires M1, *in vitro et in vivo* (Krausgruber *et al.*, 2011; Weiss *et al.*, 2013). Le rôle important d'IRF-5 dans les réponses immunitaires à l'infection microbienne et à

l'inflammation a été révélé chez des souris déficientes en IRF-5. Ces souris ont montré une résistance au choc létal d'endotoxines (Takaoka *et al.*, 2005) ainsi qu'une sensibilité à l'infection par *L. donovani,* qui était associée à un développement altéré des réponses Th1 et à une forte réduction de l'infiltration de cellules inflammatoires (Paun *et al.*, 2011; Takaoka *et al.*, 2005). Plus récemment, il a été signalé qu'IRF-5 induisait directement l'apoptose des lymphocytes T $CD4^+$ et des hépatocytes dans les maladies chroniques (Alzaid *et al.*, 2016; Fabie *et al.*, 2018). Le mécanisme d'induction de l'apoptose nécessite la régulation à la hausse de DR5, Fas, la voie du ligand induisant l'apoptose liée au facteur de nécrose tumorale (TRAIL) ou la perte du potentiel de membrane mitochondriale, qui sont tous régulés par IRF-5 (Alzaid *et al.*, 2016; Cevik *et al.*, 2017; Fabie *et al.*, 2018; Hu & Barnes, 2009).

IRF-5 a également été décrit comme un suppresseur de tumeur. IRF-5 est directement activé par p53 et transloqué vers le noyau à la suite de dommages à l'ADN, suggérant un rôle de ce facteur de transcription dans les réponses aux dommages de l'ADN (Barnes *et al.*, 2003). IRF-5 inhibe la croissance des cellules tumorales en favorisant l'expression des gènes pro-apoptotiques, notamment p21, Bak, la caspase 8, la DAP kinase 2 et Bax, dans une voie distincte de la p53 (Barnes *et al.*, 2003; Hu *et al.*, 2005). IRF-5 inhibe également le potentiel de transformation de virus oncogéniques tels que le virus Epstein-Barr et le virus de l'herpès associé au sarcome de Kaposi, et inhibe la croissance des cellules tumorales (Bi *et al.*, 2011b; Xu *et al.*, 2011). Il inhibe également la réplication du virus de l'hépatite C et inhibe la progression du carcinome hépatocellulaire en empêchant la migration et l'invasion des hépatocytes (Cevik *et al.*, 2017). De plus, l'expression d'IRF-5 n'est pas détectée dans la leucémie lymphoïde aiguë ou chronique, le cancer gastro-intestinal, et est associée au développement du cancer du sein et à la propagation métastatique, suggérant un rôle putatif d'IRF-5 en tant que suppresseur de tumeur (Barnes *et al.*, 2003; Bi *et al.*, 2011a; Yamashita *et al.*, 2010). En revanche, IRF-5 présente un niveau d'expression élevé dans les carcinomes thyroïdiens, favorisant ainsi le potentiel de prolifération et de tumorigenèse des cellules tumorales thyroïdiennes (Massimino *et al.*, 2012).

Chez l'homme, les polymorphismes pour IRF-5 sont associés à un risque accru de diverses maladies auto-immunes, telles que le lupus érythémateux systémique (SLE) (Graham *et al.*, 2006), la polyarthrite rhumatoïde (PR) (Dieguez-Gonzalez *et al.*, 2008; Stahl *et al.*, 2010), sclérodermie systémique (ScS) (Carmona *et al.*, 2013), la sclérose en plaques (MS) (Kristjansdottir *et al.*, 2008) et les maladies inflammatoires de l'intestin (MICI) (Dideberg *et al.*, 2007). Chez les patients atteints de SLE, une augmentation significative de l'expression d'IRF-5 a été observée dans le sang par rapport aux donneurs sains (Hellquist *et al.*, 2009). En outre, l'expression accrue d'IRF-5 est liée à l'haplotype à risque de SLE et à une

production élevée d'IFN de type I chez les patients atteints de SLE (Niewold *et al.*, 2008). Le modèle de souris *Irf5*$^{-/-}$ SLE a révélé l'importance d'IRF-5 dans la régulation des niveaux d'expression d'IFN de type I (Feng *et al.*, 2012), limitant les réponses Th2 (Feng *et al.*, 2012; Xu *et al.*, 2012), favorisant le recrutement de monocytes inflammatoires (Yang *et al.*, 2012), la modification de la commutation de classe d'IgG (Feng *et al.*, 2012) et le développement de plasmocytes (Lien *et al.*, 2010). IRF-5 était également fortement exprimé dans le modèle murin d'arthrite induite par antigène; Dans ce modèle, IRF-5 régule le recrutement des neutrophiles sur les sites de la blessure en contrôlant l'expression et la sécrétion de chimokines (Weiss *et al.*, 2015).

6.2. Hypothèse

La leishmaniose viscérale (LV), plus connue sous le nom de kala-azar, est une maladie potentiellement mortelle caractérisée par une fièvre persistante de faible intensité, une hépatosplénomégalie, une pancytopénie, une hypergammaglobulinémie et une immunosuppression (Kaye *et al.*, 2004; Kumar & Nylen, 2012). *L. donovani*, une espèce de parasites protozoaires intracellulaires appartenant au genre *Leishmania*, est l'un des agents étiologiques de la LV (Kumar & Nylen, 2012). Ce parasite protozoaire intracellulaire infecte et prolifère de manière systémique dans les macrophages et autres cellules phagocytaires des organes internes, généralement la rate, le foie et la moelle osseuse. Les lymphocytes T CD4$^+$ et CD8$^+$ produisant de l'IFN-γ sont nécessaires pour contrôler l'infection. L'IL-12 dérivée des CD est nécessaire pour promouvoir les réponses protectrices des lymphocytes T CD4$^+$ et CD8$^+$. Les cellules myéloïdes jouent un rôle important pendant la LV expérimentale. En effet, *L. donovani* active fortement la myélopoïèse d'urgence dans la moelle osseuse, ce qui favorise la production et la libération de monocytes inflammatoires dotés d'un phénotype régulateur. Les monocytes et les neutrophiles sont constamment recrutés dans la rate, où ils acquièrent des fonctions de cellules suppressives dérivées de cellules myéloïdes (MDSC) (Abidin *et al.*, 2017; Hammami *et al.*, 2017). Dans une étude précédente, nous avions signalé le rôle essentiel d'IRF-5 dans l'induction d'une inflammation lors d'une infection par *L. donovani*. Nous avons constaté qu'en l'absence d'IRF-5, non seulement les souris n'avaient pas développé de réponse Th1, mais avaient également une infiltration inflammatoire fortement réduite dans la rate et le foie (Paun *et al.*, 2011). Ce défaut dans la réponse Th1 n'était pas dû une fonction intrinsèque d'IRF-5 dans les lymphocytes T CD4$^+$, car les souris *Irf5*$^{-/-}$ spécifiques des lymphocytes T développaient une réponse Th1 plus fortes que leurs homologues de type sauvage (Fabie *et al.*, 2018).

Par conséquent, nous avons émis l'hypothèse que l'expression d'IRF-5 dans les cellules myéloïdes est nécessaire pour favoriser et maintenir la réponse protectrice Th1 et initier une inflammation. Cette hypothèse est également basée sur la littérature qui montre que l'expression d'IRF-5 favorise la polarisation des macrophages inflammatoires (M1) à la fois *in vitro* et *in vivo*, nécessaires au développement des Th1 (Alzaid *et al.*, 2016; Krausgruber *et al.*, 2011). De plus, les souris dépourvues d'IRF-5 dans les cellules myéloïdes n'ont pas réussi à développer de lymphocytes T $CD4^+$ sécrétant de l'IFNγ dans le contexte de maladies chroniques du foie (Alzaid *et al.*, 2016). L'objectif de mon projet sera donc d'étudier le rôle d'IRF-5 dans les cellules myéloïdes à l'aide de souris possédant une délétion cellulaires spécifique et du modèle expérimental de leishmaniose viscérale. Tout d'abord, je vais étudier l'expression d'IRF-5 dans les cellules myéloïdes au cours d'une infection aiguë par *L. donovani*. Ensuite, j'examinerai le rôle d'IRF-5 dans les cellules myéloïdes dans la réponse immunitaire contre l'infection par *L. donovani*, en tenant compte du recrutement cellulaire, du développement des réponses protectrices et inhibitrices des lymphocytes T $CD4^+$ et de la charge parasitaire hépatique et splénique. Pour tester notre hypothèse, nous avons établi deux objectifs:

1. Caractériser l'expression d'IRF-5 dans les cellules myéloïdes pendant la LV expérimentale.

2. Étudier le rôle d'IRF-5 dans les cellules myéloïdes pendant une infection par *L. donovani*.

6.3. Résultats

6.3.1. L'absence d'expression d'IRF-5 dans les cellules myéloïdes n'affecte pas l'évolution de l'infection à *L. donovani*

Comme les cellules myéloïdes, en particulier les monocytes, s'accumulent dans la rate des souris infectées au cours de la progression de la LV (Hammami *et al.*, 2017), nous avons voulu voir si l'expression d'IRF5 par les cellules myéloïdes influe sur l'évolution de l'infection par *L. donovani*. Pour ce faire, nous avons infecté des souris C57BL/6 *Irf5*$^{flox/flox}$ *LysM-Cre*$^+$ (ici appelées *LysM-cre*) ou leurs souris *Cre*-littermates (appelées ici WT) et C57BL/6 *IRF5*$^{flox/flox}$ *CMV-Cre*$^+$ (*CMV-cre*) avec des amastigotes de *L. donovani*. Les hépato- et splénomégalies ont été évaluées à différents moments de l'infection pour les différentes souches de souris. Comme prévu, nous avons observé une augmentation du poids de la rate chez les souris WT par rapport aux souris naïves au cours de l'infection, en particulier au 28éme jour, lorsque la rate des souris WT est cinq fois plus grosse que celles des souris naïves. En accord avec Paun et al., les souris *CMV-cre* n'ont pas développé de

splénomégalie. De manière surprenante, les souris *LysM-cre* ont révélé un poids de la rate similaire à celui des souris WT. Aucune différence significative dans le poids du foie n'a été observée entre les trois groupes. Bien que les souris *Irf5*$^{-/-}$ aient une rate plus petite, elles étaient plus sensibles à *L. donovani* en ayant une charge parasitaire plus élevée dans la rate et le foie que les souris WT (Paun *et al.*, 2011), 2011). Par la suite, nous avons examiné si les souris *LysM-cre* étaient plus sensibles au parasite. Bien que la charge parasitaire splénique chez les souris *CMV-cre* soit légèrement supérieure, elle ne différait pas significativement du groupe WT. De plus, nous avons observé des charges parasitaires similaires dans la rate des souris *LysM-cre* et WT pendant toute la durée de l'infection. En revanche, les souris *CMV-cre* ont développé une charge parasitaire significativement plus élevée dans le foie que les souris WT, tandis qu'un niveau d'infection similaire a été observé dans le foie des souris WT et *LyM-cre*. L'ensemble de ces résultats nous permettent de dire que l'expression d'IRF-5 dans les cellules myéloïdes n'affecte pas l'évolution de l'infection par *L. donovani* ni dans la rate, ni dans le foie.

6.3.2. L'expression d'IRF-5 dans les cellules myéloïdes n'est pas requise pour les réponses des cellules T dans la rate après l'infection à *L. donovani*

Chez les souris WT, les lymphocytes T CD4$^+$ IFNγ$^+$ spléniques sont déjà détectés au jour 14, bien que leur fréquence soit faible (environ 1,5% des lymphocytes T CD4$^+$ totaux); ces réponses atteignent un pic au jour 21 p.i (environ 6% des lymphocytes T CD4$^+$ totaux) et diminue à nouveau à 3% au jour 28 p.i. Les souris LysM-Cre et CMV ont développé des réponses de lymphocytes T CD4$^+$ producteurs d'IFNγ similaires dans la rate par rapport aux souris WT (Fig. 7A et 7D). La fréquence des lymphocytes T CD4$^+$ TNF$^+$ ou co-exprimant IFNy et TNF étaient également équivalentes parmi les trois groupes de souris pendant toute la durée de l'infection (Fig. 7B, C, E et F). Dans son ensemble, l'expression d'IRF-5 dans les cellules myéloïdes n'affecte pas la réponse Th1 dans la rate lors de l'infection par *L. donovani*.

Nous avons observé une expansion significativement plus importante des cellules Tr1 chez les souris *CMV-cre* comparées aux souris WT. Cependant, les souris *LysM-cre* ont présenté des fréquences Tr1 similaires à celles du groupe WT (Fig. 8). Ainsi, la délétion conditionnelle d'IRF-5 dans la population de cellules myéloïdes ne semble pas affecter le développement des lymphocytes T CD4$^+$ IFNγ$^+$ IL-10$^+$ au cours de la LV.

6.3.3. L'IRF-5 dans les cellules myéloïdes n'est pas nécessaire pour les réponses Th1 dans le foie après l'infection à *L. donovani*

Alors que le développement de lymphocytes T $CD4^+$ $IFNy^+$ est significativement altéré chez les souris *CMV-cre* au jour 28 p.i. Les souris *LysM-cre* ont été capables de générer une forte réponse Th1, comparable à celles observées dans le groupe WT (Fig. 9A et 9D). De même, la fréquence des lymphocytes T $CD4^+$ produisant uniquement du TNF était maximale au jour 14 p.i (environ 2,3% des lymphocytes T $CD4^+$ totaux) chez les souris WT et diminuait progressivement au cours de l'infection. De plus, les lymphocytes T $CD4^+$ des *CMV-cre* ont généré significativement moins de cellules produisant uniquement du TNF par rapport aux souris WT et aucune différence n'a été détectée entre les souris *LysM-cre* et WT (Fig. 9B et 9E). De même, nous n'avons pas observé de différence dans la fréquence des coproducteurs d'IFNγ et de TNF entre le groupe WT et le groupe *Lysm-cre*; cependant, les souris *CMV-cre* ont présenté une réduction significative de cette population de cellules au jour 28 p.i. (Fig. 9C et F). Ainsi, l'ablation conditionnelle d'IRF-5 dans les cellules myéloïdes en utilisant le modèle *LysM-cre* n'affecte pas le développement des réponses des lymphocytes T lors d'une infection par *L. donovani* dans le foie.

L'expression d'IRF-5 par les cellules $CD11b^+$ ne semble pas contribuer au recrutement ou à la différenciation des cellules myéloïdes dans la rate lors de la LV chronique.

6.3.4. LysM-Cre n'a pas réussi à supprimer Irf5 dans les cellules myéloïdes spléniques après j14 p.i.

Nous avons été intrigués par le fait que tous les paramètres que nous avons évalués chez les souris *LysM-cre* infectées par *L. donovani* étaient étonnamment similaires aux souris WT mais pas aux souris *CMV-cre*. Nos résultats n'ont pas non plus montré de différence dans le développement des macrophages et des Th1, contrairement aux études précédentes menées par d'autres groupes (Krausgruber *et al.*, 2011; Weiss *et al.*, 2013). Nous avons alors suspecté que l'efficacité de délétion de la recombinase cre était en quelque sorte diminuée dans les cellules myéloïdes spléniques de souris *LysM-cre.* Pour répondre à cette question, nous avons infecté des souris WT et *LysM-cre* avec des amastigotes de *L. donovani* et suivi l'expression d'*Irf5* par qPCR dans des cellules spléniques $CD11b^+$ isolées à différents moments de l'infection. 10^6 cellules ont été utilisées pour extraire l'ARN total et effectuer une qPCR pour déterminer l'efficacité de délétion d'IRF-5. Comme nous le soupçonnions, nous avons seulement observé une réduction partielle de l'expression des niveaux d'ARNm d'IRF-5 dans les cellules myéloïdes *LysM-cre* au jour 14 p.i, en revanche, aucune délétion n'a été observée dans les cellules myéloïdes spléniques *LysM-cre* isolées à partir de souris naïves ou infectées aux jours 21 et 28 p.i (Fig. 11). Pour confirmer ces résultats, nous avons également évalué l'expression d'IRF-5 au niveau protéique en utilisant la technologie ImageStream. Cependant, nous avons eu du mal à détecter le signal d'IRF-5

dans les cellules myéloïdes malgré le fait que nous avons pu observer IRF-5 dans d'autres types de cellules, tels que les lymphocytes T $CD4^+$ et $CD8^+$.

Nos résultats sont en accord avec une étude (Abram *et al.*, 2014) rapportant que *LysM-cre* aurait une efficacité de délétion de 37% dans les cellules spléniques $CD11b^+$. Il est intéressant de noter que *LysM-cre* favorise une délétion significative dans les neutrophiles et les macrophages tissulaires tels que les macrophages broncho-alvéolaires et péritonéaux, mais n'est pas efficace dans les macrophages de la pulpe rouge et ceux de la zone marginale. *LysM-cre* induit également une délétion de 40% dans les monocytes inflammatoires et résidents et de 10% dans les populations de cellules dendritiques spléniques. Par conséquent, nos résultats démontrent seulement que l'expression d'IRF-5 dans les neutrophiles ne joue pas un rôle dans l'induction de la réponse Th1 ou dans la migration des cellules myéloïdes vers la rate. Le rôle d'IRF-5 dans d'autres cellules myéloïdes spléniques telles que les monocytes, les macrophages ou les cellules dendritiques ne peut être élucidé en utilisant des souris *LysM-cre*.

6.3.5. Les souris *Irf5$^{flox/flox}$-Cd11c cre$^+$* présentent un niveau de recrutement des cellules myéloïdes plus faible, mais des réponses Th1 similaires

Comme les souris spécifiquement déficientes pour IRF5 avec LysM ne sont pas un bon modèle pour étudier le rôle d'IRF5 dans les monocytes/macrophages dans la rate et que la grande majorité des cellules spléniques $CD11b^+$ durant la LV étaient $CD11c^+$ (Hammami *et al.*, 2017; Hammami *et al.*, 2015) nous avons décidé d'utiliser des souris spécifiquement déficientes pour IRF-5 avec CD11c pour étudier le rôle d'IRF5 dans les cellules myéloïdes, en particulier les monocytes, au cours de la LV chronique. Nous avons infecté des souris WT et *Irf5$^{flox/flox}$-CD11c Cre$^+$* (*CD11c-cre*) avec des amastigotes de *L. donovani* et avons observé les populations de cellules myéloïdes spléniques et la réponse Th1 tout au long de l'infection. Au jour 28 p.i, les cellules $CD11b^+$ ont été massivement recrutées dans la rate des souris WT, mais pas dans la rate des souris *CD11c-cre* (Fig 12A). Il est à noter que les souris *CD11c-cre* ne présentaient pas non plus de splénomégalie, ce qui est une caractéristique visible de VL chez les souris WT (Fig.12B). Nous avons ensuite examiné si l'absence d'IRF-5 dans les cellules myéloïdes affectait les réponses immunitaires protectrices contre *L. donovani*. Bien que moins de cellules $IFNy^+$ aient été détectées dans la rate des souris *CD11c-cre* au jour 28 p.i. par rapport aux souris WT, la différence n'était pas significative. Les souris WT et *CD11c-cre* ont développé des fréquences équivalentes de lymphocytes T CD4 producteurs d'IFNγ dans le foie pendant toute la durée de l'infection (Fig.12C-D). L'ensemble de ces résultats montrent que l'expression d'IRF-5 dans les cellules

myéloïdes est nécessaire pour le recrutement des cellules myéloïdes, mais pas pour le développement de la réponse Th1 au cours de la LV.

6.4. Disscusion

L. donovani favorise la myélopoïèse d'urgence dans la moelle osseuse et induit un recrutement spectaculaire de cellules myéloïdes dans la rate, ce qui entraîne une splénomégalie (Abidin *et al.*, 2017; Hammami *et al.*, 2017). De plus, les réponses inflammatoires et le développement de lymphocytes T $CD4^+$ protecteurs au cours d'une infection par *L. donovani* dépendent largement de l'expression d'IRF-5 (Paun *et al.*, 2011), mais pas par les lymphocytes T $CD4^+$ (Fabie *et al.*, 2018). Cela suggère que le développement des Th1 et l'initiation de réponses pro-inflammatoires au cours de la LV sont probablement régulés par l'expression d'IRF-5 dans des cellules présentant l'antigène. Cependant, nous n'avons pas pu prouver notre hypothèse en utilisant des souris *LysM-Cre–Irf5*$^{-/-}$. Nos résultats démontrent que ces souris ne constituent pas un modèle adéquat pour étudier la délétion spécifique de gènes dans les cellules myéloïdes spléniques au cours d'une infection par *L. donovani*. Par conséquent, nous avons utilisé un autre promoteur pour déléter spécifiquement *Irf5* dans les cellules myéloïdes, à savoir le promoteur CD11c. Fait intéressant, l'expression d'IRF-5 dans les cellules myéloïdes régule la migration ou la différenciation des cellules myéloïdes dans la rate, mais n'est pas impliquée dans le développement et le maintien des lymphocytes Th1.

La délétion conditionnelle avec *LysM-Cre* a été utilisée avec succès pour étudier le rôle de gènes spécifiques dans plusieurs publications. *LysM-Cre* supprime efficacement et spécifiquement les gènes dans les cellules myéloïdes dérivées de la moelle osseuse (Imtiyaz et al., 2010), les macrophages dans les poumons (McCubbrey *et al.*, 2017), les cellules de Kupffer dans le foie (Alzaid *et al.*, 2016) et les cellules myéloïdes résidant dans la peau ou les intestins (Andrianne *et al.*, 2017; Malik *et al.*, 2018). En revanche, l'efficacité de suppression de *LysM-Cre* dans les populations myéloïdes spléniques était d'environ 35%. Plus précisément, une suppression de 75% a été réalisée dans les neutrophiles, moins de 40% dans les macrophages spléniques MZM et de la pulpe rouge et environ 10% dans les cDC ou les pDC (Abram *et al.*, 2014). Fait intéressant, la délétion dans les monocytes sanguins n'était également que d'environ 40% ou moins. Après l'infection par *L. donovani*, les monocytes sont les principales cellules qui sont progressivement recrutées dans la rate au cours d'une infection chronique (Hammami *et al.*, 2017). De plus, ces cellules peuvent se différencier en macrophages et en différents sous-ensembles de DC. Nos résultats sont en accord avec la littérature. Entre nos mains, le modèle murin *LysM-Cre* ne permet pas

d'étudier les fonctions des gènes dans les cellules myéloïdes de la rate, sauf peut-être chez les neutrophiles. Mais dans notre modèle, l'expression d'IRF-5 dans les neutrophiles n'est pas nécessaire pour induire une réponse inflammatoire à la suite d'une infection par *L. donovani*.

Le modèle murin *CD11c-Cre* a démontré une bonne efficacité d'ablation dans les cellules myéloïdes spléniques, qui expriment fortement CD11c au cours d'une infection chronique (Hammami *et al.*, 2017; Hammami *et al.*, 2018; Hammami *et al.*, 2015). Nos résultats démontrent que les souris dépourvues d'IRF-5 dans les cellules $CD11c^+$ présentent une altération massive du recrutement des cellules myéloïdes dans la rate par rapport aux souris WT. De plus, chez ces souris, *L. donovani* ne parvient pas à induire une splénomégalie, contrairement à ce qui a été observé chez les souris $Irf5^{-/-}$ (Paun *et al.*, 2011). Nous ne savons pas ce qui cause ce défaut de recrutement cellulaire. Un nombre inférieur de cellules myéloïdes dans la rate de souris $Irf5^{-/-}$ spécifiques de CD11c pourrait être dû à une altération du recrutement dans la rate (en raison d'une expression altérée de chimiokines), à une réduction de la myélopoïèse dans la moelle osseuse ou à une augmentation de la susceptibilité à la mort cellulaire. Le recrutement de cellules dans l'organe cible est régulé par l'IL-23 ou des chimiokines produites par les macrophages (Town *et al.*, 2009; Weiss *et al.*, 2015). Cependant, les souris $Irf5^{-/-}$ expriment des taux d'IL-23 supérieurs à ceux des souris WT (Paun *et al.*, 2011). Dans le même temps, il est connu que les macrophages exprimant IRF-5 régulent l'expression de CXCL1 et d'autres chimiokines, qui sont des chimioattractants pour les cellules myéloïdes au niveau des sites d'inflammation (Weiss *et al.*, 2015). De plus, la différenciation des monocytes en macrophages $CD64^+$ est régulée par IRF-5 (Weiss *et al.*, 2015). Nous pouvons probablement exclure le fait que les cellules myéloïdes déficientes en IRF-5 sont plus sujettes à l'apoptose, car IRF-5 est en fait un médiateur connu de la mort cellulaire (Couzinet *et al.*, 2008; Fabie *et al.*, 2018; Hu & Barnes, 2009). Ainsi, la plus petite infiltration de cellules inflammatoires au cours de la LV chronique en l'absence d'IRF-5 dans les cellules myéloïdes résulte plus probablement d'une diminution du recrutement ou de la différenciation.

Nos résultats ont également montré que l'expression d'IRF-5 dans les cellules myéloïdes n'est pas essentielle au développement de la réponse Th1 au cours de la LV. Nos résultats sont en désaccord avec la littérature qui a montré que la polarisation en macrophages M1 dépendant d'IRF-5, favorise le développement de la réponse Th1 (Krausgruber *et al.*, 2011). Cela pourrait être dû au fait que ces expériences ont été menées *in vitro*. Il est donc possible *qu'in vivo*, d'autres facteurs de transcription et d'autres voies compensent l'absence d'IRF-5. Parallèlement à IRF-5, d'autres membres de la famille des facteurs de transcription IRF, notamment IRF-3 et IRF-7, sont connus comme des régulateurs clefs de l'expression de

plusieurs cytokines pro-inflammatoires et de l'IFN de type I (Ikushima *et al.*, 2013; Tamura *et al.*, 2008). Une étude a démontré que la combinaison de ces trois facteurs de transcription induisait l'expression de gènes stimulés par l'IFN de type I en réponse à l'infection par le virus du Nil occidental (Lazear *et al.*, 2013). Cependant, les souris *Irf7*$^{-/-}$ ont montré une fréquence comparable de lymphocytes T CD4^{+} produisant de l'IFNγ par rapport aux souris WT pendant la LV (Paun *et al.*, 2011), ce qui suggère que les fonctions d'IRF-3 et d'IRF-5 pourraient se compenser pendant la VL. Le facteur NF-κB est un autre facteur susceptible de compenser la perte d'IRF-5. NF-κB est un régulateur majeur de la transcription de cytokines pro-inflammatoires telles que TNF, IL-1, IL-6, IL-23 et diverses chimiokines (Taniguchi & Karin, 2018). NF-κB peut également être activé par la stimulation des TLR via la voie de signalisation MyD88. Il semble que NF-κB et IRF-5 partagent de nombreux sites de liaison parmi les promoteurs de cytokines pro-inflammatoires, ce qui suggère une relation étroite dans la co-activation et la fonction au niveau de promoteurs cibles (Moynagh, 2005). Spécifiquement, NF-κB p50 et IRF-5 sont décrits comme des co-médiateurs pour l'expression de l'IFN-β et IL-6 lors de la stimulation du TLR9 dans des pDC humains (Steinhagen *et al.*, 2013). De plus, une autre sous-unité de NF-κB, ReIA, interagit avec IRF-5 pour induire l'activation de la transcription des cytokines inflammatoires dans les macrophages et les cellules dendritiques humains (Krausgruber *et al.*, 2010; Saliba *et al.*, 2014). Cependant, NF-κB peut rester actif même lorsque IRF-5 est inhibé par une inhibition chimique ou par la kinase Lyn (Ban *et al.*, 2016; Cushing *et al.*, 2017). Ceci suggère qu'une fonction de compensation de NF-κB pourrait également se produire dans les cellules myéloïdes déficientes en IRF-5 au cours d'une infection par *L. donovani*.

Dans des études précédentes, nous avions montré qu'IRF-5 régulait le développement de l'inflammation et la réponse Th1 au cours d'une LV chronique (Paun *et al.*, 2011), mais que cela ne dépendait pas d'un effet intrinsèque d'IRF-5 dans les lymphocytes T CD4^{+} (Fabie *et al.*, 2018). Nos résultats révèlent que l'absence d'IRF-5 dans les cellules myéloïdes n'a pas d'incidence sur la différenciation des lymphocytes T CD4^{+} IFNγ^{+}. Le manque d'IRF-5 pourrait être compensé par d'autres voies d'activation ou bien par l'expression d'IRF-5 dans les lymphocytes B. Il a été démontré que les lymphocytes B sont impliqués dans le maintien de l'inflammation au cours de la LV chronique via la régulation positive de cytokines inflammatoires et l'induction de l'hypergammaglobulinémie (Silva-Barrios *et al.*, 2016). De plus, IRF-5 est connu pour réguler l'activation, la prolifération, la différenciation et la production d'anticorps des cellules B naïves de l'homme (De *et al.*, 2017). De plus, les souris dépourvues d'IRF-5 présentent une hypergammaglobulinémie et des taux d'IFN de type I diminués, ainsi qu'une augmentation de la réponse Th2 (Feng *et al.*, 2012). Ainsi, l'expression d'IRF-5 dans les lymphocytes B peut favoriser la différenciation de la réponse

Th1. Des études complémentaires sont nécessaires pour étudier le rôle d'IRF-5 dans les lymphocytes B pendant la LV.

6.5. Conclusion

En conclusion, nous proposons que le modèle de souris *LysM-Cre* n'est pas adéquat pour étudier la délétion de gènes dans les cellules myéloïdes de la rate. Ce modèle ne peut pas être utilisé pour étudier les délétions de gènes au cours de la LV, probablement parce que la majorité des cellules recrutées dans la rate lors de l'infection par *L. donovani* sont des monocytes et que *LysM-Cre* ne permet pas d'induire une délétion des gènes dans les monocytes. En utilisant des souris *Irf5*$^{flox/flox}$*-Cd11c-Cre*, nous observons un recrutement diminué des cellules myéloïdes dans la rate au jour 28 post-infection. Cependant, l'expression d'IRF-5 dans les cellules myéloïdes n'est pas requise pour le développement de la réponse Th1 ou pour le contrôle du parasite. Ces résultats suggèrent un possible rôle d'IRF-5 dans les lymphocytes B lors de la promotion de l'inflammation et de la réponse protectrice des lymphocytes T CD4^{+} contre *L. donovani*.

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APPENDIX: Additional Contribution

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IRF-5 Promotes Cell Death in CD4 T Cells during Chronic Infection

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Résumé de l'article

Le facteur de transcription « Interferon regulatory factor 5 » (IRF-5) a des fonctions importantes au niveau de la réponse immunitaire innée et initie la réponse pro-inflammatoire lors d'infections par des agents pathogènes. IRF-5 est constitutivement exprimé dans plusieurs types cellulaires dont les cellules dendritiques plasmacytoïdes, les monocytes et les lymphocytes B. Nous avons précédemment montré qu'IRF-5 est également exprimé dans les lymphocytes T au cours l'infection. Cependant, le rôle d'IRF-5 dans les lymphocytes T est encore inconnu. Dans cette étude, nous démontrons que l'expression d'IRF-5 est augmentée dans les lymphocytes T CD4 (IFN)-γ^+ durant l'infection avec *L. donovani*. Ce facteur de transcription est induit par le matériel de cellules apoptotiques à travers la voie du « Toll-like receptor 7 » (TLR7) et promeut l'expression du « death receptor 5 » (DR5). L'activation d'IRF-5 sensibilise les lymphocytes T CD4 à la mort cellulaire. La destruction tissulaire et l'inflammation chronique étant des caractéristiques communes aux infections persistantes, l'activation d'IRF-5 dans les lymphocytes T CD4 pourrait représenter un mécanisme d'action général menant à la suppression des réponses protectives des lymphocytes T CD4, favorisant ainsi l'établissement d'une infection chronique.

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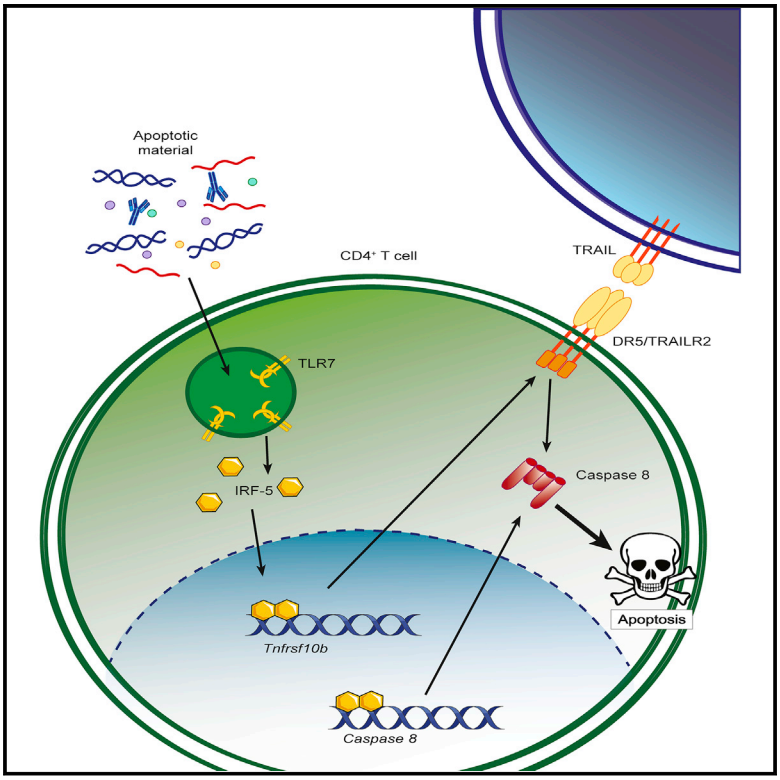
IRF-5 Promotes Cell Death in CD4 T Cells during Chronic Infection

Graphical Abstract



Highlights

- Apoptotic cell material triggers TLR7 in IFNγ$^+$ CD4 T cells during chronic infection
- Signaling via TLR7 induces the upregulation and activation of IRF-5 in CD4 T cells
- IRF-5 promotes the upregulation of DR5 and caspase 8
- Inflammatory tissue damage sensitizes protective CD4 T cells to cell death

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In Brief

Fabié et al. report a role for IRF-5 in IFNγ$^+$ CD4 T cell death during chronic visceral leishmaniasis. IRF-5 is activated by apoptotic cell material, derived from inflammatory tissue damage, via TLR7, resulting in upregulation of death receptor 5 and caspase 8 and cell death of protective CD4 T cells.

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IRF-5 Promotes Cell Death in CD4 T Cells during Chronic Infection

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SUMMARY

The transcription factor interferon regulatory factor 5 (IRF-5) plays an important function in innate immunity and in initiating pro-inflammatory responses against pathogens. IRF-5 is constitutively expressed in several cell types, including plasmacytoid dendritic cells, monocytes, and B cells. We have previously reported that IRF-5 is also expressed in T cells during infection. The role of IRF-5 in T cells is yet unknown. Here, we demonstrate that IRF-5 is increasingly expressed in interferon (IFN)-γ^+ CD4 T cells over the course of *L. donovani* infection. This transcription factor is induced by apoptotic material via Toll-like receptor 7 (TLR7) and promotes the expression of death receptor 5 (DR5). IRF-5 activation sensitizes CD4 T cells to cell death. Because tissue disruption and chronic inflammation are common characteristics of persistent infections, activation of IRF-5 in CD4 T cells may represent a common pathway that leads to suppression of protective CD4 T cell responses, favoring the establishment of chronic infection.

INTRODUCTION

The transcription factor interferon regulatory factor 5 (IRF-5) has been implicated in the antiviral immune response because of its involvement in the transcriptional activation of both type I interferon (IFN) genes and genes encoding key pro-inflammatory cytokines in antigen-presenting cells (Barnes et al., 2001, 2004; Takaoka et al., 2005). Besides its important role in innate immunity, IRF-5 also appears to be a critical regulator of DNA-damage-induced apoptosis and tumor suppression (Bi et al., 2014; Couzinet et al., 2008). In human, various *IRF5* polymorphisms are also linked to autoimmune diseases, including lupus erythematosus (Graham et al., 2006), rheumatoid arthritis (Dieguez-Gonzalez et al., 2008), and inflammatory bowel disease (Dideberg et al., 2007). IRF-5 is constitutively expressed by several cell types, such as plasmacytoid dendritic cells, monocytes, and B cells. Activation of IRF-5 occurs after phosphorylation, following which this transcription factor delocalizes from the cytoplasm to the nucleus. IRF-5 can be activated by Toll-like receptor 7 (TLR7) and TLR9 via the MyD88 signaling pathway, directly by viral infections and type I IFN (Schoenemeyer et al., 2005), or by apoptotic and/or necrotic material (Stone et al., 2012).

Visceral leishmaniasis (VL) is a potentially lethal chronic disease that causes hepatosplenomegaly, anemia, cachexia, hypergammaglobulinemia, and immunosuppression (Kaye et al., 2004). Mice infected with the protozoan parasite *Leishmania donovani*, a causative agent of VL, also develop hepatosplenomegaly (Kaye et al., 2004). Although infection in the liver is self-resolving, the parasite persists in the spleen of infected mice. Chronic infection in the spleen is associated with disruption of the splenic architecture and massive recruitment of pro-inflammatory cells. IFN-γ-producing CD4 T cell responses are essential for controlling parasite growth but only develop 2 to 3 weeks after infection and gradually show signs of functional exhaustion soon thereafter. CD8 T cells also contribute to protection; however, these cells undergo very limited expansion and become dysfunctional during chronic infection (Hammami et al., 2015; Joshi et al., 2009). Protective T cell responses are also suppressed by interleukin-10 (IL-10) derived from IFN-γ^+IL-10$^+$ CD4 T cells (Ranatunga et al., 2009; Stäger et al., 2006) and B cells (Bankoti et al., 2012) and by myeloid-derived suppressor cells (Hammami et al., 2017).

We have previously reported that *Irf5*$^{-/-}$ mice are more susceptible to *L. donovani* infection. Susceptibility was associated with a significant reduction of inflammatory cell infiltration in the liver and spleen and with a severe impairment in the development of Th1 cells (Hammami et al., 2015; Paun et al., 2011). IRF-5 was indeed required for inducing IL-12 production by dendritic cells and sustaining Th1 responses. Our results were in agreement with Krausgruber et al. (2011), who demonstrated the critical role of this transcription factor in determining lineage commitment of inflammatory macrophages by promoting IL-12 while repressing IL-10. Interestingly, we noticed that IRF-5 mRNA was also upregulated in total splenic T cells mainly during chronic infection (Paun et al., 2011). The role of IRF-5 in T cells is yet unknown.

Here, we show that IRF-5 is upregulated and activated in CD4 T cells during chronic VL. This transcription factor is induced by TLR7 triggering. IRF-5 activation results in the upregulation of death receptor 5 (DR5) and, ultimately, in CD4 T cell death.

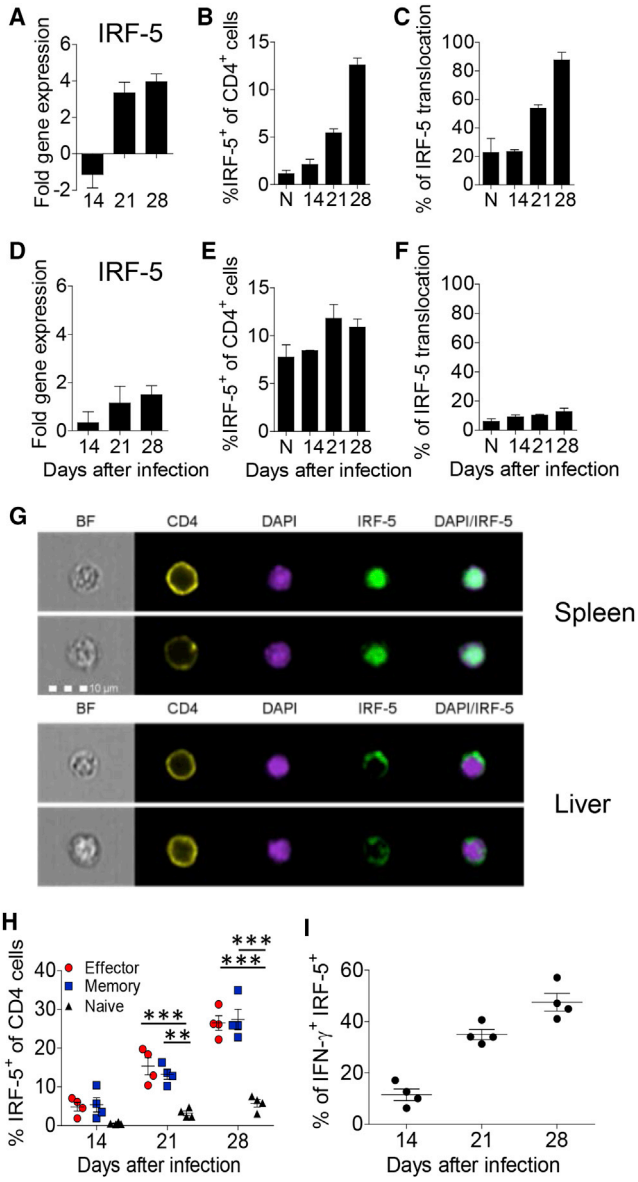


Figure 1. IRF-5 Is Activated in Splenic CD4 T Cells during Chronic Visceral Leishmaniasis

Mice were infected with 2 × 10^7 amastigotes intravenously and euthanized at various time points after infection.

(A) Real-time PCR analysis of splenic CD4 T cells from *L. donovani*-infected mice measuring mRNA for IRF5.

(B) Percentage of splenic CD4 T cells expressing IRF5 after *L. donovani* infection.

(C) Percentage of IRF5 nuclear translocation in splenic CD4 T cells.

(D) Real-time PCR analysis of IRF-5 mRNA expression levels in hepatic CD4 T cells.

(E and F) Percentage of hepatic CD4 T cells expressing IRF5 (E) and percentage of hepatic CD4 T cells showing IRF5 nuclear translocation (F).

(G) Representative images of CD4 T cells expressing IRF5: CD4 (yellow); nucleus (purple); and IRF5 (green). The last column represents co-expression of nuclear staining and IRF5. Upper row: examples of IRF-5 nuclear localization in spleen are shown; lower row: examples of cytoplasmic localization in liver are shown.

(H) Percentage of splenic effector ($CD44^+$ $CD62L^-$), memory ($CD44^+$ $CD62L^+$), and naive ($CD44^-$ $CD62L^+$) CD4 T cells expressing IRF5 over the course of infection.

(I) Percentage of IRF-5^+ within the IFN-γ^+ CD4 T population.

Data represent mean ± SEM of one of 3 or 4 independent experiments; n = 3–5. **p < 0.01; ***p < 0.001.

RESULTS

IRF-5 Is Activated in Splenic CD4 T Cells during Chronic VL

We have previously reported that IRF-5 mRNA is expressed in splenic T cells in *L. donovani*-infected mice (Paun et al., 2011). Because effector CD8 T cells are not functional (Hammami et al., 2015; Joshi et al., 2009) and do not express IRF-5 during chronic VL (data not shown), we decided to concentrate our analysis on CD4 T cells. Splenic CD4 T cells upregulated IRF-5 mRNA from day 21 (d21) post infection (p.i.) on (Figure 1A). Image Stream flow cytometry analysis revealed that about 5%–12% of splenic CD4 T cells expressed IRF-5 on the protein level (Figure 1B) and that this transcription factor was increasingly translocated to the nucleus over the course of infection (Figures 1C, 1G [upper rows], S1A, and S1B), indicating that it was activated. Although a significant percentage of CD4 T cells expressed IRF-5 in the liver (Figures 1D and 1E), this transcription factor remained in the cytoplasm and was not translocated to the nucleus (Figures 1F, 1G [lower rows], S1A, and S1B). Next, we wanted to identify splenic CD4 T cell subpopulations expressing IRF-5. As shown in Figure 1H, effector and memory cells, but not naive CD4 T cells, were positive for IRF-5 during chronic VL (Figure S1C). A closer analysis of subpopulations expressing IRF-5 revealed that 40%–50% of IFN-γ-producing CD4 T cells were expressing this transcription factor at d21 and d28 p.i. (Figure 1I). Hence, IRF-5 appears to be active in splenic, but not hepatic, IFN-γ-producing CD4 T cells during chronic *L. donovani* infection.

Cell-Specific IRF-5 Ablation in T Cells Results in Higher Frequencies of IFN-γ-Producing CD4 T Cells during VL

To better characterize the function of IRF-5 in CD4 T cells, we generated T-cell-specific *Irf5*$^{-/-}$ mice. We first compared the frequency of CD4 T cells and T cell precursors in the thymus of naive *Irf5*$^{flox/flox}$-*Cre*$^+$ (IRF-5 deficient) to that of *Cre*$^-$ (IRF-5 sufficient) littermate controls to ensure that the absence of IRF-5 did not alter T cell development. The percentage of various precursors and of CD4 T cells present in the thymus was comparable in both groups of mice (Figures 2A, S2A, and S2B), as was the frequency of CD4 T cells in the spleen (Figures 2B and S2C). This confirmed that T cell development was normal in our T-cell-specific IRF-5-deficient mice and that we could use these mice to investigate IRF-5 function in T cells during VL. Because total *Irf5*$^{-/-}$ mice develop defective Th1 responses following *L. donovani* infection (Paun et al., 2011), we sought to determine whether this transcription factor was required for IFN-γ production by CD4 T cells. Hence, we stimulated naive CD4 T cells from *Cre*$^+$ and *Cre*$^-$ mice with anti-CD3/CD28 in the presence of recombinant IL-12 and monitored IFN-γ production. The absence of IRF-5 did not seem to affect the differentiation into IFN-γ-producing Th1 cells (Figure 2C), suggesting that defective Th1 responses in *L. donovani*-infected total *Irf5*$^{-/-}$ mice was more a

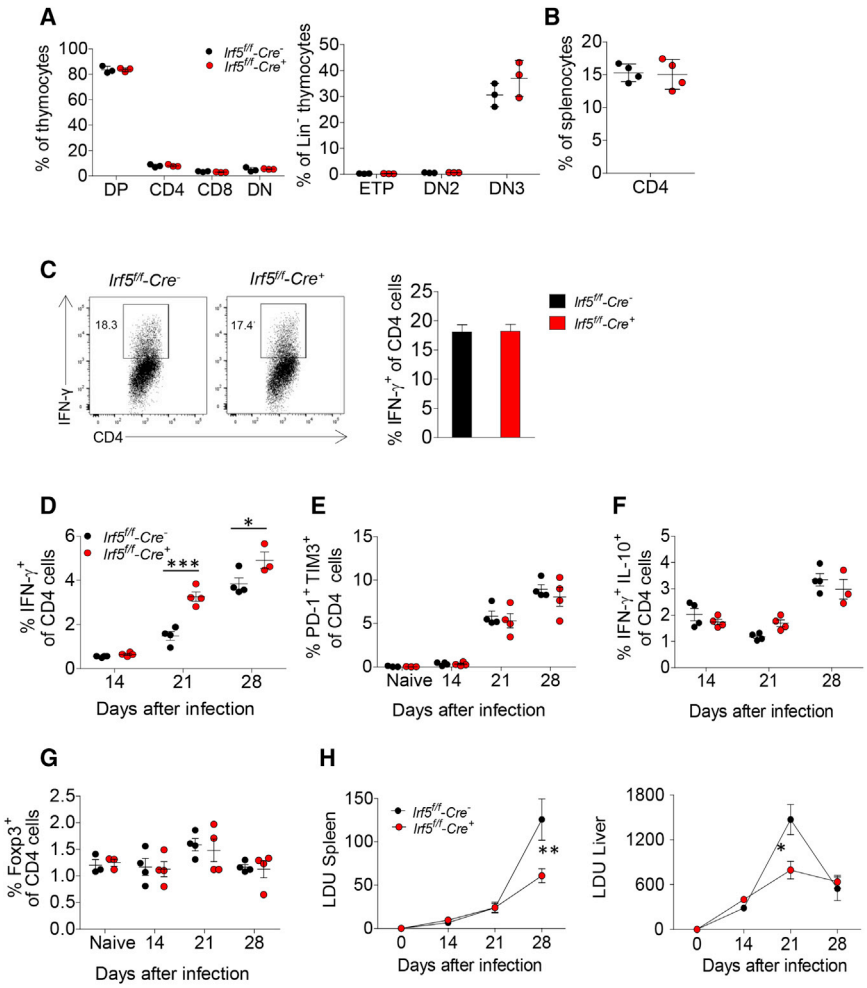


Figure 2. Cell-Specific IRF-5 Ablation in T Cells Results in Higher Frequencies of IFN-γ-Producing CD4 T Cells during VL

Thymus and spleen from naive *Irf5*$^{f/f}$*-Cre*$^{-}$ and *Irf5*$^{f/f}$*Cre*$^{+}$ mice were harvested to assess T cells development.

(A) Percentage of thymic T cells in *Irf5*$^{f/f}$*-Cre*$^{-}$ and *Irf5*$^{f/f}$*-Cre*$^{+}$ mice, showing CD4^{+}CD8^{+} double positive (DP), CD4^{+} or CD8^{+} single positive, and CD4^{-} CD8^{-} double negative (DN) cells (left panel). T cell progenitors were first gated on Lin^{-} lineage negative (B220, CD3ε, CD11b, GR1, Ter119, and CD8α) and then separated according to c-Kit (CD117) and CD25 expression as follows: c-Kit^{+} CD25^{-} ETP; c-Kit^{+} CD25^{+} DN2; and c-Kit^{-} CD25^{+} DN3 (right panel).

(B) Percentage of splenic CD4 T cells in naive *Irf5*$^{f/f}$*-Cre*$^{-}$ and *Irf5*$^{f/f}$*-Cre*$^{+}$ mice.

(C) CD4^{+} T cells from *Irf5*$^{f/f}$*-Cre*$^{-}$ and *Irf5*$^{f/f}$*-Cre*$^{+}$ mice were stimulated with anti-CD3/CD28 in the presence of IL-12. Representative FACS plots and percentage of IFN-γ^{+} CD4 T cells are shown.

(D–G) Mice were infected with 2 × 10^{7} amastigotes intravenously and euthanized at various time points after infection. Graphs show frequency of (D) IFN-γ^{+}CD3^{+} CD4^{+}, (E) PD-1^{+} TIM3^{+}CD3^{+} CD4^{+}, (F) IFN-γ^{+} IL-10^{+}CD3^{+} CD4^{+}, and (G) FoxP3^{+}CD3^{+} CD4^{+} T cells in infected *Irf5*$^{f/f}$*-Cre*$^{-}$ and *Irf5*$^{f/f}$*-Cre*$^{+}$ mice.

(H) Graphs represent the splenic (left) and hepatic (right) parasite burden at different times of *L. donovani* infection.

Data are shown as the mean ± SEM of one of 3 independent experiments; n = 3–5. *p < 0.05; **p < 0.01; ***p < 0.001.

consequence of antigen-presenting cell (APC) malfunction rather than an intrinsic T cell effect, in agreement with Krausgruber et al. (2011).

We then infected *Cre*$^{+}$ and *Cre*$^{-}$ mice with *L. donovani* and monitored CD4 T cell responses over the course of infection. Interestingly, similar frequencies of splenic IFN-γ^{+} CD4 T cells were detected in both groups of mice at d14 p.i.; however, at d21 and d28 p.i., IFN-γ-producing CD4 T cells were significantly more abundant in the spleen of T-cell-specific *Irf5*$^{-/-}$ mice (Figures 2D and S2D). We next investigated whether this increase was a consequence of fewer CD4 T cells becoming exhausted during chronic VL. The frequency of exhausted PD-1^{+} TIM3^{+} CD4 T cells did not vary between both groups of infected mice (Figure 2E). To rule out the possibility that IRF-5 was affecting the survival of regulatory T cells and that this had an indirect impact on the frequencies of IFN-γ^{+} CD4 T cells, we examined Tr1 and Tregs responses. No differences were observed in the percentage of IFN-γ^{+} IL-10^{+} Tr1 cells (Stäger et al., 2006; Figure 2F) or Foxp3^{+} CD4 T cells (Figures 2G and S2D), concurring with our previous observation that IRF-5 was mainly active in IFN-γ^{+} CD4 T cells. Overall, these results indicate that IRF-5 may be involved in regulating the survival of IFN-γ^{+} CD4 T cells.

Because Th1 responses are required to control parasite growth (Bankoti and Stäger, 2012), it was not surprising that *Cre*$^{+}$ mice displayed a significantly lower splenic and hepatic parasite burden (Figure 2H).

TLR7 Is Required to Induce IRF-5 in CD4 T Cells during VL

In the following experiment, we wanted to identify which pathway was responsible for IRF-5 upregulation in IFN-γ^{+} CD4 T cells to better understand the role this transcription factor might have in these cells. IRF-5 can be induced, among others, by IFN-I and TLR7. IFN-I is upregulated during chronic VL (Silva-Barrios et al., 2016), and TLR7 can be triggered by the parasite (Paun et al., 2011; Silva-Barrios et al., 2016). Hence, we performed adoptive transfer experiments using *Ifnar*$^{-/-}$ and *Tlr7*$^{-/-}$ CD4 T cells and monitored the expression of IRF-5 in endogenous and adoptively transferred cells during the course of *L. donovani* infection. When we transferred *Ifnar*$^{-/-}$ CD4 T cells into congenic mice, we noticed that IRF-5 was upregulated (Figure 3A) and translocated (Figure 3B) at similar levels as endogenous wild-type (WT) CD4 T cells. Moreover, no differences were observed in the percentage of

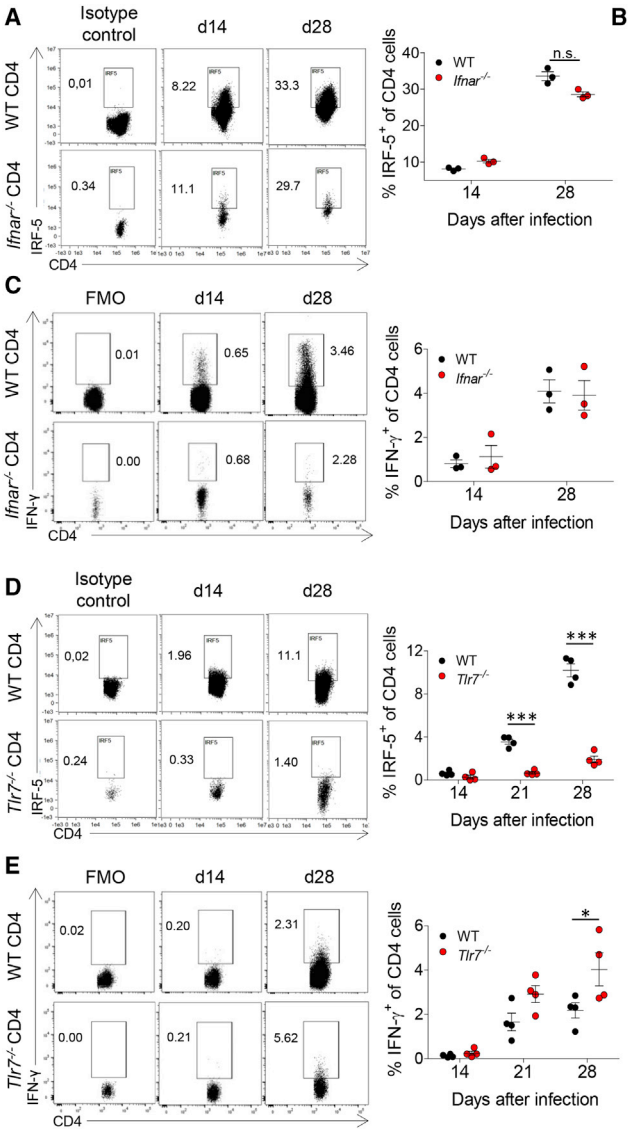


Figure 3. TLR7 Is Required to Induce IRF-5 in CD4 T Cells during VL

CD4 T cells from *Ifnar*$^{-/-}$ and *Tlr7*$^{-/-}$ mice were adoptively transferred into CD45.1 congenic mice a day prior to infection with 2 × 10^7 *L. donovani* amastigotes.

(A–C) Graphs show (A) the percentage of endogenous (WT) and adoptively transferred (*Ifnar*$^{-/-}$) CD4 T cells expressing IRF5, (B) the percentage of IRF5 nuclear translocation, and (C) the frequency of splenic IFN-γ$^+$ CD3$^+$ CD4$^+$ T cells over the course of *L. donovani* infection.

(D and E) Graphs represent (D) the percentage of endogenous (WT) and adoptively transferred (*Tlr7*$^{-/-}$) CD4 T expressing IRF5 and (E) the percentage of splenic IFN-γ$^+$ CD3$^+$ CD4$^+$ T cells over the course of *L. donovani* infection.

Data are shown as the mean ± SEM of one of 3 independent experiments; n = 3–4. *p < 0.05; ***p < 0.001.

IFN-γ-producing cells between WT and *Ifnar*$^{-/-}$ CD4 T cells (Figure 3C). In contrast, adoptively transferred *Tlr7*$^{-/-}$ CD4 T cells failed to upregulate IRF-5 (Figure 3D), suggesting that TLR7 is upstream of IRF-5 in CD4 T cells. Similarly to *Irf5*$^{-/-}$ cells (Figure 2D), significantly more *Tlr7*$^{-/-}$ CD4 T cells expressed IFN-γ at d28 p.i. (Figure 3E).

TLR7-Induced IRF-5 Promotes the Upregulation of DR5 and Induces Cell Death in CD4 T Cells from *L. donovani*-Infected Mice

Several studies have reported the expression of various TLRs, including TLR7, on murine T cell subsets (Caramalho et al., 2003; Fukata et al., 2008; Gelman et al., 2004; Tomita et al., 2008). To better identify the role of TLR7 in inducing IRF-5, we first assessed its expression on CD4 T cells over the course of *L. donovani* infection. The expression kinetics of TLR7 mRNA was similar to the one observed for IRF-5: at d14 p.i., it was expressed at similar levels as in CD4 T cells from naive mice; however, by d21 and d28 p.i., CD4 T cells from infected mice significantly upregulated TLR7 (Figure 4A). We next investigated whether TLR7 was responsible for IRF-5 induction in CD4 T cells during VL. CD4 T cells were purified from the spleen of *L. donovani*-infected mice at d14, d21, and d28 p.i. and treated *in vitro* with the TLR7 agonist imiquimod (IMQ). IRF-5 mRNA expression was evaluated 24 and 30 hr after stimulation (Figure 4B). As expected, treatment with IMQ did not induce IRF-5 in CD4 T cells purified from the spleen at d14 p.i., because the cells do not yet express TLR7 (Figure 4B). In contrast, when we stimulated cells enriched at d21 and d28 p.i., we saw an upregulation of IRF-5 at 24 and 30 hr post-treatment (Figure 4B).

When we perform adoptive transfer experiments using CD4 T cells, we notice that a large percentage of adoptively transferred wild-type CD4 T cells downregulates CD4 (Figure 4C). About 70% (at d21) and 50% (at d28) of the adoptively transferred CD4 T cells present in the spleen are CD4$^{lo/neg}$. Most of these cells also downregulated CD3 (Figure S3A), and about 30% of the cells were DR5 and annexin V positive (Figures S3B and S3C). Interestingly, the majority of the CD4$^{lo/neg}$ T cells upregulated B220 (Figure S3D), a marker that has been associated with pre-apoptotic stages in T cells (Oka et al., 2000; Park et al., 2002; Renno et al., 1998).

Surprisingly, adoptive transfer of *Tlr7*$^{-/-}$ or *Irf5*$^{-/-}$ CD4 T cells does not lead to the same results and only a small percentage of cells downregulate CD4 at d21 and d28 p.i. (Figure 4C).

IRF-5 has been described to transcriptionally regulate death receptor 5 (DR5 or TRAIL receptor 2) in human colorectal carcinoma cell lines, sensitizing cells to TRAIL-induced apoptosis

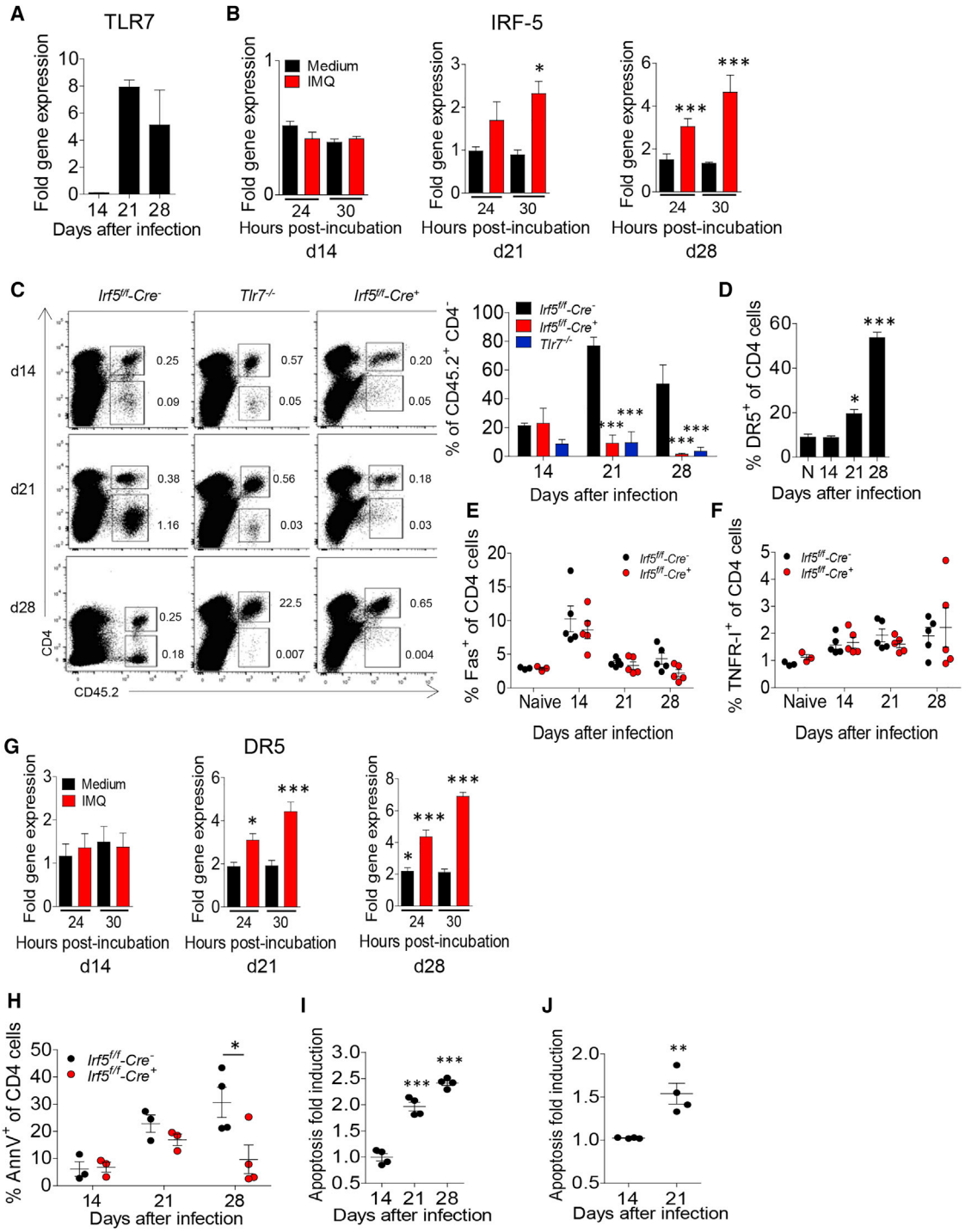


Figure 4. TLR7-Induced IRF-5 Promotes the Upregulation of DR5 and Induces Cell Death in CD4 T Cells from *L. donovani*-Infected Mice

Mice were infected with 2 × 10^7 amastigotes intravenously and euthanized at various time points after infection.

(A) Real-time PCR of splenic CD4 T cells from infected mice measuring mRNA for TLR7.

(B) Purified CD4 T cells from infected mice were incubated with medium or imiquimod. Real-time PCR analysis of IRF5 mRNA levels in CD4 T cells from infected C57BL/6 mice.

(C) *Irf5*$^{f/f}$*-Cre*$^+$, *Irf5*$^{f/f}$*-Cre*$^-$, and *Tlr7*$^{-/-}$ CD4 T cells were adoptively transferred into congenic CD45.1 mice a day prior to *L. donovani* infection. Representative dot plots (left panels) and percentages (right panel) of adoptively transferred CD45.2$^+$ cells from the various donors are shown.

(D–I) Mice were infected with 2 × 10^7 amastigotes intravenously and euthanized at various time points after infection. Graphs show frequency of (D) DR5$^+$, (E) Fas$^+$, and (F) TNFR-I$^+$ CD4 T cells in the spleen of naive and *L. donovani*-infected mice.

(legend continued on next page)

(Hu and Barnes, 2009). Thus, we were wondering whether DR5 and cell death were also promoted following TLR7 stimulation of CD4 T cells. We first assessed whether DR5 was expressed on CD4 T cells during VL, and we noticed that this molecule was upregulated on CD4 T cells from d21 p.i. on (Figures 4D and S4A). We also monitored the expression of Fas receptor (FasR), another known IRF-5 target (Hu and Barnes, 2009), on CD4 T cells from *L. donovani*-infected *Irf5*$^{flox/flox}$-*Cre*$^{+}$ and *Cre*$^{-}$ mice. No differences were observed between *Cre*$^{+}$ and *Cre*$^{-}$ mice (Figures 4E and S4B). Similar results were obtained when we assessed tumor necrosis factor (TNF) receptor-I (TNFR-I) expression (Figures 4F, S4C, and S4D), suggesting that DR5, but not FasR or TNFR-I, may possibly be a target of IRF-5 in CD4 T cells.

Indeed, DR5 was also upregulated on CD4 T cells purified at d21 and d28 p.i., but not on those from d14 p.i. following TLR7 stimulation with IMQ (Figure 4G), implying that the TLR7-IRF-5-DR5 pathway could predispose CD4 T cells to cell death. Hence, we monitored the expression of annexin V on CD4 T cells in *L. donovani*-infected *Cre*$^{+}$ and *Cre*$^{-}$ mice (Figures 4H and S4E). CD4 T cells in IRF-5-sufficient mice increasingly showed signs of apoptosis over the course of VL, and the frequency of CD4 T cells expressing annexin V was significantly lower at d28 p.i. in *Cre*$^{+}$ mice. Moreover, apoptosis was also induced in CD4 T cells purified at d21 and d28 p.i. following incubation with IMQ (Figure 4I), although annexin V expression was not altered in CD4 T cells from d14-p.i.-infected mice. To determine whether IRF-5 induction in CD4 T cells made cells more prone to TRAIL-mediated death, we treated CD4 T cells purified at d14 and d21 p.i. from *L. donovani*-infected mice with recombinant TRAIL. Cell death was assessed after 6 hr by fluorescence-activated cell sorting (FACS). As shown in Figure 4J, treatment with recombinant TRAIL (rTRAIL) did not alter cell survival rate in CD4 purified at d14 p.i. when DR5 is still expressed at low levels (Figure 4D). In contrast, when we incubated CD4 T cells from d21-infected mice, we noticed a significant increase in cell death in rTRAIL-treated cells.

Taken together, these results indicate that the TLR7-IRF-5-DR5 axis sensitizes splenic CD4 T cells to TRAIL-induced cell death during VL.

IRF-5 Does Not Affect CD4 T Cell Proliferation

IRF-5 was reported to be involved in cell cycle regulation in tumor cells (Barnes et al., 2003). Thus, we assessed whether there was a difference in the proliferative capacity between *Cre*$^{-}$ and *Cre*$^{+}$ CD4 T cells. Carboxyfluorescein succinimidyl ester (CFSE)-labeled naive *Cre*$^{-}$ and *Cre*$^{+}$ CD4 T cells were stimulated with anti-CD3/CD28 *in vitro* for 5 days. No differences in the percentage of CFSE$^{lo/int}$ cells were observed between the two groups over the 5-day period (Figure 5A), indicating that IRF-5 deficiency did not confer a proliferative advantage. To test whether *Cre*$^{-}$ and *Cre*$^{+}$ CD4 T cells had similar proliferative capacities during *L. donovani* infection, we performed a double adoptive transfer experiment, in which IRF-5-sufficient CD45.1 and IRF-5-deficient CD45.2 CD4 T cells were co-transferred into CD45.1/CD45.2 recipients a day prior to *L. donovani* infection. The proliferative capacity was monitored based on the Ki67 expression. As shown in Figure 5B, IRF-5-sufficient and deficient cells had similar Ki67 expression levels, suggesting that the absence of IRF-5 did not alter cell division in CD4 T cells *in vivo* as well. Interestingly, though, the number of IRF-5-deficient CD4 T cells present in the spleen at d21 and d28 p.i. was significantly higher than that of IRF-5-sufficient cells (Figure 5C). Because both cell types had similar proliferative capacities (Figure 5B), this implies that wild-type CD4 T cells were dying.

TLR7-Mediated IRF-5 Activation and CD4 Cell Death Are Not a Consequence of Microbial Sensing

The next important question that arose was to determine whether TLR7 on CD4 T cells was directly triggered by *L. donovani* and whether other pathways were also involved in inducing IRF-5. Hence, we incubated CD4 T cells purified from d14, d21, and d28 p.i. with CpG, lipopolysaccharide (LPS), parasites, and parasite RNA or DNA and monitored IRF-5 and DR5 expression by flow cytometry. Because apoptotic and/or necrotic material has also been described to induce IRF-5 in monocytes of systemic lupus erythematosus (SLE) patients (Stone et al., 2012) and splenic tissue disruption occurs during chronic VL (Smelt et al., 1997), we incubated CD4 T cells with supernatant (SN) of apoptotic splenocytes previously incubated with staurosporine. As for IMQ, cells purified at d14 p.i. did not upregulate IRF-5 (Figure 6A) or DR5 (Figure 6B) with any of the stimuli. Only IMQ and the supernatant of apoptotic splenocytes induced IRF-5 and DR5 expression 30 hr after incubation on CD4 T cells enriched at d21 and d28 p.i. (Figures 6A, 6B, S5A, and S5B). Similarly, only the incubation of CD4 T cells with IMQ and the SN of apoptotic splenocytes led to an increase in cell death (Figure 6C), and all other treatments did not alter the survival of the cells at any time point observed. This suggests that microbial sensing or TLR9 and 4 were not involved in inducing IRF-5 expression and cell death in CD4 T cells during VL, but this pathway was rather triggered by tissue disruption and TLR7.

Apoptotic Cell Material Induces IRF-5 via TLR7

Our results indicate that TLR7 and tissue disruption can both induce IRF-5 in CD4 T cells from *L. donovani*-infected mice. Hence, we next investigated whether the SN from apoptotic cells was inducing IRF-5 via TLR7. CD4 T cells were purified from *L. donovani*-infected C57BL/6 mice at d14 and d21 p.i. and incubated with medium alone, IMQ, or SN from apoptotic cells. Cells

(G) Real-time PCR analysis of DR5 mRNA levels in CD4 T cells purified from the spleen of infected mice and incubated with medium or IMQ.
(H) Percentage of apoptotic CD4^{+} T cells in infected *Irf5*$^{f/f}$-*Cre*$^{-}$ or *Irf5*$^{f/f}$-*Cre*$^{+}$ mice.
(I) Apoptosis fold induction in splenic CD4 T cells from infected mice 30 hr after IMQ treatment. Apoptosis fold induction was calculated as follows: (cell death in IMQ treated sample − *ex vivo* cell death)/(medium control cell death − *ex vivo* cell death).
(J) Apoptosis fold induction in CD4 T cells from d21-infected mice following a 6-hr-incubation with recombinant TRAIL; fold induction was calculated as (cell death in rTRAIL treated sample − *ex vivo* cell death)/(medium control cell death − *ex vivo* cell death).
Data represent the mean ± SEM of one of 3 independent experiments; n = 4. *p < 0.05; **p < 0.01; ***p < 0.001.

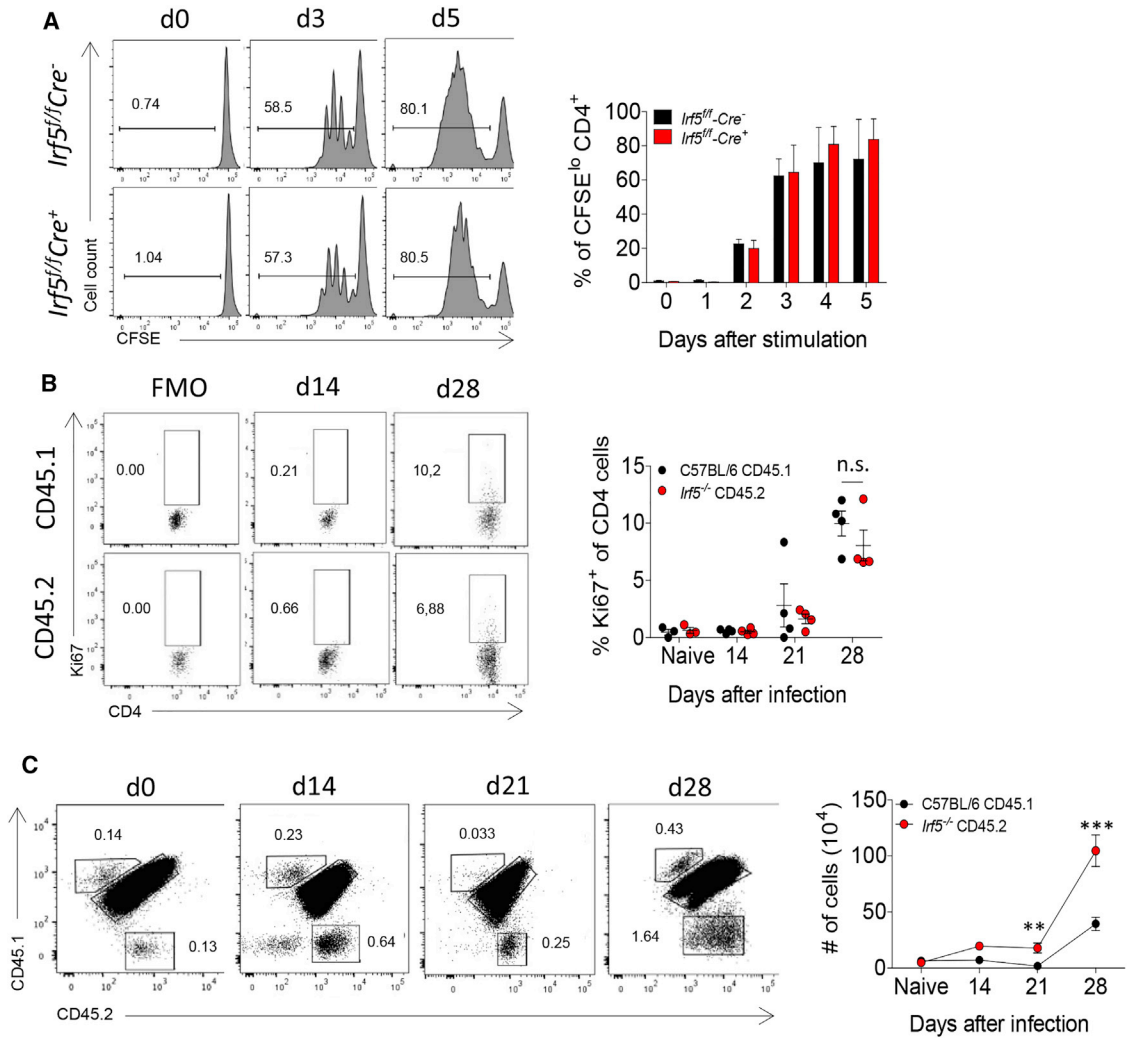


Figure 5. IRF-5 Does Not Affect CD4 T Cell Proliferation during *L. donovani* Infection

(A) CFSE-labeled, IRF-5-sufficient, and -deficient CD4 T cells were stimulated with anti-CD3/CD28; CFSE dilution was used as readout for proliferation. Representative histograms (left panel) and percentages (right panel) of $CFSE^{lo}$ CD4 T cells are shown.

(B and C) 5×10^6 CD45.1 C57BL/6 and $Irf5^{-/-}$ CD4 T cells were co-transferred into CD45.1/CD45.2 recipients a day prior to *L. donovani* infection. Mice were then euthanized at various time points after infection. (B) Representative dot plots (left panel) and percentages (right panel) of $Ki67^+$ CD4 T cells; (C) representative dot plots (left panel) and average numbers (right panel) of CD45.1 WT ($Irf5^{f/f}$-Cre^-) and CD45.2 *Irf5*-deficient ($Irf5^{f/f}$-Cre^+) CD4 T cells.

Data represent the mean ± SEM. $^{**}p < 0.01$; $^{***}p < 0.001$.

were also treated or not with synthetic oligodeoxynucleotides with immunoregulatory sequences (IRSs) that specifically block signaling via TLR7, IRS661 (Barrat et al., 2005). Thirty hours later, IRF-5 expression was assessed by Image Stream. As expected, IRF-5 induction following IMQ treatment of d21 p.i. CD4 T cells was inhibited by IRS661 (Figure 7A). Interestingly, the TLR7 antagonist also prevented the upregulation of IRF-5 following incubation of CD4 T cells purified at d21 p.i. with SN from staurosporine-treated cells (Figure 7A), suggesting that apoptotic material also signals via TLR7. No differences in the level of IRF5 expression were detected after any treatment of CD4 T cells purified at d14 p.i.

To identify the SN component(s) that triggers TLR7, we purified RNA and DNA (Figure S6) from the SN of apoptotic cells and analyzed whether IRF-5 was induced by these two components. As expected, treatment of splenic Cre^- CD4 T cells from d21-infected mice with IMQ or SN resulted in the upregulation of IRF-5 mRNA, which was abrogated by the TLR7 agonist IRS661 oligodeoxynucleotide (ODN) (Figure 7B). In contrast, IRF-5 mRNA levels did not change when RNA or DNA purified from the SN of apoptotic cells was added to the same Cre^- CD4 T cells (Figure 7B), suggesting that pure RNA or DNA from apoptotic cell material is not involved in the TLR7-dependent IRF-5 induction in CD4 T cells.

Similarly to IRF-5, DR5 expression was also not induced following incubation of Cre^- CD4 T cells with RNA or DNA purified from the SN of apoptotic cells, but it was induced, as expected, by IMQ and the SN in cells purified at d21 p.i. (Figure 7C).

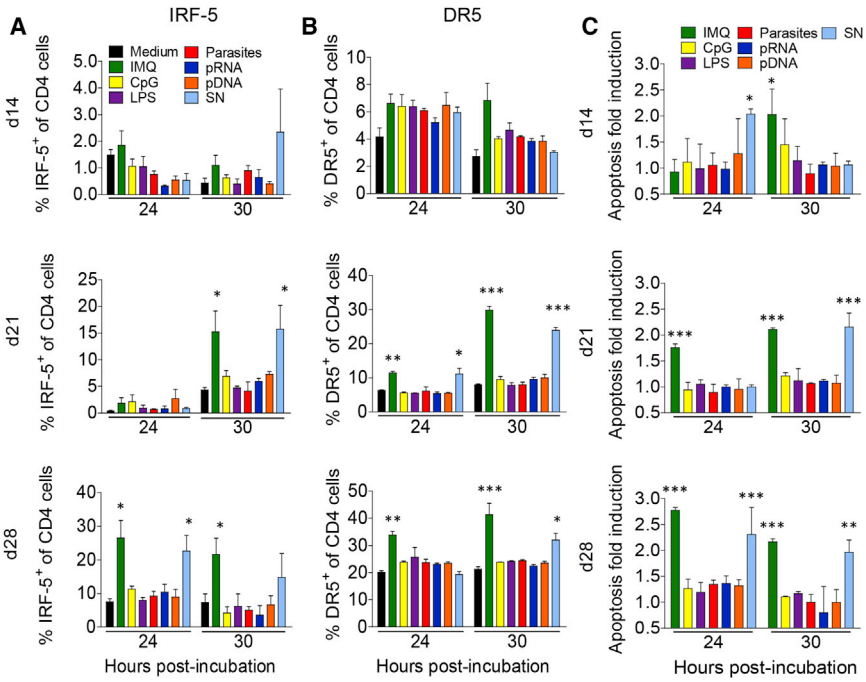


Figure 6. TLR7-Mediated IRF-5 Activation and CD4 Cell Death Are Not a Consequence of Microbial Sensing

Mice were infected with 2 × 10^7 amastigotes intravenously and euthanized at various time points after infection. CD4 T cells were purified from the spleen of infected mice and incubated with medium, IMQ, CpG, LPS, living parasites (P), parasite RNA (pRNA), parasite DNA (pDNA), or supernatant from apoptotic cells (SN). Graphs show the percentage of splenic CD4 T cells from infected mice expressing (A) IRF5, (B) DR5, and (C) the fold induction of apoptosis in CD4 T cells from infected mice after treatments. Data represent the mean ± SEM of one of 3 independent experiments; n = 4. *p < 0.05; **p < 0.01; ***p < 0.001.

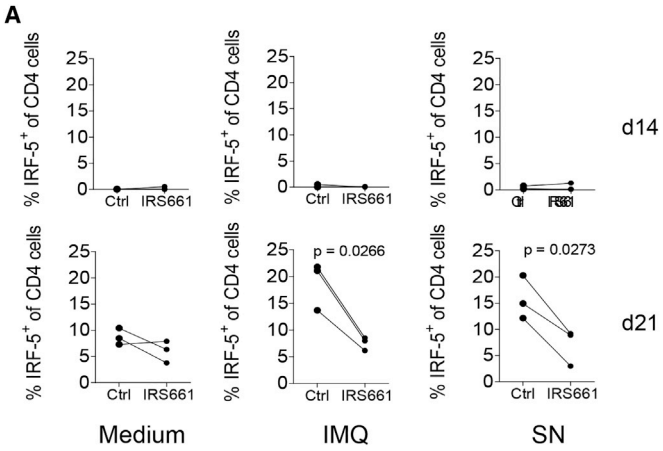
Treatment with IRS661 ODN abrogated DR5 induction. To note, DR5 was not upregulated in CD4 from *Cre*$^+$ mice even when treated with IMQ and SN, confirming that IRF-5 is required in this pathway. We next determined the level of caspase 8 mRNA expression (Figure 7D). Caspase 8 is another reported downstream target of IRF-5 (Hu and Barnes, 2009) and is part of the TRAIL-mediated cell death signaling pathway. Treatment of CD4 T cells purified from d21-infected *Cre*$^-$ mice with IMQ or SN resulted in a significant caspase 8 induction, which was blocked by IRS661 ODN. No effect on caspase 8 mRNA expression levels was observed when the same cells were incubated with RNA or DNA purified from the SN of apoptotic cells (Figure 7D). As for DR5, *Cre*$^-$ CD4 T cells and IRF-5-deficient CD4 T cells purified from infected mice at d14 or d14 and d21 p.i., respectively, did not upregulate caspase 8 expression under any condition tested (Figure 7D). Taken together, our results suggest that RNA or DNA from staurosporine-treated cells cannot trigger TLR7 to induce IRF-5 in CD4 T cells and that IRF-5 is a crucial component in this pathway.

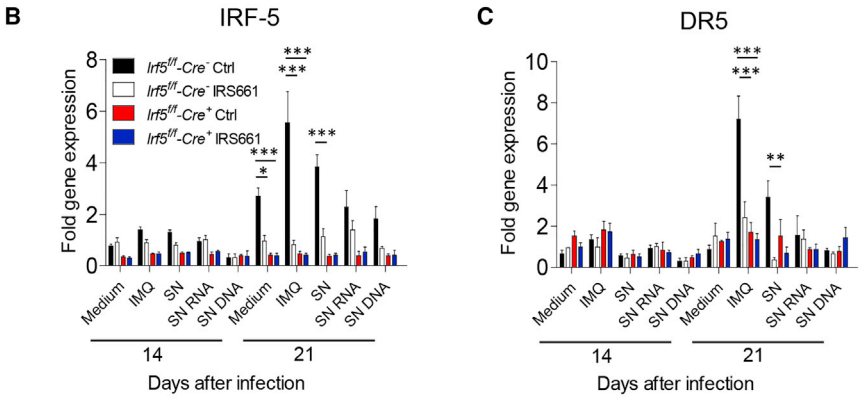
We finally sought to understand whether the lack of IRF-5 was somehow altering TLR7 expression on CD4 T cells, and this would additionally contribute to the reduced cell death observed in IRF-5-deficient CD4 T cells. To this end, we monitored TLR7 expression on CD4 T cells at d14, d21, and d28 p.i. in *L. donovani*-infected *Cre*$^-$ and *Cre*$^+$ mice. As shown before (Figure 4A), TLR7 expression was increasingly upregulated over the course of infection in *Cre*$^-$ CD4 T cells (Figure 7E). In contrast, TLR7 mRNA was maintained on a stable level in IRF-5-deficient CD4 T cells (Figure 7E), suggesting that IRF-5 is not only directly involved in the TLR7-IRF-5-DR5 pathway, but it also participates in a feedforward loop to upregulate TLR7 expression.

DISCUSSION

Chronic infections are typically associated with anti-inflammatory responses aimed at regulating T cell responses and limiting tissue injuries. However, these responses often favor pathogen persistence and may result in immune suppression. In the present study, we propose IRF-5 as an important player in the regulation of pro-inflammatory CD4 T cells. We show that TLR7-mediated IRF-5 activation is enhancing DR5-dependent induction of apoptosis of protective IFN-γ-producing CD4 T cells in *L. donovani*-infected mice. T-cell-specific IRF-5 ablation resulted in stronger IFN-γ responses and ultimately lower parasite burden. Our results also suggest that this pathway is triggered by tissue disruption.

Inflammation is essential for controlling intracellular pathogen growth. However, excessive and/or chronic inflammation often leads to tissue damage and initiates anti-inflammatory tissue-protecting pathways. In the case of VL, disruption of the splenic microarchitecture occurs from d14 p.i. on and leads to an impaired capacity of naive T cells and dendritic cells to migrate to T cell area (Ato et al., 2002) and a progressive loss of B cell germinal centers (Smelt et al., 1997). TNF is largely responsible for initiating this process as well as for inducing IL-10 (Ato et al., 2002; Engwerda et al., 2002). Tissue damage is accompanied by a massive infiltration of inflammatory cells and by splenomegaly (Hammami et al., 2017). In this environment, we observed a gradual upregulation and activation of IRF-5 in IFN-γ$^+$ CD4 T cells. Increased IRF-5 expression was paralleled by an upregulation of TLR7 and DR5. Although high IFN-I levels have been linked to DR5 and lymphocytes death (Hardy et al., 2007; Herbeuval et al., 2006), we did not see a direct effect of IFN-I on IRF-5 upregulation and CD4 T cell death in *L. donovani*-infected mice. In contrast, IRF-5 appeared to be induced by TLR7. TLR9 and 4, which are both expressed in T cells (Rahman et al., 2009), do not seem to promote IRF-5 expression in CD4 T cells. The function of innate sensors in cells of adaptive immunity is still a fairly unexplored field. TLR signaling pathways in T cells were shown to be necessary for





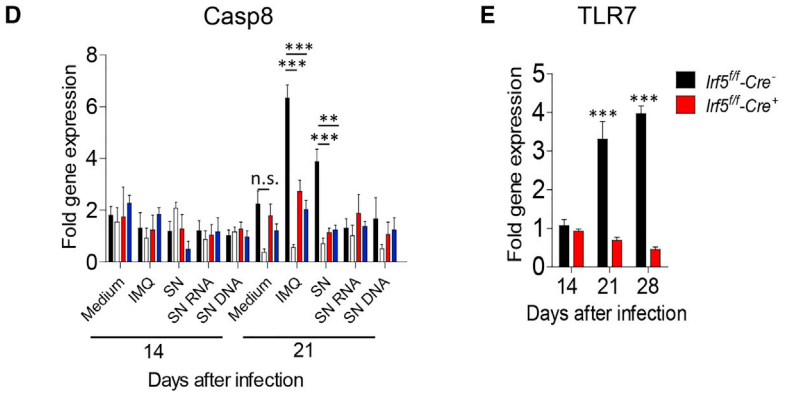


Figure 7. Inhibition of TLR7 Impairs IRF-5-Mediated Cell Death Pathway

(A–D) CD4 T cells from infected mice were treated with IRS661 or ODN control for 30 min prior to a 30-hr incubation with medium alone, IMQ, supernatant from apoptotic cells, or RNA or DNA purified from the supernatant of apoptotic cells. Graphs represent (A) expression of IRF-5 and real-time PCR measuring (B) IRF-5, (C) DR5, and (D) caspase 8 mRNA expression levels.

(E) TLR7 mRNA expression levels in Cre^- and Cre^+ CD4 T cells over the course of *L. donovani* infection.

Data are shown as the mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

effective clonal expansion, in that they act synergistically with T cell receptor (TCR)-induced signals to enhance proliferation, survival, and cytokine production in effector cells (Rahman et al., 2009). Nevertheless, TLR7 was reported to induce anergy in human CD4 T cells (Dominguez-Villar et al., 2015). A similar antiproliferative activity was also recently attributed to stimulator of IFN genes (STING) in T lymphocytes (Cerboni et al., 2017). We also observed a reduction in proliferation of CD4 T cells from *L. donovani*-infected mice in the presence of IMQ (data not shown); however, the most remarkable effect of TLR7 triggering was the induction of IRF-5 and subsequently DR5, a known target of IRF-5 (Hu and Barnes, 2009). The TLR7-IRF-5 pathway was sensitizing CD4 T cells to TRAIL-mediated cell death. Interestingly, IRF-5 and DR5 were mainly expressed in IFN-γ^+ CD4 T cells, suggesting that induction of apoptosis via IRF-5 preferentially affected pro-inflammatory cells. This observation prompted us to believe that this pathway may be in place to protect inflammation-mediated tissue injuries.

A major question that arose during our study pertained to the nature of the ligand responsible for triggering TLR7 in CD4 T cells: is the TLR7 pathway activated following microbial sensing? *Leishmania* typically resides in parasitophorous vacuoles inside macrophages (Arango Duque et al., 2014). Although parasites have been seen inside or on various cells other than macrophages (Abidin et al., 2017; Bankoti et al., 2012; Ribeiro-Gomes et al., 2012; Romano et al., 2017; Silva-Barrios et al., 2016), they were never reported to be internalized by T cells. However, there is still the remote possibility though that dead parasites or RNA from dead parasites could be internalized by T cells and trigger TLR7. Our results refute this possibility: *Leishmania* or leishmanial DNA/RNA does not trigger TLR7 to induce IRF-5 in T cells, suggesting that IRF-5-induced cell death during VL is not a consequence of microbial sensing.

Because splenic tissue disruption is a characteristic feature of VL (Silva-Barrios et al., 2016) and, in SLE patients, IRF-5 can be induced by apoptotic and/or necrotic material (Stone et al., 2012), we were not surprised to observe that the supernatant of apoptotic splenocytes induced IRF-5 expression and consequently cell death of CD4 T cells from *L. donovani*-infected mice. Interestingly, RNA and DNA released by apoptotic cells were not involved in this induction, suggesting that other, yet

unidentified supernatant components may trigger the TLR7 pathway in T cells. Hence, local tissue damage mediated by persistent inflammation leads to suppression of protective T cell responses during chronic VL. The fact that IRF-5 is not activated in hepatic CD4 T cells supports our conclusions. Indeed, this organ clears *L. donovani* infection by generating a very efficient granulomatous response without major disruption of the hepatic microarchitecture. It is well documented that dying cells can regulate adaptive immunity by the release of damage-associated molecular pattern (DAMP). However, DAMPs are mainly known for promoting inflammation and provide immunogenic signals enhancing T cell priming (Yatim et al., 2017). In our model of chronic infection, tissue damage appears to dampen pro-inflammatory CD4 T cell responses rather than promoting inflammation. This discrepancy probably depends on the cell and receptor recognizing DAMPs and on the stage of infection; innate sensors may have very different functions in T cells in a chronic inflammatory environment.

Although the activation of the TLR7-IRF-5-DR5 axis in T cells protects the spleen by reducing inflammation, it is also beneficial to the establishment of persistent *L. donovani* infection. Indeed, IFN-γ^+ CD4 T cells are essential for controlling parasite growth during chronic VL (Engwerda et al., 1998). In *L. donovani*-infected mice, IFN-γ-producing CD4 T cells are first detectable at d14 p.i. and reach maximal expansion at d28 p.i. In the liver, high frequencies of parasite-specific IFN-γ^+ CD4 T cells are observed (Paun et al., 2011); in contrast, small percentages of IFN-γ-producing CD4 T cells are typically detected in the spleen. Because progressive tissue damage and upregulation of TLR7 also occur during this time in the spleen, we believe that IRF-5-mediated CD4 T cell death severely undermines the expansion and, later, the maintenance of protective responses in this organ. This highlights a tissue-specific inhibition of CD4 T cell responses in *L. donovani*-infected mice. It remains to determine though whether IRF-5-mediated death of effector CD4 T cells is restricted to *Leishmania*-specific responses or extends to all effector CD4 T cells present in the spleen and therefore is accountable for the general immune suppression observed in VL patients.

Another observation that requires further investigation concerns the regulation of TLR7 expression in CD4 T cells. Our results indicate that IRF-5 is involved in the upregulation of this receptor. However, we do not yet know the exact role of IRF-5 in this feedforward loop or the initial signal(s) responsible for inducing and activating IRF-5. Because TLR7 is also upregulated on T cells during chronic viral infections in humans (Dominguez-Villar et al., 2015; Hammond et al., 2010), dissecting the dynamic of IRF-5 and TLR7 induction in T cells could help identify potential therapeutic targets aimed at reversing suppression of protective T cell responses during persistent infections.

In conclusion, we propose IRF-5 as an important player in the regulation of IFN-γ^+ CD4 T cell responses during chronic VL. Activation of this transcription factor is TLR7 dependent and appears to be induced by apoptotic and/or necrotic material. In IFN-γ^+ CD4 T cells, IRF-5 promotes the expression of DR5 and caspase 8, sensitizing these cells to TRAIL-mediated cell death. Because tissue disruption is a common characteristic of persistent infections, this regulatory pathway may not only be activated during VL but could be a generalized mechanism present in other chronic infectious diseases.

EXPERIMENTAL PROCEDURES

Mice and Parasites

B6.129S7-*Rag1*^{tm1Mom} and congenic B6-Ly5.1 mice were purchased from The Jackson Laboratory. C57BL/Bl6-*Tlr7*$^{-/-}$ mice and *Ifnar*$^{-/-}$ mice were a kind gift from Drs. Alain Lamarre (INRS-Institut Armand Frappier) and Jörg Hermann Fritz (McGill University). Mice with a targeted IRF-5 mutation in T cells were generated by crossing *Irf5*$^{flox/flox}$ mice (Hammami et al., 2015) with mice expressing the cre-recombinase under the Lck promoter. All mice were housed at the Institut National de la Recherche Scientifique (INRS) animal facility under specific pathogen-free conditions and used at 6–10 weeks of age. *Leishmania donovani* (strain LV9) were maintained by serial passage in B6.129S7-*Rag1*^{tm1Mom} mice, and amastigotes were isolated from the spleen of infected animals. Mice were infected by injecting 2×10^7 amastigotes intravenously via the lateral tail vein. Splenic parasite burden was determined by examining methanol-fixed, Giemsa stained tissue impression smears. Data are presented as *Leishmania donovani* units (LDUs) (Silva-Barrios et al., 2016).

Study Approval

Experiments involving mice were carried out under protocols approved by the Comité Institutionnel de Protection des Animaux of the INRS-Institut Armand Frappier (1510-02 and 1602-02). These protocols respect procedure on good animal practice provided by the Canadian Council on Animal Care. No differences in the course of *L. donovani* infection were observed between male and female mice, so male and female mice were used indiscriminately. Nevertheless, male mice were always matched and compared to male mice for the various experimental groups.

In Vivo Th1 Polarization

CD4 T cells were enriched by magnetic cell sorting (MACS) using a CD4 T cell isolation kit (Miltenyi Biotech) and activated for 5 days with plate-bound 1 μg/mL anti-CD3 (clone 145-2C1C; eBioscience) and 2 μg/mL anti-CD28 (clone 37.51; eBioscience) in the presence of 2 ng/mL IL-2 and 30 ng/mL IL-12 (PeproTech). Cells were then restimulated for 4 hr with phorbol 12-myristate 13-acetate (PMA)/ionomycin in the presence of Brefeldin A (Sigma), harvested, and analyzed by flow cytometry as detailed below.

In Vitro CD4 T Cell Proliferation

CD4 T cells were enriched by MACS using a CD4 T cell isolation kit (Miltenyi Biotech) and then stained with CFSE as previously described (Joshi et al., 2009). CFSE-labeled CD4 T cells were activated with plate-bound 1 μg/mL anti-CD3 (clone 145-2C1C; eBioscience) and 2 μg/mL anti-CD28 (clone 37.51; eBioscience) in the presence of 2 ng/mL IL-2 and analyzed by flow cytometry as detailed below.

Adoptive Transfer Experiments

C57BL/6, C57BL/6-Ly5.1, *Irf5*$^{-/-}$, *Tlr7*$^{-/-}$, and *Ifnar*$^{-/-}$ mice were used as CD4 T cells donors. CD4 T cells were enriched by MACS from splenocytes of naive animals using a CD4 T cell isolation kit (Miltenyi Biotech). 5×10^6 donor CD4 T cells were injected into the lateral tail vein of congenic B6-Ly5.1 or B6-Ly5.1-Ly5.2 mice the day prior to infection with *L. donovani* amastigotes. Mice were sacrificed at the indicated time points.

Flow Cytometry

The following antibodies were used for surface staining: BV421-conjugated anti-CD3 (clone 145-2C11; BD Biosciences); BV421-conjugated anti-CD4 (clone GK1.5; BD Biosciences); fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (clone GK1.5; BD Biosciences); phycoerythrin (PE)-conjugated anti-CD4 (clone GK1.5; BD Biosciences); FITC-conjugated anti-CD45.2 (clone 104; BD Biosciences); APC-conjugated anti-CD8 (clone 53-6.7; BD Biosciences); APC-conjugated streptavidin (BD Biosciences); APC-R700-conjugated anti-CD117 (clone 2B8; BD Biosciences); APC-conjugated annexin V (BD Biosciences); PE-conjugated anti-PD-1 (clone J43;

eBioscience); biotin-conjugated anti-DR5 (clone MD5.1; eBioscience); PE-conjugated anti-CD25 (clone PC61.5; eBioscience); PE-Cy7-conjugated anti-TIM3 (clone B8.2C12; BioLegend); PE-conjugated anti-Fas (clone SA367H8; BioLegend); APC-conjugated anti-CD120a (TNFR-I; clone 55R-286; BioLegend); BV650-conjugated CD45.2 (clone 104; BD Biosciences); and Zombie Aqua Fixable Viability Kit (BioLegend). Intracellular staining was performed as previously described (Hammami et al., 2015). Briefly, splenocytes were either restimulated with bone-marrow-derived dendritic cells (BMDCs) pulsed with fixed parasites or PMA/ionomycin in the presence of Brefeldin A (BD Biosciences). After fixation, cells were permeabilized and stained with the following antibodies: APC-conjugated anti-IFNγ (clone XMG1.2; BD Biosciences); PE-conjugated anti-IL-10 (BD Biosciences); AF488-conjugated anti-FoxP3 (clone FJK-16 s; eBioscience); PE-Cy7-conjugated anti-Ki67 (clone 16A8; BioLegend); and propidium iodide (Sigma). Flow cytometric analysis was performed with a BD LSRFortessa cell analyzer (Becton Dickinson). Samples were analyzed with Flowjo and DIVA software.

ImageStream Flow Cytometry

The following antibodies were used for surface staining: PE-conjugated anti-CD4 (clone GK1.5); biotin-conjugated anti-CD4 (clone GK1.5); biotin-conjugated anti-CD45.2 (clone 104; cat no. 553771); PE-Cy7-conjugated streptavidin; APC-conjugated streptavidin; PE-conjugated anti-CD62L (clone MEL-14); APC-conjugated anti-CD44 (clone IM7); and biotin-conjugated anti-CD3 (clone 145-2C11) from BD Biosciences. For intracellular staining, cells were stimulated with BMDCs pulsed with fixed parasites in the presence of Brefeldin A (BD Biosciences) and stained with surface antibodies. After fixation, cells were permeabilized and stained with APC-conjugated anti-IFN-γ (clone XMG1.2; BD Biosciences) and AF488-conjugated anti-IRF5 (R&D Systems). DAPI (Invitrogen) was used to stain the nucleus of cells immediately before acquisition. Samples were acquired using the ImageStreamX MKII flow cytometer and analyzed with IDEAS software (Amnis, Seattle, WA, USA). 200,000–500,000 gated cell singlets were analyzed for each sample. Nuclear localization of IRF5 was measured using a morphology mask to determine a similarity score, which quantifies the correlation of pixel values of the DAPI and IRF5 images on a per cell basis. A similarity score >1 was used as a cutoff for nuclear localization. Cells in individual bins were visually inspected to confirm subcellular localization (values < or >1).

Real-Time qPCR Analysis

RNA from isolated CD4 T cells from *in vitro* or *in vivo* experiments was extracted using the RNeasy mini kit (QIAGEN) as per manufacturer's instructions. Reverse transcription was carried out using the iScript cDNA synthesis kit (Bio-Rad) as previously described (Hammami et al., 2015). Real-time PCR analysis was performed using iTaq Universal SYBR Green Supermix (Bio-Rad). *Dr5*, *Irf5*, *Tlr7*, and *Hprt* were amplified using the following primers: *Hprt*, 5′-GTT GGA TAC AGG CCA GAC TTT GTT G-3′ and 5′-GAT TCA ACC TTG CGC TCA TCT TAG GC-3′; *Tnfrsf10b* (*DR5*), 5′-TGA CGG GGA AGA GGA ACT GA-3′ and 5′-GGC TTT GAC CAT TTG GAT TTG-3′; *Irf5*, 5′-TAG AGG CTA CCC AGG AGC AA-3′ and 5′-GCC CAC TCC AGA ACA CCT TA-3′; *Tlr7*, 5′-GGC ATT CCC ACT AAC ACC AC-3′ and 5′-TTG GAC CCC AGT AGA ACA GG-3′; and *Casp8*, 5′-CTC CGA AAA ATG AAG GAC AGA-3′ and 5′-CGT GGG ATA GGA TAC AGC AGA-3′. All PCRs were carried out with the Stratagene mx3000P real-time PCR system. Data were normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT) and expressed as fold gene regulation compared to naive controls.

In Vitro CD4 T Cell Stimulation and Recombinant TRAIL Treatment

CD4 T cells were purified following manufacturer's instructions from splenocytes of infected and naive mice by MACS using a CD4 isolation kit (Miltenyi Biotech). Purified CD4 T cells (purity >95%) were incubated at 37°C for 24 hr or 30 hr with 5 μg/mL imiquimod (Invivogen), 3 μg/mL CpG (Invivogen), 10 μg/mL LPS (Sigma), amastigotes at a MOI of 1:10, 500 ng/mL parasite RNA, 500 ng/mL parasite DNA, 10% v/v supernatant of staurosporine-treated cells, 500 ng/mL RNA, or 500 ng/mL DNA purified from supernatant of staurosporine-treated cells. ODNs IRS661 (5′-TGCTTGCAAGCTTGCAAGCA-3′) were used as TLR7 antagonist; 5′-TCCTGCAGGTTAAGT-3′ ODNs were used as control (Barrat et al., 2005). ODNs were prepared in sterile water and utilized at 5.6 μg/mL. Cells were treated with IRS661 30 min before to receive other treatments. For TRAIL treatment, CD4 T cells were purified as mentioned above and incubated at 37°C for 6 hr with 100 ng/mL recombinant murine TRAIL (Peprotech). Cells were cultured in RPMI-1640 (Gibco; Invitrogen) supplemented with 10% fetal bovine serum (FBS).

Statistical Analysis

Statistical analysis was performed using paired Student's t test or multi-way ANOVA. Statistical analysis was conducted using Graphpad Prism (GraphPad). Differences were considered to be statistically significant when $p < 0.05$. All experiments were conducted independently at least three times.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.06.107.

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AUTHOR CONTRIBUTIONS

A.F. performed experiments, analyzed data, and wrote the manuscript; S.S. conceived the project, designed the experimental approach, interpreted data, and wrote the manuscript; X.D.-L. performed experiments; L.T.M. and A.H. performed experiments and analyzed data; and J.v.G. provided key expertise and interpreted data.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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