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L'activation immune innée des cellules B: découverte des mécanismes et des réponses immunitaires au cours de l'infection expérimentale à *L. donovani*

Innate immune B cell activation: uncovering mechanisms and immune responses during experimental *L. donovani* infection

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To my little sisters, not that little anymore, for being my principal and constant motivation to keep moving forward

RÉSUMÉ

Les cellules B peuvent remplir une large gamme de rôles dans la réponse immunitaire et sont des composants essentiels pour la défense de l'hôte. Cependant, dans un large spectre de maladies inflammatoires chroniques, les cellules B peuvent être nuisibles. Nonobstant, les mécanismes sous-jacents qui expliquent l'exacerbation des maladies d'une façon dépendante des cellules B ne sont pas très étudiés. La plupart des pathologies associées à une réponse délétère par des cellules B sont liées à la sécrétion d'anticorps et de cytokines (Matsushita, 2018). La Leishmaniose viscérale (LV) est une maladie inflammatoire chronique négligée dans laquelle l'activation des cellules B est préjudiciable à l'hôte et est causée par des parasites appartenant au complexe de *Leishmania donovani*. L'hypergammaglobulinémie, caractéristique de la LV, résulte de l'activation polyclonale des cellules B, est une caractéristique commune à plusieurs pathologies. À ce jour, il n'existe aucun traitement efficace ni prophylaxie immunitaire contre la LV (Silva-Barrios *et al.*, 2018). Par conséquent, des mécanismes élucidant menant à l'activation des cellules B polyclonales pourraient contribuer au développement de nouvelles approches thérapeutiques contre la LV et à un large éventail de pathologies chroniques dans lesquelles l'hypergammaglobulinémie est une caractéristique principale.

Dans le modèle murin de LV, les souris déficientes en cellules B sont très résistantes à l'infection par *L. donovani* (Smelt *et al.*, 2000). Bien que ce fait soit connu depuis de nombreuses années, les recherches sur le rôle et la fonction des cellules B restent très limitées. Les parasites *Leishmania* affectent les réponses des cellules B principalement en induisant l'activation polyclonal des cellules B conduisant à l'hypergammaglobulinémie et à la production de cytokines immunomodulatrices. Bien que la recherche dans ce domaine soit limitée, un intérêt croissant est apparu au cours des dernières années (Silva-Barrios *et al.*, 2018). Cependant, de nombreuses questions restent à résoudre. À cet égard, notre laboratoire a précédemment démontré que l'activation des cellules B par *L. donovani* entraînait la production d'IL-10, impliquée dans l'inhibition partielle des réponses protectrices de Th1, favorisant l'exacerbation de la maladie. La sécrétion d'IL-10 peut résulter de l'activation de plusieurs voies de signalisation dans les cellules B. Dans le modèle murin de LV, la production d'IL-10 par des cellules B implique la signalisation à travers la voie de MyD88, une protéine adaptatrice de presque tous les TLR (Bankoti *et al.*, 2012). Cependant, au cours de ces premières étapes de reconnaissance, les voies déclenchées en amont de MyD88 par *L. donovani* sont encore inconnues. De plus, une production des anticorps de faible affinité contre *Leishmania* caractérise l'hypergammaglobulinémie dans la LV (Silva-Barrios *et al.*, 2018). L'hypergammaglobulinémie favorise la progression de la maladie et

l'établissement d'un stade inflammatoire chronique. Cependant, les connaissances sur les effets néfastes des anticorps sur la LV ne sont pas exhaustives.

Par conséquent, ce projet de thèse vise à identifier les mécanismes d'activation qui entraînent une réponse des cellules B délétère et les conséquences immunitaires résultant a cette activation dans un modèle inflammatoire chronique dans lequel les cellules B sont nuisibles, étant la LV un modèle idéal pour atteindre cet objectif. L'intérêt principal était de caractériser la contribution des réponses immunitaires médiées par les cellules B au cours de la LV expérimentale et d'identifier les voies de signalisation qui conduisent à l'activation des cellules B et favorisent l'hypergammaglobulinémie.

La présente étude donne de nouvelles informations sur le rôle délétère des cellules B dans un modèle sous-jacent infectieux chronique qui favorise l'activation des cellules B et contribue à la progression de la maladie. Nous fournissons des preuves de la nécessité d'activer les cellules B innées pendant la LV afin de favoriser l'exacerbation de la maladie. En effet, le déclenchement par les cellules B des voies innées impliquant de TLR endosomaux et IFN-I conduit à la sécrétion de cytokines et à l'hypergammaglobulinémie. En outre, nous déterminons que *L. donovani*, en déclenchant une activation immunitaire innée sur les cellules B, peut augmenter l'expression de plusieurs cytokines telles que l'IL-10, qui supprime les réponses Th1 et, de manière remarquable, l'IFN-I, qui augment l'expression de TLR endosomaux, la sécrétion de cytokines et l'hypergammaglobulinémie. En outre, dans le cadre de l'infection expérimentale à *L. donovani*, nous démontrons que l'hypergammaglobulinémie favorise l'établissement d'un microenvironnement inflammatoire aux stades chroniques de l'infection, qui contribue au succès du parasite chez l'hôte. Nous avons montré que l'hypergammaglobulinémie induit un microenvironnement de cytokines qui favorise les réponses Tr1 suppressives et supprime la différenciation des cellules Th1 au cours de l'infection. Dans la LV et plusieurs pathologies chroniques, l'hypergammaglobulinémie est impliquée dans la médiation de l'inflammation et favorise l'exacerbation de la maladie. Les mécanismes impliquant l'activation innée des cellules B représentent une caractéristique commune au-delà de différents types de maladies chroniques, notamment les maladies auto-immunes.

L'activation immunitaire innée médié par l'activation de l'axe TLR-IFNAR, qui armement à la production d'anticorps, est un mécanisme commun qui reste commun parmi des maladies inflammatoires chroniques. Des études ultérieures visant à comprendre comment les anticorps modulent le microenvironnement inflammatoire pourraient aider à clarifier la biologie de ces

caractéristiques communes et à donner un nouvel aperçu qui pourrait favoriser le développement de futures stratégies thérapeutiques.

Mots clés: L'hypergammaglobulinémie, cellules B, activation polyclonal, activation innée, IFN-I, TLR, infection chronique.

ABSTRACT

B cells can accomplish a wide range of immunological roles and are critical components for host defense. However, in a broad spectrum of chronic inflammatory diseases can B cells detrimental. However, the underlying mechanisms that account for B cell-dependent disease exacerbation are poorly understood. Most of the pathologies associated with a detrimental B cell response are related to antibodies and cytokines secretion (Matsushita, 2018). Visceral Leishmaniasis (VL) is a chronic inflammatory neglected disease, where B cell activation results prejudicial to the host. VL is caused by parasites belonging to the *Leishmania donovani* complex. Hypergammaglobulinemia, a hallmark of VL, results from polyclonal B cell activation, a common feature among several infections. To date, there are no effective treatments or immune prophylaxis available against VL (Silva-Barrios *et al.*, 2018). Therefore, elucidating mechanisms that lead to polyclonal B cell activation might contribute to the development of novel therapeutic approaches against VL and a wide range of chronic diseases where hypergammaglobulinemia is a hallmark.

In experimental VL, B cell-deficient mice are highly resistant to *L. donovani* infection (Smelt *et al.*, 2000). Although this fact has been known for many years, research on the role and function of B cells remains very limited. *Leishmania* parasites affect B cell responses mostly by inducing polyclonal B cell activation, which leads to hypergammaglobulinemia, and by the production of immunomodulatory cytokines. Although research in this field is limited, increasing interest has arisen during the past years (Silva-Barrios *et al.*, 2018). However, many questions remain to be addressed. In this regard, our laboratory has previously demonstrated that B cell activation by *L. donovani* results in IL-10 production, which is involved in partial inhibition of protective Th1 responses, promoting disease exacerbation. IL-10 secretion can result from the activation of several singling pathways in B cells. In experimental VL, B cell-derived IL-10 requires Myd88 signalling, the adaptor molecule of almost all TLRs (Bankoti *et al.*, 2012). However, the pathways that are triggered upstream of MyD88 by *L. donovani* are still unknown. Another feature that characterizes hypergammaglobulinemia in VL is the production of low-affinity antibodies against *Leishmania* (Silva-Barrios *et al.*, 2018). The role of these antibodies in favouring disease progression and the establishment of a chronic infection as well as the mechanisms underlying their enhanced production remains an open question.

In consequence, this thesis project aims to identify activation mechanisms that lead to impaired B cell responses and the immune consequences that result from deleterious B cell activation in a chronic inflammatory model where B cells are detrimental, namely VL. Particularly, this project aims to characterize the contribution of B cell-mediated immune responses during experimental VL and identify pathways that lead to B cell activation and promote hypergammaglobulinemia

The current study provides new insights into the disease-exacerbating role of B cells during a chronic parasitic infection, uncovering mechanisms that promotes B cell activation and contribute to disease progression. We provide evidence for the requirement of innate B cell activation during VL to promote disease exacerbation. Indeed, B cell triggering of innate pathways involving endosomal TLRs and IFN-I leads to cytokine secretion and hypergammaglobulinemia. Also, we determine that *L. donovani* by triggering innate immune activation of B cells can enhance the expression of several cytokines, such as IL-10 which suppresses Th1 responses and IFN-I, which enhances TLR expression, cytokine secretion and hypergammaglobulinemia. In addition, we demonstrate that hypergammaglobulinemia supports the establishment of an inflammatory microenvironment at chronic stages of the infection that favours parasite growth. We have shown that hypergammaglobulinemia promotes suppressive Tr1 responses, and inhibits protective Th1 cells. Similarly, to other chronic diseases, hypergammaglobulinemia is involved in mediating inflammation and promoting disease exacerbation during VL. Innate B cell activation, mediated by the TLR-IFNAR axis and leading to antibody production, also occurs in various autoimmune disease. Further studies are required to understand how antibodies modulate the inflammatory microenvironment and could provide new insight for the development of future treatment strategies.

Key words: hypergammaglobulinemia, B cells, polyclonal B cell activation, innate activation, IFN-I, TLR, chronic infection.

SYNTHÈSE

Au cours de l'histoire, la réponse immunitaire a été classifiée comme étant soit cellulaire ou humorale. En conséquence, les cellules B sont traditionnellement associées à l'immunité humorale, mais elles sont également essentielles à l'immunité cellulaire. De ce fait, les cellules B peuvent remplir une vaste gamme de rôles dans une réponse immunitaire, mais elles sont considérées principalement comme des régulateurs positifs de la réponse et des acteurs essentiels à l'élimination des agents pathogènes, principalement en raison de leur capacité à produire des anticorps de haute affinité. L'augmentation des taux d'anticorps - conséquence de l'activation des cellules B polyclonales - est une caractéristique commune de diverses maladies chroniques, notamment la leishmaniose viscérale (LV). Dernièrement, l'étude de la biologie et de la contribution des cellules B dans différents modèles d'infections et de maladie associée à l'auto-immunité s'est considérablement intensifiée. Cependant, un large éventail de questions demeure sans réponse.

Les cellules B contribuent à la pathogenèse dans plusieurs cas de maladies inflammatoires chroniques. Cependant, les mécanismes sous-jacents impliquant les cellules B dans l'exacerbation de maladies sont limités. La plupart des pathologies associées à une réponse immunitaire dans laquelle les cellules B ont une fonction délétère sont liées à la sécrétion d'anticorps et de cytokines immunomodulatrices, ou à des réponses immunologiques médiées par des anticorps (Matsushita, 2018). La LV est une maladie caractérisée par une réponse inflammatoire chronique exacerbé par les cellules B. Cette dernière est causée par des parasites appartenant au complexe *Leishmania donovani*. L'hypergammaglobulinémie, une des caractéristiques principales de la LV, résulte d'une réponse immunitaire délétère des cellules B qui est également commune à plusieurs pathologies. De plus, dans la plupart des cas, l'activation des cellules B est essentielle pour conduire à une réponse immunitaire altérée de celles-ci (Silva-Barrios *et al.*, 2018). À ce jour, il n'existe aucun traitement efficace, ni prophylaxie immunitaire contre la LV. Ainsi, l'identification des mécanismes qui déclenchent la dérégulation des cellules B pourraient fournir de l'information nécessaire qui contribuerait au développement de nouvelles approches thérapeutiques contre la LV.

Les souris déficientes en cellules B sont résistantes à l'infection par *L. donovani*. Dans ce modèle, la multiplication du parasite est contrôlée dans certains organes cibles de la LV, particulièrement la rate et le foie, dans les 21-28 jours suivant l'infection (Smelt *et al.*, 2000).

Bien que ce fait soit connu depuis de nombreuses années, les recherches sur le rôle et la fonction des cellules B au cours de la LV sont limitées. Les parasites *Leishmania* affectent les réponses des cellules B principalement en induisant leur activation, ce qui entraîne la production de cytokines immunomodulatrices et l'activation de cellules polyclonales, induisant une hausse remarquable du taux d'anticorps circulants. Un intérêt croissant a pu être observé au cours des dernières années (Silva-Barrios *et al.*, 2018). Cependant, de nombreuses questions restent sans réponse. À cet égard, notre laboratoire a précédemment démontré que l'activation des cellules B par *L. donovani* entraîne la production d'IL-10, qui est impliqué dans l'inhibition partielle des réponses protectrices de Th1, favorisant l'exacerbation de la maladie. La sécrétion d'IL-10 peut résulter de l'activation de plusieurs voies de signalisation dans les cellules B.

Au cours de la LV, la production d'IL-10 par les cellules B implique la signalisation via la protéine Myd88, une protéine adaptatrice participant à la signalisation des récepteurs de type toll (TLR) (Bankoti *et al.*, 2012). Cependant, au cours de ces premières étapes de reconnaissance, les voies déclenchées en amont de MyD88 par *L. donovani* sont inconnues.

De plus, la LV est caractérisée par une hypergammaglobulinémie, une augmentation de la production d'anticorps de faible affinité, ne reconnaissant pas *Leishmania*. L'hypergammaglobulinémie favorise la progression de la maladie et l'établissement d'un stade inflammatoire chronique. Cependant, les connaissances sur les effets néfastes des anticorps lors de la LV ne sont pas exhaustives. En outre, la contribution des immunoglobulines hypermutées à la pathogenèse de la maladie et à sa progression chronique dans le modèle expérimental de *L. donovani* est encore inconnue.

Ce projet de thèse vise à identifier les mécanismes d'activation entraînant des réponses cellulaires B délétères, ainsi que les conséquences immunitaires qui en résultent. La LV, étant un modèle inflammatoire chronique dans lequel les cellules B sont nuisibles, est donc un modèle idéal pour l'atteinte de cet objectif. L'intérêt principal de ce projet est de caractériser la contribution des cellules B aux réponses immunitaires au cours de la LV expérimentale, et d'identifier les voies qui conduisent à l'activation des cellules B favorisant l'hypergammaglobulinémie.

Au cours de ce parcours, nous avons démontré que l'activation des cellules B par *L. donovani* contribue à l'établissement d'un microenvironnement inflammatoire qui favorise la persistance des parasites. Ce projet a identifié pour la première fois dans un modèle parasitaire des voies de signalisation impliquées dans l'activation des cellules B menant à la production d'hypergammaglobulinémie et de cytokines. Nous avons aussi exploré la contribution de

l'hypergammaglobulinémie lors de l'infection expérimentale de *L. donovani*. Nous avons constaté que des TLR endosomaux réagissent à *L. donovani* dans les cellules B, induisant des cytokines. L'expression des TLR endosomaux est ensuite augmentée due à une signalisation autocrine via le biais de récepteur aux interférons de type I (IFN-I), ce qui favorise l'hypergammaglobulinémie et exacerbe la LV. Enfin, l'hypergammaglobulinémie favorise l'établissement d'un microenvironnement pro-inflammatoire chronique contribuant au succès du parasite *L. donovani*.

Dans cette étude, nous avons constaté que, tout comme l'IL-10, *L. donovani* induit dans les cellules B l'expression d'IL-1, l'IL-6 et l'IFN-I. Nous avons démontré que l'expression des cytokines par les cellules B exposées au parasite *in vitro* et au cours de l'infection implique le déclenchement de TLR endosomaux et la signalisation des IFN-I. Nous avons trouvé que des cellules B déficientes en IFNAR (récepteur des IFN-I) ou en TLR endosomal, exposés à *L. donovani*, n'ont pas réussi à augmenter l'expression de l'ARNm des cytokines.

Les cytokines produites par des cellules B pourrait avoir des différentes conséquences dans la réponse immune. Dans certains cas, l'effet pourrait être protecteur ou pourrait favoriser l'exacerbation de la maladie. Cependant, dans le cas de la LV, la production d'IL-10 par les cellules B de la zone marginale induite par les vois de signalisation impliquant MyD88 favorise la persistance de *L. donovani* chez l'hôte (Bankoti *et al.*, 2012).

Afin d'éliminer ou de contrôler la multiplication de parasites, divers modèles infectieux causés par des parasites protozoaires requièrent une réponse efficace de l'IFN-γ (interféron de type II). Le rôle de l'IFN- γ a été principalement étudié dans la modulation de la réponse immunitaire cellulaire contre les infections à protozoaires. Néanmoins, la contribution des IFN-I à la défense contre les parasites n'est pas élucidée. De plus en plus de publications suggèrent une fonction pléiotropique des IFN-I lors d'infections à protozoaires, en particulier au cours de la réponse immunitaire innée (Silva-Barrios & Stäger, 2017). Dans le model murin de LV, nous avons observé que la production d'IFN-I par les cellules B dépend de TLR endosomaux et est associée à l'augmentation de l'hypergammaglobulinémie, favorisant l'infection a *L. donovani*.

En effet, des souris déficientes en TLR endosomaux ou en signalisation d'IFN-I spécifiquement dans les cellules B sont plus résistantes à l'infection causée par *L. donovani*, comparé à leurs homologues de type sauvage. La contribution des IFN-I dans l'exacerbation des infections à protozoaires est un sujet fascinant qui nécessite une étude approfondie. Jusqu'à présent, dans

ce projet de recherche, nous avons démontré que les IFN-I dérivés de cellules B contribuent à la pathogenèse de LV en contexte expérimentale, en favorisant la persistance du parasite.

En plus de la production de cytokines, nous avons constaté que *L. donovani* déclenche l'expression de TLR endosomaux dans les cellules B. *L. donovani* augmente l'expression de TLR3, TLR7 et TLR9 dans les cellules B exposées au parasite et au cours de l'infection. Nous avons observé que, dans les cellules B exposées au parasite et lors de l'infection dans le modèle murin, la régulation à la hausse des TLR endosomaux implique une signalisation par les IFN-I. Pour la première fois dans un modèle d'infection chronique, nous avons observé que les amastigotes de *L. donovani* induisent l'expression des IFN-I dans les cellules B via des TLR endosomaux. Ainsi, nous avons démontré que les IFN-I étaient impliqués dans une boucle de régulation positive qui régule positivement les TLR endosomaux et augmente l'expression de l'IL-10 ainsi que d'autres cytokines dans les cellules B, contribuant à la production d'anticorps et favorisant des taux élevés d'IgG dans la LV expérimentale. Nous avons constaté que les souris ayant subi une ablation spécifique de L'IFNAR dans des cellules B ne développe pas d'hypergammaglobulinémie à la suite d'une infection par *L. donovani*. Différents modèles de maladies infectieuses et chroniques suivent ce mécanisme qui implique une boucle de rétroaction positive entre la régulation à la hausse des TLR endosomaux et la signalisation par l'IFNAR afin d'augmenter la production d'anticorps. Ces résultats suggèrent une signature commune entre des maladies inflammatoires chroniques où l'hypergammaglobulinémie est une caractéristique principale.

Une de nos observations clés liées à la biologie des cellules B dans la LV est le fait qu'une réduction des niveaux d'anticorps est en corrélation avec une diminution de la sensibilité à *L. donovani*. En effet, les souris déficientes en lymphocytes B ne développent pas d'hypergammaglobulinémie et démontrent une résistance à l'infection. Dans ce projet de recherche, nous avons observé une diminution de la charge parasitaire chez les souris chimères avec une ablation spécifique des TLR endosomaux ou de la signalisation IFNAR dans les cellules B, qui ont également failli au développement de l'hypergammaglobulinémie. Au cours de la LV expérimentale et de la LV humaine, l'activation des cellules B polyclonales menant à l'hypergammaglobulinémie est caractérisée par la présence de taux élevés d'IgG, avec une faible affinité pour le parasite, augmentant les probabilités de développement de l'auto-immunité (Louzir *et al.*, 1994).

Malgré le développement d'hypergammaglobulinémie, les anticorps de haute affinité spécifiques contre *Leishmania* ne semblent pas être produits lors des stades chroniques de l'infection. Dans

le modèle expérimental de la LV dans des macaques, la réduction du nombre de cellules T folliculaires (TFh) affecte l'évolution des anticorps de haute affinité au stade chronique de l'infection (Rodrigues *et al.*, 2014). De plus, il est bien connu que les complexes immuns d'IgG favorisent l'exacerbation de la maladie dans la LV. L'un de ces mécanismes consiste à augmenter la production d'IL-10 dans les macrophages (Miles *et al.*, 2005). Malheureusement, les recherches sur le rôle des anticorps dans l'infection à *L. donovani* sont limitées. À cette fin, nous avons étudié la contribution des IgG hypermutées et de l'hypergammaglobulinémie à la pathogenèse de la maladie dans le modèle murin de l'infection par *L. donovani* en utilisant des souris déficientes en AID (*Activation-induced cytidine deaminase*). Dans la LV, les anticorps à faible affinité diluent les réponses immunitaires protectrices induites par des anticorps de haute affinité au cours de l'infection.

Les processus d'hypermutation somatique (SHM) et recombinaison de classe (CSR) des anticorps sont absents chez les souris déficientes en AID. Ainsi, la production d'anticorps de haute affinité est confinée dans ce modèle. Comme prévu, nous avons constaté que les souris déficientes en AID, un modèle murin dans lequel les cellules B ne peuvent pas générer d'anticorps de haute affinité génétiquement diversifiés (Muramatsu *et al.*, 2000), ne développent pas d'hypergammaglobulinémie. Dans ce modèle, la charge parasitaire diminue considérablement dans la rate et le foie. Ce résultat confirme que l'hypergammaglobulinémie, caractéristique de la LV et d'autres maladies chroniques, est cruciale à la progression de l'infection, contribuant à un microenvironnement inflammatoire qui favorise la persistance de *L. donovani*.

Des réponses immunitaires de type Th1 sont nécessaire au cours de la LV afin de contrôler la multiplication de *L. donovani* et la progression de la maladie. Nous avons constaté que les souris déficientes en AID développent une réponse améliorée des réponses de type Th1, par rapport aux souris de type sauvage, corrélant avec une diminution de la charge parasitaire. En accord avec cette observation, dans notre modèle chimérique d'ablation spécifique de TLRs endosomaux ne menant pas au développement de l'hypergammaglobulinémie, la production de l'IFN-γ par les lymphocytes Th1 T ont été légèrement améliorés. Les parasites *L. donovani* lors de stades chroniques parviennent à amortir les réponses immunitaires Th1 lors de la LV, principalement en induisant l'expression d'IL-10. Dans ce contexte, les cellules T CD4 + qui coproduisent de l'IFN-γ et de l'IL-10, également appelées cellules Tr1, sont une source majeure de l'IL-10 (Stäger *et al.*, 2006). Au cours de l'infection, le nombre des cellules Tr1 est considérablement réduit chez les souris déficientes en AID infectées par *L. donovani*, ce qui suggère que des modifications de l'environnement splénique telles qu'une diminution de l'hypergammaglobulinémie et du profil des

cytokines pourraient affecter les résultats de cette population suppressive. Enfin, les réponses des cellules Tr1 suppressives diminuent aux stades chroniques de la LV chez les souris avec une ablation des TLR endosomaux fonctionnels chez les cellules B.

Dans la phase chronique de l'infection nous avons constaté que le microenvironnement splénique distinctif caractérisé par l'expression l'IFN-I, IL-10 et TNF. Ce qui est intéressant cependant, c'est que dans les souries déficientes en AID infectées par *L. donovani* l'expression de ces cytokines était diminuée aux stades chroniques de l'infection. Lors de stades chroniques de la LV, chez les souris qui ne développent pas d'hypergammaglobulinémie et dans laquelle les cellules Tr1 suppressives sont presque absentes, l'expression de l'IL-10 par des splénocytes est significativement réduite par rapport aux souris de type sauvage. Le microenvironnement immunitaire détermine en partie la divergence des réponses effectrices des cellules T. La réponse des cellules T effectrices sont principalement orchestrées par un large gradient de cytokines et des chimiokines qui varient entre les stades de l'infection dans lequel l'amorçage des cellules T est produit (Caza & Landas, 2015).

Au cours de ce projet, nous avons démontré que les cellules B participent à la promotion d'un microenvironnement splénique approprié favorisant la persistance de *L. donovani* permettant l'établissement d'une LV chronique. Nous avons constaté que l'hypergammaglobulinémie contribue au développement d'un environnement pro-inflammatoire au cours de l'infection. Dans notre modèle, au cours de la phase aiguë, les souris déficientes en AID présentent un microenvironnement pro-inflammatoire splénique plus robuste, caractérisé par la régulation à la hausse des IFN-I, de l'IL-6 et du TNF, une régulation qui diminue aux cours des stades chroniques de l'infection, contrairement à leurs homologues de type sauvage, où un microenvironnement splénique pro-inflammatoire persiste aux stades chroniques de l'infection.

Par conséquent, une réponse altérée des cellules B conduit au développement et à la progression d'une large gamme de pathologies, caractérisées par une réponse immunitaire délétère associée à la sécrétion d'anticorps et de cytokines immunomodulatrices ou de réponses immunologiques médiées par des anticorps. Jusqu'à présent, la compréhension des mécanismes impliqués dans l'exacerbation de maladies renforcées par des réponses altérées des cellules B demeure limitée.

Nonobstant, la présente étude donne de nouvelles connaissances sur le rôle négatif des cellules B dans un modèle infectieux chronique qui favorise l'activation des cellules B et contribue à la progression de la maladie. Nous avons démontré que l'activation des mécanismes de la réponse innée dans des cellules B par *L. donovani* favorise l'exacerbation de la maladie. En effet, dans les

cellules B, le déclenchement des voies innées impliquant les TLR endosomal et les IFN-I conduit à la sécrétion de cytokines et à l'hypergammaglobulinémie.

En outre, nous avons constaté que *L. donovani*, en déclenchant une activation immunitaire innée chez les cellules B, peut induire l'expression de plusieurs cytokines telles que l'IL-10, qui supprime les réponses protectives Th1 et, de manière remarquable, les IFN-I mènent à l'augmentation de l'expression des TLRs endosomaux, la sécrétion de cytokines et l'hypergammaglobulinémie. Additionnement, dans le cadre d'une infection expérimentale à *L. donovani*, nous démontrons que l'hypergammaglobulinémie favorise l'établissement d'un microenvironnement inflammatoire aux stades chroniques de l'infection qui contribue au succès du parasite chez l'hôte. Nous avons montré que l'hypergammaglobulinémie favorise un microenvironnement de cytokines approprié et favorise le développement les réponses suppressives de Tr1 au cours de l'infection.

Dans la LV, comme plusieurs pathologies chroniques, l'hypergammaglobulinémie est impliquée dans la médiation de réponses inflammatoires et favorise l'exacerbation de la maladie. Les mécanismes impliquant l'activation innée des cellules B représentent une caractéristique commune entre différents types de maladies chroniques, notamment les infections virales et l'auto-immunité. L'activation immunitaire innée à travers l'axe TLR-IFNAR, menant à la hausse de la production d'anticorps, est un mécanisme connu pour certaines maladies autoimmunitaires. Des études ultérieures visant à comprendre comment les anticorps modulent le microenvironnement inflammatoire pourraient aider à clarifier la biologie de ces caractéristiques communes et à donner un nouvel aperçu du développement de futures stratégies de traitement.

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ADAM10:	a disintegrin and metalloproteinase domain-containing protein 10
ADCC:	antibody-dependent cellular cytotoxicity
ADCP:	antibody-dependent cellular phagocytosis
ADE:	antibody-dependent enhancement of disease
AID:	activation-induced cytidine deaminase, also known as AICDA
AIM2:	interferon-inducible protein 2
ANAs:	antinuclear antibodies
APC:	antigen presenting cell
APRIL:	a proliferation-inducing ligand, also known as tumor necrosis factor ligand superfamily.
BAFF:	B-cell activating factor
BAFFR:	BAFF activating factor receptor
Bcl-xL:	B-cell lymphoma-extra-large protein
BCMA:	B-cell maturation antigen B-cell maturation antigen
BCR:	B cell receptor
BTK:	Bruton's tyrosine kinase
Blnk:	B-cell linker protein also known as SLP-65, BASH, and BCA
CC:	Chemokine
CCL:	Chemokine ligand
CD:	Cluster of Differentiation
cDCs:	conventional dendritic cells
cGAS:	Cyclic GMP-AMP synthase
CL:	cutaneous *Leishmania*sis
CLP:	common lymphoid precursor
CLRs:	C-type lectin receptors
CMV:	cytomegalovirus
CR:	complement receptors
CSR:	class switch recombination
CTLs:	cytotoxic T cells
CXC:	chemokine receptors

DAG: diacylglycerol

DAMPs: Damage-associated molecular patterns

DCs: Dendritic cells

EAE: autoimmune encephalomyelitis

EBF: early B cell factor

EBV: Epstein Barr virus

ERK: extracellular signal–regulated kinase

E2A: E2A immunoglobulin enhancer-binding factors E12/E47), also known as TCF3

Fab: the antigen-binding fragment of an antibody

FAS: apoptosis antigen 1 receptor

FAS-L: apoptosis antigen 1 ligand

Fc: the fragment crystallizable region, the tail region of an antibody

FcR: Fc receptor

FDCs: follicular dendritic cells

Flt3-L: fms like tyrosine kinase 3 ligand

Foxp3: forkhead box P3 protein

Gal-1: Galectin-1

GCs: germinal centers

GM-CSF: granulocyte-macrophage colony-stimulating factor

HBV: Hepatitis B virus

HCV: Hepatitis C virus

HIFs: Hypoxia-inducible factors

HIF-1α: Hypoxia-inducible factor 1-alpha

HIV: human immunodeficiency virus

I

ICAM-1: Intercellular Adhesion Molecule 1

IFN-I: type I interferons

IFN-II: type II interferons

IFNAR: interferon-α/β receptor

Ig: immunoglobulin
IGF-I: insulin-like growth factor-I
IKAROS: Ikaros family zinc finger protein 1
IL: interleukin
iNOS: inducible nitric oxide synthases
iNTK: invariant natural killer T cell
IP3: Inositol trisphosphate
IRFs: Interferon regulatory factors
ITAMs: tyrosine-based activation motifs

JKN: c-Jun N-terminal kinase

LCMV: Lymphocytic choriomeningitis virus
LiTXN1: cytosolic tryparedoxin of *Leishmania infantum*
LLO: lysteriosin O
LPS: Lipopolysaccharides
LTα: Lymphotoxin alpha protein
LTα1β2: Lymphotoxin alpha1/beta2 Protein
Lyn: Tyrosine-protein kinase lyn

MAC: membrane attack complex
MADCAM1: Mucosal Vascular Addressin Cell Adhesion Molecule 1
MAPK: mitogen-activated protein kinase
MAPKK: mitogen-activated protein kinase kinase
MCL: mucocutaneous *Leishmania*sis
MDH: malate dehydrogenase
MDSC: myeloid derived suppressor cells
MHC: the major histocompatibility complex
MID: *Moraxella catarrhalis* IgD-binding protein
MIP- 1α: Macrophage Inflammatory Protein 1 alpha
MMM: marginal zone metallophilic macrophages
MS: multiple sclerosis
MPPs: multipotent progenitor cells
MyD88: Myeloid differentiation primary response 88

MZ: marginal zone
MZM: marginal zone macrophages
MZPs: marginal zone B cells precursors

NETs: Neutrophil extracellular traps
NK: natural killer
N-FAT: Nuclear factor of activated T-cells
NLRs: NOD-like receptors
NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells
Notch2: Neurogenic locus notch homolog protein 2

PAMPs: Pathogen-associated molecular pattern
Pax 5: paired box protein 5
PD1: programmed cell death protein 1
PD-L1: Programmed death-ligand 1
PI3K: Phosphoinositide 3-kinases
PKC: protein kinase C
PLCγ2: Phospholipase C gamma 2
PRRs: pattern recognition receptors
p38: P38 mitogen-activated protein kinas

RAC: proline recemase
RAG: Recombination activating gene protein
RLRs: retinoic acid-inducible gene-I-like receptors
RIG: retinoic acid-inducible gene I

SKF: src-kinase family
Syk: spleen tyrosine kinase
SHM: somatic hypermutation
SH2: Src Homology 2 domain
SIR2: sirtuin 2
SLE: Systemic lupus erythematosus
SLO: secondary lymphoid organs
STAT: signal transducer and activator of transcription protein

S1PR1: Sphingosine-1-phosphate receptor 1

S3a: ribosomal protein S3a

TACI: Transmembrane activator and CAML interactor

Taok3: Serine/threonine-protein kinase TAO3

TCR: T cell receptor

TD: thymus-dependent

TdT: Terminal deoxynucleotidyl transferase

TFh: T follicular helper cell

TGFβ: transforming growth factor beta

TNF: Tumour necrosis factor

Th T helper

TI: thymus-independent

TI-I: thymus-independent antigen I

TI-II: thymus-independent antigen II

TIR: Toll/interleukin-1 receptor

TLRs: toll-like receptors

T1: transitional 1 B cell

T2: transitional 2 B cell

T3SS: Type three secretion system

VAV1: Vav Guanine Nucleotide Exchange Factor 1

VCAM1: vascular adhesion Molecule 1

VL: visceral *Leishmania*sis

WHO: World Health Organization

CHAPTER 1:

INTRODUCTION

Historically immune responses have been classified as either cellular or humoral. Accordingly, B cells have traditionally been associated with humoral immunity although we now know that they are equally critical to cellular immunity. In this regard, B cells can accomplish a wide range of immunological roles. However, due to their capacity to produce specific antibodies, they have been considered mostly as positive regulatory components of immune responses and key elements for the elimination of pathogens. Increased levels of antibodies -a consequence of polyclonal B cell activation- is a common feature of several chronic diseases, including visceral *Leishmania*sis. Recently, the study on the biology and contribution of B cells in different infection and autoimmune models has notably intensified. However, a wide range of questions remain unanswered. In my thesis work, I will investigate some of these questions, namely the mechanisms of induction of polyclonal B cell activation and hypergammaglobulinemia, and the role of hypergammaglobulinemia in the immune response to pathogens. The experimental model of visceral leishmaniasis was used to answer these questions

Part I: B cell Biology

Besides controlling and eliminating pathogens, B cell contributes to maintaining the synergy between the innate and the adaptive immune systems (Pieper *et al.*, 2013). Defects in this balance can induce illness or disease, such as autoimmune diseases, immunodeficiency disorders, and hypersensitivity reactions. An overactive immune response (hypersensitivity reactions), an inappropriate reaction to self (autoimmunity) or ineffective immune responses (immunodeficiency) are generally responsible for these immune-associated diseases (Owen *et al.*, 2014).

1. B cells

B cells are versatile and diverse. They are involved in antigen presentation and are classified as professional antigen-presenting cells (APCs), playing a relevant role in T cell activation and co-stimulation. Also, under different conditions of activation, B cells secrete various cytokines and chemokines that contribute to the immune response against pathogens by inducing or controlling

inflammation. Notably, B cells serve as regulatory cells that modulate both cellular and humoral responses (Pieper *et al.*, 2013).

Antigen recognition in B cells is known to be mainly mediated by the surface B cell receptor (BCR). However, other molecules such as pattern recognition receptors (PRRs) on B cells can recognize conserved antigens of different pathogens (i.e. pathogen-associated molecular patterns [PAMPs]). Antigen recognition by B cells triggers B cell activation and subsequent responses that regulate their survival, proliferation and differentiation. Consequently, plasma cells or memory B cells will develop. In this context, BCR signalling regulates several B cell responses. For example, the formation of the immunological synapse, antigen internalization and presentation, cytoskeletal re-organization and antigen affinity maturation (Hoffman *et al.*, 2016).

1.1. B cell receptor (BCR)

B cells activation is commonly triggered by the binding of the antigen to the BCR. Additionally, B cell functioning and development are primarily affected by BCR signalling. The BCR is composed of immunoglobulin (Ig) molecules coded in the heavy and light chain loci of Igs. These loci are composed of a series of variable (V) gene elements followed by several diversity (D) segments (present only on the heavy chain gene), joining (J) segments and constant region (C) exons. In mature B cells two Ig isotypes, the membrane-bound-IgM (5 Ig molecules) and the membrane-bound IgD (4 Ig molecules) compose the BCR (Lu *et al.*, 2018).

The BCR itself does not contain any signalling motifs but instead is linked to the Igα/Igβ heterodimer and contains immunoreceptor tyrosine-based activation motifs (ITAMs) (Packard & Cambier, 2013). The early molecular events triggered by BCR-antigen binding have been well-characterized. BCR activation promotes phosphorylation of ITAMs by the tyrosine-protein kinase Lyn (Lyn), the first kinase in the BCR signalling pathway. Lyn, a member of the src-kinase family (SKF) is the predominant SFK expressed in B cells. After this first phosphorylation occurs a second kinase, the spleen tyrosine kinase (Syk) is recruited through its Src-homology 2 (SH2) domain to the phosphorylated Igα–Igβ heterodimer (Packard & Cambier, 2013). Double phosphorylation of ITAM tyrosines leads to Syk binding via its tandem SH2 domains, resulting in the activation and phosphorylation of Syk. Therefore, Syk activation leads to BCR signal propagation. This event takes place via a group of proteins associated with the adaptor protein B-

cell linker (Blnk), forming the signalosome. Activated Blnk assists as a scaffold for the assembly of the other components including Bruton's tyrosine kinase (BTK), the proto-oncogene vav1 (VAV 1), and phospholipase C-gamma 2 (PLCγ2). These components are critical regulators in several B cell responses triggered by phosphoinositides such as phosphoinositide-3-lkinase (PI3K) (Packard & Cambier, 2013; Pierce & Liu, 2010).

Additionally, and following BCR ligation, tyrosine residues of the cytoplasmic tail of CD19 are phosphorylated by Lyn to create binding sites for the SH2 domains of the p85 adaptor subunit of PI3K as well as other SH2 domain-containing effectors. Activated PLCγ2 cleaves the membrane-associated phosphoinositide PI(4,5)P2, releasing the second messenger inositol 1,4,5 triphosphate (IP3) and diacylglycerol (DAG) inducing Ca^{2+} release. Activation of transcription factors such as the nuclear factor kappa B (NF-κB) and the nuclear factor of activated T cells (N-FAT) by protein kinase C (PKC) requires Ca^{2+} efflux. DAG activates different PKC isotypes which trigger members of the mitogen-activated protein kinase (MAPK) family (i.e. extracellular signal-regulated kinase [ERK], c-Jun NH2-terminal kinase [JNK/SAPK], and the P38 mitogen-activated protein kinase [p38]). The overall result of these processes drives B cell activation, antigen presentation, cytokine production, and cell proliferation and differentiation (Burger & Wiestner, 2018; Packard & Cambier, 2013; Pierce & Liu, 2010).

1.2. Innate receptors

Like other antigen-presenting cells, B cells are also capable of innate sensing. Activation of these innate pathways modulates key B cell responses, such as antibody production, antigen presentation and cytokine secretion among other functions in B cell survival and development. The vertebrate innate immune sensing system comprises a wide range of molecules including plasma and endosomal membrane-bound Toll-like receptors (TLRs), surface-expressed C-type lectin receptors (CLRs), as well as cytosolic retinoic-acid-inducible gene I (RIG-I)-like receptors (RLRs), Nod-like receptors (NLRs), Cyclic GMP-AMP synthase (cGAS), and several cytosolic DNA receptors including absence in melanoma 2 (AIM2). These cytosolic and/or surface receptors can sense a wide range of foreign conserved antigens and self-molecules inducing the activation of various signalling pathways (De Nardo, 2015).

2. Toll-like receptors

Initially characterized in drosophila, TLRs were the first PRRs to be described and remain being some of the most studied to date (Hashimoto *et al.*, 1988; Voogdt & van Putten, 2016). TLRs are a family of receptors conserved through the evolution among invertebrates and vertebrate species. They can sense foreign and endogenous molecules characterized for a distinctive conserved pattern including PAMPs and damage-associated molecular patterns (DAMPs). TLRs are type-I transmembrane glycoproteins that share basic characteristics. Structurally, they are composed by extracellular leucine-reach repeats that sense PAMPs and DAMPs, a transmembrane domain, and a cytosolic Toll/Interleukin-1 receptor (TIR) domain that is involved in activation of downstream signalling pathways (Botos *et al.*, 2011).

To date, 13 different TLRs have been identified in mammals. TLR1, TLR2, TLR4-6, and TLR10 are expressed on the plasma membrane while TLR3, TLR7-9 and TLR11-13 are to be found in the endosomal compartment. Synthesis of TLRs takes place in the endoplasmic-reticulum (ER), they are mobilized through the Golgi and recruited to either the cell surface or intercellular compartments such as endosomes. The location of most TLRs relies on ER-resident proteins including the protein associated with toll-like receptor 4 (PRAT4A) and the Unc-93 homolog B1 (UNC93B1) (Blasius & Beutler, 2010). The transmembrane protein UN93B1 is involved in the trafficking of intracellular TLRs from the ER to endosomes. In addition, UNC93B1 can play a regulatory role in dampening TLR7 overactivation by employing TLR9 (Fukui *et al.*, 2009). This transmembrane protein regulates differential transport of TLR7 and TLR9 into signalling endosomes, a mechanism used to prevent autoimmunity that relies in the D34 aminoacidic from UNC93B1 (Sasai & Iwasaki, 2011)

In general, each TLR is able to sense molecules with a similar nature such as lipoproteins (TR1-2-6 and 10), double-stranded RNA (TLR3), lipopolysaccharides (TLR4), flagellin (TLR5), profilin (TLR11-12), single-stranded RNA (TLR7 and TLR8), unmethylated CpG Oligodeoxynucleotide DNA (TLR9), and bacterial ribosomal RNA (TLR13) (Voogdt & van Putten, 2016). Following molecule sensing, TLRs recruit TIR domain-containing adaptor proteins such as the Myeloid differentiation primary response 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF). This recruitment initiate signal transduction pathways that leads to the

activation of NF-κB, interferon regulatory factors (IRFs), or MAP kinases to regulate the expression of cytokines, chemokines, and IFN-I (Kawasaki & Kawai, 2014) **(Figure 1)**.

2.1. TLR signalling

Engagement between TLRs and their ligands results in the early recruitment (TIR-dependent) of several adaptor molecules including MyD88, TIR domain-containing adaptor protein (TIRAP), TRIF-related adaptor molecule (TRAM), and TRIF (Vidya et al., 2018). TLR adaptor proteins are diverse and crucial in the stimulation of cytokine, chemokine and IFN-I production which will ultimately be determinant in the host response to microbial infection (Kawai & Akira, 2011; Vidya et al., 2018) **(Figure 1).** TLR adaptor proteins are required for recruitment and activation of kinases such as IRAK4, IRAK1, IRAK2, TBK1, and IKKε. Additionally, TLR adaptor proteins participate in the activation of ubiquitin ligases including the TNF receptor-associated factor 6 (TRAF6) and pellino-1(Kawai & Akira, 2011). These molecules are essential for downstream signalling propagation which results in the engagement of the NF-κB, IFN-I, p38 MAP kinase (MAPK), and JNK MAPK pathways (Kawasaki & Kawai, 2014).

2.1.1. MyD88-dependent activation

With the exception of TLR3, all TLRs require the activation of the adaptor protein MyD88 to initiate downstream signaling pathways (Kawasaki & Kawai, 2014). Additionally, members of the IL-1 receptor family also transmit signals through this adaptor protein (Kawai & Akira, 2011). Following TLR engagement, the N-terminal death-domain of MyD88 allows the interaction with IRAK4 inducing IRAK1, IRAK2 and subsequently TRAF6 activation (Kawasaki & Kawai, 2014). Modifications on TRAF6 by K63-linked autoubiquitylation enables the recruitment of IκB kinase (IKK) through a ubiquitin-binding domain of the IKKγ subunit (Kawasaki & Kawai, 2014). The IKK proteins phosphorylate IkBs and results in nuclear translocation of NF-KB (Kawasaki & Kawai, 2014). In parallel, ubiquitinylated TRAF6 can be recognized by TGF-β- activated kinase (TAB2), leading to activation of the associated TAK1 kinase, which then phosphorylates the IKKβ subunit (Kawasaki & Kawai, 2014). Pellino-1 can modify IRAK1 with K63-linked ubiquitin, allowing IRAK1 to recruit IKK directly which results in the activation of a MAPKs cascade leading to the translocation of AP1 (Kawasaki & Kawai, 2014). In addition, IRFs can be associated directly with MyD88 and TRAF resulting in the induction of gene transcription of proinflammatory cytokines and IFN-I (Vidya et al., 2018).

2.1.2. TRIF-dependent activation

TRIF-dependent TLR signaling is crucial for downstream signaling of TLR3 , however TLR4 can also signal through this adaptor protein (Kawasaki & Kawai, 2014). After TLR engagement, TLR3 can directly interact with TRIF while TLR4 requires the bridging adaptor protein TRAM in order to activate the downstream signaling pathway (Kawasaki & Kawai, 2014). TRIF engagement to TLR3 or TLR4 allows the recruitment and association of TRAF6 and the TNF receptor-associated factor 3 (TRAF3) (Kawasaki & Kawai, 2014). TRIF/TRAF3 association leads to TBK-1-mediated activation of IRF3 inducing the expression of IFN-I genes while TRIF/TRAF6 association triggers RIP-1 kinases and initiates NF-kB activation (Kawasaki & Kawai, 2014).

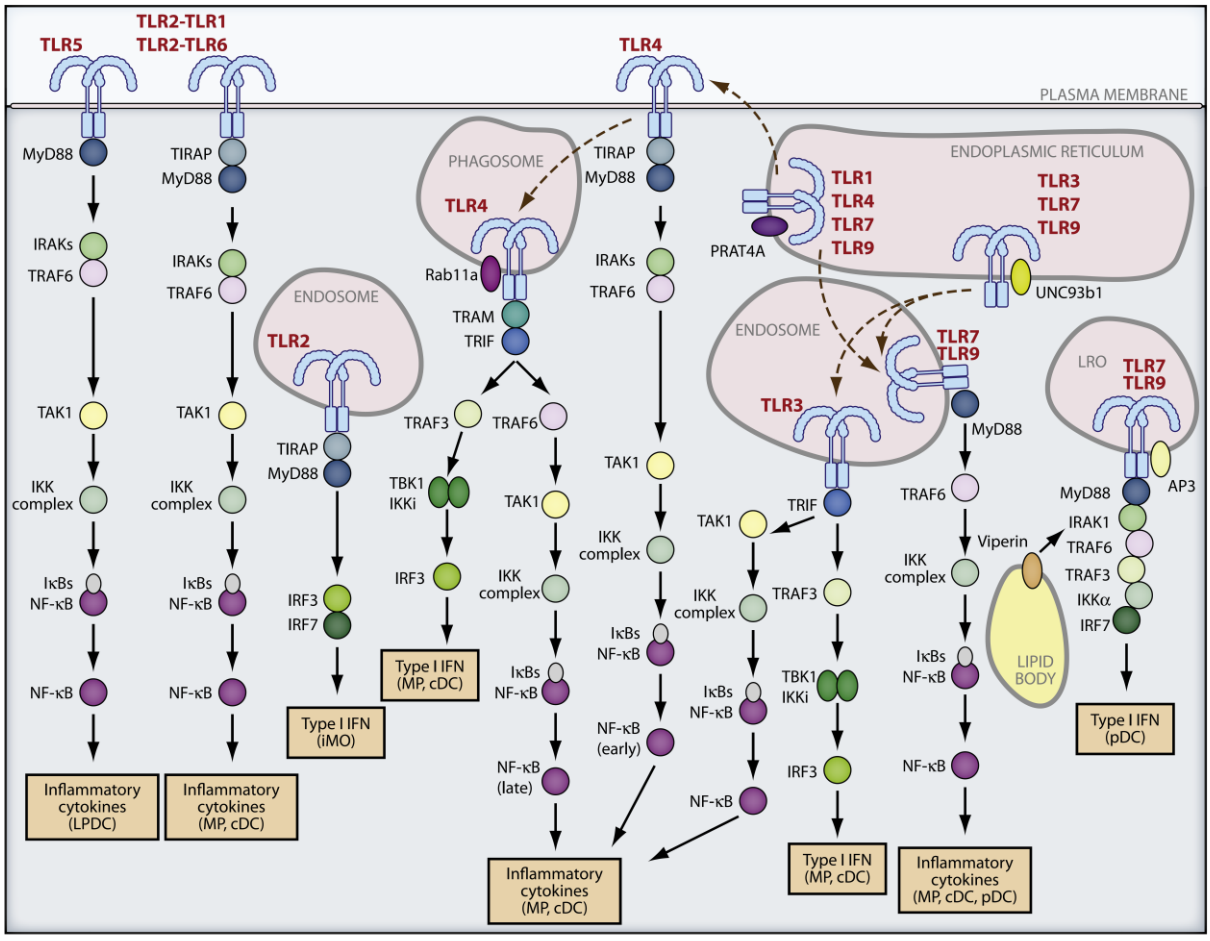

Figure 1. TLR trafficking and signaling (Kawai & Akira, 2011). Individual TLRs initiate overlapping and distinct signaling pathways in various cell types such as macrophages (MP), conventional DC (cDC), plasmacytoid DC (pDC), lamina propria DC (LPDC), and inflammatory monocytes (iMO). PAMP engagement induces conformational changes of TLRs that allow homo- or heterophilic interactions of TLRs and recruitment of adaptor proteins such as MyD88, TIRAP, TRIF, and TRAM. TLR5, which is highly expressed on the cell surface of LPDC, uses MyD88 and activates NF-κB through IRAKs, TRAF6, TAK1, and IKK complex, resulting in induction of inflammatory cytokines. Heterodimers of

TLR1-TLR2 and TLR2-TLR6 are also expressed on the cell surface and induce NF-κB activation through recruitment of TIRAP and MyD88 in macrophages and cDCs. In iMO, TLR2 is found to be expressed within the endosome and induce type I IFN via IRF3 and IRF7 in response to viruses. TLR4, which is expressed on the cell surface, initially transmits signals for the early-phase activation of NF-κB by recruiting TIRAP and MyD88. TLR4 is then transported into Rab11a-positive phagosomes that contain bacteria, where it recruits TRAM and TRIF and activates TRAF3-TBK1-IRF3 axis as well as late-phase NF-κB activation for the induction of type I IFN. Both early- and late-phase activation of NF-κB is required for the induction of inflammatory cytokines. TLR3, TLR7, and TLR9 are localized mainly to the ER in the steady state and traffic to the endosomal compartment, where they engage with their ligands. UNC93B1, which interacts with these TLRs in the ER, mediates this trafficking. The translocation of TLR7 and TLR9 from the ER to the endosome is also regulated by PRAT4A, which also supports the translocation of TLR4 and TLR1 to the cell surface. A member of ER-resident gp96 functions as a general chaperone for most TLRs including TLR1, TLR2, TLR4, TLR5, TLR7, and TLR9 (not shown here). TLR3 activates the TRIF-dependent pathway to induce type I IFN and inflammatory cytokines in macrophages and cDCs. In pDCs, TLR7 and TLR9 activate NF-κB and IRF7 via MyD88 to induce inflammatory cytokines and type I interferon, respectively. The activation of NF-κB during TLR7 and TLR9 signaling is initiated from the endosome whereas IRF7 activation is initiated from the lysosome-related organelle (LRO) after TLR7 and TLR9 are transported from the endosome to this vesicle in a manner dependent on AP3. MyD88-dependent IRF7 activation in pDCs is mediated by activation of IRAK1, TRAF6, TRAF3, and IKKα and is facilitated by IFN-inducible Viperin expressed in the lipid body. In cDCs and macrophages, TLR7 and TLR9 induce inflammatory responses by activating NF-κB via MyD88 but fail to activate IRF7.

2.2 B cells and TLRs

Although B cells primarily recognize antigens via the BCR, a new body of literature indicates that they may also use innate mechanisms for pathogen recognition. TLR expression by B cells has mostly been described in human and mouse B cells. A large proportion of studies have examined mRNA expression (Barr *et al.*, 2007; Gururajan *et al.*, 2007). However, relatively few have directly quantified protein expression (Kuraoka *et al.*, 2017). In both species, TLR expression in B cells is broadly diverse. TLRs composition and levels of expression vary based on the activation status, B cell subset, and physiological location (Barr *et al.*, 2007; Buchta & Bishop, 2014; Genestier *et al.*, 2007). In humans, memory B cells display enhanced levels of TLR1,6,7,9 and 10; while peripheral and plasma B cells upregulate TLR4, 3, and 7 compared to naïve B cells. Similarly to human, mouse B cells subsets express TLR1-3,4,6, and 7. However, TLR4 is highly expressed compared to human B cells (Buchta & Bishop, 2014).

In B cells, research based on TLRs suggest that they participate in the initiation of adaptive immune responses and promoting antibody class switch (Gavin *et al.*, 2006; Kuraoka *et al.*, 2017). However, TLRs seem to affect various B cell functions, from cytokine production, to antigen-presentation to subset commitment just to name a few. For instance, several reports have now demonstrated that exposing B cells to TLR agonists results in the production of various cytokines, such as interleukin (IL) 10 (IL-10), IFN-γ, IL-6, and IFN-I (Barr *et al.*, 2007). The role of TLRs in B cell development is controversial. Some literature showed that TLR signalling appears to be

dispensable at the early stage. However, TLRs are required for B cell subset commitment (Hua & Hou, 2013). In addition, in mouse and human B cells, TLR3 and 7 expression can be enhanced by stimulation through the BCR, CD40, or cytokine receptors such as the IFN-I receptor (IFNAR) (Chang *et al.*, 2007; Hua & Hou, 2013).

Aditionally, TLR appear to enhance antigen presentation in B cells. TLR engament on B cell enhances the expression of chemokine receptors and surface costimulatory molecules requiered for antigen presentation (Buchta & Bishop, 2014). TLRs also cooperate with additional B cell receptors to modulate B cell survival. For example, TLR7 and TLR9 upregulate the expression of anti-apoptotic factors (cmyc and Bcl-xL), protecting B cells from apoptosis (Benson, 2007). A consequence of an enhanced TLR signalling in B cells is the promotion of autoreactive B cells. Enhanced TLR expression by B cells is a common hallmark among several pathologies, such as autoimmune disorders and cancer (Farrugia & Baron, 2017; Grimmig *et al.*, 2016). In addition, a wide range of pathogens exploits this pathway in order to succeed within the host (McGuire & Arthur, 2015). Understanding TLR-mediated effector functions in B cells might lead to for the discovery of novel treatment strategies.

3. Humoral immune response

Following antigen encounter, B cell activation drives humoral responses. Two pathways lead to B cell activation and promote humoral immune responses that vary depending on the nature of the antigen and the B cell subset involved. Typically, non-proteinaceous antigens such as lipids, nucleic acids and glycoproteins stimulate antibody production in the absence of T cells. This type of antigens is referred to as thymus-independent (TI) antigens. In contrast, the antibody response to proteinaceous antigens requires T cell participation. These antigens are defined as thymus-dependent (TD) antigens (Crotty, 2015).

3.1. T cell-independent B cell activation

Antibodies generated upon TI-antigen activation are typically IgM and are characterized by their low affinity. They are divided as type I and type II antigens. TI antigens type I (TI-I) are mitogens

that can activate both immature and mature B cells and induce antibody production in a polyclonal manner, regardless of antigen specificity. (Genestier *et al.*, 2007). TI-I are structurally conserved molecules found in various pathogens, such as bacteria derived lipopolysaccharides (LPS); these molecules are also known as PAMPs (Defrance *et al.*, 2011). As mentioned before, immune cells, including B cells, can recognize PAMPs via PRRs located on the cell surface or within the cell (Janeway, 1989; Janeway Jr & Medzhitov, 2002). PRRs are critical molecules in sensing self and non-self-antigens. TLR family, one of the most studied PRRs, is involved in TI-I antigen recognition. Besides PAMPs, TLRs can recognize endogenous molecules known as DAMPs, mostly released from damaged tissues or dead cells that act as endogenous stress signal (Vidya *et al.*, 2018). Indeed, defects in self-antigen recognition results in many auto-immune disorders. (Vidya *et al.*, 2018).

In contrast to TI-I antigens, T-independent type II (TI-II) antigens are commonly polysaccharides, such as bacterial capsular polysaccharides, characterized by the presence of repetitive surface epitopes that are abundant on their structure. TI-II antigens engage the BCR and induce antigen-specific B cell responses in mature B cells (Janeway Jr & Medzhitov, 2002). However, continuous cross-linking between the BCR and the antigen can induce cell anergy; in this state B cells become unresponsive to antigen stimulation. Consequently, epitope density on TI-II antigens is critical for TI B cell activation. At low epitope density, antigen-BCR cross-linking is insufficient to induce B cell activation. However, at extremely high epitope density, B cells become unresponsive to antigen stimulation (Vos *et al.*, 2000). TI-I and TI-II antigens also own an intrinsic capacity to directly induce B cell proliferation (Vos *et al.*, 2000).

3.1. T cell-dependent B cell activation

TD antigens are generally monomeric soluble proteins that display single or few epitopes to antigen-specific B cells. TD antigens require cognate T cell help for the induction of highly specific antibody responses generated through germinal centre (GC) reactions (Parker, 1993). B cell activation triggered by TD antigens occurs in two distinct phases. These stages are defined based on the anatomical region of secondary lymphoid organs (SLO) where the activation occurs. The early phase takes place in the T cell area and primary follicles. During this phase, B cell

proliferation, initial antibody secretion, and isotype switching occur. The late phase occurs in GCs within lymphoid follicles where affinity maturation and B cell memory formation take place (MacLennan *et al.*, 1997; Song & Matthias, 2018).

TD activation in B cells initiates when the antigen-specific cell recognizes and binds the antigen through the BCR. BCR-antigen interactions enhance the expression of co-stimulatory molecules, such as CD40, CD80, and CD86, on the surface of the B cell. In this context, activated B cells can internalize the antigen-BCR complex through Fc receptor-mediated endocytosis. Following internalization, antigen processing occurs and peptide fragments are presented on the cell surface via the major histocompatibility complex class II (MHC-II) molecules to cognate $CD4^+$ T cells. Therefore, $CD4^+$ T cells can recognize the MHC-peptide complex on the surface of the B cell, and bind CD80 via CD28 expressed on the T cell surface promoting T cell activation and proliferation. Moreover, activated T cells express CD40-Ligand (CD40L or CD154), which binds to CD40 on the B cell surface, favouring co-stimulation between the B and T cell. The interaction between CD40-CD40L has several effects: it promotes transcription of immunoglobulin genes, enhances cytokine secretion from T cells and induces B cell proliferation.
In addition, cytokines derived from activated T cells enhance proliferation and differentiation of B cells, promote isotype class switching and are involved in determining the isotype of the antibody produced. Furthermore, after completion of T cell-dependent B cell maturation in GCs, some of the antigen-activated B cells develop into memory B cells that are capable of mounting rapid antibody responses upon secondary exposure to an antigen. (MacLennan *et al.*, 1997).

4. B cells diversity

B cell populations in mammals comprise several subtypes with diverse functionalities. B cell types are divided base on their origin into two main lineages: B1 and B2 cells **(Figure 2).** In mice, early precursors of B1 cells arise from the fetal liver and persist as a self-renewing population. B1 cells are located after birth mainly in pleural and peritoneal cavities and are typically known to be involved in the innate immune system (Baumgarth, 2011; Berland & Wortis, 2002). On the other hand, B2 cells are derived from the bone marrow (BM) with continued output during life. Within

the BM, elimination of autoreactive cells occurs by a process known as central tolerance (Nemazee, 2017). Immature B2 cells that leave the bone marrow undergo further selection in the spleen (peripheral tolerance) and become mature cells that disperse throughout the secondary lymphoid organs (Gavin *et al.*, 2004). B2 cells represent an essential component of the adaptive immune response, but also are critical contributors to the innate immunity.

The dynamics of the expression of surface molecules (that varies depending on the state of B cell development) is a relevant feature of B cell identification that allows discriminating between B cell subsets and other immune cell types, facilitating the study of B cell biology **(Figure 2 and Table 1)** (Silva-Barrios *et al.*, 2018).

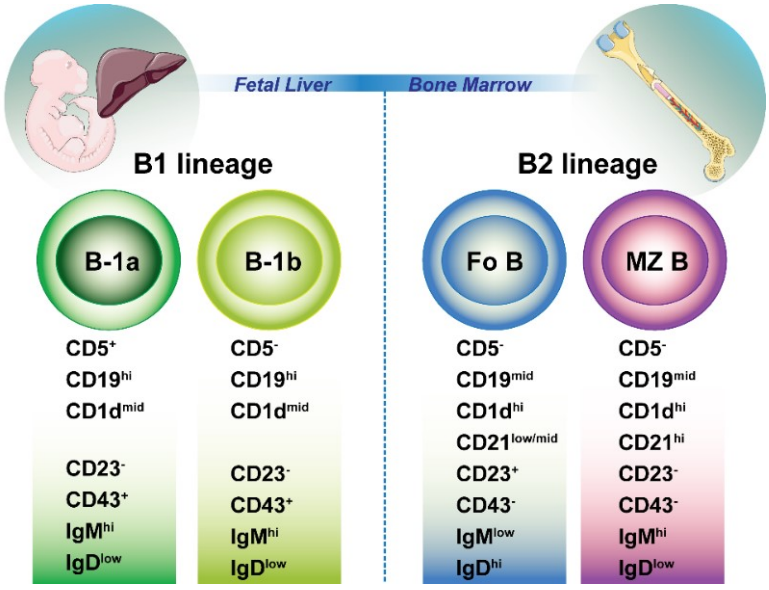

Figure 2. Murine B cell lineages and subtypes. Based on their origin in mouse B cells are divided into two main lineage B1 and B2. B1 B cells, which comprises B-1a and B-1b cells are derived from the fetal liver at early stages of development. In contrast, B2 lineage, which is mainly composed by follicular B cells (Fo B) and Marginal Zone B cells (MZ B) are derived from bone marrow precursors. The differential expression of surface molecules facilitates their identification.

4.1. B1 linage

B1 cells are a heterogeneous pool of self-renewing cells, mostly studied in the mouse. However, innate immune cells with similar phenotypical capacities are observed in other mammalian species, such as human and rabbit. Based on the differential expression of the surface receptor CD5, B1 cells are classified in two cell subsets: **B-1a** ($CD5^+$) and **B-1b** ($CD5^-$) cells **(Figure 2)** [21]. CD5 expression was initially thought to be a restricted receptor on the surface of T cells. In

B cells, CD5 is linked to natural IgM production, participating as a negative regulator of the BCR signalling (Baumgarth, 2011; Berland & Wortis, 2002; Lalor, 1991). Another surface molecule that is expressed by B1 cells is CD43; is also present in thymocytes and peripheral blood T-cells, but not by the B2 lineage. B1 cells secrete polyclonal antibodies, known as natural antibodies that can recognize a wide range of PAMPs. Natural antibodies are produced in a TI manner and are polyreactive or poly-specific. B1 cells primarily produce IgM and preferentially class switch to IgA (Kaminski & Stavnezer, 2006; Tarlinton *et al.*, 1995) . Additionally, B1 cells recognize self-antigens, act as APCs, and secrete a wide range of cytokines and chemokines (Baumgarth, 2016). Also, B1 cells are essential contributors to the immunoglobulins secreted at mucosal sites, including the intestinal and respiratory tracts (Baumgarth, 2016).

4.1.1. *B-1a cells*

Expression of CD5 by B-1a cells is a well-known signature constitutively associated with membrane-bound IgM (Sen *et al.*, 2002). In B-1a cells, CD5 negatively regulates BCR-mediated activation by limiting signals after an antigen-BCR engagement (Bikah *et al.*, 1996) and has also been associated with cell survival (Montecino-Rodriguez & Dorshkind, 2012). Peritoneal and pleural cavities are the main location for B-1a cells. However, they can also be found in the intestinal lamina propria, spleen, lung, and bone marrow (Baumgarth, 2016). B-1a cells are about 1% of the total B cell pool. In both mice and humans, $CD19^{+}CD5^{+}$ B cells are the main source of natural antibodies (Wang *et al.*, 2015). Moreover, they are considered as an important source of immunoglobulin A (IgA), contributing significantly to the response against enteric pathogens (Wang *et al.*, 2015; Woof & Kerr, 2006).

T-independent antigens can directly activate B-1a lymphocytes, particularly TI-1 antigens, promoting cell proliferation and differentiation without T cell stimulation and independently from the BCR specificity. TI-1 antigens activate B-1a lymphocytes mostly by triggering TLRs. B-1a activation by TI-1 antigens leads to clonal cell activation and represents an important process in the early IgM response against several pathogens (Genestier *et al.*, 2007). Most of the research carried out on B-1a cells biology have focused on their role in autoimmunity (Deng *et al.*, 2016; Miles *et al.*, 2018). However, the attention on the role of B1 cells in favouring or controlling infection is increasing (Aziz *et al.*, 2015).

4.1.2. *B-1b cells*

B-1b cells share common features with B-1a cells. They are located mostly in the peritoneal and pleural cavities and share the expression of cell-surface markers such as CD43 (**Figure 1**). As mentioned earlier, one of the phenotypic markers to date that defines these two B1 subsets is the differential expression of CD5 that is present on B-1a but not on B-1b cells. Additional differences between these two subsets exist regarding developmental pathways (Montecino-Rodriguez & Dorshkind, 2012) and cytokine responses. However, the study of B-1b lymphocytes in areas that are not the peritoneal and pleural cavities (i.e. spleen) represents a challenge due to the difficulty to discriminate this population based on the specificity of the phenotypic surface markers described to date. In the past years, adoptive transfer and chimeric mice experiments allowed the identification of several features of B1-b lymphocytes. Most of the research carried out in the field has focussed on function, migration and development (Baumgarth, 2016; Bondada *et al.*, 2000; Montecino-Rodriguez & Dorshkind, 2012).

In B1 cells, TLR-mediated stimulation induces a rapid and transient down-regulation of integrins. This results in an efficient movement of B1 cells from the peritoneal cavity in a chemokine-dependent fashion to secondary lymphoid organs (Colombo *et al.*, 2010; Haas *et al.*, 2005). Efficient activation of B1 cells allows differentiation into antibody-secreting cells inside the lymph nodes (Watanabe *et al.*, 2000), a pivotal event in the early protective immune response. Like B-1a, B-1b cells also secrete IgM and IgA. Antibody responses in these cells have mostly been studied in bacterial and viral infection models.

B-1b lymphocytes respond to TI-2 antigens, such as capsular polysaccharides, in contrast to B-1a cells that are activated mainly by TI-1 One of the distinguishing characteristics of B-1b cells is being efficient and long-lasting after antigen contact. (Baumgarth, 2016; Colombo *et al.*, 2010; Haas *et al.*, 2005). In summary, B1 cells have a unique developmental, phenotypical, and functional characteristic along with their tissue distribution. B1 cells play an important role in immunity against pathogens, but are also implicated in autoimmunity.

4.2. B2 lineage

The B2 lineage is composed of two main subtypes of B cells: Marginal Zone B cells (MZ) and Follicular B cells (Fo), based on their location in the spleen **(Figure 3)**. MZ B cells are distributed in the marginal sinus of the spleen and are considered innate-like B cells due to functional similarities with B1 cells (i.e. polyreactive BCR). Following activation, MZ B cells mainly produce polyreactive IgM in a TI manner. However, MZ B can also produce high-affinity antibodies in a TD

manner. Over the years, MZ B cells are considered a key player in the elimination of blood-borne pathogens. Additionally, this B cell subset participates in the elimination of cells that undergo apoptosis (Cerutti *et al.*, 2013).

Fo B cells, the conventional B lymphocytes of the adaptive immune response, are the most abundant B cell subtype. Fo B cells in most of the cases develop through GC reactions and generate long-lasting high-affinity IgG antibodies in a TD manner. High-affinity antibodies are a critical feature for the classic humoral response participating in the protection following vaccination or infection (Crotty, 2015).

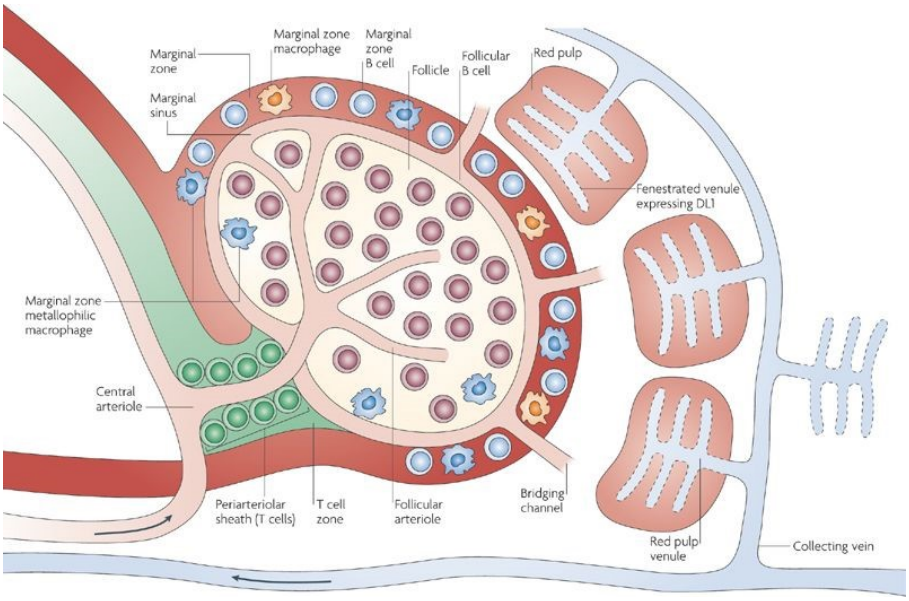

Figure 3. A schematic view of the anatomy of the spleen. The afferent splenic artery branches into central arterioles, which are enclosed by white-pulp areas; these white-pulp areas consist of the T-cell zone (also known as the periarteriolar lymphoid sheath, PALS), arterioles and B-cell follicles. The arterioles end in cords in the red pulp, from where the blood runs into venous sinuses, which collect into the efferent splenic vein. The larger arteries and veins run together in connective-tissue trabeculae, which are continuous with the capsule that surrounds the spleen. **(Mebius & Kraal, 2005; Pillai & Cariappa, 2009)**

4.2.1. *Marginal Zone B cells*

Mouse MZ B cells, in contrast to Fo B cells, do not recirculate. MZ B cells are confined to the outer white pulp of the spleen, between the marginal sinus and the red pulp (**Figure 3**). Retention of this B cell subset in the marginal zone requires the interaction of αLβ2 integrin and α4β1 integrin (expressed by MZ B cells) with intercellular adhesion molecule 1 (ICAM1) and vascular cell

adhesion molecule 1 (VCAM1) expressed on stromal cells (Lu & Cyster, 2002). Although these cells were considered sessile for a long time, several studies found that MZ B can transport antigen in immune complexes from the neighbourhood of the marginal sinus to follicular B cells in the splenic follicle. This process depends on the expression of the C-X-C motif chemokine receptor (CXCR) - 5 (CXCR5) and the sphingosine-1-phosphate receptor 1 (S1PR1). MZ B cells can bind immune complexes through complement receptors 1 and 2 (CR and CR2). This shuttling process is considered a possible mechanism by which antigens are effectively delivered, from the blood to follicular dendritic cells (FDCs) (Batista & Harwood, 2009; Cinamon *et al.*, 2008). In humans, MZ B cells can recirculate, they are also present in peripheral blood (Weller *et al.*, 2004). Like innate B1 cells, the BCR of MZ B cells can recognize and respond to a wide range of conserved molecular signatures that are often shared by exogenous and endogenous antigens, such as PAMPs and DAMPs. Following BCR recognition, MZB cells produce polyreactive antibodies and cytokines. Overall, MZ B cells are considered like important contributors to the innate immune response (Cerutti *et al.*, 2013; Martin & Kearney, 2002).

From the total B cell pool in the spleen, about 5-6 % are MZ B cells. MZ B express high levels of CD21 (also known as, CR2) **(Figure 2)** and respond vigorously to blood-borne-pathogens. CD21 on the MZ B cells surface facilitates the transport of immune complexes from the circulation to the splenic follicles promoting antigen presentation to FDCs (Cerutti *et al.*, 2013). MZ B cells also express CD1d, which increases upon cell activation. CD1d in MZ B cells facilitates the presentation of lipid antigens to invariant natural killer T (iNKT) cells in a BCR-independent manner (Barral *et al.*, 2008). iNKT cells are involved in the control of cancer, infections and autoimmunity. Upon activation, iNKT cells enhance the expression of diverse co-stimulatory molecules and produce a wide range of cytokines and chemokines, which have a powerful impact on immune cells. MZ B cells, but also B1 cells, are essential contributors to iNKT activation (Salio *et al.*, 2014).

In the spleen, the marginal zone allows MZ B cells to be in contact with macrophages, dendritic cells (DCs) and granulocytes in a stromal reticular cell network. This strategic microarchitecture favour MZ immune cells to rapidly interact with blood-borne pathogens (Mebius & Kraal, 2005). Activation of MZ B cells can occurs by direct interaction with pathogens or by pathogen-derived antigens via BCR or PRRs eliciting TI and/or TD antibody responses. Additionally, antigens associated with complement opsonin also leads to MZ B cell activation via CD21 (Cerutti *et al.*, 2013; Dempsey *et al.*, 1996).

In the presence of co-stimulatory signals from innate or adaptive immune cells, antigen-activated MZ B cells rapidly differentiate into antibody-secreting cells via a T cell-independent pathway, similarly to B1 cells (Cerutti *et al.*, 2013). Additionally, MZ B cells express increased levels of MHC class II, CD80 and CD86 molecules. Antigen presentation and T follicular helper (TFh) cell activation by B cells requires the expression of these molecules. MZ B cells can also generate long-lived plasma cells that secrete high-affinity antibodies via a canonical follicular T-dependent pathway (Cerutti *et al.*, 2013). Since MZ B cells can undergo class switching through a process that requires the enzyme activation-induced cytidine deaminase (AID), they can produce either IgG or IgA (Cerutti, 2008; Guinamard *et al.*, 2000; Puga *et al.*, 2012). Therefore, mouse MZ B cells are a functional cell population that respond to multiple types of pathogens challenges, including TI carbohydrate antigens and TD protein antigens.

MZ B cells also express different TLR, which together with BCR activation induce the production of low-affinity antibodies by mouse MZ B cells (Cerutti *et al.*, 2013; Pone *et al.*, 2012). This early activation of MZ B cells links the temporal gap required to produce high-affinity antibodies by Fo B cells, bridging the innate with the humoral response. In human and mouse, the development of autoimmunity is often linked to a deregulated production of antibodies and raise of MZ B cells number (Rawlings *et al.*, 2012).

4.2.2. *Follicular B cells*

The largest proportion of B cells that recirculate and home in secondary lymphoid organs are Fo B cells; they represent about 70% of the total B cells in the spleen (Baumgarth, 2011). The proximity of Fo B cells to T cell adjacent areas within the follicles gives an advantage in the activation dynamics between T helper cells and Fo B cells. This strategic location favours the participation of Fo B cells in the TD immune response to produce high affinity antibodies. Additionally, Fo B cells can recirculate to the bone marrow. Within this lymphoid organ, they can be activated by blood-borne pathogens, eliciting a TI response that generates specific IgM antibodies (Cariappa *et al.*, 2007; Cariappa *et al.*, 2005).

Prominent expression of the surface molecules CD23, IgD and the low low-affinity receptor for IgE (FcεRII), are one of the main characteristics of mature Fo B cells. Additionally, the limited expression of CD21 (CR2) on the surface of Fo B cells allows differentiating this population from

MZ B cells. Based on the expression of these surface markers, Fo B cells are classified in type I and type II. Phenotypically Fo B type I are $CD23^{+}IgD^{hi}IgM^{low}CD21^{mid}$ while Fo B type II are typically $CD23^{+}IgD^{hi}IgM^{hi}CD21^{mid}$. Type I and II Fo B cells can recirculate equally. However, the requirements for their development are different. Fo B type II can potentially reconstitute the MZ B pool during diseases caused by blood-born-pathogens (Pillai & Cariappa, 2009).

Like B1 and MZ B cell subsets, TLRs expression by Fo B cells is quite diverse. In contrast to innate and innate-like B cells, most of the Fo B cell subset lack the intrinsic ability to differentiate into antibody-secreting cells if exclusively triggered through TLRs (Genestier *et al.*, 2007). As mentioned before, TD responses are required by Fo B cells in most of the cases. At early times post-TD-activation, Fo B can differentiate into short-lived antibody secreting cells. At this point, they have limited migration capacities and remain close to the site of activation. Following GC reactions, Fo B cells B differentiate into plasmablast producing long-lasting high-affinity antibodies and into memory cells. During this activation and differentiation process, B cell survival and maintenance are promoted by molecular interactions of the cytokines B-cell activating factor (BAFF) and the A proliferation-inducing ligand (APRIL), together with their respective receptors: the B-cell maturation antigen (BCMA), transmembrane activator and CAML interactor (TACI), and BAFF receptor (BAFFR) (MeinI *et al.*, 2018).

Table 1. B cell subsets generalities (Silva-Barrios *et al.*, 2018).

Tissue location	B cell subtype	Function	Mouse-specific markers	Human-specific markers
Secondary lymphoid organs (SLO)	Transitional B cells	Naive B cell pool renewal	$IgM^{hi}IgD^{var}CD1d^{lo}CD19^{+}$ $CD93^{+}CD21^{var}CD23^{var}$	$IgD^{+}CD27^{-}$ $CD10^{var}CD24^{var}CD38^{var}$
	Marginal zone B cells	Blood-borne antigen trapping and early antibody secretion	$IgD^{lo}IgM^{hi}CD9^{+}CD19^{+}CD$ $21^{hi}CD23^{lo}CD24^{hi}CD38^{hi}$ $CD44^{+}B220^{+}CD80^{lo}CD8$ $6^{+}CD1d^{hi}$	$IgD^{lo}IgM^{hi}CD1c^{+}CD21^{hi}CD$ $23^{-}CD27^{+}CD1d^{hi}CXCR5^{+}$
	Regulatory B cells	Immunoregulation	$IgM^{hi}IgD^{var}CD19^{+}CD1d^{hi}$ $CD21^{hi}CD23^{var}CD24^{hi}C$ $D5^{+}CD93^{var}TIM\text{-}1^{+}IL\text{-}10^{+}$	$IgM^{hi}CD19^{+}CD1d^{hi}CD5^{+}C$ $D10^{var}CD27^{var}CD24^{hi}CD3$ $8^{var}CD138^{var}IL\text{-}10^{+}$
	Activated B cells	Cognate antigen recognition and T cell priming	$IgD/M^{var}BLA\text{-}$ $1^{hi}CD19^{+}CD23^{var}CD38^{var}$ $GL\text{-}$ $7^{+}CD80^{+}CD86^{+}CD95^{+}C$ $D24^{hi}MHC\text{-}II^{hi}$	$IgD/M^{hi}CD23^{var}CD38^{+}CD4$ 4^{+}

	Germinal centre B cells	Antigen-dependent antibody affinity maturation	IgD/M^{var}BLA-1^{var}CD19^{+}CD23^{var}CD38^{-}GL-7^{+}CD80^{+}CD86^{var}CD95^{+}CD24^{hi}MHC-II^{hi}	IgM/G/A/E^{+}IgD^{-}CD10^{+}CD20^{+}CD27^{+}CD23^{-}CD38^{hi}CD44^{-}CD77^{var}
	Plasmablast	High affinity antibody secretion	IgM/G/A/E^{+}IgD^{var}CD19$^{lo/-}$CD138^{+}CD93^{+}MHC-II^{+}Ki-67^{+}	IgM/G/A/E^{+}IgD^{-}CD19^{lo}CD20^{-}CD27^{hi}CD38^{hi}CD138^{lo} Ki-67^{+}
	Plasma cells (short-lived)	Early antibody production	mIg^{-}sIg^{hi}B220^{lo}CD19$^{lo/-}$CD44^{hi}CD138^{+}CD86^{-}CD93^{+}MHC-II^{var}CXCR5^{lo}CCR7^{lo}CXCR4^{hi}CXCR6^{hi}	IgM/G/A^{+}CD27^{+}CD38^{hi}CD138^{+}CXCR4^{hi}CD19^{lo}CD20^{-}CD45^{lo}MHC-II^{lo}
Peritoneal and pleural cavities	B-1a B cells	Natural antibody secretion	IgM^{hi}IgD^{lo}CD5^{+}CD9^{+}CD11b^{+}CD19^{+}CD23^{lo}CD43^{+}B220^{lo}CD138^{-}	IgM^{var}IgD^{var}CD5^{var}CD11b^{var}CD19^{+}CD20^{+}CD27^{+}CD43^{+}B220^{+}CD86^{var}CD70^{-}CD10^{var}
	B-1b B cells	Early antibody secretion	IgM^{hi}IgD^{lo}CD5^{-}CD9^{+}CD11b^{+}CD19^{+}CD23^{lo}CD43^{+}CD45^{lo}CD138^{-}	
Bone marrow (BM) only	Plasma cells (long-lived)	Long term antibody production	mIg^{-}sIg^{hi}B220^{lo}CD19^{lo}MHC-II^{lo}CD138^{+}CXCR4^{+}	IgG/A/M^{+}CD27^{hi}CD38^{hi}CD138^{hi}CXCR4^{hi}CD19^{lo}CD20^{-}CD45^{lo}MHC-II^{lo}
Alternance between SLO and BM	Follicular B cells	Adaptive immune responses	IgD^{hi}IgM^{lo}CD19^{+}CD21^{+}CD23^{hi}CD38^{+}CD43^{-}CD44^{-}B220^{+}CD80^{-}CD86^{-}CD1d^{lo}	IgD/M^{+}CD23^{var}CD27^{lo}CD38^{lo}CD44^{+}CD138^{lo}CXCR4^{lo}CD19^{+}CD20^{+}CD45^{+}MHC-II^{hi}
	Memory B cells	Immunological recall readiness	IgG/A/E^{+}IgD/M^{var}CD19^{+}CD21^{var}CD23^{var}CD38^{+}CD44^{+}B220^{+}CD80^{var}CD86^{+}	IgG/A/M^{+}IgD^{-}CD19^{+}CD20^{+}CD27^{+}CD21^{hi}CD23^{-}CD38^{lo}CD44^{+}CD138^{-}

5. B cell ontogeny

At early stages of development, B cells originate in the bone marrow, with exception of B1 cells that arise from the fetal liver (Baumgarth, 2011; Baumgarth, 2016). B cell proliferation and differentiation in the primary lymphoid organs require a suitable microenvironment provided mostly by stromal cells. This niche is composed of essential molecules, such as adhesive integrins,

growth factors, chemokines, and cytokines. IL-7, insulin-like growth factor-I (IGF-I), IL-6, IL-11, granulocyte-macrophage-colony-stimulating factor (GM-CSF), Fms-like tyrosine kinase 3, thrombopoietin, and CXC motif chemokine ligand (CXCL)- 12 (CXCL12) are key effector components in this process (Melchers, 2015; Svensson *et al.*, 2015).

The migration of multipotent progenitor cells (MPPs) first into the fetal liver and then into the bone marrow is the first step in B cell development. Afterwards, MPPs differentiate into the common lymphoid precursor (CLP), which could also give rise to T and natural killer (NK) cells (Tobón *et al.*, 2013). One of the essential proteins involved in B lymphocyte lineage commitment is the paired box protein 5 (Pax5). Pax5 expression is critical for the establishment and maintenance of the global lineage-specific architecture of B cells (Johanson *et al.*, 2018). Besides Pax5, other molecules are also required: IL-7, Fms-like tyrosine kinase 3 ligand (Flt3-L), transcription factors including PU.1, Ikaros family zinc finger protein 1 (IKAROS), the E2A immunoglobulin enhancer-binding factors E12/E47E2A (E2A or TCF3), early B cell factor 1(EBF), and the interferon regulatory factor 8 (IRF8) (Cobaleda *et al.*, 2007; Johanson *et al.*, 2018; Melchers, 2015; Tobón *et al.*, 2013). Maturation of B cells occurs through sequential developmental steps within the bone marrow before their release to circulation and effective differentiation into mature B cells, (**Figure 4**).

BCR generation is a critical step in B cell development. A diverse repertoire of BCRs is required to ensure the selectivity and specificity of the adaptive immune response. The rearrangement process of the heavy- and light-chain segments (variable V, diversity D, joining J) of the immunoglobulin gene, define the developmental stages at early phases of B cell maturation. During the first stage of this process, proB cells rearrange the D and J segments of the heavy chain, followed by a second rearrangement that allows joining the upstream V region to the DJ segment (Janeway Jr *et al.*, 2001). The terminal deoxynucleotidyl transferase (TdT) and recombinase activating genes 1 and 2 (RAG1/2) mediate nucleotide insertion of VDJ gene rearrangement. In lymphoid progenitors, in response to cytokines produced by stromal cells, TdT and RAG1/2 enzymes are induced. TdT catalyzes the addition of non-germline-encoded N nucleotides in the junctions of these rearranging gene segments and is critical for the development of a diverse antigen receptor repertoire in adult B cell development. In mice, fetal-derived B cell lineage does not express TdT (Nemazee, 2017).

The entry to the following stage, the pre-B cell stage, determines the functional rearrangement of the μ-heavy chain. Pre-B cells express on their surface the pre-BCR complex that includes the signalling components Ig-α and Ig-β. Pre-BCR signalling is required for continued B cell development, leading to decrease RAG1/2 protein levels and an increase in proliferation to form large pre-B cells. Pre-BCR signalling is required for continued B cell development, leading to decrease RAG1/2 protein levels and an increase in proliferation to form large pre-B cells. Ultimately, large pre-B cells proliferation stops and expression of RAG1/2 proteins is enhanced. A subset of pre-B cells differentiates to resting, known as small pre-B cells, as all the pre-B cells do when they cannot form a pre-BCR. At this phase, light chain rearrangement is induced defining the small pre-B cell pool. In the pre-B cell stage, the rearrangement of the gene segments that includes the κ and ʎ chains occurs. κ and ʎ chains in combination with the successful μ-heavy chain rearrangement will give rise to an IgM molecule, expressed on the surface of the immature B cells (Pieper *et al.*, 2013).

Immature B cells leave the bone marrow to home to the spleen, where they differentiate into transitional 1 (T1) and 2 (T2) B cells and finalize early development by differentiating into mature B cells. T1 B cells acquire cell surface expression of IgD and CD23, and become T2 B cells **(Table 1)** (Pillai & Cariappa, 2009). Signalling via BAFF and BAFFR is required to induce the transition from T1 to T2 B cells. Besides BAFF, the developmental process involves other molecules, such as APRIL, BAFFR, TACI and BCMA (Naradikian *et al.*, 2014). T3 stage of B cells initially was considered as a pre-stage of mature B cells. However, recently is suggested that T3 B cells may be autoreactive cells that are targeted for deletion through apoptosis due to interactions with self-antigens (Simon *et al.*, 2016).

In the spleen, antigen-based selection of transitional B cells also occurs to ensure the survival of mature B cells with low avidity to self-antigens and the destruction of B cells with high affinity to self-antigens. Transitional B cells represent the final stage before differentiation into more mature pre-immune B cell pools, specifically Fo B cells, MZ B cells, germinal centre B cells (GC B), and memory B cells. The T2 population, as originally described, contains considerable numbers of MZ B cell precursors (MZPs) (Srivastava *et al.*, 2005). Fo B or MZ B cells maturation from T1/T2 B cells requires essential signals such as differential self-antigen-BCR activation, cytokines like BAFF, the disinterring and metalloproteinase-10 (ADAM10) , expression of the transcription factor including Neurogenic locus notch homolog protein 2 (Notch2), activation of the Bruton's tyrosine

kinase (BTK), and the nuclear factor kappa beta (NF-κB) signalling (Meinl *et al.*, 2018; Pillai & Cariappa, 2009).

The strength of BCR signalling is one of the most important parameters that govern Fo B and MZ B cell development. A weak BCR triggering often induce MZ B cells while a relative strong BCR signalling will result in Fo B cell commitment, promoting the survival of Fo B type I (Allman & Pillai, 2008; Yam-Puc *et al.*, 2018). A strong signalling through the BCR will activate BTK in transitional B cells, triggering the canonical NF-κB signalling pathway and inducing the development of type I Fo B cells. A very strong BCR signalling will induce apoptosis (Pillai & Cariappa, 2009). Recently it was demonstrated that BCR ligation by endogenous or exogenous ligands induced the surface expression of the metalloproteinase ADAM10 on T1 B cells in a Taok3-dependent manner. Indeed, T1 B cells that express the surface protein ADAM10 are more receptive to Notch ligands favouring the differentiation towards MZ B cells (Hammad *et al.*, 2017). BAFF mediated signalling are essential for guaranteeing B cell survival, yet do not determine the fate of B cell subtype commitment (Pillai & Cariappa, 2009). Although Fo B cell maturation from T2 B cells mostly occurs in the spleen, about 25% still takes place in the bone marrow (Cariappa *et al.*, 2007).

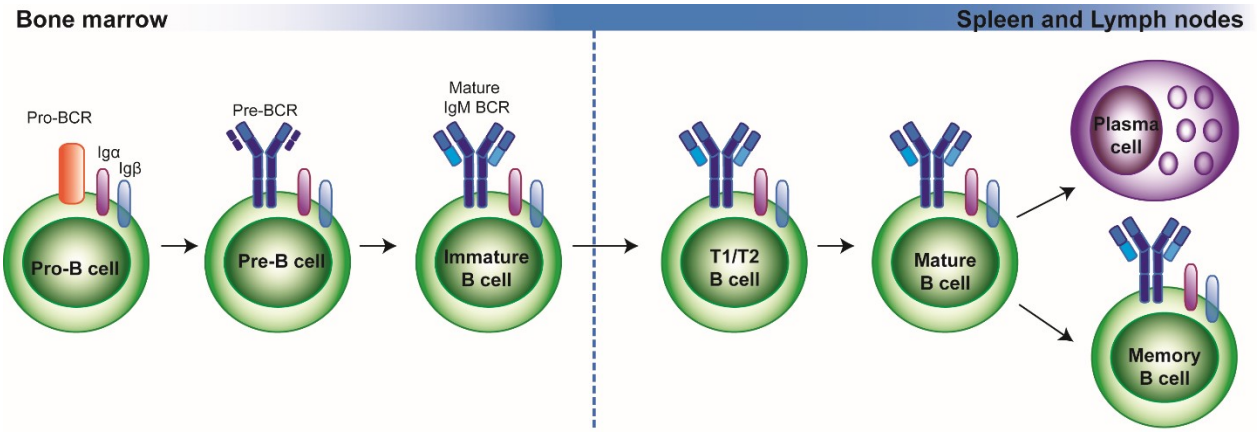

Figure 4. A general overview of B cell development. Development of B cells takes place from the bone marrow and is completed in peripheral lymphoid tissues. In the bone marrow B cell ontogeny progresses sequentially through pro-B, pre-B, and immature B cell stages and expression of surface IgM, mature B-cell receptor (BCR). Immature B cells with a selective BCR that can discriminate self antigens entry to the transitional B-cell pool. Transitional (T1/T2) B cells depend on diverse survival signal that induce to transitional B cells differentiate into mature B cells in the spleen. Mature B cells that are activated by foreign antigen and enter GC reactions give rise to isotype-switched memory B cells and plasma cells.

In the mouse, Fo B cells recirculate and populate various secondary lymphoid tissues, in contrast to MZ B cells that have limited circulation and are mostly retained in the spleen. Mature Fo B and MZ B cells, activated by foreign antigens, give rise to isotype-switched plasma and memory B

cells. In the case of Fo B cells, these events mainly occur in the GCs within the B cell follicles of secondary lymphoid organs while for MZ B cells they take place within the marginal sinus **(Figure 4)** (Cerutti *et al.*, 2013; De Silva & Klein, 2015).

Based on their functional characteristics, GCs are divided into two areas: the light and the dark zone. B cells residing in either zone express specific surface markers (**Table 1**). Enhanced B cell proliferation and somatic hypermutation (SHM) of the immunoglobulin V regions genes occurs within the dark zone of GCs. This event will provide the basis for the generation of high-affinity BCR (Victora & Nussenzweig, 2012). B cells expressing a high affinity-BCR then migrate from the dark to the light zone, where is tested the BCR modified in the light zone. TFh and FDCs are also involved in this process. Within the light zone, FDCs present MHC-associated antigens to GC B cells. The antigens are then captured by B cells via the BCR, processed and presented to TFh cells. TFh cells drive B cell proliferation by providing co-stimulation to GCs B cells via CD40-CD40 ligand interaction and the production of IL-21. GCs do not form in the absence of TFh and antibody defects are common under this condition (Suan *et al.*, 2017). BCR affinity-is directly associated with higher antigen capture and leads to a higher density of peptide–MHC complex presentation on the surface of the B cell. B cells with high-affinity antibody specificities predominantly differentiate into plasmablast (O'Connor *et al.*, 2006). Most of the GC B cells in the light area with a deficient BCR affinity are selected to recirculate to the dark zone to undergo an extra round of SHM or class switch recombination (CSR) to improve their BCR affinity or are deleted by apoptosis. Recirculation between the dark and the light zone within a short time allows the generation of high-affinity memory B cells and plasmablast. Plasmablast home to the bone marrow via CXCR4, where they reside in specific niches. Within the bone marrow, CXCL12, IL-6 and APRIL produced by stromal cells ensure the survival of plasmablast. They secrete antibodies and maintain serological memory independent of further antigen exposure (Tokoyoda *et al.*, 2010). Memory B cells can recirculate and rapidly differentiate into plasma cells (GC-independent memory) or re-enter GCs upon antigen re-encounter (extrafollicular and GC-dependent memory), resulting in further diversified secondary antibody responses (Takemori *et al.*, 2014; Tokoyoda *et al.*, 2010).

GC-dependent and independent memory cells survive equally over long periods of time. Even if the development of both memory B-cell types requires classical T-cell help, functional maturation occurs through distinct but related transcriptional programs. GC-dependent memory B cells generation requires TFh-cell help while GC-independent memory cells do not (Takemori *et al.*, 2014).

6. B cell functions

In the past years, additional B cell functions besides antibody production have been identified. Namely, B cells can act as antigen presenting cells and produce several cytokines and chemokines. These features have positioned B cells as new key players in the regulation of immune responses.

6.1. Antibody production by B cells

B1 and MZ B cells are known to have a polyreactive membrane-bound antibodies and have the capacity to secreted antibodies with wide-ranging specificity. They produce the first wave of antibodies in a TI manner to quickly limit pathogen invasion. In contrast, Fo B cells typically require T cell activation to differentiate into antibody-secreting plasma cells and generate high-affinity mono-reactive antibodies (Cerutti *et al.*, 2013; Popi *et al.*, 2016). During the initial encounter with a pathogen, plasma cells will provide protection by secreting pathogen-specific antibodies that can have a neutralizing capacity (Panda & Ding, 2015).

Antibodies, also known as immunoglobulins, are glycosylated proteins that are present on the surface of B cells or can be secreted to neutralize or eliminate their target antigens. A single immunoglobulin molecule is structurally composed of two pairs of polypeptide chains, one pair of light (L) low molecular weight chains (κ or λ), and one pair of heavy (H) chains (γ, α, μ, δ, and ε), typically all four joined by disulphide bonds to form a Y-shaped macromolecule (Gunn & Alter, 2016).

Antibody diversity in vertebrates produces a broad repertoire of molecules that are involved in fighting against pathogens. CSR and SHM are the two central mechanisms that rule Ig diversity. Interestingly, during a lifetime in humans up to 10^9 different antibodies can be produced. Ig genes are the main target of the SHM and CSR process. However, when other genes are involved, this process may lead to the development of B-cell lymphomas. The recombination-activating gene 1 and 2 (RAG1/2); and AID are essential for triggering V-D-J gene integration and inducing Ig

variation during SHM and CSR, (Casellas *et al.*, 2016; Methot & Di Noia, 2017). RAG1/2 are also involved in T cell receptor (TCR) arrangement during T cell development (Famili *et al.*, 2017).

6.2. Antibodies classes

The large repertoire of antibodies produced comprise five main classes or isotypes of immunoglobins (IgM, IgD, IgG, IgA and IgE) that are generated by CSR. Heavy chains on Ig isotypes serve as a structural identification pattern, specifically the C-terminal region, which remains constant and do not participate in antigen binding **(Table 2)** (Xu *et al.*, 2012). The conserved C-terminal region in the molecule, also known as Fc (the fragment crystallizable region of the antibody), is essential for the effector function of each isotype because it binds to several proteins, including complement, lectin-like molecules, and Fc-receptors found on all innate immune cells (Akula *et al.*, 2014). Besides binding and directly neutralizing pathogens, antibodies have additional effector functions that contribute to the pathogen clearance. Antibodies are involved in several processes such as infected cells elimination, toxins clearance, promoting phagocytosis, increase antigen presentation and regulating inflammation (Chan *et al.*, 2015; Lu *et al.*, 2018). Antibody-mediated effector functions will be further detailed in the text.

Table 2. Isotypes and main function of Immunoglobulin classes

Characteristics	Isotype						
	IgM	IgG				IgA	IgE
		IgG1	IgG2	IgG3	IgG4		
Heavy chain	μ	γ1	γ2	γ3	γ4	α	E
Polysaccharide antigens	++	+	+++	+/-	+/-	++	++
Protein antigens	+	++	+/-	++	++	+	+
Placental transfer	-	+++	+	++	+/-	-	-
Neutralization	+	++	++	++	++	++	-
Classic Pathway of complement activation	+++	++	+	+++	-	-	-
Sensitization for killing by NK cells	-	-	++	-	++	-	-
Biding to macrophage and phagocyte by Fc receptors	-	+	-	+	+/-	+	+
Binding to mast cells and basophils	-	+	-	+	-	-	+++
+ symbolizes relative presence –relative absence							

As previously mentioned, at early stages of B cell development, prior to antigen exposure, the successful rearrangement of the V, D, and J genes segment to produce unique Ig heavy and light chain variable (V) regions leads to the initial generation of the antibody repertoire. This process

allows tuning affinity and specificity of the Ig. Before B cells have undergone SHM, innate IgM is produced (Methot & Di Noia, 2017).

Secreted pentameric IgM, mainly present in the blood, simultaneously binds various antigens on 10 different sites. Additionally, IgM binds to complement allowing this Ig to drive the clearance of some pathogens at early stages of infection, a feature that compensates in part for their low affinity. This complex isotype is highly efficient at activating the complement system, opsonizing antigens and promoting phagocytosis, enhancing inflammatory responses, and destabilizing the surface membrane of several pathogens. After SHM, IgM is also produced, however other isotypes are more abundant at later stages of the humoral response (Baumgarth, 2016; Díaz-Zaragoza *et al.*, 2015).

At later stages of the immune response, IgG is one of the most abundant isotypes. Monomeric IgG immunoglobulins are usually high-affinity antibodies. They are classified in four subclasses based on structural differences: IgG1, IgG2, IgG3 and IgG4). The structural differences in IgG subclasses favour differential effector functions (Redpath *et al.*, 1998). IgG is present in the blood and the extracellular fluid. Like secreted IgM, IgG can neutralize toxins, viruses and bacteria, opsonize them for phagocytosis and activate the complement system.
B cells secrete monomers or dimers of IgA, monomers are within the blood and extracellular fluids while dimeric IgA, the most common form, is mainly in the lamina propria from several epithelial tissues (Vidarsson *et al.*, 2014). There are two subclasses of IgA: IgA1, which predominates in serum, and IgA2, more abundant in secretions (i.e. saliva and tears), where they neutralize toxins and viruses and block bacterial crossing across the intestinal epithelium (Gunn & Alter, 2016; Macpherson *et al.*, 2018).

Like IgM, IgD is a membrane receptor on naïve mature B cells, but it can also be secreted. IgD synergizes with IgM at early stages of infection, particularly within mucosal tissues. (Lutz *et al.*, 1998). IgD binds to basophils and mast cells, which promotes the production of antimicrobial peptides and inflammatory cytokines (Chen *et al.*, 2009). The remaining Ig isotype is IgE, a monomeric immunoglobulin. This isotype is the least abundant in the blood. During infection, IgE effector function mainly targets parasites, such as helminths (i.e. *Schistosoma mansoni*) and certain protozoan (i.e*. Plasmodium falciparum*). IgE are capable of triggering a marked inflammatory reaction, mainly by binding to Fc receptors found on the surface of mast cells and basophils (Wu & Zarrin, 2014).

Physiological deregulation in the elimination (negative selection) or functional inactivation (anergy) of high-affinity, self-reactive B lymphocytes results in the development of immune-inflammatory related pathologies, including hypersensitivity. Hypersensitivities are classified in four main types. Immunoglobulins contribute to direct exacerbation of three of these inflammatory pathologies, particularly IgE, IgM and IgG isotypes (Gell & Coombs, 1963).

Type I hypersensitivity is the most frequent form, also known as allergy. This immune overreaction occurs in short periods of time (minutes) and is induced by the re-exposure to a specific antigen (allergen). IgE participates in this type of hypersensitivity. Following exposure to the same allergen, cross-link the IgE on mast cells and basophils, which causes a release of vasoactive biomolecules, such as histamine, leukotriene, and prostaglandin (Owen *et al.*, 2014).

IgM and IgG isotypes participate in type II hypersensitivity reactions. In contrast to type I, type II requires a longer time to develop (hours). IgM and IgG bind antigen on a target cell, a host cell that in this case is sensed as foreign, creating complexes that activate the complement system and induce the membrane attack complex (MAC) (Bayly-Jones *et al.*, 2017). This, provokes cell lysis and death by destabilization of the cellular membrane. Autoimmune hemolytic anemia is a representative example of Type II hypersensitivity (Owen *et al.*, 2014).

In type III hypersensitivity reactions, IgG and IgM antibodies also participate. This type of reaction can take hours, days, or even weeks to develop. In contrast to type I hypersensitivity, IgG and IgM antibodies bind to soluble proteins forming circulating immune complexes that can deposit in tissues, leading to complement activation, inflammation, neutrophil infiltration and mast cell degranulation (Owen *et al.*, 2014). Examples of type III hypersensitivity reactions include systemic lupus erythematosus (SLE) (Kaul *et al.*, 2016). Type IV hypersensitivity involves over-inflammatory reactions mediated by T helper cells (Th), specifically memory Th1 T cells inducing tissue damage.

6.3. Antibody-mediated effector functions

Antibodies act by a diverse number of mechanisms that can involve different components of the innate and adaptive immune system. Most of the effector functions of antibodies are based on the

differential modifications of the Ig constant domain, including the Fab (the fragment antigen-binding) and Fc domain. This regions provides specific effector signals to dictate the associated immune response **(Figure 5)**.

6.3.1. *Antibody-mediated neutralization*

The simplest antibody-mediated effector function is antigen neutralization. The Fab domain of the Ig binds directly to specific targets, including pathogens and toxins (Lu *et al.*, 2018). Neutralization prevents the direct interaction between the pathogen and the receptors from the host cell, preventing infection and disease progression. Neutralizing antibodies have been mainly studied in viral infections (Corti *et al.*, 2017). However, this mechanism also applies to neutralization of toxins from pathogenic bacteria, such as diphtheria toxin. Antibody-mediated neutralization is a broadly used tool in several immune therapies (Corti *et al.*, 2017; Lu *et al.*, 2018). Indeed, antibodies that have an enhanced capacity to drive antibody-dependent cellular cytotoxicity (ADCC) are associated with specific-antibody mediated protection in influenza (Ackerman *et al.*, 2016) and HIV (He *et al.*, 2016). Recently, ADCC-based therapeutic approaches were reported to prevent or treat HIV (Bruel *et al.*, 2016; Lee *et al.*, 2015; Lee *et al.*, 2016). Specifically, viral replication can be suppressed with passive administration of potent HIV-specific neutralizing monoclonal antibodies in the absence of antiretroviral therapy in patients (Caskey *et al.*, 2017; Lu *et al.*, 2016; Lynch *et al.*, 2015; Scheid *et al.*, 2016). Overall, the goal is to induce the host immune system to target latently infected cells for killing by ADCC (Bruel *et al.*, 2016).

6.3.2. *Antibody-mediated complement activation*

An important component of the innate immune system is the complement. This system is constituted by a pool of proteins with diverse enzymatic activities that are present in the blood and tissues from various mammals. Interaction between these molecules leads to the initiation of a signalling cascade that induce a series of inflammatory responses (Ghebrehiwet *et al.*, 2016).

Complement mediated immune responses can be triggered by three pathways: the classical, the mannan-binding lectin, and the alternative pathway. These three pathways converge in the generation of a crucial enzymatic activity, the C3 convertase that leads complement system activation (Ghebrehiwet *et al.*, 2016). The three main consequences of complement activation are

opsonization of pathogens, the recruitment of inflammatory and phagocytic cells, and direct killing of pathogens (Lu *et al.*, 2018; Reddy *et al.*, 2017).

In the classical pathway, pentameric IgM and IgG clusters can recruit and bind complement (Diebolder *et al.*, 2014; Lu *et al.*, 2018). Several immune cells can recognize complement-antibody complexes via complement receptors. For instance, naïve B cells can capture these complexes and transfer them to FDCs within the GC, contributing in part to the development of the humoral response (Phan *et al.*, 2007). Also, these interactions enhance BCR signalling and B cell survival (Lu *et al.*, 2018).

6.3.3. *Antibody-dependent cellular cytotoxicity*

Antibodies can bind to antigens located on the surface membrane of infected or damaged cells, mediating cell lysis of the targeted cell by activation of effector immune cells. This effector mechanism is known as ADCC. Classical ADCC results from the crosslinking of FcγRIIIa on NK cells, where IgG1 and IgG3 isotypes have a higher affinity for FcγRIIIa. This crosslinking leads to perforin and/or granzyme secretion and induces cell death of the target cell (Wu *et al.*, 1997). However, other immune cells, such as macrophages, DCs, neutrophils, and eosinophils can mediate ADCC. ADCC is not a prerogative of IgG, data obtained from *Schitosoma mansoni* model suggest that IgA and IgE can mediate ADCC (Gounni *et al.*, 1994). The latter may protect against extracellular organisms, including helminths, through the non-classical induction of degranulation by eosinophils and platelets that promote pathogen clearance. ADCC is used as a tool in several therapeutic approaches against chronic diseases (Lu *et al.*, 2018).

6.3.4. *Antibody-dependent cellular phagocytosis*

Clearance of antibody-pathogen complexes from the circulation mediated by mononuclear phagocytes (monocytes, macrophages and DCs) and granulocytes (neutrophils, eosinophils, basophils and mast cells) upon molecular recognition by complement receptors and/or Fc receptors is known as antibody-dependent cellular phagocytosis (ADCP). Besides pathogen clearance, ADPC can facilitate the triggering of several signalling pathways that promote cytokine secretion, production of lipid mediators and enhance antigen presentation (Lu *et al.*, 2018; van Egmond *et al.*, 2015; Yende & Wunderink, 2011). Interestingly, FcR-mediated phagocytosis can regulate macrophage polarization towards the inflammatory (M1) and/or the regulatory (M2)

phenotype (van Egmond *et al.*, 2015; Yende & Wunderink, 2011). In DCs, internalization of antibody-pathogen complexes drives maturation and differentiation, and promotes antigen presentation (Guilliams *et al.*, 2014). While in neutrophils, ADCP induces the formation of neutrophil extracellular traps (NETs) that can kill extracellular pathogens (van Kessel *et al.*, 2014). This simultaneous immune response in most of the cases promotes an inflammatory response required for pathogen control.

6.3.5. *Antibody-dependent enhancement of disease*

Pathogens have evolved various strategies over time to take advantage of host defense mechanisms. Pathways that involve complement, antibodies and FcR mediated responses are commonly hijacked by pathogens (Lu *et al.*, 2018; Sarantis & Grinstein, 2012). This mechanism is known as antibody-dependent enhancement of disease (ADE).

ADE is a recurrent mechanism used by some viruses to favour disease progression. Flaviviruses such as Dengue and Zika exploit this mechanism. Indeed, a primary infection with Flavivirus may result in deficient protective humoral immunity. Infection of host cells with partially antibody-opsonized viruses can cause severe infection (Lu *et al.*, 2018). Precisely, cross-reactive antibodies originated from the first infection form immune complexes with the second viral serotype that stabilize the virus; this stabilization promotes viral uptake within macrophages leading to hemorrhagic fever and shock syndrome (Ayala-Nunez *et al.*, 2016; Beltramello *et al.*, 2010).

ADE is not limited to viral infections; parasites also use this mechanism to promote disease. In *Leishmania* infection, IgG-bound-parasites are phagocytized via-FcRs, resulting in IL-10 production that favours parasite replication. IL-10 downregulates nitric oxide synthase and inhibits Th1 cell and IFN-γ responses, required for parasite elimination (Kaye & Scott, 2011). Therefore, studies carried on in specific antibody effector function in several pathologies will provide a better understanding of humoral immune responses, providing new treatment approaches.

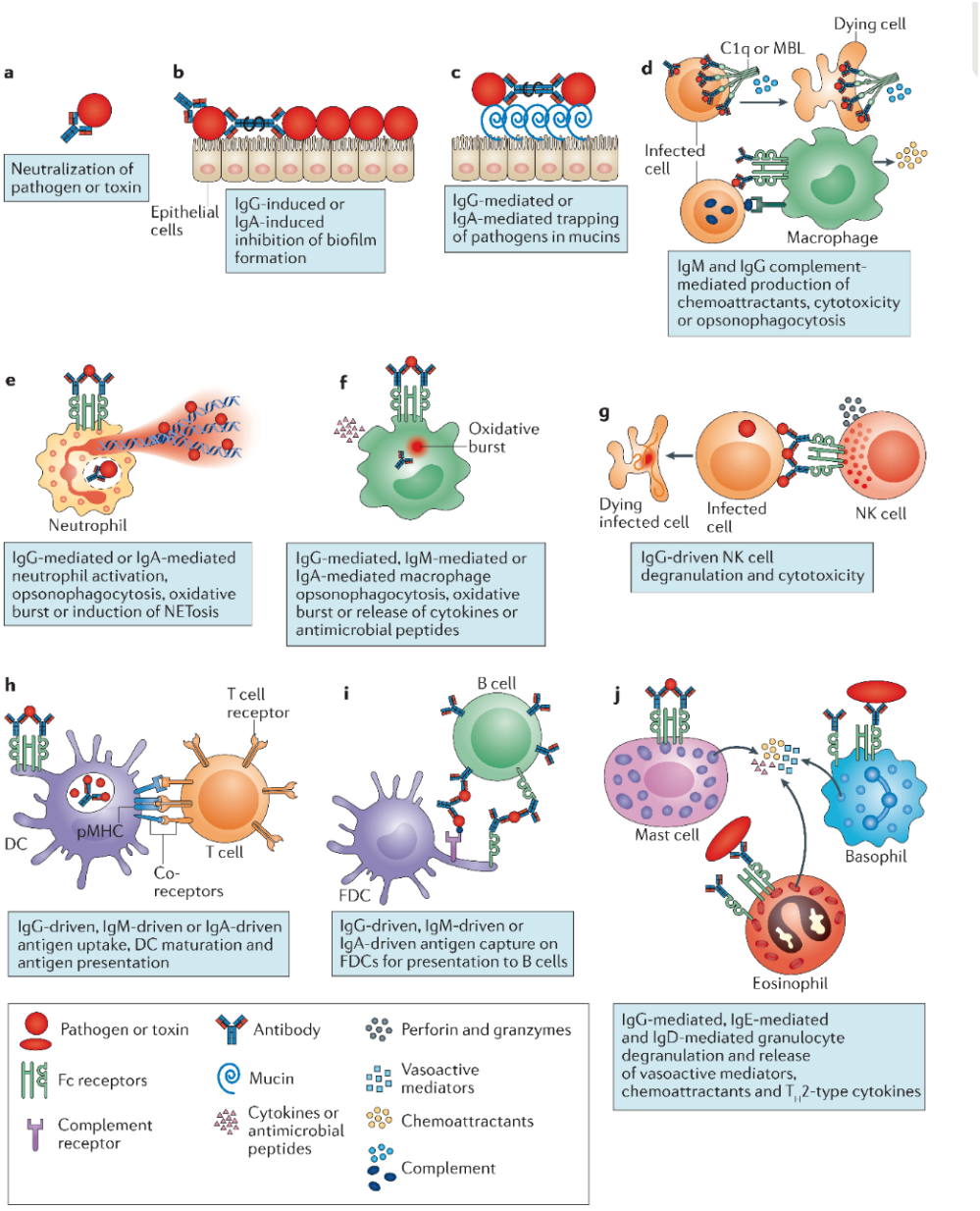

Figure 5. Antibody effector functions (Lu *et al.*, 2018). Antibodies can deploy a plethora of effector functions over the course of an infection. These include but are not limited to the following: **a** | The direct neutralization of toxins or microorganisms. **b** | The neutralization of microbial virulence factors, such as those involved in quorum sensing and biofilm formation. **c** | The trapping of pathogens in mucins. **d** | The activation of complement to drive phagocytic clearance or destruction, generate chemoattractants or anaphylatoxins such as C3a and C5a or complement fragment opsonins such as C3b or induce lysis through the membrane attack complex. **e** | The activation of neutrophil opsonophagocytosis, oxidative bursts, production of lytic enzymes and chemoattractant, or the formation of neutrophil extracellular traps (NETs) of chromatin and antimicrobial proteins. **f** | The induction of macrophage opsonophagocytosis, oxidative bursts or antimicrobial peptide release. **g** | The activation of natural killer (NK) cell degranulation to kill infected cells. **h** | The enhancement of antigen uptake, processing and presentation by dendritic cells (DCs) to T cells. **i** | The presentation of antigens by follicular dendritic cells (FDCs) to B cells. **j** | The degranulation of mast cells, basophils and eosinophils to release vasoactive substances, chemoattractants and T helper 2 (TH2)-type cytokines in the setting of allergens or parasitic infections. Fc, crystallizable fragment; MBL, mannose-binding lectin; pMHC, peptide–MHC complex.

6.4. Cytokine production by B cells

Cytokines and chemokines are essential players in the cross-talk between the innate and the adaptive immune system, as well as in the development of the immune response. Cytokines alone or in combination with other extracellular mediators can trigger a complex intracellular array of signalling pathways and modulates the activation or inactivation of inflammatory responses and induction of cell proliferation, among other functions (Turner *et al.*, 2014). In addition to antibody production, B cells can be involved in modulating immune responses by the production of several cytokines and chemokines.

In the past years, a wide range of cytokines and modulatory molecules produced by B cells has been identified, such as lymphotoxin α1β2 (LTα1β2), IL-2, tumour necrosis factor (TNF), interferon (IFN) gamma (IFN-γ), chemokine [c-c motif] ligand (CCL) - 3 (CCL3), GM-CSF, IL-17, IL-10, IL-6, Galectin-1 (Gal-1) among others. B cell-derived cytokines can be involved in different functions such as B cell development, organogenesis of lymphoid tissue, regulation of T cell responses, allergies, and autoimmunity (Shen & Fillatreau, 2015). This multifunctional role can either promote or protect from disease development (Lund & Randall, 2010). B cell responses and cytokine production are mostly regulated by their immune microenvironment, which determines B cell capacity to produce effector cytokines with inflammatory or anti-inflammatory properties (Shen & Fillatreau, 2015).

6.4.1. *B cell-derived cytokines contribution to organ biogenesis*

During lymphoid organogenesis B cells are one of the first cells that populate the spleen. In this organ, they secrete TNF and LTα1β2 which are required for the development of FDCs. Notably, FDCs are essential for the organization of B cells within the follicle, through the production of the B cell-attracting CXCL13. This mechanism represents a positive feedback loop between B cells and FDCs. In contrast to the spleen, in lymph nodes and Peyer's patches, FDC development requires TNF but not LTα1β2 expression by B cells. Additionally, LTα1β2 derived from B cells contributes to the organization of the stroma of the T cell zone and development of the splenic MZ (Zindl *et al.*, 2009). TNF secreted by B cells also contributes to the marginal zone organization, by promoting the establishment of CD169+ macrophages and enhancing the local expression of the addressin cell adhesion molecule 1 (MADCAM1) (Mebius & Kraal, 2005). Also, LTα1β2 during

acute immune responses against different pathogens and chronic diseases is associated with secondary lymphoid organ remodelling and homeostasis (Ben-Neriah & Karin, 2011; Kumar *et al.*, 2010). In this context, B cells impact the generation and homeostasis of diverse organs.

6.4.2. *B cell-derived cytokines in the regulation of T cell responses*

B cells influence a wide range of aspects of T cell responses. Cytokines derived from B cells can either promote or suppress effector capacities of T cells. Effector functions of heterogenic $CD4^+$ T cells are an essential component of the adaptive immune response. They can enhance or suppress the activity of different immune cells through the release of a wide range of cytokines and costimulatory interactions. As mentioned before, effector $CD4^+$ T cells are indispensable in B cell antibody class switching, the activation and promotion of cytotoxic T cells activity and enhancing killing-capacity of phagocytic cells, such as macrophages. $CD4^+$ T cell polarization and plasticity into different effector subtypes is an event that is highly dependent on the cytokine microenvironment, which allows individuals to respond to environmental stimuli in a context-dependent manner (i.e. infectious and autoimmune diseases, cancer, among others). $CD4^+$ T cells subpopulations have been an important research subject over the past years. Known $CD4^+$ T cell subsets includeTh1, Th2, Th17, Th9, Th25, TFh, and regulatory T cells (Treg) (Cher & Mosmann, 1987; Mosmann *et al.*, 1986; Mosmann & Coffman, 1989). However, $CD4^+$ T cell effector cells, defined as Th1 and Th2 cells, based on their cytokine production, are the most studied populations (Caza & Landas, 2015; Cher & Mosmann, 1987).

Th2 CD4+ T cells are known to promote humoral immune responses and host defence against extracellular parasites, and to potentiate allergic responses and asthma. IL-4, IL-25 and IL-33 are involved in polarization and maintenance of Th2 cells (Buchholz *et al.*, 2016). However, LTα1β2 produced by B cells is known to be involved in the modulation of Th2 $CD4^+$ cells effector responses. B cell-derived LTα1β2 promotes T cell priming outside the T cell zone, modulating the localization of DCs and T cells by enhancing the local upregulation of the lymphoid chemoattractant CXCL13 (León *et al.*, 2012). Additionally, in some infectious models, IL-2 secreted by B cells promotes Th2 cell memory. However, the effect of IL-2 produced by B cells in Th2 cells differs according to the disease model (León *et al.*, 2012).

Th1-type immune responses, participate in the elimination of intracellular parasites and tumour immunity, and perpetuate inflammatory autoimmune responses. Th1 cells are primarily

responsible for activating and regulating the development and persistence of cytotoxic T cells (CTL) that can directly kill tumours (Lai *et al.*, 2011). Through IFN-γ production, they also induce the activation of macrophages and enhance activity of the nitric oxide synthases (iNOS). Increased iNOS activity leads to the killing of intracellular pathogens, such as *Leishmania spp*., *Listeria monocytogenes* and *Mycobacterium spp*. IL-12 is essential for inducing Th1 $CD4^+$ T cells development (Buchholz *et al.*, 2016; Silva-Barrios *et al.*, 2018). B cell-derived cytokines can promote or suppress IFN-γ - mediated Th1 effector functions on $CD4^+$ T cells. In *Toxoplasma gondii* infection, B cell-derived-TNF and CCL3 are known to contribute to an efficient Th1 response (Menard *et al.*, 2007). Another cytokine produced by B cells that is known to enhance Th1 responses in *Salmonella enterica* and *L. monocyogenes* experimental models is IFN-γ (Bao *et al.*, 2014; Barr *et al.*, 2010). In the atherosclerosis autoimmune model, GM-CSF produced by B cells enhance detrimental Th1 responses, and contribute to disease pathology (Hilgendorf *et al.*, 2014).

B cell-derived cytokines can also suppress T cell effector functions, dampening inflammatory immune responses. IL-10 is one of the most studied B cell-derived suppressive cytokines in different chronic diseases and infectious models (Hilgenberg *et al.*, 2014). Suppression of Th1 responses by B cell-derived IL-10 could be detrimental in the context of infectious diseases, like for instance VL, or protective in autoimmune models such as multiple sclerosis (Bankoti *et al.*, 2012; Kalampokis *et al.*, 2013). IL-10 derived from B cells is mostly induced by TLR triggering (Bankoti *et al.*, 2012; Lampropoulou *et al.*, 2008; Silva-Barrios *et al.*, 2018), but BCR and CD40 engagement can also be involved (Fillatreau *et al.*, 2002).

Interleukin-6 (IL-6) is another cytokine produced by B cells upon activation (Arkatkar *et al.*, 2017). IL-6 is involved in hematopoiesis and is required in the final maturation of B-cells into antibody-producing plasma cells, facilitates $CD4^+$ T cell differentiation into TFh, and promotes GC formation (Chavele *et al.*, 2015; Kishimoto, 2010). For example, in the intranasal model of influenza virus infection, B cell-derived IL-6 promotes the transcription of *Il21* in $CD4^+$ T cells, facilitating their differentiation into TFh cells (Karnowski *et al.*, 2012). Also, IL-6 production by B cells drives autoimmune GC formation in a mouse model of systemic lupus erythematosus (SLE) (McHugh, 2017). B cell-derived IL-6 participates in the activation of Th1 and Th17 cells, an essential mechanism of B cell–driven pathogenesis in the experimental model of autoimmune encephalitis (EAE) (Barr *et al.*, 2012).

Th17 cells are known to be one of the major producers of IL-17. One of the main roles of IL-17 is the induction and regulation of proinflammatory immune responses. However, B cells can produce IL-17 as well. In the experimental model of *Trypanosoma cruzi*, B cells are the main source of IL-17 during infection. B cell-derived IL-17 contributes to protective responses against the parasite (Bermejo *et al.*, 2013; Boari *et al.*, 2012; da Matta Guedes *et al.*, 2010).

Besides IL-17, during *T. cruzi* infection Gal-1 is produced by B cells. Gal-1, a member of the family of β-galactoside-binding proteins, plays a role in modulating cell-cell and cell-matrix interaction. Gal-1 has been shown to mediate immunosuppression of $CD8^+$ T cells, triggering apoptosis and reducing IFN-γ secretion during *T. cruzi* infection (Zuñiga *et al.*, 2001). In summary, cytokines produced by B cells regulate a wide range of T cell functions in various models, from infectious diseases to autoimmunity.

6.5. B cells as antigen presenting cells

APCs are a diverse group of immune cells that are involved in the cellular immune response. They are provided with an antigen-sensing, -processing, and -presentation capacity. The group of classical APCs include DCs, macrophages, Langerhans cells and B cells.

A common feature of APCs is the constitutive expression of MHC-II molecules and the ability to express costimulatory molecules. In the case of B cells, during B cell development the expression of MHC-II is initiated at early stages (Chen & Jensen, 2004). Besides constitutive expression of MHC-II and costimulatory molecules, B cells have other characteristics that confer them their ability to serve as APCs. For instance, their proximity to T cells areas in secondary lymphoid organs, which facilitates their participation in the generation or regulation of adaptive immune responses (Batista & Harwood, 2009).

Following antigen recognition, APCs can internalize the antigen by phagocytosis, pinocytosis, and receptor-mediated endocytosis. In B cells, receptor-mediated phagocytosis involves antigen recognition and capture by the BCR. Following internalization into endosomal compartments, antigens are processed into peptides associated to MHC molecules that re-emerge on the cell surface. This feature allows B cells to concentrate very small quantities of antigen and present it effectively to $CD4^+$ and $CD8^+$ T cells (Batista & Neuberger, 2000). Additionally, B cells express the

costimulatory molecules CD80, CD40 and CD86, and the cytokines IL-6 and IFN-γ required for an efficient T cell activation and long-lasting T cell memory (Ng *et al.*, 2010).

6.6. Polyclonal B cell response

An effective immune response usually involves the production of a wide range of diverse antibodies against the same antigen by a pool of B cells that proliferates and differentiates in antibody-secreting cells. This type of immune response is known as polyclonal B cell activation, where a heterogeneous pool of antibodies is produced against diverse epitopes of a specific antigen, conferring in most of the cases enhanced protection against various pathogens. Cytokine secretion and the upregulation of costimulatory and receptor molecules, such as MHC-II, CD25, CD69, CD80, and CD86 on the B cell surface, occurs during polyclonal B cell activation (Mims, 2018) .

Over the course of a normal immune response, polyclonal B cell activation confers an immunological advantage to the host contributing to pathogens clearance. However, it also increases the probabilities of developing autoimmunity. A strong polyclonal B cell response results in hypergammaglobulinemia and generation of autoantibodies. Hypergammaglobulinemia is a hallmark in a wide spectrum of infections and autoimmune disorders. Several pathogens are known to induce hypergammaglobulinemia, including virus, bacteria, and protozoan parasites (Mims, 2018). For example, viruses such as HCV, LCMV, HIV induce hypergammaglobulinemia. Also, bacteria, such as *Mycobacterium tuberculosis* and *Coxiella burnetii* (Mims, 2018), and protozoan parasites, including *Plasmodium spp.*, *Leishmania spp.* and *Trypanosoma* spp., p polyclonally activate B cells and promote hypergammaglobulinemia (Silva-Barrios *et al.*, 2018). In most of the cases, this mechanism favours pathogen success and disease progression. In autoimmune diseases, hypergammaglobulinemia is characterized by high levels of auto-antibodies. Systemic lupus erythematosus (SLE), is one of the most studied autoimmune pathologies involving hypergammaglobulinemia (Kaul *et al.*, 2016).

7. Deleterious B cell functions

In the past years, the study of mechanisms that drive a non-protective B cell phenotype in various disease models gained increased interest. B cells have been shown to be detrimental in several diseases, including immune disorders and infectious diseases.

7.1. B cells and chronic diseases

In some chronic diseases such as autoimmunity and cancer, B cell-mediated immune responses can contribute to disease exacerbation. Various effector functions have been associated with disease aggravation including polyclonal B cell activation, induction of cytokine production, and impairment of B cell survival, among others.

7.1.1. B cells in autoimmunity

An uncontrolled development of autoreactive B cells that produce autoantibodies with harmful effects to the host leads to autoimmunity. In autoimmune diseases, B cells are responsible for disease progression mostly through the production of autoreactive antibodies, deposition of immune complexes, presentation of autoantigens, enhancement of pathogenic $CD4^+$ T cell responses, and production of proinflammatory cytokines. Nevertheless, B cells with a regulatory phenotype (Bregs) are beneficial in various autoimmune disorders since they contribute to control the highly inflammatory microenvironment. Bregs control inflammation by secreting regulatory cytokines such as IL-10, IL-35 and TGFβ (Fillatreau *et al.*, 2002). B cells depletion at early stages of the disease in some autoimmune models like SLE and multiple sclerosis (MS) enhances immunopathology; in contrast, depletion at later stages decreases disease severity (Matsushita, 2018). Understanding the regulatory and effector B cell balance is essential for the development of effective therapeutic approaches.

Cytokine production by effector B cells such as IFN-γ, IL-2, IL-4, IL-6, IL-12, IL-17, TNF, GM-CSF, contribute differently to disease progression. B cell-derived IL-6 is known to induce TFh, Th17, and plasma cell differentiation (Caza & Landas, 2015). In the mouse model for EAE, specific B cell ablation of IL-6 decreased detrimental Th17 responses and EAE severity. (Barr *et al.*, 2012). IFN-γ produced by B cells in autoimmunity induces macrophage activation and contributes to

inflammation (Matsushita, 2018). In the experimental arthritis model, B cell-derived IFN-γ is known to suppress regulatory T cells promoting disease exacerbation (Olalekan *et al.*, 2015); whereas GM-CSF produced by B cells in MS promotes macrophages activation, contributing to disease progression (Li *et al.*, 2015b).

High levels of autoantibodies are the hallmark of autoimmune diseases. Autoantibodies can target a wide range of molecules such as nucleic acid, lipids, and proteins present within the cell (nucleus and cytoplasm), on the cell surface or in the extracellular matrix. Antinuclear antibodies (ANAs) and rheumatoid factors (RFs) were the first to be described in SLE patients, in the late 1940s (Mackay, 2010). ANAs are the most common group of autoantibodies. They can recognize different nuclear components released into the extracellular environment upon cell death. (Suurmond & Diamond, 2015). Interestingly many antigens recognized by autoantibodies are ligands for B cell TLRs. TLR triggering is associated with antibody production by autoreactive B cells in SLE, particularly TLR7 and TLR9 (Christensen *et al.*, 2006; Han *et al.*, 2015). In experimental SLE, mice deficient in TLR7, TLR9 or MyD88 are partially protected against the development of ANAs and have decreased lupus-like symptoms (Christensen *et al.*, 2006; Ehlers *et al.*, 2006; Hua *et al.*, 2014). TRL7 and TLR9 have opposite regulatory roles in experimental SLE. In the absence of TLR9, SLE symptoms are exacerbated, activation of lymphocytes and plasmacytoid DCs are enhanced, and serum IgG and IFN-α increase. In contrast, TLR7-deficient mice shown decreased lupus-like symptoms, less lymphocyte activation, and reduced serum IgG (Christensen *et al.*, 2006; Han *et al.*, 2015).

TLR triggering results in the production of a wide range of cytokines, including type I interferon (IFN-I). The IFN-I family include two main classes of related cytokines: IFN-α, which comprises 13 different subtypes encoded by 13/14 different genes, and IFN-β, a product encoded by a single gene. A wide variety of cells can produce and respond to IFN-I. Therefore, this confers to B cells several autocrine and paracrine effects that have been extensively characterized mainly in viral infections. However, they also play a role in modulating immune responses in bacterial and protozoan infections (Kovarik *et al.*, 2016; Silva-Barrios & Stäger, 2017). On the cell surface, IFNAR mediates IFN-I signalling. The crosstalk between IFNs and their specific receptors elicits an intracellular signalling cascade that mainly enhances inflammatory responses (Silva-Barrios & Stäger, 2017). Several genes that contribute to the risk of developing the disease have been identified in SLE. These include IFN-I signalling related genes such as IFN regulatory factor 5 (IRF5). Mutations in IRF5 are related to enhanced levels of IFN-I family molecules (Kaul *et al.*,

2016). In B cells, a positive feedback loop involving IFN-I and TLR7 promotes antibody production (Green *et al.*, 2009). The contribution of antibodies to disease development are not only dependent upon their direct binding to end-organ tissue antigens, but also through indirect mechanisms, including immune complexes formation and deposition, complement activation, and FcR mediated activation. These represent common features of many immunopathological disorders.

7.1.2. B cells in cancer

In the context of cancer B cells can either promote anti-tumour immunity or have detrimental effects allowing tumour progression. For instance, B cells can modulate the function of their antigenic target on the cancer cell, opsonize tumour cells for the presentation and cross-presentation of tumour antigens by DCs, mediate complement cascade activation, or contribute to NK cell mediated tumour killing via ADCC. Even though antibodies against tumour antigens are present in the serum of cancer-affected individuals, the role of humoral immune responses in cancer is controversial (Sarvaria *et al.*, 2017).

In some cases, B cells can promote anti-tumour immunity. Findings obtained from experimental mouse models demonstrated that B cells facilitate T-cell-mediated immune responses that inhibit tumour development (DiLillo *et al.*, 2010). Indeed, these responses were impaired in B cell-depleted mice carrying B16 melanoma tumours, suggesting the requirement of T and B crosstalk in anti-tumour immunity (Sorrentino *et al.*, 2011). In addition, it has been reported that activated B cells impair tumour progression through ADCC (Li *et al.*, 2009), enhance IFN-γ production and promotes NK anti-tumour mediated responses (Jones *et al.*, 2008). However, most of these results were based on tumour prevention rather than the treatment of established tumours (Sarvaria *et al.*, 2017). Additionally, in cancer patients, the presence of infiltrated B cells within the tumour correlates with improved survival and lower relapse (Al-Shibli *et al.*, 2008; Nedergaard *et al.*, 2008; Nielsen *et al.*, 2012). However, further studies are required to fully understand how B cells regulate tumour growth.

In contrast, other studies showed that B cells favour tumour progression. Indeed, anti-IgM antibody-mediated B cell depletion in tumour-bearing mice reduced tumour burden (Barbera-Guillem *et al.*, 2000). Also, B-cell-deficient mice showed stronger T cell responses against adenocarcinoma tumour cells (Qin *et al.*, 1998). In addition, B cells can promote tumour progression trough the production of anti-tumour antibodies. Most of antibodies found in cancer

patients target autoantigens that are present in the tumour cells as well as in unmutated host cells. However, some antibodies are against tumour antigens and target the mutated suppressor tumour protein p53. Cancer cells that bear a mutant p53 are no longer able to control cell proliferation which results in inefficient DNA repair and the emergence of a genetically unstable daughter cell (Yue *et al.*, 2017). Pioneer research in this field showed that adoptive transfer of tumour-specific antibodies amplified tumour growth in murine models and B cell absence impaired tumour development (Brodt & Gordon, 1982; Kaliss, 1958; Ran & Witz, 1972).

Like for SLE, immune complexes can be circulating or present in tumorous tissues. In several human cancers, immune complexes contribute to disease progression (Gunderson & Coussens, 2013; Yuen *et al.*, 2016). Different experimental models support the pathogenic role of B cells in promoting tumour progression (De Visser *et al.*, 2005). Immune complexes, deposited in premalignant tissue, activate Fcγ receptors on resident and infiltrating myeloid cells (Andreu *et al.*, 2010). They also mediate complement pathway activation, and contributing to a pro-angiogenic program of tissue remodelling that ultimately results in hyperproliferation and malignant progression (Andreu *et al.*, 2010; Yuen *et al.*, 2016).

In addition to antibodies, cytokines derived from B cells can be detrimental in cancer models. B cells infiltrating within the tumour, can provide LTα1β2, a survival factor that can induce angiogenesis (Ammirante *et al.*, 2010). Moreover, B cell-derived LTα1β2, activates non-canonical and canonical NF-κB signalling and STAT3 in the remaining cancer cells, resulting in growth and tumour progression (Ammirante *et al.*, 2010; Yuen *et al.*, 2016). In bladder cancer, B cell-derived IL-8 enhances metastasis. (Ou *et al.*, 2015). IL-10 producing Bregs in cancer can suppress anti-tumour immune responses by inhibiting effector cells such as CTLs and NK cells (Sarvaria *et al.*, 2017). Additionally, B cell-derived TGFβ induces $CD4^+$ T cells to become $Foxp3^+$ $CD4^+$ T regulatory cells (Tregs), which suppress Th1 cells, NK cells, and cytotoxic $CD8^+$T cells (Sarvaria *et al.*, 2017). Although current immunotherapeutic strategies focus extensively upon the augmentation of T-cell-mediated immunity, B cells are increasingly being appreciated as crucial players in cancer therapies (Gunderson & Coussens, 2013)

7.2. B cells and infectious diseases

A wide range of pathogens can hijack B cells function to succeed within the host. Through the co-evolution between pathogens and the host, pathogens developed diverse strategies targeting the B cell compartment. These include polyclonal B cell activation, induction of cytokine production, impairment of B cell development, inhibition or promotion of B cell survival, and the use of B cells as a reservoir.

7.2.1. Viral infections

Several viruses target B cells as host reservoir like the Epstein-Barr virus, and suppress protective B cell responses, like cytomegaloviruses or the measles virus. Epstein-Barr virus (EBV), a member of the Herpesviridae family, is one of the most studied viruses that targets B cells. EBV viral glycoproteins gp350 and gp42 mediate the entry into B cells by directly binding to CD21 (CR2) and MHC-II. This interaction triggers endocytosis of the viral particle by the targeted B cell (Hatton *et al.*, 2014). Within B cells, EBV induces the transition of the infected cell into a long-lasting resting memory B cell phenotype. It is suggested that EBV uses this mechanism to avoid antibody-mediated immune responses and immune surveillance(Hatton *et al.*, 2014). Influenza virus, cytomegalovirus (CMV) and hepatitis C virus (HCV) are other examples of viruses that infect B cells (Nothelfer *et al.*, 2015).

Several viruses mediate pathogenic activation and proliferation of B cells. In HCV infection, the viral glycoprotein E2 binds to CD81 on B cells, resulting in enhanced activation and proliferation of B cells (Rosa *et al.*, 2005). The E2-HCV glycoprotein also enhances AID activity and SHM, which results in the generation of antibodies with decreased affinity and reduced antibody-mediated effector capacity(Machida *et al.*, 2008). Similarly, during HIV-1 infection, although HIV-1 does not infect B cells, it has been reported that the viral protein gp120 can induce AID and CSR from IgM to IgA isotypes in tonsillar B cells (Perisé-Barrios *et al.*, 2012). In addition, HIV-1 and co-activation of BAFF signalling induces polyclonal B cell activation in a T-independent manner (He *et al.*, 2006).

Induction of regulatory B cells is another mechanism used by some viruses to succeed within the host. Most of the studies on the role of Bregs in viral infections are predominantly on the human immunodeficiency virus (HIV) and hepatitis B virus (HBV) models. In HBV, IL-10-producing B cells inhibit protective antigen-specific $CD8^+$ T cells and induce suppressive $Foxp3^+$ Tregs (Gong *et al.*,

2015; Thimme *et al.*, 2003). Likewise, in HIV infection IL-10 producing Bregs contributes to disease pathology through $CD8^+$ T cell impairment. Interestingly, the impairment of protective $CD8^+$ responses in HIV^+ patients correlates with the prevalence of TLR and CD40 ligands on B cells, which promote IL-10 production and PD-L1 expression by B cells (Liu *et al.*, 2014b; Siewe *et al.*, 2013).

B cell survival is commonly induced by viruses that produce B cell lymphomas, such as EBV and HCV. The 2A EBV membrane protein activates SRC kinases downstream of BCR signalling, preventing apoptosis of the infected cell (Hatton *et al.*, 2012). Similarly, during EBV infection the viral protein E2 enhances the expression of BCL-2 on infected cells, protecting cells from FAS-mediated apoptosis (Chen *et al.*, 2011).

7.2.2. Bacterial infections

Like some viruses, several gram-positive and gram-negative bacteria use B cells as a reservoir. Bacteria such as Br*ucella sp*p., *Moraxella* spp., *Salmonella* spp., *Yersini*a spp., *Shigella* spp., and *Listeria* spp. are known to target B cells. Remarkably, specific B cell populations in some cases are the major target of certain bacteria. For instance *Brucella abortus*, a gram-negative bacterium, target splenic MZ B cells (Goenka *et al.*, 2012). Within the bone marrow, subspecies of *Salmonella enterica* can infect B cell precursors (Castro-Eguiluz *et al.*, 2009). Additionally, circulating memory B cells are also targeted by *Salmonella enterica* subspecies (Souwer *et al.*, 2009). BCR, complement receptors and Fc-mediated phagocytosis are the central mechanisms described in bacterial internalization by B cells (Nothelfer *et al.*, 2015). BCR-dependent internalization of *S. Typhimurium*, a *Salmonella enterica* subspecies, is mediated by the type III secretion system (T3SS) of the bacterium. In some cases, unspecific BCR crosslinking between pathogen superantigens and the BCR in the target B cell occurs in certain bacterial infections (Souwer *et al.*, 2009). *Moraxella catarrhalis* infect tonsillar B cells, the *Moraxella* IgD binding protein (MID) unspecific crosslinking BCR favouring bacterial internalization (Jendholm *et al.*, 2009).

Bacteria also induce polyclonal B cell activation to impair beneficial host immune responses mediated by specific antibodies. Impairment of a protective response mediated by high-affinity antibodies occurs in Borrelia burgdorferi infection. B cell activation by this bacterium leads to the expansion of non-switched IgM secreting plasma cells in a T-independent manner (Hastey *et al.*,

2012). *Moraxella catarrhalis* MID antigen also induces polyclonal B cell activation, triggering TLR9 activation, which results in the production of non-specific antibody (Singh *et al.*, 2012).

Bacterial virulence factors can inhibit B cell proliferation in some cases. The lethal toxin from *Bacillus anthracis* directly bind to B cells and cleaves the mitogen-activated protein kinase kinases (MAPKKs), which results in the inhibition of B cell proliferation and antibody secretion (Fang *et al.*, 2006). Some bacteria induce regulatory B cells. During *Listeria* infection, for example, MZ B cells play a key role suppressing anti-bacterial $CD4^+$ T-cell responses. MZ B cells produce IL-10 in a TLR2- and TLR4-dependent manner upon cell activation and inhibit protective immunity in an IL-10-dependent manner (Shen & Fillatreau, 2015).

B cell survival and death pathways can be hijacked during bacterial infection as well. During *Listeria monocytogenes* infection, a *Listeria* derived virulence factor called listeriolysin O (LLO) induces B cell apoptosis. LLO damages the B cell membrane causing cell death (Bhunia & Feng, 1999). Another bacterium that promotes cell death is *Francisella tularensis. F. tularensis* enhances caspase 3 and 8 expression in B cells promoting apoptosis in infected and uninfected B cells. (Zivna *et al.*, 2010). In contrast, *S. Typhimurium* induces B cell survival by downregulating inflammasome components which favours bacterium survival and dissemination (Perez-Lopez *et al.*, 2013).

7.2.3. Protozoan parasites infections

Even though there is no evidence that protozoan parasites actively infect B cells, the successful survival inside the host of several parasites relies on the manipulation of B cell functions. Cytokine secretion, polyclonal B cell activation, and induction of high levels of low-affinity antibodies are the main mechanisms used by protozoan parasites to hijack protective immune responses. Hypergammaglobulinemia is a hallmark of *Plasmodium* sp., *Trypanosoma* sp., and *Leishmania* sp. infections and results from polyclonal B cell activation (Nothelfer *et al.*, 2015; Silva-Barrios *et al.*, 2018).

During *Plasmodium falciparum* infection, the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) function as a TI antigen, PfEMP1induces polyclonal B cell activation and enhances TLRs signalling in B cells (Simone *et al.*, 2011). *Trypanosoma cruzi* is also known to induce polyclonal B cell activation, which results in the production of low-affinity antibodies and self-

reactive antibodies (Peralta *et al.*, 1981). In the experimental mouse model, the development of autoantibodies requires BAFF signalling in B cells (Bermejo *et al.*, 2010). B cell activation during *T. cruzi* infection is triggered by several *T. cruzi*-derived metabolic proteins, such as trans-sialidase (TcTrans-sialidase), mitochondrial malate dehydrogenase (TcMDH), and *T. cruzi*-proline racemase (TcPRAC). These parasite-derived proteins can induce T-independent polyclonal B cell activation and promote the generation of low-affinity antibodies (Silva-Barrios *et al.*, 2018). *Trypanosoma brucei* is another example of a protozoan that induces antigen-specific humoral responses but also triggers polyclonal B cell activation and secretion of nonspecific and self-reactive antibodies [23]. Lastly, *Leishmania* also elicits polyclonal B cell activation and the secretion of low-affinity antibodies. Several *Leishmania*l proteins that cause polyclonal B cell activation have been identified. Among these, one can find *L. major*-sirtuin (LmSIR2), *L. major*-ribosomal protein (LmS3a), *L. infantum* protein LiTXN1 (Menezes Cabral *et al.*, 2008).. Nevertheless, to date, the direct contribution of polyclonal B cell activation and hypergammaglobulinemia to *Leishmania* immunity remains unknown (Cordeiro-da-Silva *et al.*, 2001; Silvestre *et al.*, 2006).

Manipulation of B cell maturation is another common strategy observed in different protozoan infections. *T. cruzi* induces a marked reduction in B cells in the bone marrow and splenic compartment, enhancing B cell apoptosis and affecting B cell migration to the periphery (Zuniga *et al.*, 2005). In the spleen, *T. cruzi* induces the Fas receptor/Fas ligand (FasR/FasL) pathway and promotes the elimination of mature B cells (Zuñiga *et al.*, 2002). In contrast, in the bone marrow B cell death occurs in a FasR/FasL independent manner (Zuniga *et al.*, 2005). *T. brucei* also affects B cell survival, MZ B cells and Fo B cells are depleted from the spleen by an NK cell-derived elimination through a perforin-dependent lysis mechanism (Frenkel *et al.*, 2016). Like *T. cruzi*, *T. brucei* also affects the bone marrow compartment where B cell lymphopoiesis is impaired by the parasite (Bockstal *et al.*, 2011).

Another important mechanism of B cell function manipulation by protozoan parasites is the induction of cytokine production by B cells. B cell-cytokines derived affect the outcome of various protozoan infections by modulating protective T cell responses. In *T. cruzi* infection, for instance, Gal-1 has been shown suppress $CD8^+$ T cells. B cell-derived Gal-1 - triggers T cell apoptosis and reduces IFN-γ secretion (Zuñiga *et al.*, 2001). However, during *T. cruzi* infection, induction of B cell cytokines is not always detrimental. B cells also secrete the IL-17 and are considered the

major source of IL-17 during *T. cruzi* infection. This cytokine is beneficial for the anti -*T. cruzi* immune response at early stages of the infection (Bermejo *et al.*, 2013)

Finally, several studies using various *Leishmania* infection models have shown the immunoregulatory capacity of B cell-derived IL-10. In *L. major*-infected mice, B cell-derived IL-10 promotes the development of Th2 cells by suppressing protective Th1 responses (Ronet *et al.*, 2010). *L. donovani* also induces IL-10 production by B cells, particularly by MZ B cells. In this model as well, B cell-derived IL-10 inhibits protective Th1 responses contributing to disease susceptibility (Bankoti *et al.*, 2012).

Collectively, a large body of literature has shown that B cell functions can be hijacked by pathogens and that B cells are detrimental in several infection models. Several features remain common all over the various diseases, such as impairment on B cell maturation, induction of regulatory cytokines, polyclonal activation and the over induction of unspecific, non-protective antibodies.

Part II: *Leishmania* sp. and *Leishmania*sis

Leishmania species (Leishman, 1903) are an extensive group of obligate intracellular protozoan parasites belonging to the order *Trypanosoma*tida. *Leishmania* sp. are causative agents of the disease collectively known as *Leishmania*sis, a vector-borne neglected disease, classified within the seven most important tropical diseases according to the World Health Organization (World Health Organization, 2018, March 14).

1. Epidemiology and life cycle

The incidence of *Leishmania*sis affects a considerable number of people each year in 98 countries around the globe. Annually about 1.2 million new cases are reported, with a potentially fatal outcome, and over 350 million people are at risk of infection (Alvar *et al.*, 2012).

Leishmania parasite classification is based on the geographical distribution and cluster *Leishmania* species into two main groups: the old-world species, located in Europe, Africa and Asia, and the new-world species, occurring in America **(Table 3).** Around 53 species of *Leishmania* have been identified in diverse regions worldwide, 58% of this species are known to infect mammals and about 37% are considered pathogenic for humans (Bravo & Sanchez, 2003; Cupolillo *et al.*, 2000; Holakouie-Naieni *et al.*, 2017).

*Leishmania*sis may present itself in three main clinical manifestations: Cutaneous *Leishmania*sis (CL), Mucocutaneous *Leishmania*sis (MCL) and Visceral *Leishmania*sis (VL), the most severe form. In the Old World, cutaneous *Leishmania*sis is predominantly caused by *L. major, L. tropica* and *L. aethiopica*, whereas *L. donovani* and *L. infantum/L. chagasi* are responsible for the visceral disease. In the New World, members of the *L. Viannia* subgenus (including *L. V. braziliensis* and *L. V. panamensis*) and the *L. Leishmania mexicana* complex cause most of the cutaneous disease whereas *L. infantum/L. chagasi* is responsible for the visceral disease **(Table 3)** (Bravo & Sanchez, 2003; Cupolillo *et al.*, 2000; Holakouie-Naieni *et al.*, 2017).

Table 3. Geographical distribution and related clinical pathology of *Leishmania* spp.

	***Leishmania* spp.**	**Main clinical pathology**	**Main geographical distribution**
New World	*L. (Viannia) braziliensis*	CL	South America, parts of Central America, Mexico
	L. (Viannia) panamensis	CL, MCL	Northern South America, and southern central America
	L. (Viannia) peruviana	CL	Peru
	L. (Viannia) guayanensis	CL	South America
	L. (Viannia) lainsoni	CL	South America
	L. (Leishmania) colombiensis	CL	Northern South America
	L. (Leishmania) amazonensis	CL	South America
	L. (Leishmania) mexicana	CL	Central America, Mexico, USA
	L. (Leishmania) pifanoi	CL	South America
	L. (Leishmania) venezuelensis	CL	Northern South America
	L. (Leishmania) infantum	VL	Europe, North Africa, Central America, South America
Old world	*L. (Leishmania) aethiopica*	CL	Ethiopia, Kenya
	L. (Leishmania) killicki	CL	North Africa
	L. (Leishmania) major	CL	Central Asia, North Africa, middle east, East Africa
	L. (Leishmania) tropica	CL	Central Asia, middle east, parts of North Africa, central Asia, Southeast Asia
	L. (Leishmania) donovani	VL	Africa, central Asia, Southeast Asia

Arthropods belonging to the genus *Phlebotomus* (old world) and *Lutzomyia* (new world) transmit *Leishmania* parasites to a mammalian host (Claborn, 2010). During their life cycle (**Figure 6**), *Leishmania* parasites alternate between two main morphological stages: promastigotes and amastigotes. Promastigotes are the elongated, motile, extracellular stage of the parasite. A single

elongated anterior flagellum characterizes this infective form, mainly found within the invertebrate vector. In contrast, amastigotes are the replicative form within the vertebrate host. They are characterized by the lack of motility and their typical ovoid or spherical shape with a secluded flagellum (Claborn, 2010).

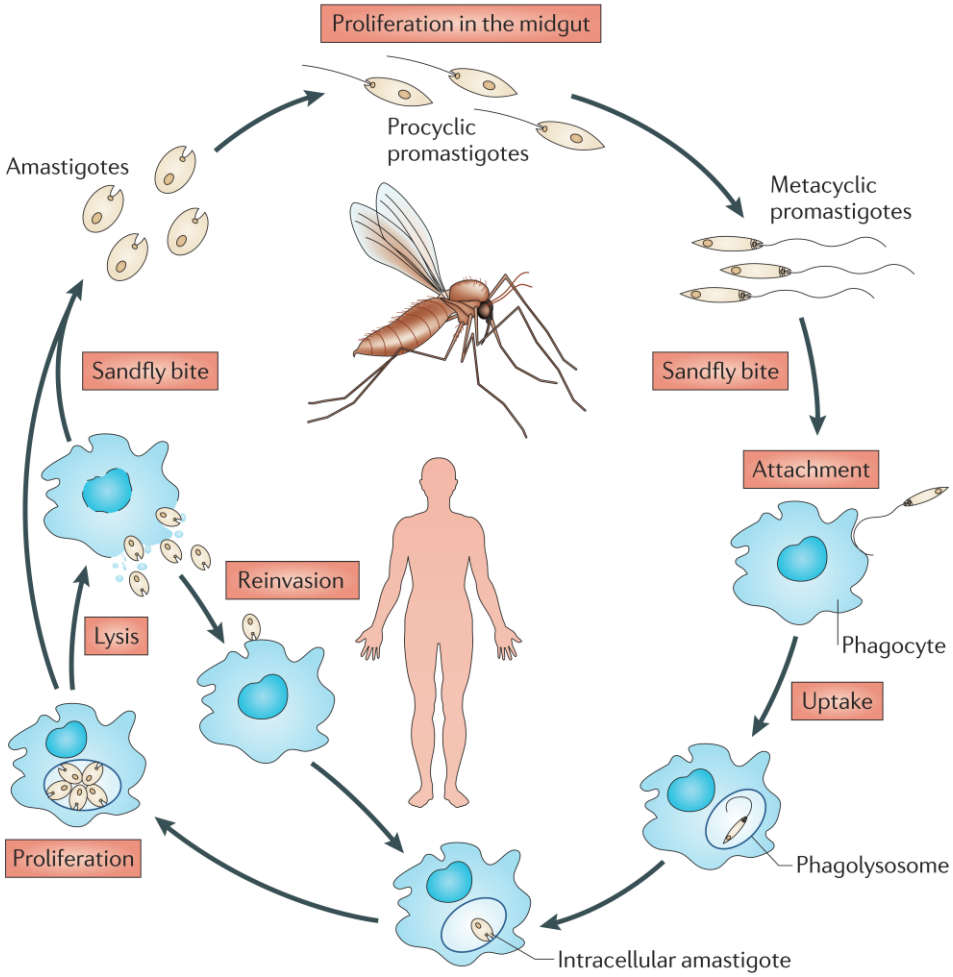

Figure 6. The life cycle of *Leishmania* parasites. *Leishmania* procyclic promastigotes differentiate in sandflies into infective, non-dividing metacyclic promastigotes, which are located ready for transmission at the stomodeal valve (an invagination of the foregut into the midgut). During blood feeding, the sandfly regurgitates metacyclic promastigotes, together with immunomodulatory parasite-derived proteophosphoglycans and various salivary components. The metacyclic promastigotes are then phagocytosed by one of several possible cell types that are found in the local environment. After establishing an intracellular residence, metacyclic promastigotes transform into aflagellated amastigotes. Amastigotes undergo replication within host cells, which rupture when too many amastigotes are present, allowing reinfection of local phagocytes. The transmission cycle is complete when infected phagocytes are taken up by another sandfly with the blood meal, and amastigotes then convert into promastigotes in the sandfly midgut. (Kaye & Scott, 2011)

In the mammalian host, promastigotes mainly reside in macrophages. However, *Leishmania* parasites can infect other professional and non-professional phagocytic cells, such as DCs, monocytes, neutrophils, and fibroblasts (Kaye & Scott, 2011). Within the phagosome of the infected cell, amastigotes multiply by evading different protective anti-*Leishmania*-host-mechanisms (Rossi & Fasel, 2017). Parasite multiplication eventually causes the lysis of the infected cell allowing amastigotes to be released, opening the possibility of spreading by infecting neighbouring phagocytic cells.

Leishmania parasites can persist at the site of the lesion or in adjacent locations (CL and MCL), but can also disseminate towards diverse target organs (VL) (Kaye & Scott, 2011; Scott & Novais, 2016). Two main factors determine the clinical manifestation of the disease, the tropism of the *Leishmania* species involved in the infection and the immune status of the host (**Table 4**). *Leishmania* amastigotes can reside in the skin and/or mucosal surfaces, resulting in cutaneous *Leishmania*sis (CL) or mucocutaneous *Leishmania*sis (MCL); alternatively, parasites can spread to internal organs such as the liver, spleen, and bone marrow, causing visceral *Leishmania*sis (VL), the most severe clinical form of the disease.

Table 4. *Leishmania*sis: immune responses in protection and pathogenesis

***Leishmania* spp.**	**Human disease**	Mouse disease	
		C57BL/6	**BALBC**
		Type of disease	**Type of disease**
Leishmania major	Self-healing or chronic cutaneous *Leishmania*sis usually caused by a single skin lesion	Self-healing	Chronic
Leishmania major (Seidman strain)	Chronic cutaneous *Leishmania*sis	Chronic	Chronic
Leishmania amazonensis	Self-healing or chronic cutaneous *Leishmania*sis usually caused by a single skin lesion, and diffuse cutaneous *Leishmania*sis	Chronic	Chronic
Leishmania mexicana	Healing or chronic cutaneous *Leishmania*sis usually caused by a single skin lesion, and diffuse cutaneous *Leishmania*sis	Chronic	Chronic
Leishmania braziliensis	Healing or chronic cutaneous *Leishmania*sis usually caused by a single skin lesion, and mucosal *Leishmania*sis	Self-healing	Self-healing
Leishmania donovani	Chronic visceral *Leishmania*sis, or kala-azar (black fever)	Chronic	Chronic

2. Visceral *Leishmania*sis

VL, also known as kala-azar (meaning “black fever”) is the most severe clinical manifestation of *Leishmania*sis and is a worldwide health problem that affects between 0.5 and 0.9 million people per year (World Health Organization, 2018, March 14). Despite the global distribution, predominantly six countries are extremely affected by this disease: India, Bangladesh, Sudan, South Sudan, Ethiopia and Brazil [3, 11]. Clinical signs of VL involve hepatosplenomegaly, characterized by an enlarged abdomen, a consequence of the augmented size of the liver and spleen, immunosuppression, and hypergammaglobulinemia. The elevated mortality in untreated patients is mostly due to secondary opportunistic infections that succeed in the immunosuppressive microenvironment present in VL patients. Tuberculosis and pneumonia are the frequent secondary infections that cause death in VL infected patients [54].

2.1. Immune response during VL

An effective immune response against VL requires balanced cooperation between innate and adaptive immunity. In the case of *Leishmania* infection, the immune response against the parasite requires the development of an efficient Th1 response (Bankoti & Stäger, 2012; Kaye & Scott, 2011). Production of IL-12 by DCs induces a Th1 T cell polarization and promotes the secretion of IFN-γ (Ghalib *et al.*, 1995; Stäger *et al.*, 2006). Secreted IFN-γ will activate macrophages and enhance TNF secretion. In this context, leishmanicidal mechanisms in phagocytic cells, mainly macrophages and monocytes, are enhanced. In experimental VL, TNF production can be protective or detrimental, depending on the organs and levels of expression. In the liver, TNF benefits host resistance; in contrast to the spleen, where it favours the establishment of chronic infection (Bankoti & Stäger, 2012). Enhanced leishmanicidal mechanisms such as the production of nitric oxide (NO) and reactive oxygen species (ROS) by activated macrophages are highly effective in killing intracellular amastigotes within the phagocytic cells (Kaye & Scott, 2011). However, in the case of VL, the disease progresses to chronicity in the spleen and bone marrow despite the presence of detectable levels of T helper-1 (Th1) cytokines. This event is in part due to immunosuppressive mechanisms that favour parasite (Bankoti & Stäger, 2012; Kaye & Scott, 2011).

Since invasive procedures are required to study target organs in VL patients, most of the information available regarding the immune modulation during the infection is built on studies carried out in animal models and most of them are based on intravenous or intraperitoneal injections of high doses of parasites. In the mouse model, the immune response is organ-specific. The compartmentalized immune response in the mouse model provides an advantage. In experimental VL, this represents a useful tool to determine diverse factors that contribute to parasite elimination and better understand the diverse mechanisms that lead to VL persistence. For instance, infection in the liver is self-resolving. The generation of protective IFN-γ-secreting $CD4^+$ and $CD8^+$ T cell responses and the formation of granulomas control parasite multiplication in the liver. In contrast to the liver, in the spleen parasite multiplication fails to be controlled. Multifactorial causes are responsible for parasite persistence.

2.1.1. Hepatic infection

Following intravenous infection, liver macrophages, also known as Kupffer cells, phagocyte most of the parasites injected. At early stages of the infection is thought that primary production of cytokines and chemokines by infected Kupffer cells are involved in monocyte and neutrophil recruitment (Cervia *et al.*, 1993; Smelt *et al.*, 2000). Because of the incapacity of Kupffer to control the parasite multiplication, the parasite burden increases considerably during the first three weeks post-infection. After this initial phase, control of parasite multiplication is due to the development of granulomas, an inflammatory structure that is formed by lymphocytes and other immune cells. Granuloma allow the encapsulation of infected Kupffer cell. IFN-γ and TNF produced by $CD4^+$ and $CD8^+$ T cells are crucial cytokines for parasite clearance. DCs-derived IL-12 initiate the development of T protectives T cell responses, inducing the production of IFN-γ, TNF and lymphotoxin alpha (LTα) by T cells (Engwerda *et al.*, 2004; Scharton-Kersten *et al.*, 1995) (Engwerda *et al.*, 2004; Scharton-Kersten *et al.*, 1995).

Granulomas allow the local concentration of inflammatory cytokines that activate infected Kupffer cells. CCL3, CCL2 and CXCL10 derived from infected Kupffer cells contribute to the recruitment of immune effector cells such as monocytes, neutrophils, and iNKT cells, which promote the parasite killing capacity of infected cells (Kaye & Beattie, 2016; Murray, 2001). iNKT cells are involved in coordinating the initial granuloma formation (Robert-Gangneux *et al.*, 2012). They express an invariant TCR that can recognize a wide range of PAMPs on the parasite surface. iNKT enhance the early IFN-γ production (Amprey *et al.*, 2004) and CXCL10 secretion that promotes the recruitment of T cells and the development of granulomas**.** Therapeutic approaches

targeting iNKT activation have been shown to be beneficial in some chronic disease models. However, in the case of VL, enhanced activation of iNKT decreases protective IFN-γ $CD8^+$ T cell responses and promotes parasite exacerbation within the liver (Stanley *et al.*, 2008). Myeloid cells can recognize PAMPs through TLRs, resulting in the activation of several signalling components such as IRFs (Thompson *et al.*, 2018). IRF-5, a member of the IRF family, is known to activate proinflammatory cytokines production, including IL-12 and TNF. In experimental VL, IRF5 is crucial for driving protective Th1 responses and granuloma formation (Paun *et al.*, 2011). T cell represents the predominant cell type in mature granulomas (Stern *et al.*, 1988). An efficient T cell response characterized by the production of IFN-γ by $CD4^+$ and $CD8^+$ T cells is essential for the success in controlling parasite multiplication in the liver. After 3-4 weeks of infection, hepatic parasite levels considerably decrease, and the parasite burden declines (Bankoti & Stäger, 2012). However, the infection is not entirely cleared in the liver; low levels of parasites contained within granulomas remain, conferring a long-term protection (Rodrigues *et al.*, 2017; Rodrigues *et al.*, 2016; Stanley & Engwerda, 2007).

2.1.2. Splenic infection

During VL, the infection persists in the spleen causing chronic inflammation and organ enlargement. A common feature between human an experimental VL is the marked splenomegaly (Kaye *et al.*, 2004). After intravenous infection, splenic macrophages phagocytize most of the parasites, and about 50% of *Leishmania* survive during the first hours post-injection. Splenic macrophages are classified in three main subtypes based on their location: red pulp macrophages, marginal zone macrophages (MZM), and marginal zone metallophilic macrophages (MMM). The splenic marginal zone plays an important role in controlling cellular trafficking within T and B areas. Compared to Kupffer cells in the liver, MZM and MMM have an improved leishmanicidal capacity that depends on IRF7 activation (Phillips *et al.*, 2010). Few hours post infection, DCs migrate from the splenic marginal zone to T cell areas at the periarteriolar lymphoid sheaths (PALS) following the encounter with parasite-derived antigens and start secreting IL-12. Non-infected DCs exposed to parasite products, also known as bystander DCs, initiate protectives immune responses in the spleen. The interaction between DCs and T cells will induce protective Th1 responses (Bankoti & Stäger, 2012).

About 2-3 weeks after infection, the first signs of disruption of the splenic microarchitecture start being visible. This outcome is caused by sustained TNF production mostly by splenic

macrophages. Although TNF is involved in the development of the splenic marginal zone (Mebius & Kraal, 2005), sustained TNF production during chronic infection causes the loss of the gp38^{+} fibroblastic reticular cell network, which guides T cells and DCs migration to the T cell area (Ato *et al.*, 2002), and the follicular DCs network in the B cell follicles of the spleen (Smelt *et al.*, 2000). The loss of the reticular cell network leads to the disruption of the MZ, the disorganization of the white pulp, and the increased of the red pulp area. Concomitantly, a marked infiltration of monocytes mostly with a proinflammatory phenotype is observed (Hammami *et al.*, 2017).

In this scenario, disruption of GCs structure occurs and T cell areas at periarteriolar lymphoid sheath (PALS) collapse. Disruption of the splenic microarchitecture has several functional consequences: cell-to-cell interactions required for effective immune responses such as T cell priming between DCs and T cells are impaired because of migratory defects of T cells and DC to the white pulp (Ato *et al.*, 2006); the long-term interaction between B cells and TFh required to produce specific anti-*Leishmania*-antibodies that might contribute to parasite elimination is hampered (Rodrigues *et al.*, 2014). Hence, disruption of the splenic microarchitecture correlates with parasite persistence (Engwerda *et al.*, 2004)

To establish chronic infection, *Leishmania* parasites hijack several components of the immune response. During experimental VL, DCs are crucial in driving protective Th1 responses, mostly by the production of IL-12. Th1 cells are essential for the control of parasite multiplication by activation of macrophages and DCs through the production of IFN-γ. However, *Leishmania* parasites hijack protectives Th1 responses in several ways. First, *Leishmania* modulates DC functions by enhancing the expression of hypoxia-inducible factor 1-alpha (HIF-1α) (Hammami *et al.*, 2018). The transcription factor HIF-1α is the master regulator of the response to decreased oxygen levels in the environment. *L. donovani* infection is characterized by a hypoxic microenvironment and the upregulation of HIF-1α, not only induced by inflammation but also by the parasite itself (Hammami *et al.*, 2018; Hammami *et al.*, 2015). In experimental VL, IRF5-mediated inflammation promotes HIF-1α expression in DCs (Hammami *et al.*, 2015). Interestingly, mice lacking HIF-1α in DCs have an enhances production of IL-12, decreased suppressive IL-10, improved Th1 responses and reduced parasite levels (Hammami *et al.*, 2018).

Another way of suppressing protective Th1 responses is via the induction of IL-10. In VL, IL-10 is a critical regulatory cytokine involved in the pathogenesis of the infection. In experimental VL, mice deficient in IL-10 are highly resistant to infection and develop enhanced Th1 responses and iNOS production [48]. Additionally to DCs, other cells can also produce IL-10 during the infection such as NK cells (Maroof *et al.*, 2008), macrophages(Murray *et al.*, 2002), CD4^{+} T cells (Stäger *et al.*,

2006), B cells (Bankoti *et al.*, 2012) and myeloid-derived suppressor cells (MDSC) (Hammami *et al.*, 2017). However, $CD4^+$ T co-producing IL-10 and IFN-γ are the most important source of IL-10 during the infection (Ranatunga *et al.*, 2009; Stäger *et al.*, 2006). Regulatory $CD4^+$ T cells that co-produce IL-10 and IFN-γ, also known as Tr1 cells. (Stäger *et al.*, 2006) and are common across diverse disease models (Zeng *et al.*, 2015). IL-27 is one of the central cytokines involved in the induction of Tr1 cells (Awasthi *et al.*, 2007). IL-27 modulates methylation pattern in the Il-10 promoter in $CD4^+$ T cells enhancing IL-10 production (Hedrich *et al.*, 2010; Ranatunga *et al.*, 2009). In experimental VL, one of the sources of IL-27 identified is CD11chi conventional DCs (cDCs). Indeed, IL-27 and IL-6 derived from cDCs induce IL-10 production by Th1 cells during the infection (Owens *et al.*, 2012; Stäger *et al.*, 2006).

Disruption of the splenic microenvironment also contributes to the impairment of protective Th1 responses during VL. IFN-γ-producing $CD4^+$ T cells increasingly express IRF5 during experimental VL (Fabié *et al.*, 2018; Paun *et al.*, 2011). IRF5 activation in Th1 cells is detrimental, since it sensitizes this population to cell death (Fabié *et al.*, 2018). Tissue disruption, specifically apoptotic cell-derived material, leads to the activation of the TLR7-IRF5 axis, promoting the expression of death receptor 5 (DR5) in IFN-γ^+ $CD4^+$ T cells (Fabié *et al.*, 2018).

$CD8^+$ T cell responses are also severely impaired during VL (Stager & Rafati, 2012). Effective host-protectives immune responses require antigen-specific $CD8^+$ T cells; in the spleen and liver,CD8 T cells contribute to control parasite multiplication (Stager & Rafati, 2012). During the first 3 days of infection, activated large proportion of bystander-activated $CD8^+$ T cells can be observed. These are induced by CD86-expressing DCs (Maroof *et al.*, 2009). This unspecific activation is followed by an antigen-specific activation. However, $CD8^+$ T cells only undergo limited clonal expansion and become exhausted upon a second wave of activation three weeks later (Stager & Rafati, 2012). Limited T cell expansion derives from altered DC functions as a consequence of HIF-1α upregulation (Hammami *et al.*, 2015). Exhaustion is associated with progressive stimulation of programmed death ligand 1 (PD-L1) by DCs (Joshi *et al.*, 2009). PD-L1 interacts with programmed cell death protein 1 (PD-1); PD-1 is also increasingly upregulated in $CD8^+$ T cells during the infection. PD-1/PDL1 interaction results in dysfunctional $CD8^+$ T cells and ultimately cell death (Joshi *et al.*, 2009).

2.1.3. Bone marrow infection

Changes in the blood cell composition are also a characteristic of VL. This complication includes anemia, thrombocytopenia, leucopenia, and neutropenia. During human and experimental VL, the bone marrow is a target organ for *Leishmania* parasites and remains chronically infected. During experimental VL, parasites growth in the bone marrow with a similar kinetic to the spleen (Cotterell *et al.*, 2000a). Persistent infection in the bone marrow takes place mostly in stromal macrophages and correlates with increased levels of myelopoiesis in the bone marrow and spleen (Abidin *et al.*, 2017; Cotterell *et al.*, 2000a; Cotterell *et al.*, 2000b). Like the spleen, a proinflammatory microenvironment occurs in the bone marrow. At the chronic stage of the infection high levels of MIP1α, IL-1, and IFN-γ-inducible factors, such as ICAM-1, CXCL10 and CXCL9 are present. Therefore, providing ideal conditions for hematopoietic stem cells precursors' expansion and enhanced myelopoiesis, which correlates with *Leishmania* persistence (Abidin *et al.*, 2017). Monocytes with a regulatory phenotype are the main output population from the active myelopoiesis taking place at the chronic stage of infection in the bone marrow. These cells exhibit an M2-like phenotype. M2 macrophages are incapable of eliminating *Leishmania* parasites (Hammami *et al.*, 2017). A recent study demonstrated that $CD4^+$ T cells expressing IFN-γ are responsible for enhancing myelopoiesis in experimental VL (Pinto *et al.*, 2017).

2.2. B cells in VL

The role of B cells during VL remains largely unexplored. Despite the limited research in this field, it is evident that in VL B cells promote disease exacerbation. Indeed, B cell-deficient mice are highly resistant to *L. donovani* infection (Smelt *et al.*, 2000). B cells promote disease in human and experimental VL in various ways. The most relevant mechanism aimed at subverting B cell functions is the induction by *Leishmania* parasites of polyclonal B cell activation (Bankoti *et al.*, 2012; Deak *et al.*, 2010). Hypergammaglobulinemia, a hallmark of VL, is a consequence of polyclonal B cell activation. In VL high levels of antibodies are produced and most of these antibodies have a low-affinity for *Leishmania* parasites. Induction of hypergammaglobulinemia is a common strategy used by several pathogens to dilute high-affinity antibodies mediated immune responses (Silva-Barrios *et al.*, 2018). In VL, antibodies and antibody- mediated responses participate in disease exacerbation. IgM production and complement activation contribute to disease susceptibility (Deak *et al.*, 2010). Besides low-affinity antibodies against *Leishmania*

parasites, polyclonal B cell activation in VL leads to the development of autoantibodies. Natural autoantibodies (Nab) recognising self-antigens - including tubulin, myosin, myoglobin and actin- can be detected in the blood of VL patients (Louzir *et al.*, 1994), increasing the probability to develop autoimmune disorders. Nevertheless, high-affinity, *Leishmania*-specific antibodies do not appear to be produced during chronic infection. This lack of high-affinity antibodies might be due to the striking decrease in TFh cells during the chronic stage of infection (Rodrigues *et al.*, 2014). Moreover, hypergammaglobulinemia favours the formation of immune complexes that promote suppressive responses during the infection. IgG immune complexes via Fc receptor mediated responses enhance IL-10 production in macrophages, promoting disease exacerbation (Miles *et al.*, 2005). In experimental VL IgM production is also associated with disease exacerbation (Deak *et al.*, 2010).

Another strategy adopted by *L. donovani is to* promote IL-10 secretion by B cells, predominantly MZB-like cells. IL-10 secreted by MZ cells partially suppresses protective Th1 responses during the infection. Interestingly, IL-10 is induced in a MyD88-dependent manner suggesting that TLR triggering might be involved (Bankoti *et al.*, 2012). However, the exact mechanism by which *Leishmania* induces polyclonal B cell activation to promotes hypergammaglobulinemia and IL-10 secretion has not yet been fully clarified and is one of the main interests in our laboratory. Several proteins from the *L. donovani* complex, such as LiTXN1 (*Leishmania infantum* cytosolic tryparedoxin) have been associated with polyclonal B cell activation and enhanced expression of IL-10 by B cells. However, *L. donovani* -specific proteins that could lead to B cell activation remain unidentified to date.

In human VL patients, IL-10 secretion by B cells depends on activation of spleen tyrosine kinase Syk, phosphatidylinositol-3 kinase, and p38 (Andreani *et al.*, 2015). Identification of alternative cytokines produced by B cells will provide additional clues about the detrimental phenotype of B cells during VL. Interestingly, microarray data obtained from VL-infected human PMBCs shows a profile of IFN-I signalling and B cell activation suggesting an important contribution of IFN-I during the infection (Gardinassi *et al.*, 2016).

CHAPTER 2:

OBJECTIVES AND GENERAL HYPOTHESIS

B cells are detrimental in a large spectrum of chronic inflammatory diseases. However, the underlying mechanisms that account for B cell-dependent disease exacerbation are poorly understood. Most of the pathologies associated with an impaired B cell immune response are related to the secretion of antibodies and immunomodulatory cytokines or antibody-mediated functions (Matsushita, 2018). VL is a chronic infectious disease in which B cell activity results prejudicial to the host. Polyclonal B cell activation and hypergammaglobulinemia are hallmarks of VL and are also common features across several chronic infections. B cell activation by *Leishmania* parasites is critical for driving impaired B cell responses and is associated with disease exacerbation (Silva-Barrios *et al.*, 2018). To date, there are no effective treatments or immune prophylaxis available against VL. Therefore, elucidating mechanisms triggering deregulation of B cell functions could contribute to the development of novel therapeutic approaches against VL.

B cells have long been known to be detrimental during VL. Indeed, B cell-deficient mice are highly resistant to *L. donovani* infection (Smelt *et al.*, 2000). In these mice, parasite multiplication is controlled in the spleen and the liver within the first 14 days post infection (Smelt *et al.*, 2000). Although this fact has been known for many years, research on the role and function of B cells during VL remains very limited. *Leishmania* parasites affect B cell responses mostly by inducing polyclonal B cell activation that results in the production of immunomodulatory cytokines and to a dramatic increase in the levels of circulating antibodies. Although research in this field is limited, increasing interest has arisen during the last years (Silva-Barrios *et al.*, 2018). However, many questions remain to be addressed.

The first question pertains to the mechanisms of B cell activation and induction of cytokine production by *L. donovani*. In this regard, our laboratory has previously demonstrated that B cell activation by *L. donovani* results in IL-10 production, which is involved in partial inhibition of protective Th1 responses. IL-10 secretion can result from the activation of several singling pathways in B cells. *Leishmania*-induced IL-10 production by B cells requires MyD88 signalling, the adaptor molecule of almost all TLRs (Bankoti *et al.*, 2012). However, the pathways triggered by *L. donovani* upstream of MyD88 are still unknown

Another aspect that needs to be clarified is the role of the large amount of low-affinity antibodies produced during the chronic stages of VL. (Silva-Barrios *et al.*, 2018). Hypergammaglobulinemia is associated with disease progression in human and mice. However, it is still unknown how

antibodies contribute to disease pathology. In addition, the impact of hypermutated immunoglobulins to disease pathogenesis and chronic progression in the experimental model of *L. donovani* also remains unknown

An interesting mechanism leading to deregulated B cell immune responses has been proposed for In patients with SLE, innate B cell activation via endosomal TLRs leads to antibody secretion and promotes disease exacerbation (Christensen *et al.*, 2006; Han *et al.*, 2015). However, it remains to be determined, whether the same applies to chronic infectious diseases. Various studies have demonstrated that B cells express TLRs and that several agonist molecules can activate these pathways leading to polyclonal activation and cytokine secretion (Barr *et al.*, 2010).

The main objective of this thesis is to characterize the contribution of B cells to the immune response during experimental VL and identify pathways that lead to B cell activation and promote hypergammaglobulinemia. We hypothesise that *Leishmania* parasites activate B cells by triggering TLRs; this activation leads to hypergammaglobulinemia that promotes chronic inflammation and favours parasite growth and disease progression.

To prove our hypothesis, two objectives will be pursued, a) to identify mechanisms of activation that lead to impaired B cell responses, giving particular emphasis to the role of TLRs; b) to evaluate the contribution of hypergammaglobulinemia to disease pathogenesis and immunopathology. To this end, we will use the experimental model of VL. Because of its characteristic hypergammaglobulinemia during chronic infection and the deleterious B cell activation, VL is an ideal model to achieve this goal. To accomplish the main objective, we structured the project in the two following aims:

1. To identify the mechanism by which *L. donovani* induces polyclonal B cell activation

L. donovani amastigotes have been shown to interact with B cells during the infection, mainly with MZ B cells. Following B cell exposure to *L. donovani* amastigotes, B cells are highly activated and form clusters retaining one or more parasites within these structures. In this part of the thesis, I will investigate pathways of B cell activation by *L. donovani* that lead to hypergammaglobulinemia giving particular emphasis to TLRs.

2. To assess the contribution of hypermutated immunoglobulins and hypergammaglobulinemia in disease pathogenesis

High levels of IgG are a hallmark of *Leishmania* infections. It is well documented that, during the disease, polyclonal B cell activation results predominantly in the production of low-affinity antibodies, as well as in an increase in the amount of circulating immune complexes (Miles *et al.*, 2005). Interestingly, hypergammaglobulinemia correlates with parasite persistence at chronic stages. Consequently, hypergammaglobulinemia might favour the development of a suitable microenvironment that contributes to parasite survival within the host. This hypothesis will be tested in the second objective of my thesis.

CHAPTER 3:

INNATE IMMUNE B CELL ACTIVATION BY *LEISHMANIA DONOVANI* EXACERBATES DISEASE AND MEDIATES HYPERGAMMAGLOBULINEMIA

Innate immune B cell activation by *Leishmania donovani* exacerbates disease and mediates hypergammaglobulinemia

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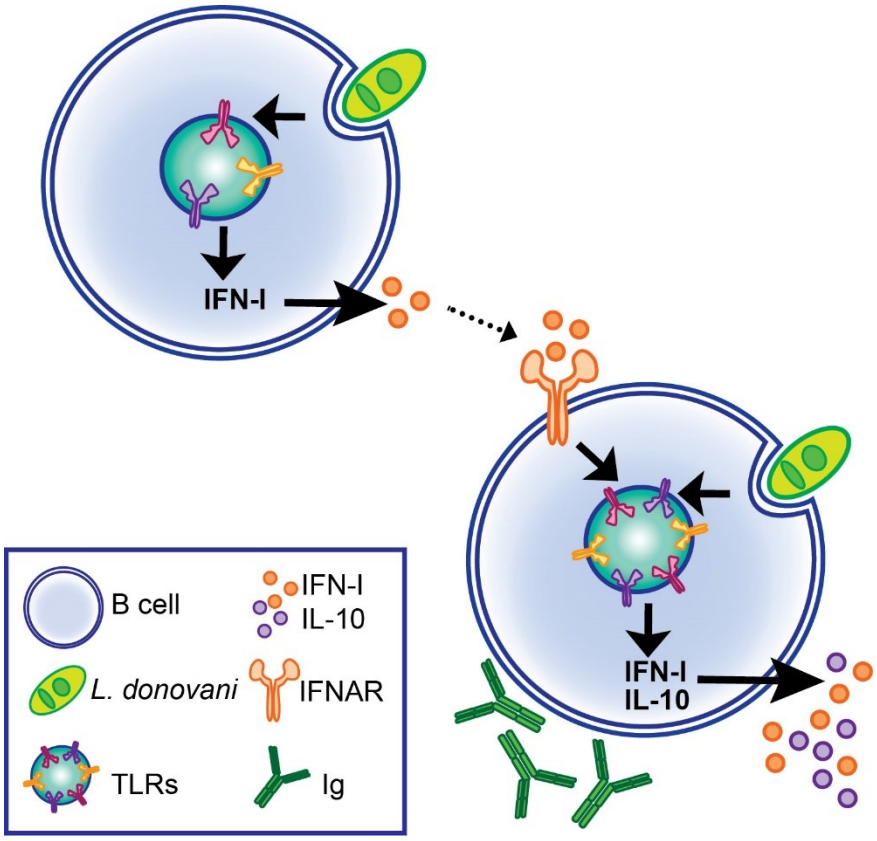
Author Contributions

S.S.-B. and S.S. conceived the project, designed the experimental approach, performed experiments, interpreted data, and wrote the manuscript. A.D. provided key expertise and interpreted data. M.S. and C.U.D. performed experiments and analyzed data. S.T.Q. and J.H.F. provided key expertise and key materials.

GRAPHICAL ABSTRACT



1. In brief

Silva-Barrios et al. report that the parasite *Leishmania donovani* triggers endosomal TLRs in B cells. This activation induces cytokine and endosomal TLR expression, which are further enhanced by signaling through the type I IFN receptor. Innate B cell activation through endosomal TLRs and IFN-I promotes disease exacerbation and hypergammaglobulinemia.

2. RÉSUMÉ

La participation des cellules B dans la réponse immunitaire par divers mécanismes indépendants des anticorps a récemment été découverte. La production des cytokines par les cellules B a été décrite pour plusieurs infections et ils semblent réguler la réponse immunitaire adaptative. Dans le cas de la Leishmaniose Viscérale l'activation des cellules B par le parasite entraîne une exacerbation de la maladie. Mais comment *L. donovani* active les cellules B reste encore inconnue. Nous montrons que les amastigotes de *L. donovani* activent les cellules B en déclenchant des TLR endosomaux; cette activation conduit à l'induction de diverses cytokines. L'expression des cytokines est complètement abrogée dans les cellules B de souris *Ifnar*$^{-/-}$ exposées à *L. donovani*, suggérant une implication de l'IFN-I dans une boucle de rétroaction positive. L'IFN-I semble également augmenter l'expression des TLR endosomaux après une exposition à *L. donovani*. L'ablation spécifique de la signalisation du TLR endosomal dans les cellules B a révélé que l'activation innée des cellules B par *L. donovani* était responsable de l'exacerbation de la maladie par la production d'IL-10 et d'IFN-I et de la promotion de l'hypergammaglobulinémie.

3. SUMMARY

Participation of B cells in the immune response by various antibody-independent mechanisms has recently been uncovered. B cells producing cytokines have been described for several infections and appear to regulate the adaptive immune response. B cell activation by *Leishmania donovani* results in disease exacerbation. How *Leishmania* activates B cells is still unknown. We show that *L. donovani* amastigotes activate B cells by triggering endosomal TLRs; this activation leads to the induction of various cytokines. Cytokine expression is completely abrogated in B cells from *Ifnar*$^{-/-}$ mice upon exposure to *L. donovani*, suggesting an involvement of IFN-I in a positive feedback loop. IFN-I also appears to enhance the expression of endosomal TLRs following exposure to *L. donovani*. Cell-specific ablation of endosomal TLR signaling in B cells revealed that innate B cell activation by *L. donovani* is responsible for disease exacerbation through IL-10 and IFN-I production and for the promotion of hypergammaglobulinemia.

4. INTRODUCTION

The main mechanisms leading to antibody production by B cells are largely known. Follicular helper CD4^{+} T cells (TFhs) are thought to play a crucial role in germinal center formation and B cell differentiation into antibody-producing plasma cells (Crotty, 2011); antigen-specific B cell receptor (BCR) recognition is required for this process. Nevertheless, other pathways have been reported to be involved in enhancing antibody production. For instance, Toll-like receptor (TLR) stimulation in B cells can regulate the magnitude of the antibody response and the amount of antigen required for initiating BCR signaling (DeFranco *et al.*, 2012; Freeman *et al.*, 2015). Moreover, *MyD88*$^{-/-}$ B cells were reported to generate decreased antibody responses (Kasturi *et al.*, 2011; Pasare & Medzhitov, 2005).

The role of B cell TLR signaling in antibody production has mainly been studied in models of autoimmune diseases. TLR7 and 9 were shown to contribute importantly to the production of anti-nuclear antibodies in various models of lupus-like disease (Christensen *et al.*, 2006; Han *et al.*, 2015). In contrast, little is known about the role of B cell TLR signaling in infectious diseases. MyD88 appears to be required for preventing lethal dissemination of commensal bacteria during colonic damage caused by dextran sulfate sodium (Kirkland *et al.*, 2012). In this model, MyD88 was essential for the production of immunoglobulin M (IgM) and complement by B cells. TLR9,

TLR7, and MyD88 expression in B cells was also shown to be involved in substantially enhancing T cell-dependent germinal center immunoglobulin G (IgG) responses following inoculation with virus-like particles (Hou *et al.*, 2011). During Salmonella typhimurium infection, however, MyD88 in B cells was mainly associated with cytokine production and led to disease susceptibility via interleukin-10 (IL-10) induction (Neves *et al.*, 2010). We have also previously reported that B cell-derived IL-10 production following *Leishmania donovani* infection was dependent on MyD88 expression in B cells (Bankoti *et al.*, 2012).

The protozoan parasite *L. donovani* is a causative agent of visceral *Leishmania*sis (VL), a chronic disease that is characterized, among others, by polyclonal B cell activation and hypergammaglobulinemia. *Leishmania* is an intracellular parasite that preferentially infects macrophages; however, it can also be found in dendritic cells, neutrophils, and fibroblasts (Kaye & Scott, 2011). In a previous study, we have shown that *L. donovani* can also be detected on marginal zone B cells (Bankoti *et al.*, 2012). In the murine model of VL, B cells play a detrimental role (Smelt *et al.*, 2000) . IgM, complement, and B cell-derived IL-10 largely contribute to disease susceptibility (Bankoti *et al.*, 2012; Deak *et al.*, 2010). Additionally, IgG immune complexes were shown to exacerbate infection by inducing IL-10 production in macrophages (Miles *et al.*, 2005). IgG is also linked to chronic infection in human VL patients, where high IgG levels are predictive of disease. However, the pathways leading to polyclonal B cell activation and hypergammaglobulinemia in VL are still unknown.

In this study, we investigate the mechanisms of B cell activation by *L. donovani*. We show that *L. donovani* amastigotes activate B cells by triggering endosomal TLRs. This activation resulted in the induction of proinflammatory cytokines, IL-10, and type I interferon (IFN) and in the upregulation of endosomal TLR expression. Upregulation of cytokine and endosomal TLR mRNA expression was dependent on the presence of type I IFN receptor (IFNAR) in B cells, suggesting an involvement of type I IFNs in a positive regulatory loop. A functional endosomal TLR pathway was also required to generate non-specific and *Leishmania*-specific antibodies. These results demonstrate that B cell activation by *L. donovani* through endosomal TLRs leads to disease exacerbation through type I IFN production and the promotion of hypergammaglobulinemia.

5. RESULTS

5.1. *L. donovani* triggers IL-10 and type I IFN expression in splenic B cells

We have previously reported that B cells can capture *L. donovani* amastigotes in vivo and in vitro (Bankoti *et al.*, 2012). Upon in vitro exposure to the parasite, B cells form clusters, upregulate the costimulatory molecule CD86 and surface IgM, and eventually die after 48 hr (Bankoti *et al.*, 2012). Confocal microscopy analysis revealed that this interaction does not result in parasite internalization but that *L. donovani* is retained on the surface in IgM-rich pockets **(Figure 1A; Movies S1 and S2)**. B cell activation also resulted in the expression of several cytokines **(Figure 1B)**, with IL-10 and IFN-β being the most highly expressed. Maximal cytokine expression was reached after 8 hr of coincubation with the parasite **(Figure S1)**. A similar pattern of cytokine expression was also observed in B cells purified from *L. donovani*-infected mice **(Figure 1C)**. Interestingly, IL-10 and IL-1a mRNAs were mainly upregulated during the early stages of infection (days 7–14), whereas type I IFN and IL-6 were prominently expressed during the chronic phase of the disease (days 21–28). We have previously reported that IL-10 secretion is MyD88-dependent (Bankoti *et al.*, 2012). The pathways upstream of MyD88 and of IFN-I induction in B cells following exposure to *L. donovani* are as yet unknown.

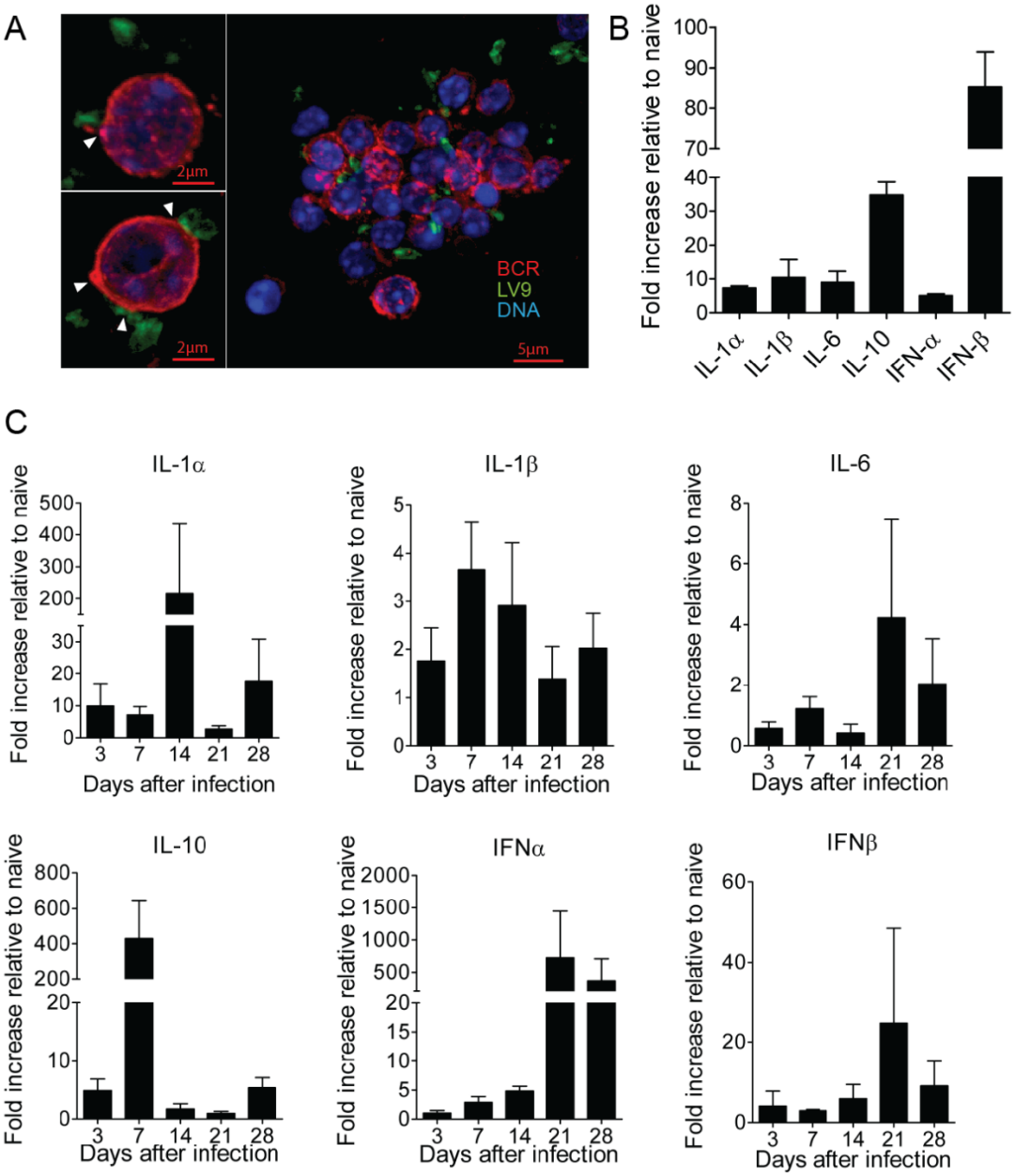


Figure 1. *L. donovani* promotes cytokine mRNA expression in B cells. (A) Naive splenic B cells were exposed to PKH67-labeled *L. donovani* amastigotes (green) at an MOI of 1:5 for 5 hr. Cells were stained with anti-IgM-AF568 (red) and Hoechst (blue) and visualized by confocal microscopy. **(B and C)** Expression analysis by qPCR of cytokine mRNA in naive splenic B cells exposed to *L. donovani* for 8 hr **(B)** and in splenic B cells purified from infected mice **(C)**. Data represent mean ± SEM from three independent experiments.

5.2. *L. donovani* activates endosomal TLRs in B cells

Because MyD88 is necessary for IL-10 production in B cells (Bankoti *et al.*, 2012), and *L. donovani* is recognized by a number of TLRs (Flandin *et al.*, 2006; Kropf *et al.*, 2004; Paun *et al.*, 2011; Schleicher *et al.*, 2007), we next investigated the role of TLRs in cytokine induction by the parasite. First, we assessed whether TLR agonists could induce a pattern of cytokines in B cells similar to that observed after exposure to *L. donovani*. Interestingly, agonists to TLR3, 7, and 9 triggered the strongest upregulation of mRNA for IL-1a, IL-1b, IL-6, IL-10, and IFN-α/β **(Figure 2A)** after 8 hr of exposure, suggesting that, although the parasite resides on the cell surface, endosomal TLRs may be triggered by *L. donovani* in B cells. We next examined, by confocal microscopy, the expression of TLR3 **(Figure 2B, top)** and TLR9 **(Figure 2B, bottom)** on splenic murine B cells exposed to the parasite. TLR3 and 9 were more intensely present at the site of contact with the parasite **(Figure 2B)**, suggesting that endosomal TLRs might be activated by *Leishmania* amastigotes. The presence of TLR7 in murine B cells was determined by western blot **(Figure 2C)**.

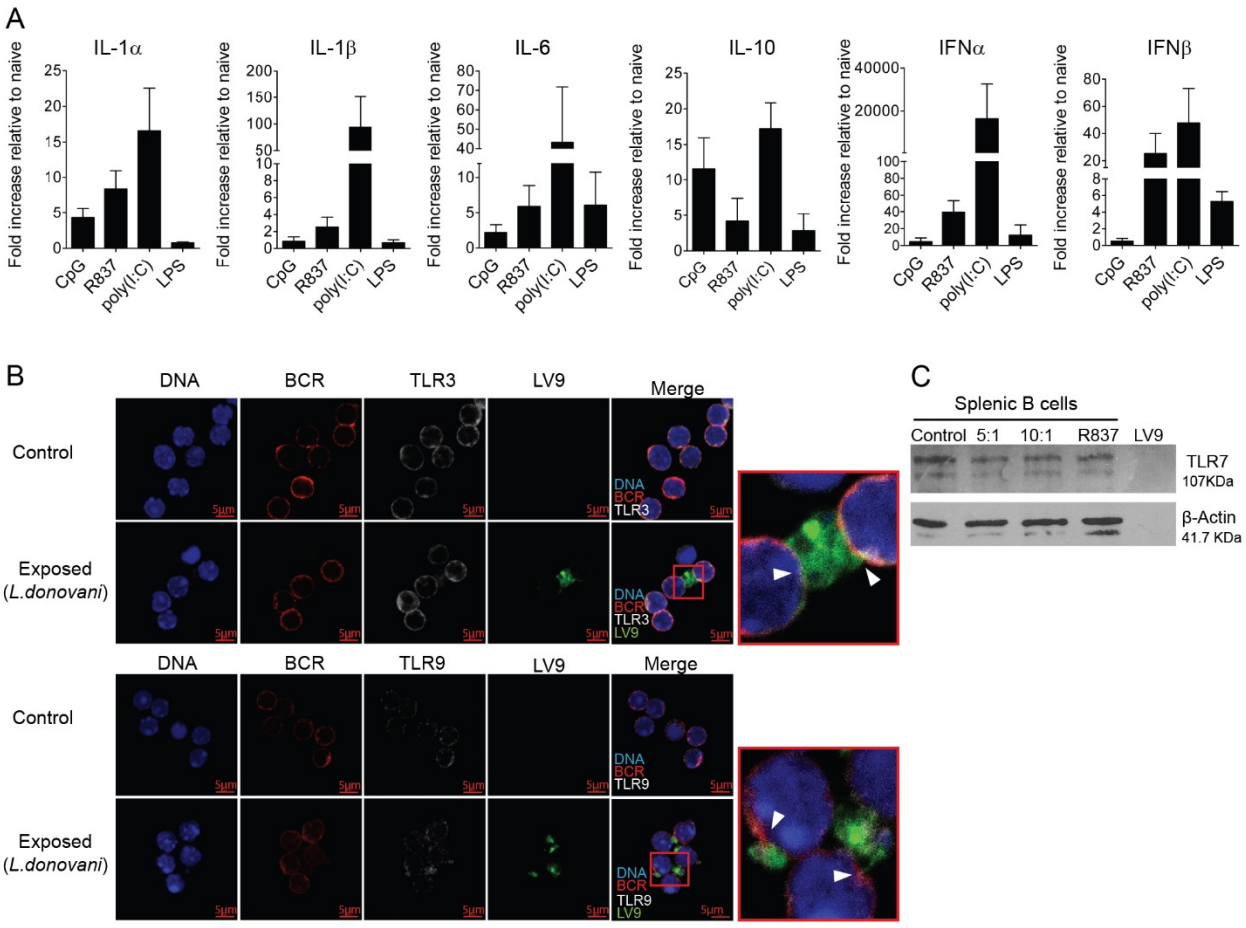


Figure 2. B Cells express endosomal TLRs and respond to TLR agonist stimulation. **(A)** Cytokine mRNA expression by qPCR in splenic B cells activated with TLR agonists for 8 hr. **(B)** TLR3 and TLR9 localization by confocal microscopy in B cells exposed or not exposed to *L. donovani* (green) and stained with anti-IgM-AF568 (red), anti-TLR3-Dylight 650 (white), or anti-TLR9-ZenonAF647 (white) and Hoechst (blue). **(C)** Immunoblot analysis for TLR7 in B cells exposed to different ratios of parasites or R837-activated and parasites alone (LV9). Data represent mean ± SEM from three independent experiments.

5.3. B cell cytokine expression following *in vitro* exposure to *L. donovani* is dependent on endosomal TLRs and the IFNAR

To confirm our hypothesis that endosomal TLRs are required for cytokine induction, we exposed splenic B cells purified from *Unc931b*$^{Letr/Letr}$ mice (Lafferty *et al.*, 2014) to the parasite in vitro and assessed cytokine expression. *Unc931b*$^{Letr/Letr}$ mice (Lafferty *et al.*, 2014) have a loss-of-function mutation, known as Letr for ‘‘loss of endosomal TLR response,’’ in UNC93B1, which is a chaperone protein for TLR3, TLR7, and TLR9. All cytokine mRNA levels measured failed to be upregulated in *Unc931b*$^{Letr/Letr}$ B cells exposed to *L. donovani* compared with wild-type (WT) B cells **(Figure 3A)**, suggesting that activation of endosomal TLR pathways was indeed responsible for cytokine expression in B cells. Our results were confirmed using TLR7 and 9 inhibitors **(Figure S2)**. Strikingly, cytokine expression was also abrogated in B cells lacking the IFN-I receptor (IFNAR) **(Figure 3A)**, implying that IFN-α/β may be involved in a positive feedback-regulatory loop. IFN-I signaling was shown to directly regulate TLR7 expression and TLR7 responsiveness by B cells (Doucett *et al.*, 2005; Green *et al.*, 2009; Thibault *et al.*, 2009). Thus, we examined endosomal TLR mRNA expression in B cells purified from C57BL/6 and *Unc931b*$^{Letr/Letr}$ mice following in vitro exposure to *L. donovani*. Activation by the parasites led to the upregulation of endosomal TLR expression in wild-type B cells **(Figure 3B)**. In contrast, endosomal TLR mRNAs failed to be upregulated in the absence of the IFNAR or in *Unc931b*$^{Letr/Letr}$ B cells **(Figure 3B)**. To determine whether endosomal TLR expression was also dependent on IFN-I signaling in vivo, we generated a mixed bone marrow chimera with a targeted ablation of IFNAR in B cells. Chimeric mice were then infected with *L. donovani*, and B cells were purified at various time points after infection. Our in vitro results were confirmed in vivo. IFN-I signaling not only regulated TLR7 expression but was also involved in the upregulation of TLR3 and 9 **(Figure 3C)**.

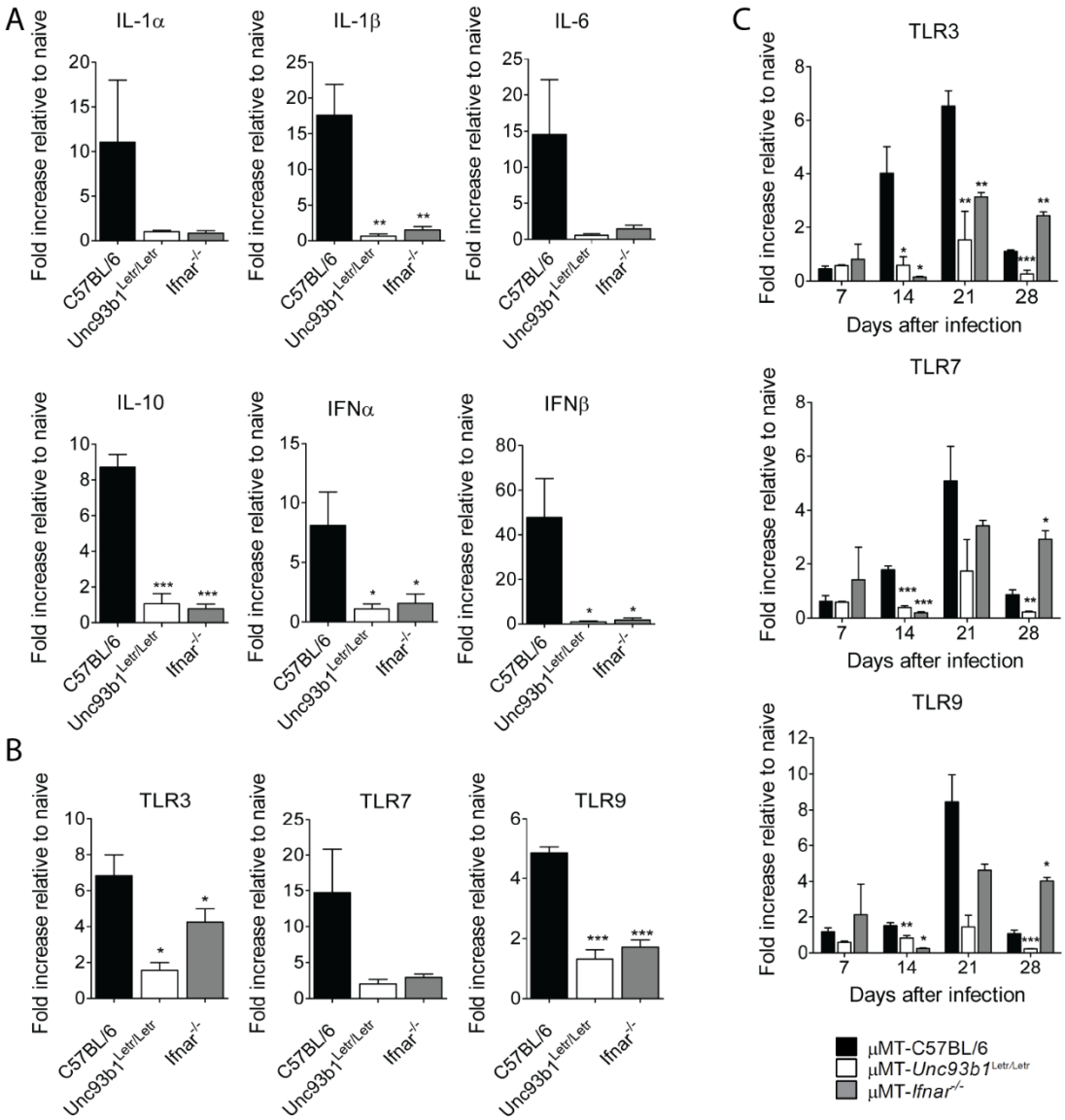


Figure 3. Functional endosomal TLRs and IFNARs are involved in cytokine and TLR mRNA expression in B Cells. (A and B) Expression analysis by qPCR of cytokine **(A)** and endosomal **(B)** TLR mRNA in B cells from C57BL/6, *Unc93b1*$^{Letr/Letr}$, and *Ifnar*$^{-/-}$ mice exposed to *L. donovani*. Data represent mean ± SEM from two independent experiments (n = 6–8). **(C)** TLR mRNA expression in B cells from infected chimeric mice assessed by qPCR. Data are expressed as fold increase to naive chimeric mice. μMT-C57BL/6 mice were used as comparison group for statistical significance. Data represent mean ± SEM of one of two independent experiments (n = 4–5). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

5.4. UNC93B1 deficiency abrogates IFN-I and IL-10 mRNA upregulation in B Cells during *L. donovani* Infection

To determine whether cytokine expression was also lost in vivo in the absence of functional endosomal TLR pathways or the IFNAR, we generated mixed bone marrow chimeras with a specific ablation of UNC93b1 or the IFNAR in B cells. B cell reconstitution levels were evaluated 6 weeks after bone marrow transfer; no significant differences were observed among the three groups **(Figure S3)**. We then purified B cells at various time points after infection from *L. donovani*-infected chimeric mice. With exception of IL-1b and IL-6, most of the cytokine expression was lost in B cells from infected μMT-*Unc931b*$^{Letr/Letr}$ chimeric mice (**Figure 4**), indicating that endosomal TLR activation was the main pathway leading to cytokine induction in vivo as well. Similar results were obtained in μMT-*Ifnar*$^{-/-}$ mice, underscoring the important role played by the IFNAR in vivo in enhancing B cell enhancing B cell activation by *L. donovani*. Interestingly, B cells from infected μMT-*Unc931b*$^{Letr/Letr}$ chimeric mice had a similar surface modulation of the costimulatory molecules CD80, CD86, and CD40 **(Figure S4)** as μMT- C57BL/6 and μMT-*Ifnar*$^{-/-}$ mice, suggesting that endosomal TLR pathways were not the only pathways triggered by *L. donovani* to activate B cells and that induction of cytokine expression was solely dependent on functional endosomal TLR signaling.

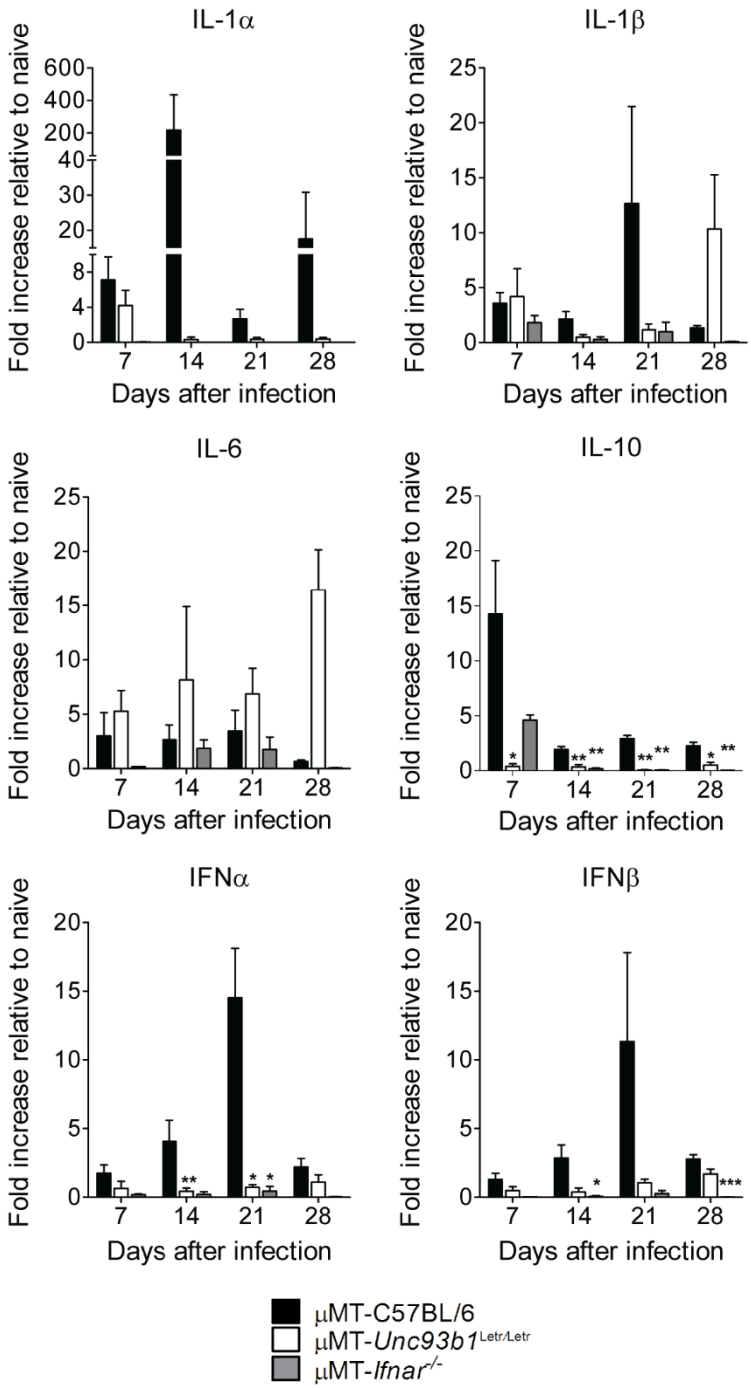


Figure 4. Cytokine mRNA expression in B cells during VL requires functional endosomal TLRs and IFNARs. Cytokine mRNA expression in B cells from infected chimeric mice was assessed by qPCR. Data represent mean ± SEM of one of two independent experiments (n = 4–5). ∗p < 0.05, ∗∗p < 0.01, ∗∗∗p < 0.001.

5.5. Lack of functional endosomal TLR signaling in B cells results in enhanced Th1 responses

We next sought to determine the biological relevance of endosomal TLR signaling in B cells during *L. donovani* infection. To this end, we infected µMT-*Unc931b*$^{Letr/Letr}$ and µMT-*Ifnar*$^{-/-}$ chimeric mice and their wild-type controls (µMT-C57BL/6) with *L. donovani* amastigotes and monitored the cellular and humoral immune responses to the parasite. We first monitored the development of protective IFN-γ-producing CD4^{+} T cells in the three groups of mice. µMT-*Unc931b*$^{Letr/Letr}$ mice displayed a trend toward increased frequencies of IFN-γ^{+} CD4^{+} T cells on days 14 and 21 post infection (p.i.) compared with the control group **(Figures 5A and 5B)**. In addition, the mean fluorescence intensity for IFN-γ in the same group of animals was significantly higher on day 21 p.i. **(Figure 5C).** A characteristic of chronic *L. donovani* infection is the appearance of CD4 T cells co-producing IFN-γ and IL-10. These cells are thought to have immune-suppressive functions and are typically associated with disease progression in humans and mice (Nylen *et al.*, 2007; Stäger *et al.*, 2006). Interestingly, the frequency of IFN-γ^{+} IL-10^{+} CD4^{+} T cells was significantly lower on day 28 p.i. in µMT-*Unc931b*$^{Letr/Letr}$ mice compared with the control group **(Figures 5D and 5E)**. Although infected µMT-*Ifnar*$^{-/-}$ mice showed stronger Th1 responses and slightly lower frequencies of IFN-γ^{+}IL-10^{+} CD4^{+} T cells, the differences were not significant at any time point analyzed.

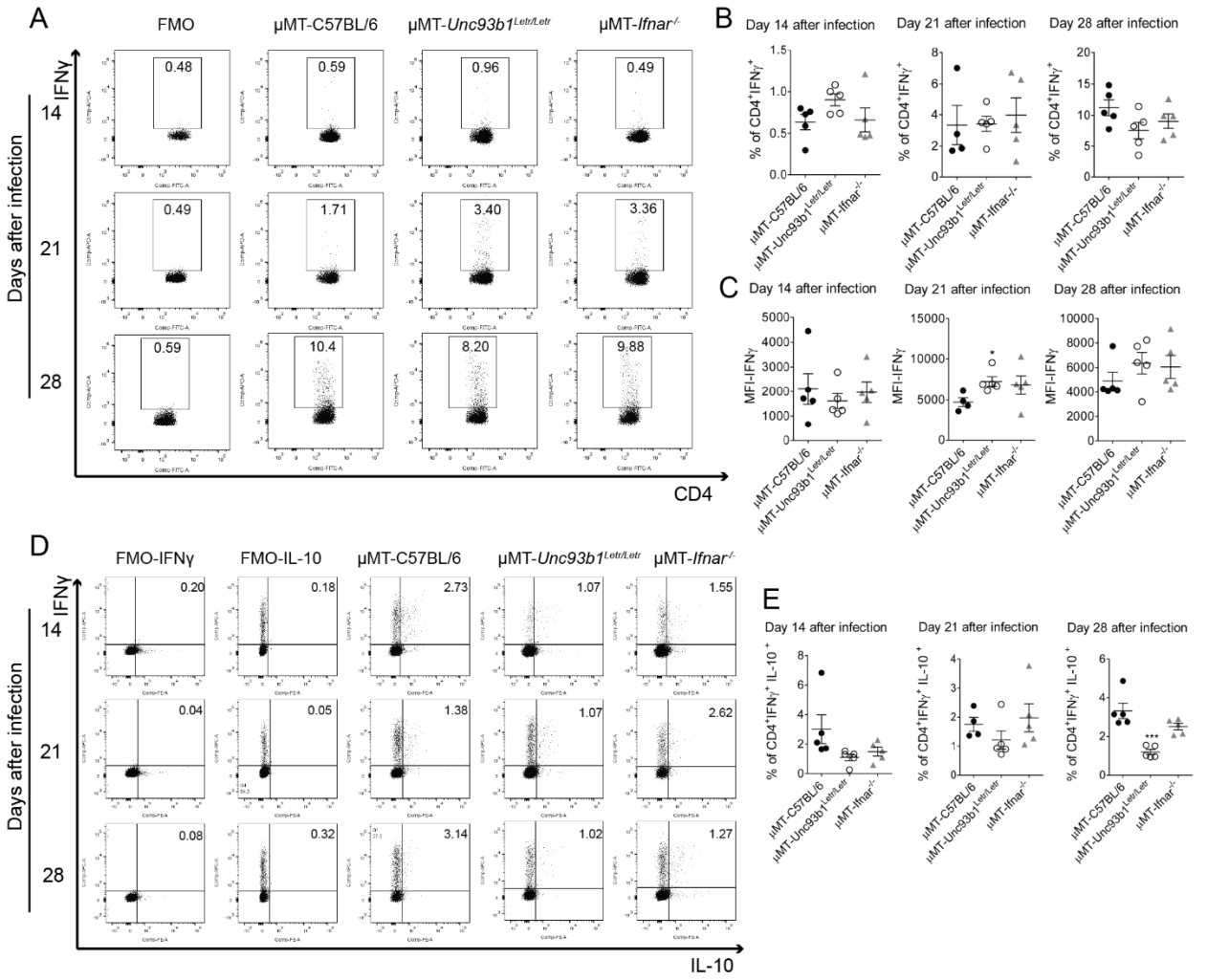


Figure 5. T cell responses in B cell-bone marrow chimeric mice during VL. Splenocytes from infected μMT-C57BL/6, μMT-*Unc93b1*$^{Letr/Letr}$, and μMT-*Ifnar*$^{-/-}$ mice were stained for CD3, CD4, IFN-γ, and IL-10 after restimulation. **(A)** Representative fluorescence-activated cell sorting (FACS) plots for IFN-γ^{+} CD4^{+}T cells. **(B and C)** Percentage of IFN-γ^{+} **(B)** and mean fluorescence intensity **(C)** of IFN-γ in CD4^{+}T cells. Splenocytes were restimulated with PMA/ionomycin for 4 hr and stained for CD3, CD4, IFN-γ, and IL-10. **(D)** Representative FACS plots for IFN-γ^{+} IL-10^{+} CD4^{+}T cells. **(E)** Percentage of IFN-γ+ IL-10+ CD4+ T cells. Data represent mean ± SEM of one of two independent experiments (n = 5). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

5.6. B cell endosomal TLR and IFN-I are involved in hypergammaglobulinemia induction during *L. donovani* Infection

TLR signaling in B cells is known to potentiate antibody production in autoimmune diseases (Han *et al.*, 2015). Because *L. donovani* induces a very strong antibody response that is detrimental to infection (Deak *et al.*, 2010; Miles *et al.*, 2005), we assessed whether ablation of TLR signaling in B cells would affect the humoral response to the parasite. First, we measured total IgG levels in the sera of infected mice over the course of infection, and we noticed an extreme reduction in serum IgG in µMT-*Unc931b*$^{Letr/Letr}$ and µMT-*Ifnar*$^{-/-}$ mice compared with the control group **(Figure 6A)**. Serum IgM levels were also significantly reduced, especially on day 14 p.i. Interestingly, both chimeric mouse groups failed to generate an IgM response following infection **(Figure 6B)**. We also found a significant reduction in *Leishmania*-specific IgG antibody levels in µMT-*Unc931b*$^{Letr/Letr}$ and µMT-*Ifnar*$^{-/-}$ mice compared with the control group **(Figure 6C)**. Collectively, our results indicate that endosomal TLR signaling in B cells strongly enhances antibody production during *L. donovani* infection and may thus contribute to hypergammaglobulinemia. The mechanism of induction partially requires signaling through the IFNAR because µMT-*Ifnar*$^{-/-}$ mice showed an intermediate phenotype with respect to the humoral response **(Figures 6A–6C)**.

To exclude a possible intrinsic B cell defect in our knockout mice, we next immunized *Unc931b*$^{Letr/Let}$, *Ifnar*$^{-/-}$, and WT control mice with ovalbumin using saponin as an adjuvant. Immunized *Unc931b*$^{Letr/Letr}$ produced a lower amount of ovalbumin-specific IgG after the first immunization compared with WT mice. However, when mice were boosted, no significant differences were observed between *Unc931b*$^{Letr/Letr}$ and WT mice **(Figure 6D)**. *Ifnar*$^{-/-}$ showed a similar response to immunization as WT mice **(Figure 6E)**. This suggests that UNC93B1- and IFNAR- deficient B cells are capable of producing antigen-specific antibodies following an immunization that does not require TLR activation.

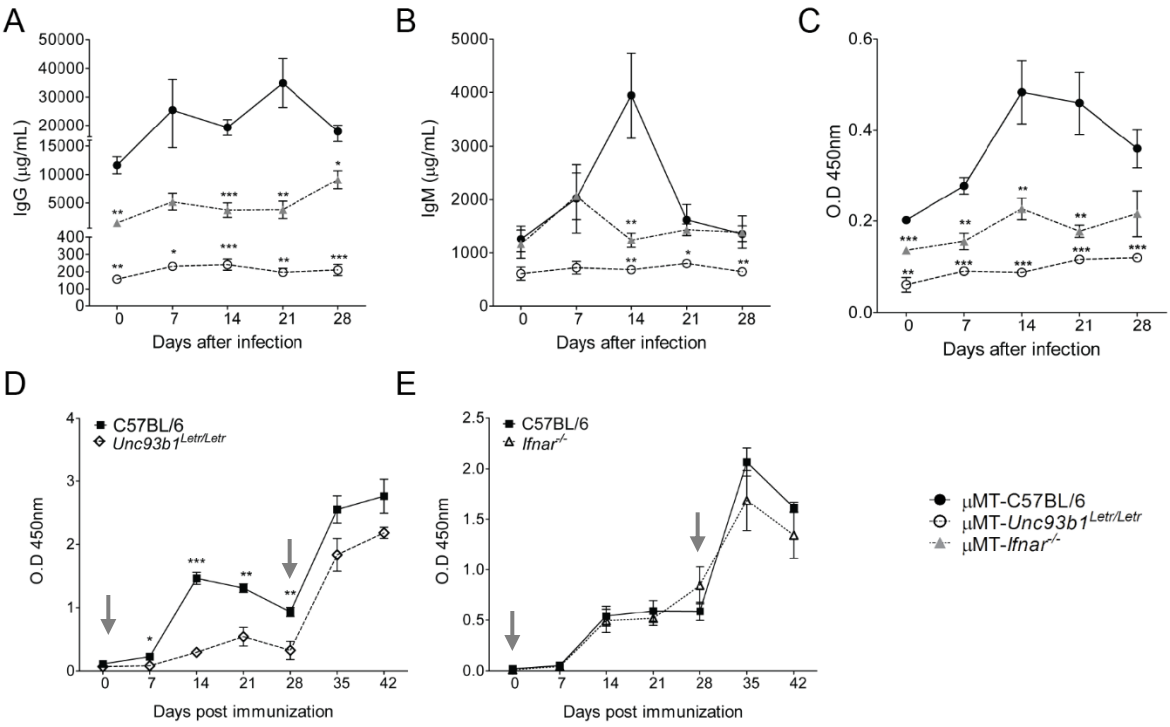


Figure 6. Endosomal TLRs and IFNARs in B Cells promote hypergammaglobulinemia. (A–C) Total levels of IgG **(A)**, IgM **(B)**, and *Leishmania*-specific IgG **(C)** in the sera of infected µMT-C57BL/6, µMT-*Unc93b1*$^{Letr/Letr}$, and µMT-*Ifnar*$^{-/-}$ mice over the course of infection. Data represent mean ± SEM of one of two independent experiments (n = 4–5). O.D., optical density. **(D and E)** C57BL/6, *Ifnar*$^{-/-}$, and Unc93b1Letr/Letr mice were immunized with ovalbumin and saponin, grey arrows indicate immunization periods. The graphs show the level of ovalbumin-specific IgG in the sera of immunized mice at various time points after immunization. Data represent mean ± SEM of one of two experiments (n = 5–6). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

5.7. B cell endosomal TLR activation by *L. donovani* exacerbates infection

Finally, we examined the contribution of B cell endosomal TLR signaling on the parasite burden. *L. donovani* typically establishes chronic infection in the spleen, whereas the parasite is eliminated in the liver. As expected, no major differences in hepatic parasite burden were observed between the three groups of chimeric mice **(Figure 7A)**, suggesting that endosomal TLR signaling in B cells does not substantially contribute to disease resolution or exacerbation in the liver. In contrast, μMT-*Unc931b*$^{Letr/Let}$ mice were remarkably resistant to *L. donovani* infection in the spleen, where we observed an 84% reduction in the splenic parasite burden **(Figure 7B)**. These results demonstrate the detrimental role of innate immune B cell activation during visceral *Leishmania*sis.

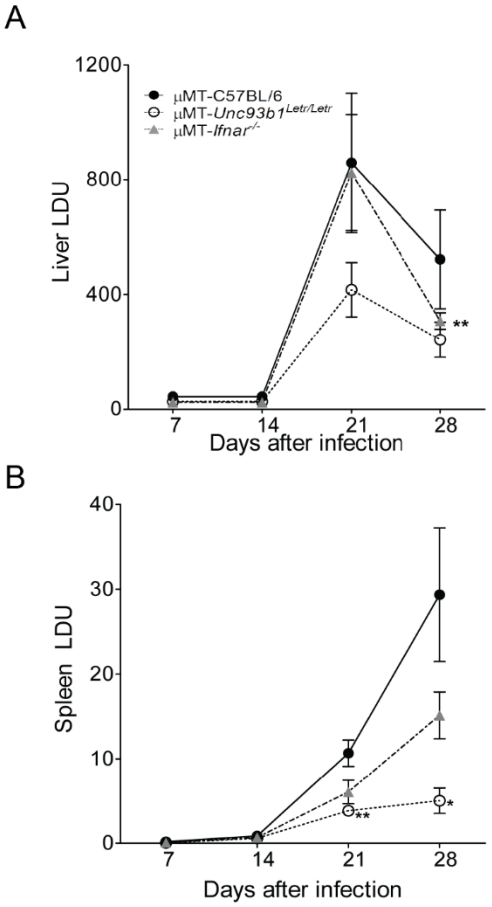


Figure 7. Endosomal TLRs in B Cells contributes to VL exacerbation. (A and B) Liver **(A)** and splenic **(B)** parasite burden in chimeric mice over the course of infection, represented as LDUs. Data represent mean ± SEM of one of two independent experiments (n = 4–5). ∗p < 0.05,∗∗p < 0.01.

6. DISCUSSION

This study identifies endosomal TLRs as a major pathway of B cell activation by *L. donovani*. Endosomal TLR activation in B cells induced cytokine expression, promoted antibody production, and led to disease exacerbation. The IFNAR was required to enhance this effect.

With exception of IL-10 (Bankoti *et al.*, 2012; Deak *et al.*, 2010; Ronet *et al.*, 2010), the role of B cell-derived cytokines in *Leishmania*sis has not yet been investigated. We have previously reported that B cell-derived IL10 is MyD88-dependent (Bankoti *et al.*, 2012). In this study, we extend our observation to demonstrate that not only IL-10 but also IL-1, IL-6, and IFN-I are downstream of endosomal TLR signaling following exposure to *L. donovani* amastigotes. B cell activation through endosomal TLRs was reported so far only for autoimmune diseases (Avalos *et al.*, 2014; Han *et al.*, 2015) or in a context of vaccination (Hou *et al.*, 2011). Lipopolysaccharides (LPS) and CpG DNA also appear to enhance signaling through the BCR in vitro (Freeman *et al.*, 2015). In contrast, MyD88 expression on B cells was shown to suppress protective immunity against Salmonella (Neves *et al.*, 2010) through IL-10 production. In this model, the pathways upstream of MyD88 were not investigated. Nevertheless, the requirement for MyD88 may suggests a role for TLRs in B cell activation and IL-10 induction. TLR signals are also required for the development of regulatory B10 cells (Yanaba *et al.*, 2009).

A large body of literature has now demonstrated that cytokines produced by B cells contribute to the regulation of the adaptive immune response and can inhibit dendritic cells (DC) and macrophage functions. In our infection model, B cell-derived cytokines probably do not play a major role in regulating T cell responses. Indeed, we only observed mild differences in T cell responses between µMT-C57BL/6, µMT-*Unc931b*$^{Letr/Letr}$, and µMT-*Ifnar*$^{-/-,}$ suggesting that B cell-derived cytokines are mainly involved in regulating innate myeloid cell functions. An interesting observation, however, was the induction of IFN-I expression by *L. donovani*. Our results indicate that IFN-α/β may act through autocrine signaling to enhance B cell activation. Indeed, in the absence of a functional IFNAR, cytokine expression was abrogated in B cells exposed in vitro to *L. donovani* or purified from infected mice. IFN-Is are known to exert pleiotropic effects on B cells (Ivashkiv & Donlin, 2014b), including enhanced IL-10 production through TLR7/8 in B cells (Liu *et al.*, 2014a), induction of marginal zone B cells (MZB) follicular shuttling (Li *et al.*, 2015a), stimulation of B cell-activating factor (BAFF) production in monocytes (Gomez *et al.*, 2015), and enhancement of antibody responses by promotion of isotype switching (Le Bon *et al.*, 2001).

In VL, we demonstrate that IFN-I signaling is also involved in the upregulation of TLR3, 7, and 9 in B cells. A positive feedback loop involving IFN-β and TLR7 has also been proposed by Green et al. (2009) (Green *et al.*, 2009). In our model, the interplay between IFN-I and endosomal TLRs has been extended to the regulation of the humoral response. The fact that treatment with IFN-I can lead to lupus-like symptoms (Gota & Calabrese, 2003b), suggests that this positive loop may apply to other diseases. A possible role for IFN-I in enhancing antibody production has already been ascribed to various models of viral infections (Heer *et al.*, 2007; Lund *et al.*, 2004a) and seem to be mainly dependent on IFNAR expression in B cells (Marzo *et al.*, 2005). However, a direct role for B cell TLRs in regulating the humoral response was only demonstrated in autoimmune diseases. For instance, TLR7 and 9 in B cells have been shown to be involved in enhancing autoantibody production in systemic Lupus Erythematosus (SLE) (Christensen *et al.*, 2006; Han *et al.*, 2015). Moreover, in patients suffering from Wiskott-Aldrich syndrome, increased TLR signaling in B cells promotes the enrichment of self-reactive transitional B cells (Kolhatkar *et al.*, 2015). Our study demonstrates that a parasite can directly activate endosomal TLRs in B cells and that this activation results in enhanced non-specific and *Leishmania*-specific antibody production.

Hypergammaglobulinemia, which commonly leads to antibody-mediated immunopathology, is a hallmark of VL. The mechanisms leading to hypergammaglobulinemia in VL are poorly understood. A recent study conducted in human VL patients revealed that serum levels of the B cell-activating factor BAFF were elevated during active disease (Goto *et al.*, 2014). However, a clear positive correlation between BAFF and IgG levels in VL patients was not found. Hence, the authors suggested that other factors may act synergistically with BAFF to promote hypergammaglobulinemia. During chronic lymphocytic choriomeningitis virus (LCMV), hypergammaglobulinemia and autoantibody production arise when B cells process viral antigen and present it to TFhs (Hunziker *et al.*, 2003). Interestingly, viral antigen processing did not require BCR, complement receptors, or Fc receptors. We also observed with *Leishmania* that induction of cytokine expression was not dependent on a specific BCR (data not shown), suggesting that perhaps *Leishmania* antigens are also internalized by a non-specific pinocytosis of either parasite exosomes or surface molecules cleaved by B cell proteases. The role of TFhs in VL has recently been clarified in a study in macaques (Rodrigues *et al.*, 2014). TFhs appear to expand during acute infection, paralleling the differentiation of activated memory B cells and the production of parasite-specific IgG. In contrast, the authors observed a contraction of germinal centers during chronic infection, confirming results reported previously in experimental VL (Smelt *et al.*, 1997). This contraction affected the production of parasite-specific IgG, whereas

hypergammaglobulinemia persisted. Hence, it appears that TFhs may play a limited role in the maintenance of hypergammaglobulinemia during VL. Nevertheless, it is possible that TLR-activated B cells may induce abortive TFh responses while enhancing the production of non-specific antibodies. Further investigations are warranted to understand the interaction between these two cell populations.

In conclusion, we discovered that a parasite can directly activate endosomal TLRs in B cells. We propose that this activation is largely responsible for promoting hypergammaglobulinemia in an IFNAR-dependent manner. Hypergammaglobulinemia and elevated levels of IFN-I are characteristics of several chronic infections and autoimmune diseases and are often associated with immunosuppression and/or increased pathology. Thus, innate activation of B cells may be an unappreciated mechanism that underlies the development of hypergammaglobulinemia and may therefore contribute to immunopathology in other chronic infections.

7. EXPERIMENTAL PROCEDURES

Mice and Parasites

C57BL/6 and μMT mice were purchased from The Jackson Laboratory. *Unc93b1*$^{Letr/Letr}$ (Lafferty *et al.*, 2014) and *Ifnar*$^{-/-}$ (Muller *et al.*, 1994) mice were bred at the animal facility of McGill University. All mice were housed at the Institut National de la Recherche Scientifique (INRS) animal facility under specific pathogen-free conditions and used at 6–12 weeks of age. Experiments involving mice were carried out under protocols approved by the Animal Care and Use Committee of the INRS, Institut Armand-Frappier. These protocols respect procedures on good animal practice provided by the Canadian Council on Animal Care.

L. donovani (strain LV9) was maintained by serial passage in B6.129S7-Rag1tm1Mom mice, and amastigotes were isolated from the spleens of infected animals. Mice were infected by injecting 2 × 10^7 amastigotes intravenously via the lateral tail vein (Stäger et al., 2003). Splenic parasite burdens were determined by examining methanol-fixed, Giemsa-stained tissue impression smears. Data are presented as number of parasites per spleen or as Leishman Donovan Units (LDUs) (Bankoti *et al.*, 2012).

B cell in vitro studies

Naive splenic B cells were purified using the B cell isolation kit (Miltenyi Biotech) according to the manufacturer's protocol. Purified B cells (purity 90%–92%) were then incubated either alone, with different ratios of parasites, or with TLR agonists: 3 μg/ml CpG (InvivoGen), 5 μg/ml R837 (InvivoGen), 3 μg/ml poly (I:C) (Amersham), and 10 μg/ml LPS (Sigma). B cells were also incubated with 1 μg/ml ODN2088 (Miltenyi) and a TLR7 and 9 inhibitors (Stunz et al., 2002). Cells were cultured in supplemented DMEM (Gibco, Invitrogen). For cytokine mRNA expression analysis, confocal microscopy, and immunoblots, cells were incubated for 8 or 24 hr at 37°C.

Quantitative Real-Time PCR

RNA from isolated B cells from in vitro or in vivo experiments was extracted using the RNeasy mini kit (QIAGEN) as described by the manufacturer. Reverse transcription was carried out using the iScript cDNA synthesis kit (Bio-Rad) following the manufacturer's protocol. Real-time PCR analysis was performed using iTaq Universal SYBR Green Supermix (Bio-Rad). The IL-1α, IL-1β, IL-6, IL-10, IFN-α, IFN-β, and HPRT genes were amplified using primers whose sequences are described below. All PCRs were carried out with the Stratagene mx3005p real-time PCR system. Data were normalized to HPRT and expressed as fold increase to naive controls.

Primers

The following primers were used to determine the relative gene expression using qPCR: *Hprt*, 5′-GTT GGA TAC AGG CCA GAC TTT GTT G-3′ and 5′-GAT TCA ACC TTG CGC TCA TCT TAG GC-3′; Il1a, 5′-CAA ACT GAT GAA GCT CGT CA-3′ and 5′-TCT CCT TGA GCG CTC ACG AA-3′; *Il1b*, 5′-AAC CTG CTG GTG TGT GAC GTT C-3′ and 5′-CAG CAC GAG GCT TTT TTG TTG T-3′; *Il6*, 5′-ACA ACC ACG GCC TTC CCT ACT T-3′ and 5′-CAC GAT TTC CCA GAG AAC ATG TG-3′; *Il10*, 5′-AGG GTT ACT TGG GTT GCC AA-3′ and 5′-CAC AGG GGA GAA ATC GAT GA-3′; *Ifna*, 5′-CAT CTG CTG CTT GGG ATG GAT-3′ and 5′-TTC CTG GGT CAG AGG AGG TTC-3′; *Ifnb*, 5′-TCA GAA TGA GTG GTG GTT GC-3′ and 5′-GCA CTT TCA AAT GCA GTA GAT TCA-3′; *Tlr3*, 5′-AAG ACA GAG ACT GGG TCT GGG-3′ and 5′-TGA AAC TTC GTC CGC AGG AA-3′; *Tlr7*, 5′-GGC ATT CCC ACT AAC ACC AC-3′ and 5′-TTG GAC CCC AGT AGA ACA GG-3′; and *Tlr9*, 5′-CTA GAT GCT AAC AGC CTC GCC-3′ and 5′-GTC CTC GCC ACT TCC ACT G-3′.

Confocal Microscopy

L. donovani parasites were stained with PKH67 (Sigma) following the manufacturer's instructions and incubated with purified naive splenic B cells (B cell isolation kit, Miltenyi). 2 × 10^6 B cells were incubated either alone or with PKH67-*L. donovani* amastigotes (at a multiplicity of infection [MOI] of 5:1 or 10:1). 8 or 24 hr post-incubation, cells were fixed with 2% paraformaldehyde for 15 min at 4°C. Permeabilization and unspecific staining were blocked using 0.1% Triton X-100, 1% bovine serum albumin, 5% goat serum, and 5% horse serum in PBS for 30 min at 4°C. For immunostaining, cells were labeled with coupled antibodies against BCR (goat anti-mouse IgM-AF568, Life Technologies), TLR3 (anti-TLR3 antibody (40C1285.6)-DyLight 650, Novus), and TLR9 (anti-TLR9 monoclonal antibody, Abcam) coupled to IgG1 Zenon-Alexa Fluor-647, Life Technologies). NucBlue (Life Technologies) was used to visualize cell nuclei, following the manufacturer's instructions. Labeled cells were washed in PBS and resuspended in 100 µl of PBS and then transferred onto a polylysine-coated coverslip. Coverslips were subsequently mounted with mounting medium (Fluoromount G, Southern Biotechnology Associates). Analysis of protein localization was performed using a Zeiss LSM780 system equipped with a 30-mW, 405-nm diode laser; 25-mW, 458/488/514 argon multiline laser; 20-mW DPSS 561-nm; laser; and 5-mW HeNe 633-nm laser mounted on a Zeiss Axio Observer Z1 and operated with Zen 2011 software (Zeiss). We used a Plan-Apochromat 63× oil differential interference contrast (DIC) 1.4 numerical aperture (NA) objective for our observations, and images were acquired via sequential acquisition.

Immmunoblot

Total cell protein extracts from 3 × 106 purified B cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich) supplemented with protease inhibitors (Complete mini, Roche). Equal amounts of protein (15 µg) were fractionated by 8% SDS-PAGE, and transferred to a nitrocellulose membrane (Hybond, Amersham). Rabbit anti-mouse polyclonal antibody against TLR7 (Imgenex) was used for the immunoblot assay. Blots were stripped (stripping buffer, Thermo Fisher), and equal loading was confirmed with a monoclonal antibody against β-actin (Abcam).

Generation of mixed bone marrow chimeras

Chimeras were generated by reconstituting lethally irradiated C57BL/6 mice with 2 × 107 bone marrow cells from μMT, *Unc93b1*$^{Letr/Letr}$, *Ifnar*$^{-/-}$, or C57BL/6 mice as described previously (Bankoti *et al.*, 2012). The bone marrow was mixed at a ratio of 4:1 for the following combinations: 80% μMT with 20% *Unc93b1*$^{Letr/Letr}$, 80% μMT with 20% *Ifnar*$^{-/-}$, and 80% μMT with 20% C57BL/6. The reconstitution level was evaluated 6 weeks after bone marrow transfer (Figure S3). Chimeric mice were infected 7–8 weeks after reconstitution with 2 × 10^7 *L. donovani*-LV9 amastigotes intravenously via the lateral tail vein.

Flow cytometry

Endogenous CD4^{+} T cell responses in infected chimera were analyzed as described previously (Nothelfer *et al.*, 2015). Briefly, splenocytes were either restimulated with bone marrow-derived dendritic cells (BMDCs) pulsed with fixed parasites or phorbol 12-myristate 13-acetate (PMA)/ionomycin in the presence of Brefeldin A (BD Biosciences). Cells were then stained with anti-CD4-fluorescein isothiocyanate (FITC), anti-CD8 Pacific Blue, anti-IFN-γ-allophycocyanin, and anti-IL-10-phycoerythrin (BD Biosciences). 350,000 cells were acquired on a BD LSRFortessa cell analyzer (Becton Dickinson), and analysis was performed using FlowJo software (Tree Star).

The expression of costimulatory molecules by B cells from infected chimeric mice was assessed using the following antibodies: FITC-conjugated anti-major histocompatibility complex class II (MHCII) (BD Biosciences), eFluor 450-conjugated anti-CD19 (eBioscience), PE-conjugated anti-CD40 (eBioscience), PE-conjugated anti-CD80 (eBioscience), and PE-conjugated anti-CD86 (eBioscience). Cells were acquired with a BD LSRFortessa cell analyzer (Becton Dickinson), and analysis was performed using FlowJo software (Tree Star).

Antibody production

Sera from infected mice were analyzed by ELISA. Total IgM and IgG levels were measured using the Mouse IgM ELISA Ready-SET-Go! kit and Mouse IgG ELISA Ready-SET-Go! kit (eBioscience) following manufacturer's recommendation. *Leishmania*-specific IgG was measured using the following protocol. Nunc Maxisorp 96-well plates (eBioscience) were coated for 2 hr at 37°C and then overnight at 4°C with 8 μg of soluble *Leishmania* antigens (SLAs) in coating buffer

(15 mM Na_2CO3 and 35 mM NaH_2CO3 [pH 9.6]). Plates were washed three times with 0.1% Tween 20 in PBS (PBS-T) and further blocked with 0.1% Tween 20 and 0.5% gelatin for 2 hr at room temperature. After washing, sera diluted in PBS (1:400) were added and incubated for 2 hr at 37°C. Wells were then washed five times with PBS-T, followed by the addition of enhanced chemiluminescence (ECL) anti-mouse IgG and horseradish peroxidase-linked species-specific whole antibody (GE Healthcare, Amersham) diluted 1:12,000 in PBS and were incubated for 1 hr at 37°C. Substrate solution (3, tetramethylbenzidine (TMB) liquid substrate, Sigma) was then added. The reaction was developed at room temperature for 15 min and stopped with 1 M H2PO4. Absorbance was measured at 450 nm in a microplate reader (model 680, Bio-Rad).

Ovalbumin Immunization

C57BL/6, *Unc93b1*$^{Letr/Letr}$, and *Ifnar*$^{-/-}$ mice were immunized with 400 µg ovalbumin (Ova) (Sigma) and 0.05% saponin (Sigma) in sterile PBS at the base of the tail. Mice were boosted 28 days after the first immunization with the same amount of protein and adjuvant. Blood was collected weekly by the lateral saphenous vein; serum was separated by centrifugation and stored at −80°C. Ova-specific IgG were measured following the protocol described by Garulli et al. (2008). A serial dilution of the sera was performed; the data represent the results of dilution at 1:200. The reaction was developed at room temperature for 5 min and stopped with 1 M H_2PO4. Absorbance was measured at 450 nm in a microplate reader (model 680, Bio-Rad).

Statistical analysis

Statistical analysis was performed using Student's t test. Differences were considered to be statistically significant when $p < 0.05$. All experiments were conducted independently at least twice.

8. ACKNOWLEDGMENTS

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Parasite Interactions is supported by the Fonds Québécois de la Recherche sur la Nature et les Technologies. Work in the laboratory of J.H.F. is supported by an operating grant from the Canadian Institutes of Health Research (MOP-114972) and a Leaders Opportunity Fund infrastructure grant from the Canadian Foundation of Innovation. J.H.F is supported by a CIHR New Investigator Award.

9. SUPPLEMENTAL INFORMATION

Movies

Movie S1 and Movie S2: Close-up on the interaction between B cells and *L. donovani* amastigotes. 3D video reconstruction from z-stacks images (Zen 2011, Zeiss software). Naïve splenic B cells were exposed to PKH67-labeled *L. donovani* amastigotes (green) at a MOI of 1:5. Cells were stained with anti-IgM-AF568 (red) and Hoechst (blue).

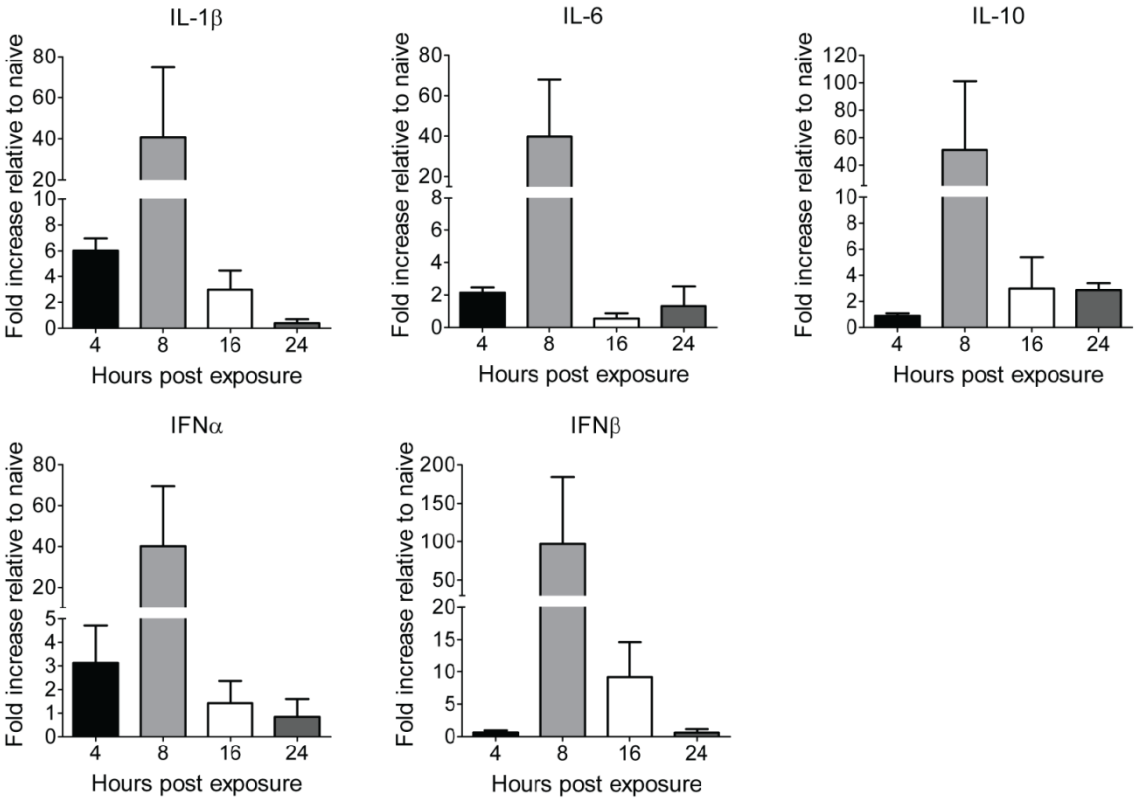

Figure S1. Modulation of mRNA expression for various cytokines in B cells exposed to *L. donovani* (Related to Figure 1). B cells were co-incubated with *L. donovani in vitro* at a MOI of 5:1. Cytokine mRNA levels were determined by qPCR at the indicated time points after incubation. Data are expressed as fold increase to unexposed B cells and represent mean ± SEM of one of 2 independent experiments, n = 5.

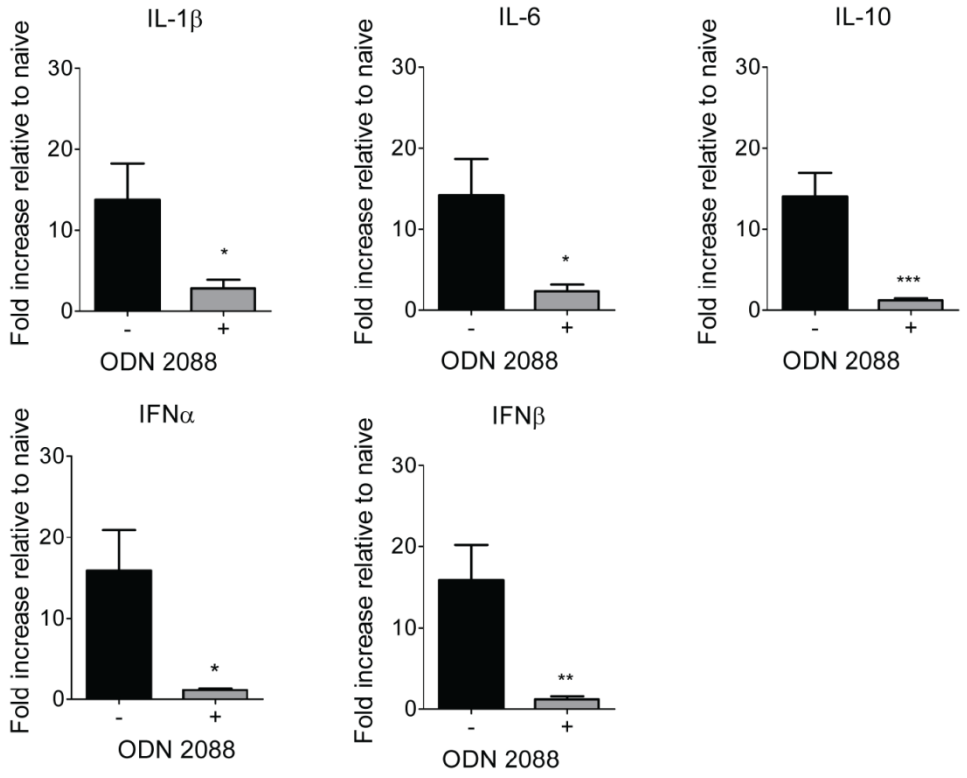

Figure S2. Cytokine mRNA levels in B cells exposed to *L. donovani* after treatment with TLR7 and 9 inhibitors (Related to Figure 3). Expression analysis by qPCR of cytokine mRNA in naïve splenic B cells treated with 1µg/ml ODN2088 and exposed to *L. donovani* for 8h. Data are expressed as fold increase to unexposed B cells (treated and untreated) and represent mean ± SEM of pool of 3 independent experiments, n = 9.

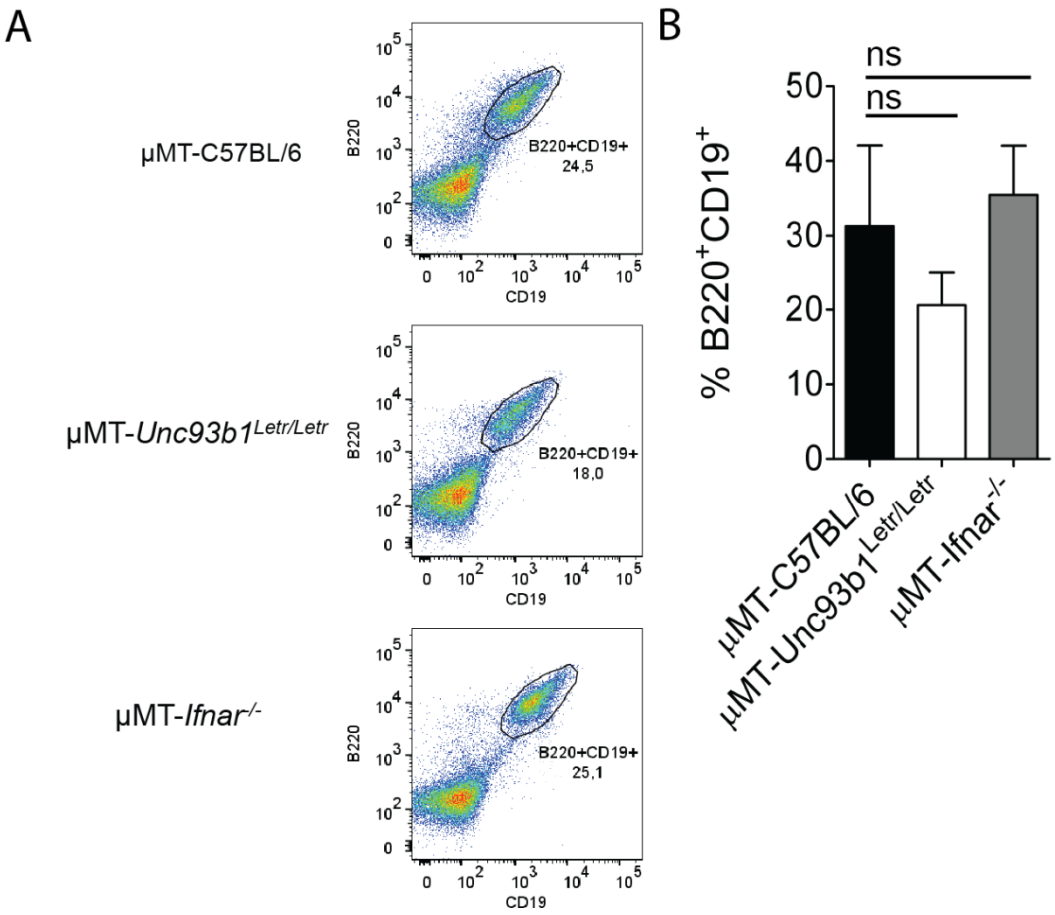

Figure S3. Percentage of B cells in the spleen of chimeric mice 6 weeks after bone marrow reconstitution (Related to Figure 4). Mice were sacrificed 6 weeks after BM transfer and the frequency of splenic B cell assessed by FACS. Graphs show the percentage of $B220^{+}$ $CD19^{+}$ cells in the spleen of chimeric mice. Data represent mean ± SEM of one of 2 independent experiments, n=3.

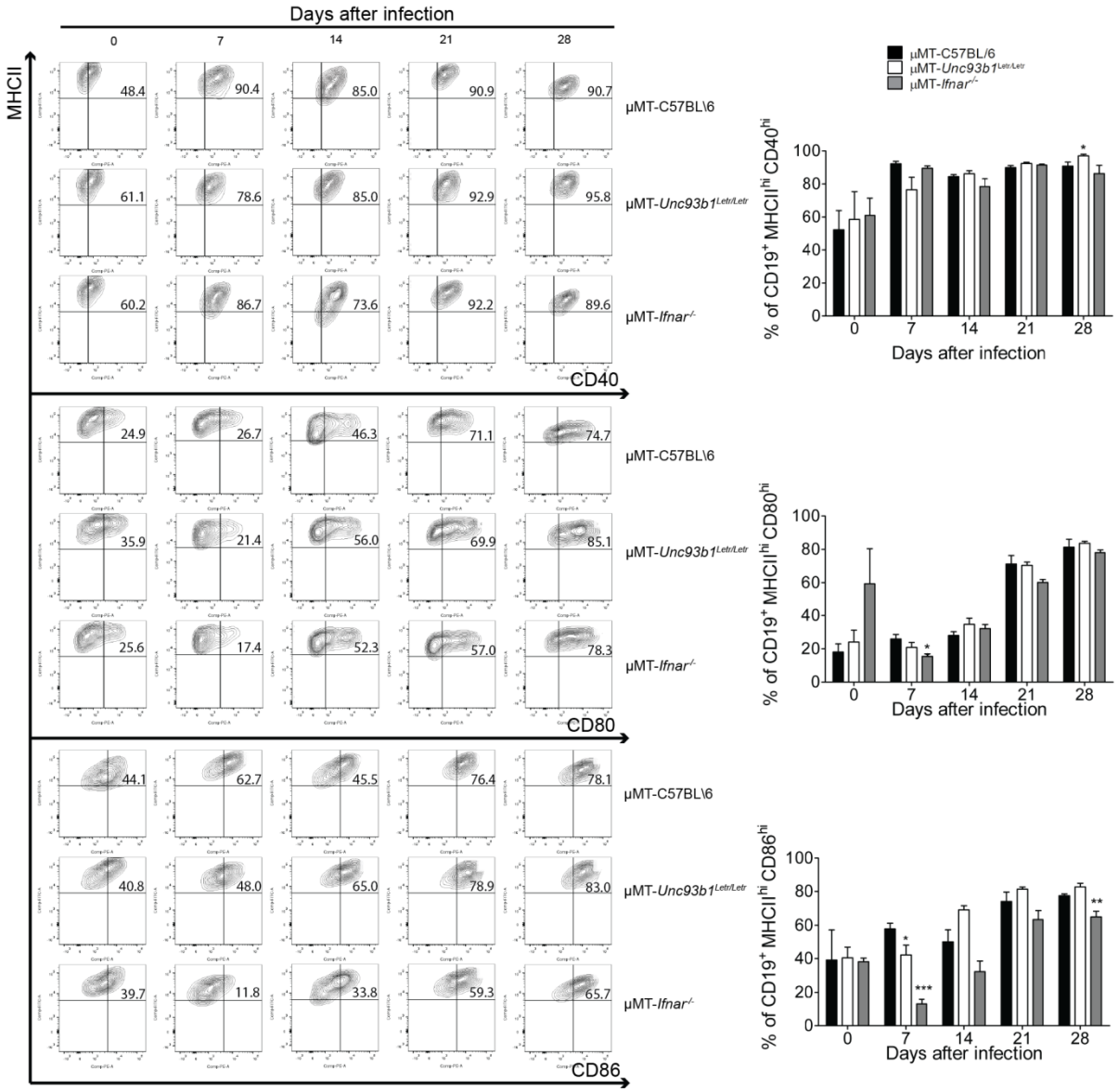

Figure S4. Modulation of co-stimulatory molecules in B cell during *L. donovani* infection (Related to Figure 4). Splenocytes from μMT-C57BL/6, μMT-*Unc93b1*$^{Letr/Letr}$, and μMT-*Ifnar*$^{-/-}$ mice were stained for CD19, MHCII, CD40, CD80, and CD86 at various time point of infection. Cells were analysed by flow cytometry. Representative FACS plots for gated CD19^{+} MHCII^{+} cells. Data represent mean ± SEM of one of 2 independent experiments, n = 5. * denotes $p<0.05$, ** denotes $p<0.01$ and *** denotes $p<0.001$.

CHAPTER 4:

HYPERGAMMAGLOBULINEMIA SUSTAINS THE DEVELOPMENT OF REGULATORY RESPONSES DURING CHRONIC *LEISHMANIA DONOVANI* INFECTION IN MICE

Hypergammaglobulinemia sustains the development of regulatory responses during chronic *Leishmania donovani* infection in mice

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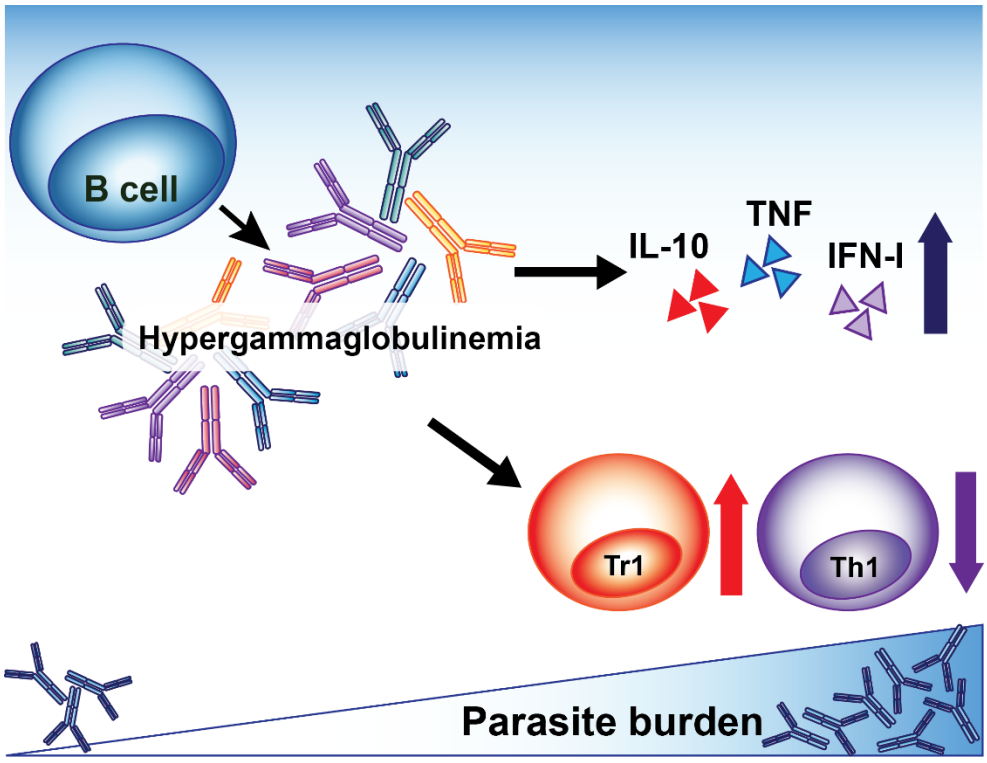
Author contributions

S.S.-B. and S.S. conceived the project, designed the experimental approach, performed experiments, interpreted data, and wrote the manuscript.

Keywords: *Leishmania donovani*, regulatory T cells, T helper cells, AID, hypergammaglobulinemia

GRAPHICAL ABSTRACT



1. RÉSUMÉ

La leishmaniose viscérale, une maladie chronique potentiellement mortelle, se caractérise par une forte production d'anticorps de faible affinité. L'hypergammaglobulinémie est une prédiction de l'évolution de la maladie chez l'homme. Néanmoins, la contribution des immunoglobulines hypermutées à la pathogenèse de la maladie n'a jamais été étudiée. À l'aide de souris Aicda-/- et du modèle expérimental d'infection à *Leishmania donovani*, nous avons démontré que l'absence d'anticorps hypermutées et la commutation isotypique était associée à une résistance accrue à la maladie, à des réponses protectrices plus fortes et à une fréquence plus faible des cellules T $CD4^+$ coproductrices d'IFNγ+ et d'IL-10+. De façon intéressant, les réponses Th1 plus fortes et l'absence de lymphocytes T $CD4^+$ coproductrices d'IFNγ+ et d'IL-10+ au cours de l'infection chronique chez les souris Aicda-/- infectées n'ont pas été causées par un effet intrinsèque médies par l'absence de Aicda dans les lymphocytes T $CD4^+$, mais par des changements dans l'environnement en particulier la composition des cytokines au cours de l'infection chronique. En effet, les expressions du TNF, de l'IL-10 et de l'IFN-β n'étaient régulées positivement qu'en présence d'anticorps hypermutées, la commutation isotypique et l'hypergammaglobulinémie aux stades chroniques de l'infection. Mises ensemble, nos résultats suggèrent que l'hypergammaglobulinémie soutient les réponses immunes inhibitrices au cours de la leishmaniose viscérale chronique.

2. ABSTRACT

Visceral leishmanisiais, a chornic, potentially fatal disease, is characterized by high production of low-affinity antibodies. In humans, hypergammaglobulinemia is prediction of disease progression. Nevertheless, the contribution of hypermutated immunoglobulins to disease pathogenesis has never been studied. Using *Aicda*$^{-/-}$ mice and the experimental model of *Leishmania donovani* infection, we demonstrate that the absence of hypermutated and class-switched antibodies was associated with increased resistance to disease, stronger protective Th1 responses and a lower frequency of regulatory IFNγ^{+}IL-10^{+} CD4^{+} T cells. Interestingly, stronger Th1 responses and the absence of IFNγ^{+}IL-10^{+} CD4^{+} T cells during chronic infection in infected *Aicda*$^{-/-}$ mice was not caused by a T cell intrinsic effect of AID, but by changes in the cytokine environment during chronic disease. Indeed TNF, IL-10 and IFN-β expressions were only upregulated in the presence of hypermutated, class-switched antibodies and hypergammaglobulinemia at later stages of infection. Taken together, our results suggest that hypergammaglobulinemia sustains inhibitory responses during chronic visceral *Leishmania*sis.

3. INTRODUCTION

Visceral leishmanisis is a chronic disease caused by the protozoan parasites *Leishmania donovani* and *L. infantum/chagasi*. VL is still causing 20,000 deaths each year and there are about 300,000 estimated new cases of VL per year worldwide. This disease is characterized by irregular bouts of fever, cachexia, hepatosplenomegaly, hypergammaglobulinemia, and anaemia, and is fatal if untreated (http://www.who.int/*Leishmania*sis/en/). In mice, VL mainly affects three organs, the liver, the bone marrow and the spleen. Although disease resolution occurs in the liver, experimental VL is characterized by persistent infection of the spleen and the bone marrow and, similarly to human patients, by immunodeficiency during the chronic stage. CD4^{+} and CD8^{+} T cells producing IFN-γ are required to control infection (Bankoti & Stäger, 2012) while B cells exacerbate disease (Bankoti *et al.*, 2012; Smelt *et al.*, 2000).

Leishmania and other *Trypanosoma*tids are known to subvert B cell responses to favour their own survival and establish chronic infections (Silva-Barrios *et al.*, 2018). In the case of VL, B cells play a central role in the pathogenesis of the disease. Indeed, B cell-deficient mice are highly resistant to infection and high IgG levels are predictive of disease in human (Smelt *et al.*, 2000). However, only few studies have investigated the underlying mechanisms by which B cells alter the course

of disease. Polyclonal B cell activation, IgM and complement appear to exacerbate infection in *L. infantum* infected mice (Deak *et al.*, 2010). We also reported that marginal zone B cells and B cell-derived IL-10 negatively affect parasite clearance (Bankoti *et al.*, 2012) and that polyclonal B cell activation and hypergammaglobulinemia are associated with disease severity in *L. donovani* infected mice (Silva-Barrios *et al.*, 2016). Nevertheless, the contribution of hypermutated antibodies to the immune response against *L. donovani* has never been studied.

Polyclonal B cell activation is responsible for excessive production of unspecific and low-affinity antibodies and typically results in hypergammaglobulinemia and can give rise to antibody-mediated immunopathology. Innate immune activation of B cells by *L. donovani* through endosomal TLRs is one of the mechanisms that lead to polyclonal B cell activation and hypergammaglobulinemia (Silva-Barrios *et al.*, 2016). This pathway strictly requires IFN-I signalling (Silva-Barrios *et al.*, 2016), which seems to be necessary for enhancing antibody production. Elevated levels of B cell activating factor BAFF were also proposed as another possible mechanism (Goto *et al.*, 2014). Interestingly, T follicular helper cells (TFh) do not seem to play a role in the induction or maintenance of hypergammaglobulinemia (Rodrigues *et al.*, 2014). Indeed, in macaques infected with *L. infantum*, TFh expand during acute infection to contract during later stages of disease, when germinal centers are gradually lost and *Leishmania*-specific antibody titers are also declining; during this time, however, hypergammaglobulinemia persist (Silva-Barrios *et al.*, 2016; Smelt *et al.*, 1997).

In the present study, we investigate how the absence of hypermutated and class-switched antibodies, which include *Leishmania*-specific and unspecific antibodies, affects the pathogenesis of visceral *Leishmania*sis. Interestingly, *Aicda*$^{-/-}$ mice were significantly more resistant to *L. donovani* infection than C57BL/6 mice. Increased resistance was associated with stronger Th1 responses and a lower frequency of IFNγ^{+}IL-10^{+} Tr1 cells. These differences were not caused by a T cell-intrinsic effect of activation induced deaminase (AID) or by a reduced level of B cell activation, but by a shift in the cytokine environment during chronic VL.

4. RESULTS

4.1. Aicda$^{-/-}$ mice are more resistant to *L. donovani* infection

We have previously demonstrated that innate B cell activation and IFN-I were promoting hypergammaglobulinemia in *L. donovani* infected mice (Silva-Barrios *et al.*, 2016). Hence, we wanted to investigate how the lack of hypermutated and class-switched antibodies and hypergammaglobulinemia would affect disease pathogenesis. To this end, we infected *Aicda*$^{-/-}$ mice with *L. donovani* and measured IgG levels in the blood over the course of infection. These mice lack the activation-induced cytidine deaminase (AID) - an enzyme that initiates somatic hypermutation and class-switch recombination in B cells- and cannot generate genetically diverse, high-affinity antibodies (Muramatsu *et al.*, 2000). As expected, the wild type (WT) control group increasingly developed hypergammaglobulinemia over the course of *L. donovani* infection; in contrast, no IgG was detected in infected *Aicda*$^{-/-}$ mice (Fig. 1A). As shown in Figure 1B, IgM levels peaked around d7 p.i. in WT mice, to decrease again later during infection. In agreement with the literature (Revy *et al.*, 2000), *Aicda*$^{-/-}$ mice displayed significantly higher IgM titers compared to WT during the whole course of infection. Interestingly, in these mice, IgM titers spiked after d21p.i., a time of infection when also *Leishmania*-specific antibodies are typically produced in WT mice (Fig. 1C). *Aicda*$^{-/-}$ mice also produced some *Leishmania*-specific antibodies (Fig. 1C) (only IgM in this case) but to a lesser extent than the WT group. We were next curious to know how the absence of antibodies would impact the parasite burden in the two main target organs, the liver and the spleen. In the absence of AID, the liver was able to better control *L. donovani* growth and a 60-70% reduction in the hepatic parasite load was observed compared to WT mice (Fig. 1D). Similarly, the splenic parasite burden was also significantly lower in *Aicda*$^{-/-}$ mice compared to the WT group (Fig. 1E). These results imply that hypergammaglobulinemia contributes to disease exacerbation in *L. donovani* infected mice.

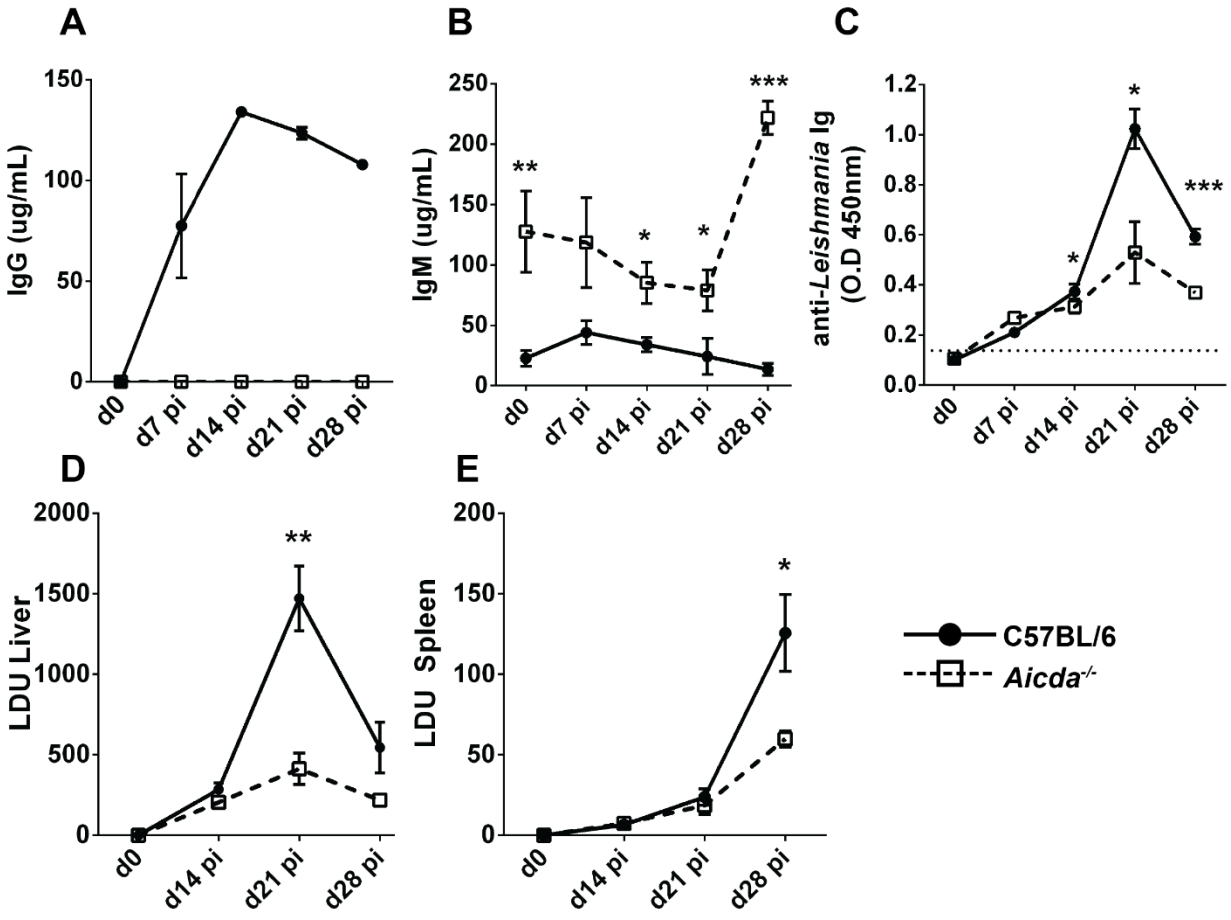


Figure 1. Hypergammaglobulinemia contributes to VL exacerbation. C57BL/6 and *Aicda*$^{-/-}$ mice were infected with *L. donovani* amastigotes and euthanized at various time points of infection. (**A–C)** Total levels of IgG (A), IgM (B), and *Leishmania*-specific Ig (C) in the sera of infected C57BL/6 and *Aicda*$^{-/-}$ mice over the course of infection were determined by ELISA. Cut off value (1.13) represented as dotted line (….) in panel C was calculated as the average value measured in C57BL/6 naïve + 3 times the standard deviation. (O.D) stands for Optical density. Liver **(D)** and splenic **(E)** parasite burden in C57BL/6 and *Aicda*$^{-/-}$ mice over the course of infection, represented as Leishman Donovan Units (LDUs). Data represent mean ± SEM of one of three independent experiments (n = 4–5 per group and time point). $*p < 0.05$, $**p < 0.01$.

4.2. Lack of AID results in stronger Th1 and severely impaired Tr1 responses following *L. donovani* infection

Because Th1 responses are required for control of *L. donovani* growth (Paun, 2011), we monitored IFN-γ and/or TNF-producing $CD4^+$ T cells over the course of infection in both groups of mice. Gating strategies and fluorescence minus one (FMO) controls are shown in supplemental figures 1A, B, and C. In WT mice $IFN\gamma^+$ $CD4^+$ T cell responses are first detected after d14p.i. and typically peak at d28p.i. (Fabie *et al.*, 2018) (Fig. 2A, left panel). In contrast, TNF single-producing $CD4^+$T cells were more frequent at d14 than at d21 and 28 p.i. (Fig. 2A, middle panel). *Aicda*$^{-/-}$ mice developed stronger IFNγ responses than the WT group (Fig. 2A, left panel) and the frequency of TNF^+ $CD4^+$ T cells declined with a delayed kinetic compared with WT mice (Fig. 2A, middle panel); despite the decline, the percentage of splenic TNF^+ $CD4^+$ T cells was still significantly higher in *Aicda*$^{-/-}$ mice. No differences between both groups were observed for IFNγ and TNF double-producing $CD4^+$ T cells (Fig. 2A, right panel).

Regulatory $CD4^+$ T cells co-producing IFNγ and IL-10 (Tr1) are thought to be the major source of IL-10 and to largely contribute to suppressing Th1 responses during chronic VL (Nylen *et al.*, 2007; Nylen & Sacks, 2007; Owens *et al.*, 2012; Ranatunga *et al.*, 2009; Stäger *et al.*, 2006). Hence, we next assessed Tr1 responses in the spleen of *L. donovani* infected mice. As previously reported (Stager *et al.*, 2006), these cells are first detected at d21 p.i. in WT mice (Fig. 2B). Although the frequency of single IL-10 producers was comparable between *Aicda*$^{-/-}$ and WT mice (data not shown), *Aicda*$^{-/-}$ mice failed to develop Tr1 cells (Fig. 2B). Taken together, our data shows that *Aicda*$^{-/-}$ mice develop stronger protective $CD4^+$ T cell responses and fail to generate Tr1 cells.

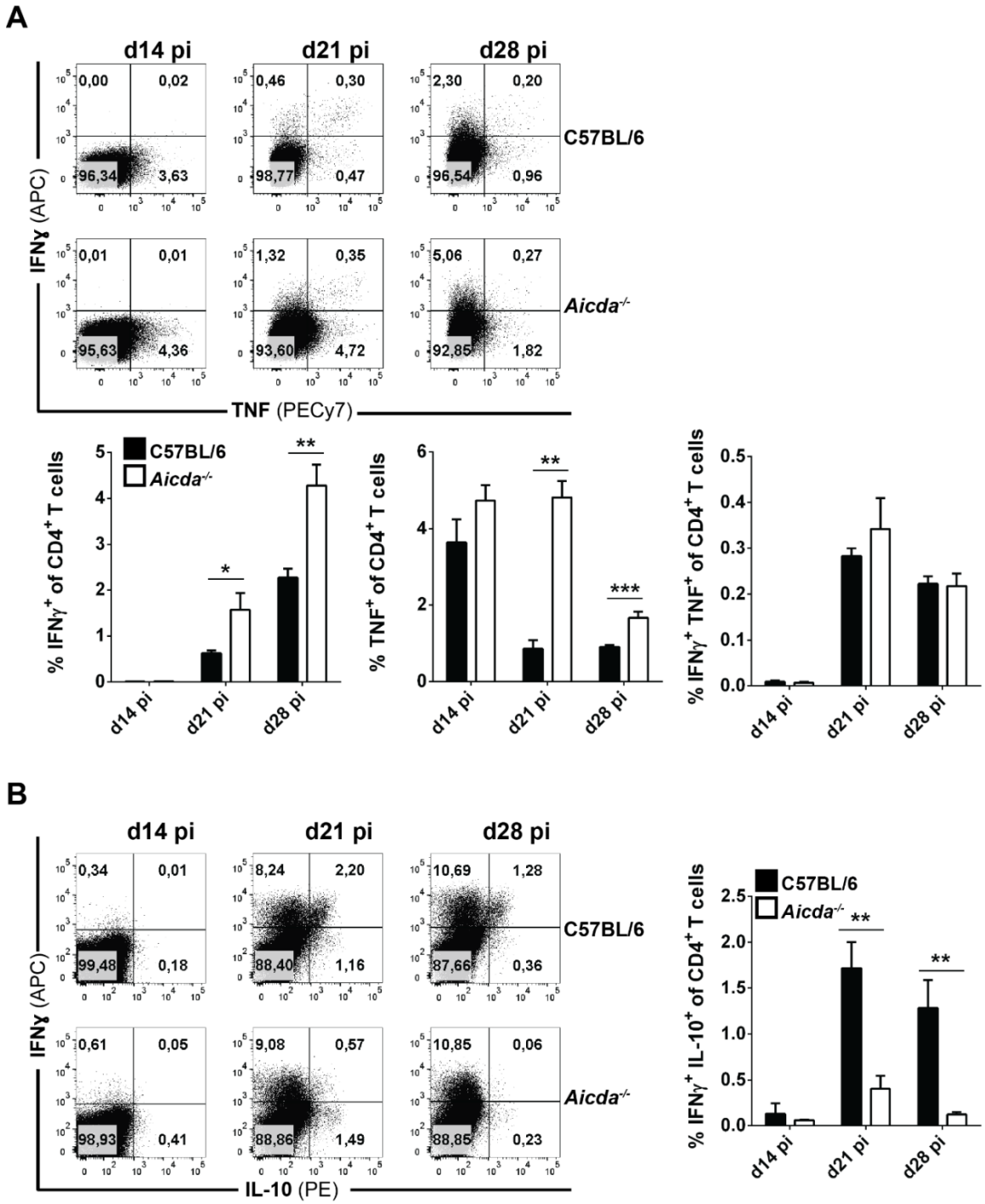


Figure 2. *Aicda*$^{-/-}$ mice develop stronger *Leishmania*-specific Th1 responses. C57BL/6 and *Aicda*$^{-/-}$ mice were infected with *L. donovani* amastigotes and euthanized at various time points of infection. Purified splenocytes were stained for CD3, CD4, IFN-γ, TNF and IL-10 after antigen-specific restimulation and analysed by flow cytometry. (A) Representative flow cytometry plots and percentages of IFN-γ$^+$ (left panel), TNF$^+$ (central panel) and IFN-γ$^+$ TNF$^+$ (right panel) CD4$^+$ T cells. (B) Representative flow cytometry plots and percentage of IFN-γ$^+$ IL-10$^+$ CD4$^+$ T cells restimulated with PMA/ionomycin. Data represent mean ± SEM of one of three independent experiments (n = 4-5 per group and time point). ∗p < 0.05, ∗∗p < 0.01, ∗∗∗p < 0.001

4.3. CD4$^+$ T cell-intrinsic AID expression does not contribute to the development of Tr1 cells

Qin et al. reported that AID can be expressed in CD4$^+$ T cells and is often associated with a particular subset that co-produces IFNγ and IL-10 (Qin *et al.*, 2011). Thus, we investigated whether the defect in Tr1 generation in *Aicda*$^{-/-}$mice was due to a T cell- intrinsic effect of AID or not. First, we determined AID expression in CD4$^+$ T cells over the course of infection. AID mRNA levels were assessed in purified splenic CD4$^+$ T cells by qPCR. An 8 fold upregulation of *Aicda* mRNA was observed at d21 p.i. compared to naïve CD4$^+$ T cells; *Aicda* mRNA expression declined thereafter to a 2-3 fold upregulation (Fig. 3A, left panel). As expected, *Aicda* mRNA expression in CD4$^+$ T cells was much lower than in B cells, where one could observe an upregulation up to 30-40 folds at d14 p.i. (Fig. 3A, right panel). To determine whether *Aicda*$^{-/-}$ CD4$^+$ T cells were able at all to differentiate into Tr1 cells, we cultured naïve CD4$^+$ T cells under Tr1-polarizing conditions. Gating strategy, purity of enriched naïve CD4 T cells and FMO controls are shown in supplemental figure 2A. The lack of AID in CD4$^+$ T cells did not affect differentiation into IFN-γ and IL-10 double producing cells in vitro (Fig. 3B); on the contrary, *Aicda*$^{-/-}$mice had a superior capacity to generate Tr1 cells and showed a greater IL-10 response (Fig. 3B). Because pathways of Tr1 induction may be different during *L. donovani* infection, we also tested the capacity of *Aicda*$^{-/-}$ CD4$^+$ T cells to differentiate into Tr1 after being adoptively transferred into WT mice a day prior to *L. donovani* infection. Gating strategy, FMO controls, and unstimulated and mock controls are shown in supplemental figure 2B. In agreement with our in vitro observation, adoptively transferred *Aicda*$^{-/-}$ CD4$^+$ T cells generated Tr1 cells at a similar frequency than endogenous WT CD4$^+$ T cells (Fig. 3C). This suggests that the lack of Tr1 generation in *L. donovani* infected *Aicda*$^{-/-}$ was not dependent on an intrinsic effect of AID in CD4$^+$ T cells, but rather on changes in the environment.

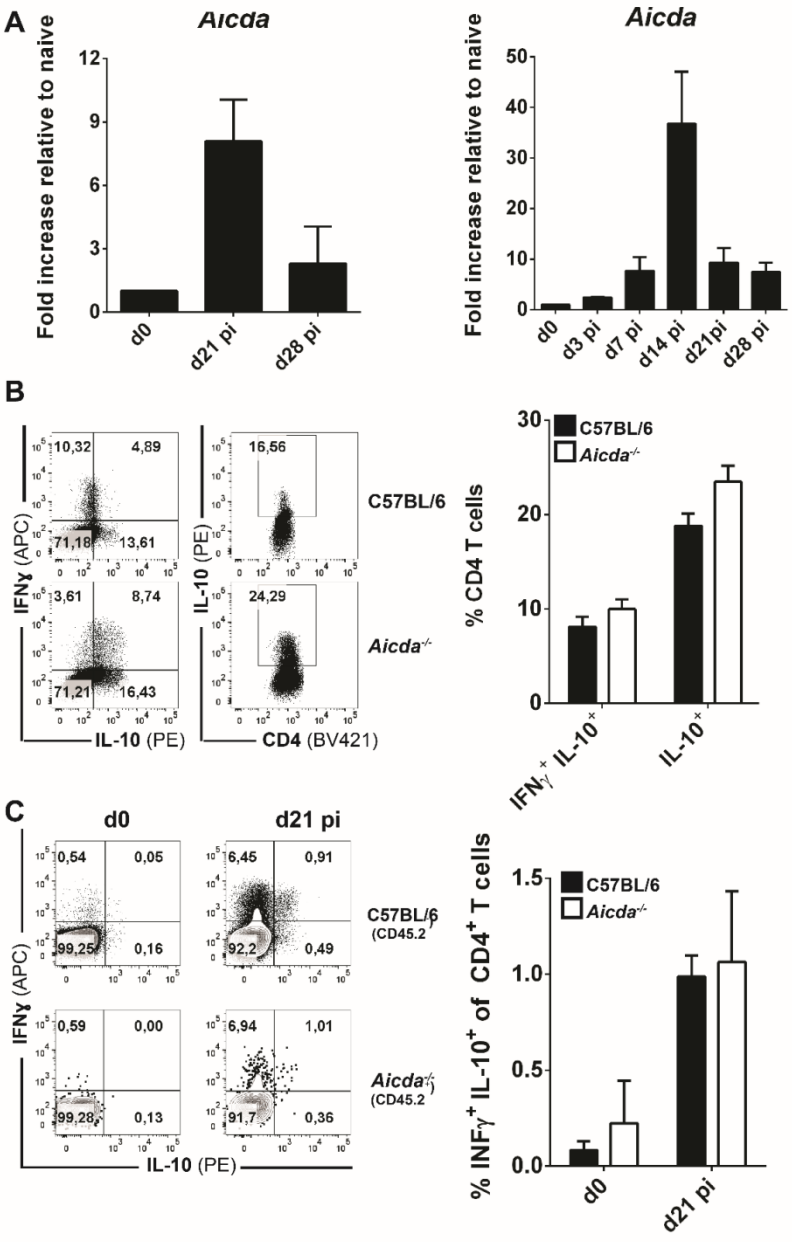


Figure 3. *Aicda* is expressed in CD4$^+$ T cell during the infection but is not required for Th1 and Tr1 responses. (**A**) C57BL/6 mice were infected with *L. donovani* amastigotes. *Aicda* mRNA expression in purified CD3$^+$ CD4$^+$ T cells (left panel) and B cells (right panel) from C57BL/6 mice was assessed by qPCR at various time points of infection. (**B**) CD3$^+$CD4$^+$ T cells were purified from naïve C57BL/6 and *Aicda*$^{-/-}$ mice. Figure shows representative flow cytometry plots for Tr1 in vitro polarization of isolated CD3$^+$ CD4$^+$ T cells from C57BL/6 and *Aicda*$^{-/-}$ mice. T cells were cultured in the presence of IL-2, anti-CD3, anti CD28 supplemented with IL-27. (**C**) A total of 2×10^4 *Aicda*$^{-/-}$ CD3$^+$CD4$^+$ T cells were adoptively transferred into congenic CD45.1- C57BL/6 mice prior to infection with *L. donovani* amastigotes. Endogenous and *Aicda*$^{-/-}$ CD4 T cells were identified by gating on CD45.2$^-$ or CD45.2$^+$ CD3$^+$ CD4$^+$ cells. Representative flow cytometry plots and percentages of IFN-γ$^+$ IL-10$^+$ CD4$^+$ T cells restimulated with PMA/ionomycin are shown. Data represent mean ± SEM of one of three independent experiments (n = 4–5 per group and time point).

4.4. B cells activation occurs in *L. donovani* infected *Aicda*$^{-/-}$ mice

Because polyclonal B cell activation and cytokine production have been associated with disease exacerbation during VL (Bankoti *et al.*, 2012; Deak *et al.*, 2010; Silva-Barrios *et al.*, 2016), we assessed B cell activation by monitoring cell surface modulation of MHCII and the costimulatory molecules CD40 (Fig. 4B and supplemental Fig. 3B), CD80 (Fig. 4C and supplemental Fig. 3B) and CD86 (Fig. 4D and supplemental Fig. 3B). The gating strategy is shown in supplemental figure 3A. Interestingly, we noticed a decline in MHCII expression in B cells from WT mice at 3 and 4 weeks after infection (Fig. 4A). This decline was significantly more pronounced in *Aicda*$^{-/-}$ mice. No differences in CD40 mean fluorescence intensity (MFI) were observed between WT and *Aicda*$^{-/-}$ mice with exception of d21p.i., when CD40 was significantly more expressed in *Aicda*$^{-/-}$ B cells (Fig. 4B). When we monitored CD80 expression, we noticed that a significantly higher CD80 MFI was found at d14, 21, and 28 p.i. in B cells from *Aicda*$^{-/-}$ mice compared to the WT group (Fig. 4C). Similar results were obtained for CD86 (Fig. 4D). This suggests that B cells are equally if not more activated in *L. donovani* infected *Aicda*$^{-/-}$ mice than in WT mice.

When we measured cytokine expression in splenic B cells purified at various time points after infection, we found no difference in the expression of IL-1β between both groups of mice (Fig. 4D). Similar results were obtained for IL-6, with exception of d28p.i., when IL-6 was slightly less expressed in B cells from *Aicda*$^{-/-}$mice compared to the WT group (Fig. 4E). In contrast, major differences were observed for IFN-β, which was more strongly upregulated in WT compared with *Aicda*$^{-/-}$ B cells (Fig. 4F). *Aicda*$^{-/-}$ B cells expressed significantly higher amounts of *Il10* mRNA at d28 p.i. (Fig. 4G).

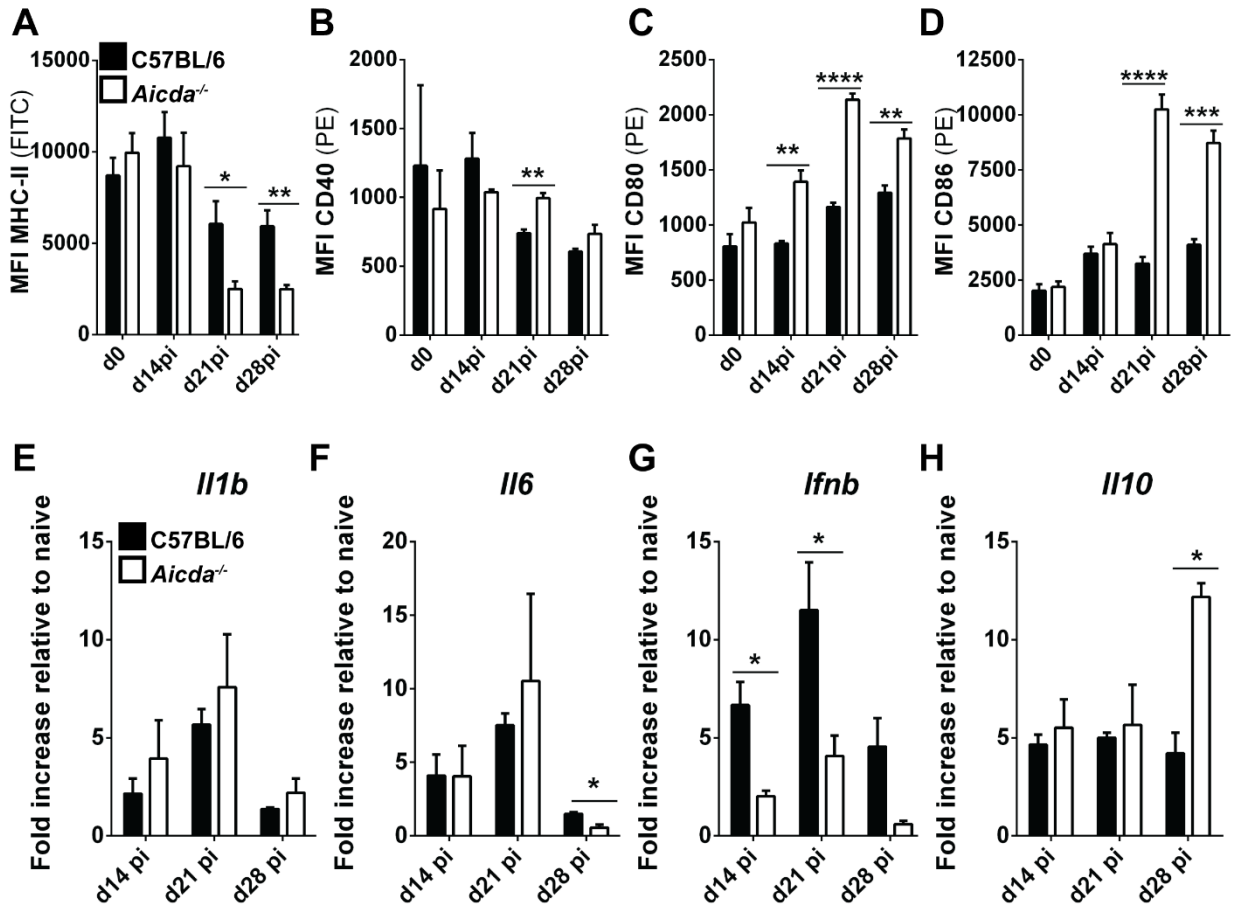


Figure 4. B cells activation occurs in *L. donovani* infected *Aicda*$^{-/-}$ mice. C57BL/6 and *Aicda*$^{-/-}$ mice were infected with *L. donovani* amastigotes and euthanized at various time points of infection. **(A-C)** Mean fluorescence intensity for CD40, CD80 and CD86 expression in gated B220+CD19+ MHCII+ B cells during *L. donovani* infection was determined by flow cytometry. Data represent mean ± SEM of one of three independent experiments (n = 4–5 per group and time point). **(D-G)** Cytokine mRNA expression in isolated B cells from infected C57BL/6 and *Aicda*$^{-/-}$ mice was assessed by qPCR. Data represent mean ± SEM of one of three independent experiments (n = 5 per group and time point). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

4.5. IFN-β and IL-10 expression are significantly reduced in *Aicda*$^{-/-}$ mice during chronic VL

Because cell recruitment could alter the splenic environment and consequently the host response to the parasite, we assessed the numbers of total splenocytes (Fig. 5A), $CD4^+$ T cells (Fig. 5B), B cells (Fig. 5C), $CD11c^+MHCII^+$ cells (Fig. 5D), and myeloid cells (Fig. 5E) in WT and *Aicda*$^{-/-}$ mice over the course of *L. donovani* infection. No major differences were observed between the two groups of mice; we only noticed a significant decrease in $CD4^+$ T cells at d21 pi in *Aicda*$^{-/-}$ compared to WT mice (Fig. 5B) and higher numbers of B cells in *Aicda*$^{-/-}$ at d14 p.i. (Fig. 5C).

The cytokine environment could also influence the development and maintenance of T cell responses. Thus, we next determined the expression of IL-6, TNF, IFN-β, and IL-10 in splenocytes at various time points of infection. Although no significant differences were observed for IL-6 (Fig. 5F), TNF was strongly upregulated in splenocytes of *Aicda*$^{-/-}$ mice during the first two weeks of infection compared to the WT group (Fig. 5G). At d28 p.i., however, *Tnf* mRNA levels in splenocytes of WT mice increased dramatically, while a decrease was noted in *Aicda*$^{-/-}$ mice starting from d21 p.i.. A similarly inverted kinetics of expression between the two groups of mice was also observed for IFN-β (Fig. 5H) and IL-10 (Fig. 5I). In *Aicda*$^{-/-}$ mice, IFN-β was mostly upregulated at the early stages of infection; in contrast, in WT mice IFN-β was highly expressed during chronic VL (Fig. 5H). TNF is not only responsible for the disruption of the splenic architecture (Engwerda *et al.*, 2002), but it also contributes to the induction of IL-10 (Ato *et al.*, 2002). In agreement with previous literature, we also noticed that IL-10 expression followed a similar kinetic than TNF (Fig. 5I). Taken together, these results suggest that hypermutated and class-switched antibodies and hypergammaglobulinemia promote IFN-β, TNF, and IL-10 expression.

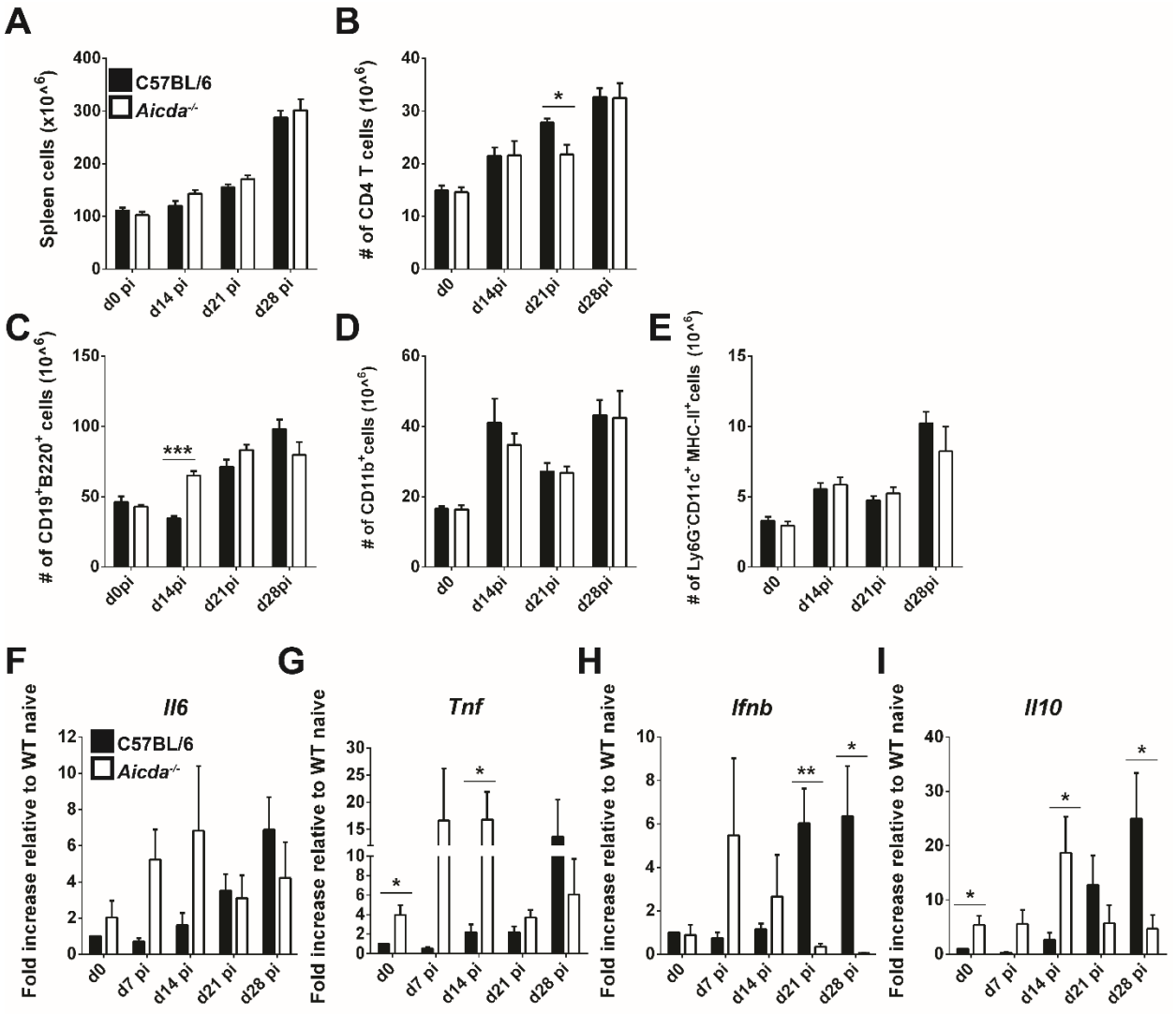


Figure 5. IFN-β and IL-10 expression are significantly reduced in *Aicda*$^{-/-}$ mice during chronic VL. C57BL/6 and *Aicda*$^{-/-}$ mice were infected with *L. donovani* amastigotes and euthanized at various time points of infection. **(A-E)** Graphs show the number of total splenocytes **(A)**, CD4^{+} T cell **(B)**, B cells **(C)**, CD11c+ MHC+ cells **(D)**, and myeloid cells **(E)** present in the spleen of both groups of mice during the course of infection analysed by flow cytometry. **(F-I)** *Il6* (**F**), *Tnf* (**G**), *Ifnb* (**H**), and *Il10* (**I**) mRNA expression in splenocytes from infected C57BL/6 and *Aicda*$^{-/-}$ mice was assessed by qPCR. Data represent mean ± SEM of two independent experiment (n = 4–5 per group and time point). $*p < 0.05$, $**p < 0.01$, $***$ $p < 0.001$

5. DISCUSSION

Hypergammaglobulinemia is a hallmark of VL and is associated with disease progression in human patients. However, the exact contribution of hypermutated antibodies and hypergammaglobulinemia to the pathogenesis of VL has not yet been explored. In the present study we show that mice unable to produce hypermutated and class-switched antibodies are significantly more resistant to *L. donovani* infection than their wild type counterpart. Resistance correlated with stronger Th1 responses and a near absence of Tr1 cells during chronic infection. Moreover, splenocytes from *Aidca*$^{-/-}$ mice expressed less TNF, IL-10 and IFN-β than C57BL/6 mice at later stages of disease.

It is well known that B cells largely contribute to the pathogenesis of experimental VL (Smelt *et al.*, 2000) and that hypergammaglobulinemia is predictive of disease progression in human. Nevertheless, the exact role of antibodies in *Leishmania*sis is not yet clear (Silva-Barrios *et al.*, 2018). Most of the studies investigating the contribution of antibodies to the immune response against *Leishmania* were conducted in B cell-deficient mice. However, B cells can regulate the immune response in many different ways besides antibody production. For instance, they can present antigen, produce cytokines, and promote T cell proliferation and effector differentiation, especially in the context of polyclonal activation (Silva-Barrios *et al.*, 2016). IgM was shown to promote disease progression in an experimental model of VL (Deak *et al.*, 2010). But, what about the role of hypermutated, class-switched antibodies and hypergammaglobulinemia? During VL, polyclonal B cell activation results in the excessive production of low-affinity, parasite-specific antibodies and in an increase in the amount of circulating immune complexes (Silva-Barrios *et al.*, 2018). Interestingly, passive transfer of circulating IgG antibodies and immune complexes in *L. infantum* infected JhD BALB/c mice results in increased susceptibility to infection (Deak *et al.*, 2010), implying that hypergammaglobulinemia may be detrimental to the host. Our results confirm this suspicion. In fact, *Aicda*$^{-/-}$, which are incapable of developing hypergammaglobulinemia, are significantly less susceptible to experimental *L. donovani* infection. Enhanced resistance to the disease was associated with stronger Th1 and nearly absent Tr1 responses.

We have previously demonstrated that B cells suppress Th1 responses (Bankoti *et al.*, 2012). This suppression was partially mediated by B cell-derived IL-10. We now show that AID-deficient mice also display stronger *Leishmania*-specific Th1 responses. Interestingly, B cells in these mice were

expressing similar or even higher levels of IL-10. Moreover, polyclonal B cell activation was detected in these mice as well and IgM levels were very high. This suggests that inhibition of protective responses most likely occurs through various pathways.

IFN-γ^{+}IL-10^{+} Tr1 cells are thought to be the major source of IL-10 during chronic VL (Maroof *et al.*, 2008) and are closely associated with disease progression in humans (Nylen *et al.*, 2007; Nylen & Sacks, 2007). When Tr1-derived IL-10 is absent or strongly reduced, enhanced Th1 responses and reduced parasite burdens are observed (Ranatunga *et al.*, 2009). In our model, Tr1 responses are detected at very low levels. A T cell-intrinsic defect of AID was not the cause for the failure of *L. donovani*- infected *Aicda*$^{-/-}$ mice to generate these responses, suggesting that the environment may be responsible. IFN-γ^{+}IL-10^{+} CD4^{+} T cells are typically induced by dendritic cells co-producing IL-10 and IL-27 (Owens *et al.*, 2012). We did not see a major difference in the expression of IL-27 in splenocytes of WT and AID-deficient mice (data not shown), but we noticed significant differences in IL-10 expression between the two groups of mice. IL-10 expression was quite low during chronic VL in *Aicda*$^{-/-}$ mice and was mainly expressed during the first two weeks of infection. In contrast, infected WT mice dramatically upregulated IL-10 mainly during chronic infection. The expression of pro-inflammatory cytokines followed a similar dynamic. *Aicda*$^{-/-}$ mice displayed a stronger pro-inflammatory profile during the first two weeks of infection compared to WT mice. IL-6, TNF and IFN-β were highly upregulated already at d7 p.i. to decline during the chronic phase. In contrast, WT mice had a stronger pro-inflammatory environment at later stages of disease. This inverse kinetic could be the key for understanding the dynamic of CD4^{+} T cell development in both groups of mice. *Leishmania*-specific Th1 responses are first detectable at d14 p.i and peak at d21-28 p.i. (Fabie *et al.*, 2018). A pro-inflammatory environment during the priming phase of disease could help promoting these responses; while a pro-inflammatory environment during peak expansion enhances tissue disruption and ultimately leads to cell death of protective responses (Fabie *et al.*, 2018). Because TNF was shown to induce IL-10 (Ato *et al.*, 2002), reduced levels of TNF in *Aicda*$^{-/-}$ mice during chronic VL would also lead to lower IL-10 responses. This would explain why Tr1 cells were hardly present in these mice. Consequently, is tempting to speculate that hypergammaglobulinemia could induce TNF, which will then promote IL-10 responses to generate Tr1 cells and suppress protective Th1 cells.

Although antigen presentation by B cells does not seem to contribute to the host immune response in *L. infantum* infected mice (Deak *et al.*, 2010), we cannot exclude the possibility that the lack of AID expression in B cells may alter BCR affinity and/or the B cell capacity to internalize antigen, which would influence the maintenance of Tr1 and/or Th1 responses.

The kinetic of IFN-I expression could also explain the differences in Th1 responses between WT and *Aicda*$^{-/-}$ mice. A recent study reports that IFN-I positively promotes post-priming accumulation of CD4^{+} T cells in a LCMV infection model (Chang *et al.*, 2017). IFN-I appears to induce expression of the glucocorticoid-induced TNFR family related gene (GITR) in monocyte-derived APCs, which on its turn upregulate OX40 and CD25, favouring therefore CD4^{+} T cell proliferation. This cytokine is hardly expressed in WT mice during the first 2 weeks of infection. In contrast, total splenocytes and B cells from AID-deficient mice upregulate IFN-I around the time when CD4^{+} T cells are primed and start expanding. If this mechanism also applies to parasitic infections, the different kinetic in IFN-I expression between the two mouse groups could also explain why *Aicda*$^{-/-}$ mice generate stronger Th1 responses. Moreover, IFN-I was also shown to impair survival of CD4^{+} T cells during chronic infection (Cheng *et al.*, 2017; Hardy *et al.*, 2007; Herbeuval *et al.*, 2005). Although we did not see a direct effect of IFN-I on CD4^{+} T cell survival in VL (Fabie *et al.*, 2018), we cannot exclude that it may indirectly cause CD4^{+} T cell death during chronic disease.

Another important function of IFN-I is the enhancement of antibody production by promotion of isotype switching (Le Bon *et al.*, 2001). We have recently demonstrated a role for IFN-I in promoting hypergammaglobulinemia during VL by directly acting on B cells (Silva-Barrios *et al.*, 2016). A similar result was obtained in an LCMV model, where IFN-I was inhibiting the production of neutralizing antibodies and promoting hypergammaglobulinemia (Daugan *et al.*, 2016b). The fact that treatment with IFN-I can lead to Lupus-like symptoms (Gota & Calabrese, 2003a) highlights the fine line between chronic infections and the development of autoimmune diseases.

Several questions still remain unanswered. The first one is about the mechanism of induction and the regulation of IFN-I expression during VL. The presence of hypermutated and class-switched antibodies seem to inhibit IFN-I production during the early stages of infection; however, the pathways involved in this are still unknown. The second question pertains to the source of IFN-I during acute and chronic infection, which is still unclear. We have shown that B cells can express IFN-I, but other cells most likely produce higher amounts than B cells. And lastly, it would be important to understand how hypergammaglobulinemia modulates TNF responses during chronic VL.

In summary, we have demonstrated that the absence of hypermutated, class-switched antibodies and hypergammaglobulinemia correlates with increased disease resistance in *L. donovani* infected mice. Antibodies appear to differentially regulate the inflammatory environment

depending on the stage of infection, influencing therefore $CD4^+$ T cell priming. A better understanding of pathways involved in the modulation of inflammation by antibodies and/or immune complexes could be beneficial for the discovery of future therapeutic targets.

6. MATERIALS AND METHODS

Mice and Parasites

C57BL/6 and congenic B6-Ly5.1 mice were purchased from The Jackson Laboratory, *Aicda*$^{-/-}$ mice were a kind gift from Dr. Alain Lamarre (INRS-Institut Armand Frappier). All mice were bred and housed at the Institut National de la Recherche Scientifique (INRS) animal facility under specific pathogen-free conditions and used at 6–12 weeks of age. Experiments involving mice were carried out under protocols approved by the Animal Care and Use Committee of the INRS, Institut Armand-Frappier. These protocols respect procedures on good animal practice provided by the Canadian Council on animal care.

Leishmania donovani (strain LV9) was maintained by serial passage in B6.129S7-*Rag1*^{tm1Mom} mice and amastigotes were isolated from the spleens of infected animals. Mice were infected by injecting $2x10^7$ amastigotes intravenously via the lateral tail vein (Stager *et al.*, 2003). Splenic parasite burdens were determined by examining methanol-fixed, Giemsa-stained tissue impression smears. Data are presented as number of parasites per spleen or as Leishman Donovan Units (LDUs) (Hammami *et al.*, 2018).

Quantitative Real-time PCR

Splenic B cells from naïve and infected mice were purified using the B cell isolation kit (Miltenyi Biotech) according to manufacturer's protocol (purity 90%–92%). Real-time PCR was used to analyze transcripts levels of *Tnf*, *Il1b*, *Il6*, *Il10*, *Ifnb*, *Aicda*, and *Hprt* genes in splenic B cells and total splenocytes. RNA was extracted using the RNeasy mini kit (QIAGEN) as described by the manufacturer. Reverse transcription was carried out using the iScript cDNA synthesis kit (Bio-Rad) following the manufacturer's protocol. Real-time PCR analysis was performed using iTaq Universal SYBR Green Supermix (Bio-Rad). The following primers were used to determine the relative gene fold expression: *Hprt*, 5'-GTT GGA TAC AGG CCA GAC TTT GTT G-3' and 5'-GATTCA ACC TTG CGC TCA TCT TAG GC-3'; *Il1b*, 5'-AAC CTG CTG GTG TGT GAC GTT C-3' and 5'-CAG CAC GAG GCT TTT TTG TTG T-3'; *Il6*, 5'-ACA ACC ACG GCC TTC CCT ACT T-3' and 5'-CAC GAT TTC CCA GAG AAC ATG TG-3'; *Il10*, 5'-AGG GTT ACT TGG GTT GCC AA-

3', and 5'-CAC AGG GGA GAA ATC GAT GA-3'; *Ifnb*, 5'-TCA GAA TGA GTG GTG GTT GC-30 and 5'-GCA CTT TCA AAT GCA GTA GAT; *Tnf,* 5'-GAC GTG GAA GTG GCA GAA GAG-3' and 5'-TGC CAC AAG CAG GAA TGA GA-3'; *Aicda*, 5'-TGC TAC GTG GTG AAG AGG AG-3' and 5'-TCC CAG TCT GAG ATG TAG CG-3'. All PCRs were carried out with the Stratagene mx3005p real-time PCR system. Data were normalized to HPRT and expressed as fold increase to naive controls.

Adoptive transfer of *Aicda* deficient CD4$^+$ T cells

CD4$^+$ T cells were enriched from splenocytes of naïve *Aicida*$^{-/-}$ mice using the negative selection CD4$^+$ T cell kit (Miltenyi Biotech) according to manufacturer's protocol. 5×10^6 isolated *Aicda*$^{-/-}$ CD4 T cells (purity 92%–94%) were injected into the lateral tail vein of congenic BL/6-Ly5.1 mice a day prior to infection (Fabie *et al.*, 2018). Animals were infected the day after with 2x10^7 *L. donovani* amastigotes.

In vitro CD4$^+$ T cell polarization

CD4$^+$ T cells were enriched from splenocytes of naïve C57BL/6 and *Aicida*$^{-/-}$ mice by negative selection using the CD4$^+$ T cell kit (Miltenyi Biotech), according to manufacturer's protocol. Tr1 cell polarization was performed as described by Ranatunga et al. (Ranatunga *et al.*, 2009). Briefly, cells were stimulated with 5 µg/ml anti-CD3 and anti-CD28 (Invitrogen), and with 2ng/ml of recombinant IL-2. For Tr1 polarization, IFN-γ and IL-4 were neutralized with 50 µg/ml anti-mouse IFN-γ and 50 µg/ml anti-mouse IL-4 **(**BD Biosciences**)**, and 50 ng/ml recombinant IL-27 (Preprotech) were added. On day 5, cells were re-stimulated for 4 h with 5 ng/ml (PMA) and 500 ng/ml Ionomycin (Sigma) in the presence of Brefeldin A (BD Biosciences). Cells were washed and stained with anti-CD4-BV421, anti-IFN-γ-APC and anti-IL-10-PE. 300,000 cells were acquired on a BD LSRFortessa cell analyzer (Becton Dickinson), and analysis was performed using FlowJo software (Tree Star).

Flow Cytometry

CD4$^+$ T cell responses in infected mice were analyzed as previously described (Hammami, 2017). Briefly, splenocytes were either restimulated with bone marrow-derived dendritic cells (BMDCs), pulsed with fixed parasites, or with 5ng/ml of phorbol 12-myristate 13-acetate (PMA), 500ng/mL of ionomycin (Sigma) in the presence of 1/1000 Brefeldin A (GolgiPlug™, BD Biosciences). For antigen-specific stimulation, 10^5 BMDC/well were pulsed overnight with fixed *L. donovani* amastigotes at a MOI 1:10 in 24-well plates. Spleen homogenates from infected mice were then

added and incubated at 37C for 4 hours. Brefeldin A was then added for further 2h before staining of surface molecules. For pharmacological stimulation, splenocytes were incubated for 4h in the presence of Brefeldin A at 37C prior to surface staining. Cells were then stained with antiCD4-fluorescein isothiocyanate (FITC), anti-CD3-BV421, followed by anti-IFN-γ-allophycocyanin (APC), and anti-IL-10-phycoerythrin (PE) (BD Biosciences) after permeabilization with 0.1% saponin. The following antibodies were used for the analysis of the adoptively transfer experiment: anti-CD3-BV421, anti-CD4-BV711, anti-CD45.2-FITC followed by anti-IFN-γ-APC and anti-IL-10-PE (BD Biosciences)

The expression of costimulatory molecules by B cells during the infection was assessed as previously described (Silva-Barrios *et al.*, 2016). The following antibodies were used: FITC-conjugated anti-major histocompatibility complex class II (MHCII) (BD Biosciences), eFluor 450-conjugated anti-CD19 (eBioscience), PE-conjugated anti-CD40 (eBioscience), PE-conjugated anti-CD80 (eBioscience), and PE-conjugated anti-CD86 (eBioscience).

Myeloid cells were analyzed by flow cytometry as previous described (Hammami, 2017). The following antibodies were used: anti-MHCII-FITC, anti-CD11c-APC, anti-CD11b-BV421, anti-Ly6C-AF700 (BD Biosciences), anti-Ly6G-PE (Biolegend), anti-F4/80-PE-Cy7 (eBioscience), 300,000- 500,000 cells were acquired on a BD LSRFortessa cell analyzer (Becton Dickinson), and analysis was performed using FlowJo software (Tree Star).

Humoral response

Sera from naïve and infected mice were analyzed by enzyme-linked immunosorbent assay (ELISA). Total IgM and IgG levels were measured using the Mouse IgM ELISA Ready-SET-Go! kit and Mouse IgG ELISA Ready-SET-Go! kit (eBioscience) following manufacturer's recommendation. *Leishmania*-specific IgG titre was determined using the protocol described by Silva- Barrios. et al.(Silva-Barrios *et al.*, 2016) Absorbance was measured at 450 nm in a microplate reader (model 680, Bio-Rad).

Statistical Analysis

Statistical analysis was performed using Student's t test. Differences were considered to be statistically significant when $p < 0.05$. All experiments were conducted independently at least three times.

7. ACKNOWLEDGEMENTS

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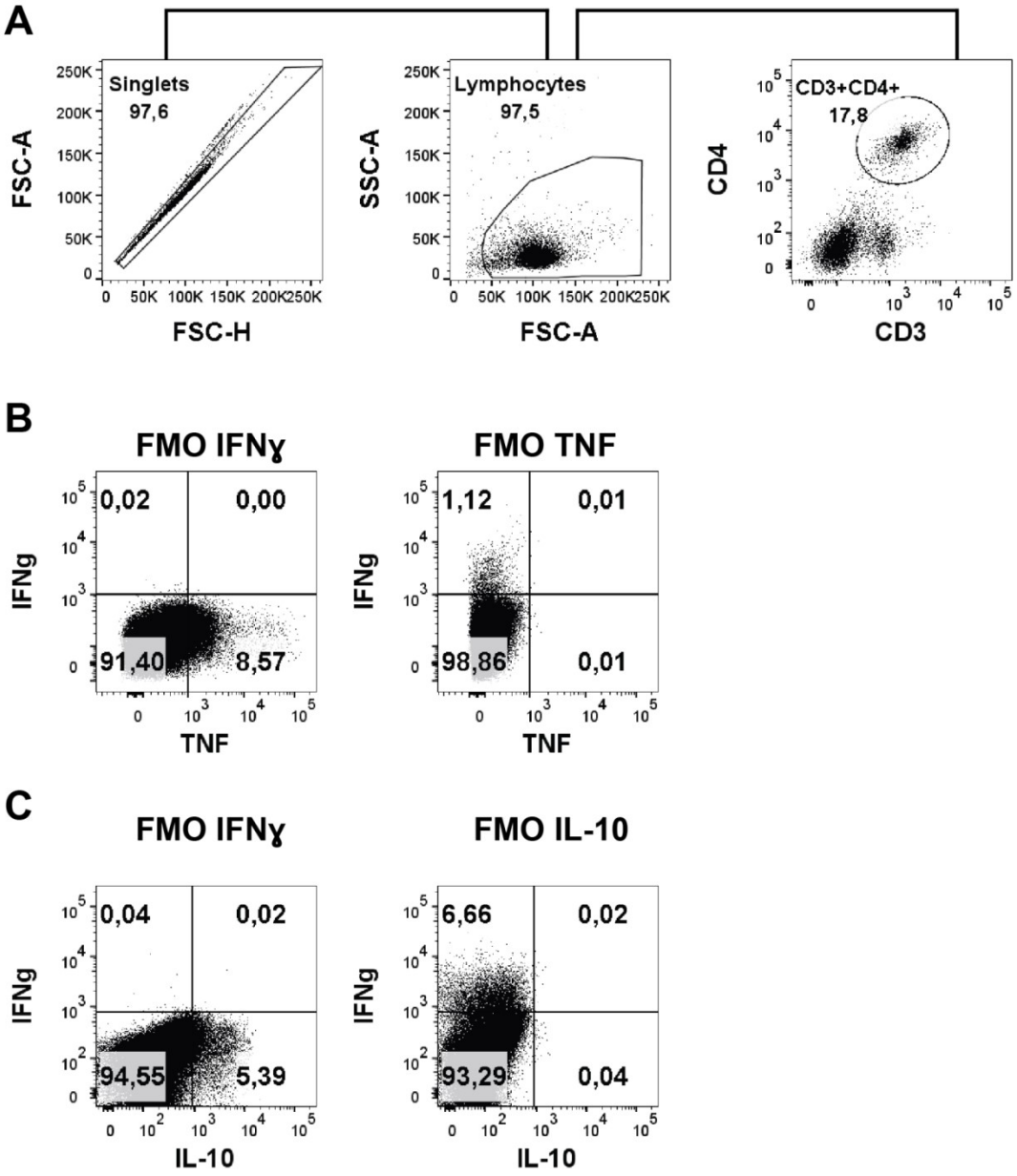
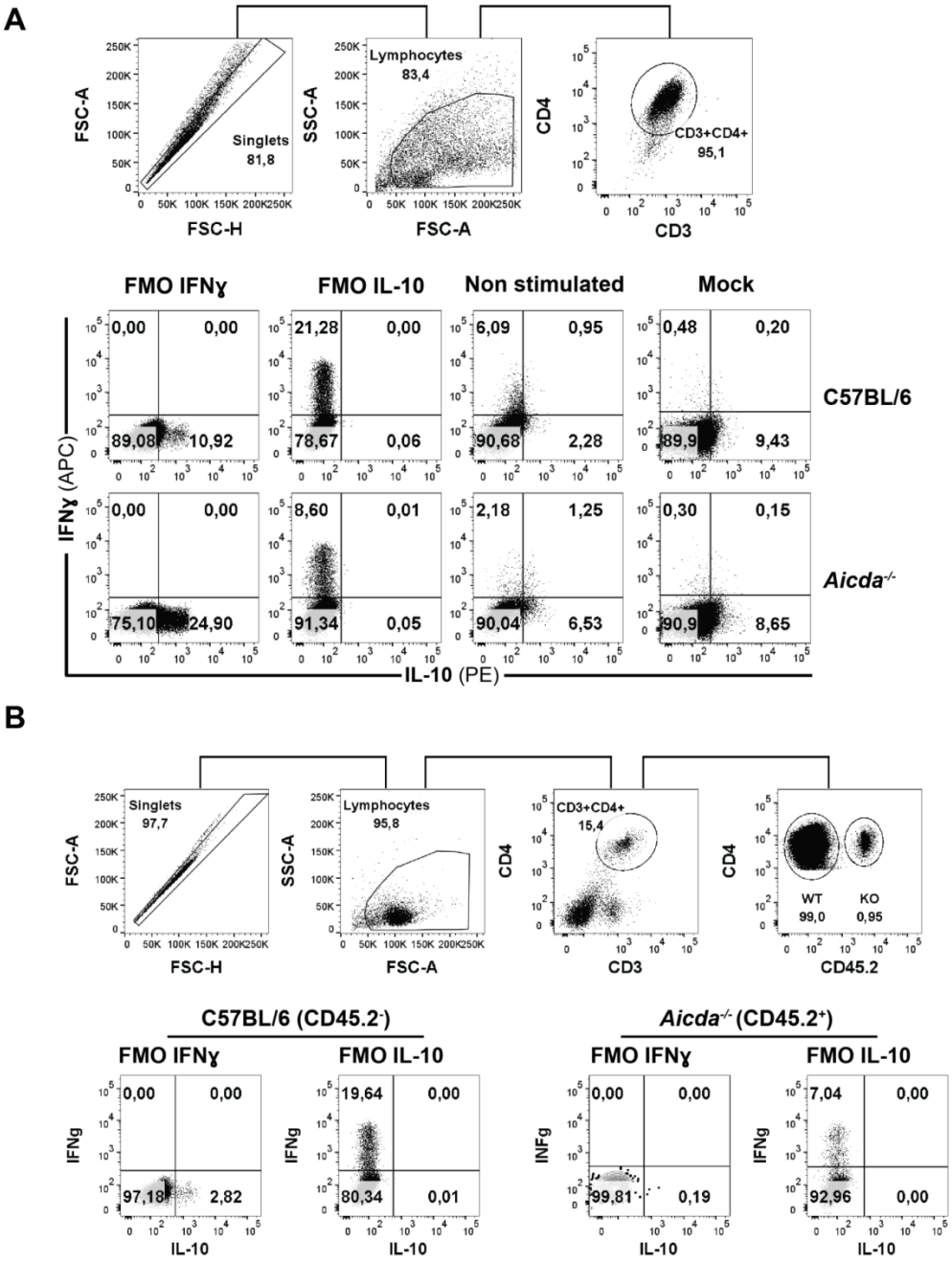
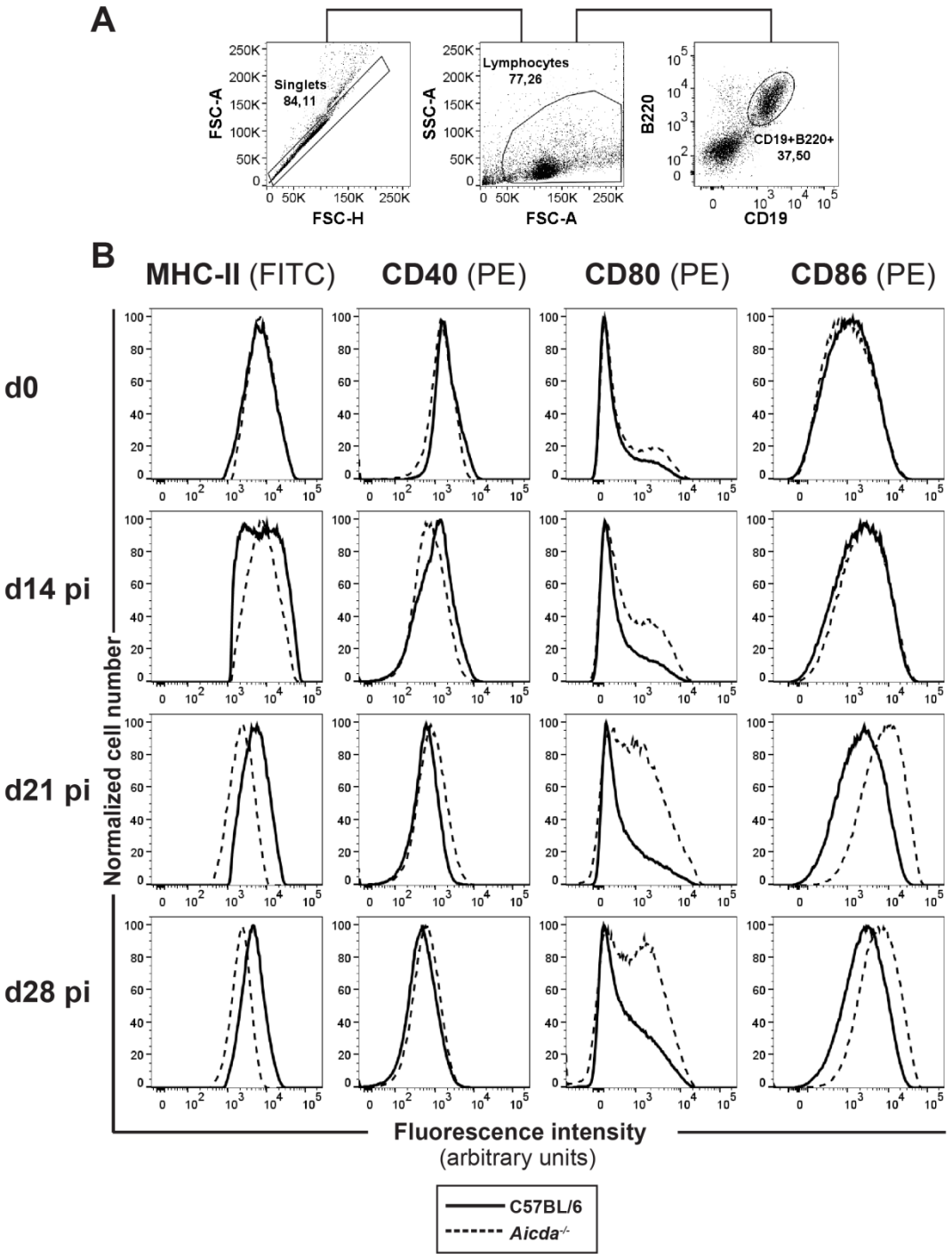
8. SUPPLEMENTAL INFORMATION



Supplemental Figure 1. (Related to figure 2)**. (A)** Representative FACS plots for gating strategy. **(B-C)** Representative FACS plots for fluorescence minus one (FMO) controls for intracellular cytokines staining. **(B)** Representatives FACS plots of IFN-γ$^+$ TNF$^+$ CD4$^+$ T cells after antigen specific restimulation. **(C)** Representatives FACS plots and percentage of IFN-γ$^+$ IL-10$^+$ CD4$^+$ T cells restimulated with PMA/ionomycin. Data represent one of three independent experiments (n = 4–5 per group and time point).



Supplemental Figure 2. (Related to figure 3). **(A-B)** Representative FACS plots for gating strategy and controls. **(A)** T cells were cultured in the presence of IL-2, anti-CD3, anti CD28 supplemented with IL-12 or IL-27. Representative FACS plots for gating strategy, fluorescence minus one (FMO) controls, and percentage of IFN-γ$^+$ IL-10$^+$ CD4$^+$ T cells not restimulated with PMA/ionomycin (but cultured in the presence of IL-27) or restimulated with PMA/ionomycin but cultured in the absence of IL-27. **(B)** A total of 2 × 10^4 *Aicda*$^{-/-}$ CD3$^+$CD4$^+$ T cells were adoptively transferred into congenic CD45.1-C57BL/6 mice before infection with *L. donovani* amastigotes Representative FACS plots for gating strategy, FMO controls, and percentage of IFN-γ$^+$ IL-10$^+$ CD4+ T cells restimulated with PMA/ionomycin. Data represent one of three independent experiments (n = 4–5 per group and time point).



Supplemental Figure 3. (Related to figure 4). **(A)** Gating Strategy to define B cells based on B220 and CD19 expression. **(B)** Representative histograms of B cells from C57BL/6 and *Aicda*$^{-/-}$ mice stained for MHCII, CD40, CD80, and CD86 at various time point of infection. Data represent one of three independent experiments (n = 4–5 per group and time point).

CHAPTER 5:
DISCUSSION AND CONCLUSION

1. DISCUSSION

This research project highlights the deleterious role of B cells in chronic infections such as VL, revealing novel immune pathways triggered by *L. donovani* in B cells. Along with this journey, we identified for the first time in a parasitic infection model signalling pathways involved in B cell activation, resulting in hypergammaglobulinemia and cytokine production. In addition, we explored the contribution of hypergammaglobulinemia to disease exacerbation during *L. donovani* infection. We found that *L. donovani* triggers endosomal TLRs in B cells inducing the expression of cytokines and the upregulation of endosomal TLRs; this pathway is dependent on IFNAR signalling and leads to IL-10 secretion, hypergammaglobulinemia, and enhanced parasite growth. In a second study, we demonstrated that hypergammaglobulinemia contributes to *L. donovani* persistence by promoting a proinflammatory microenvironment during chronic infection.

1.1. *L. donovani* induces hypergammaglobulinemia by B cells by triggering endosomal TLRs.

TLR triggering plays a critical role in the activation of interferon regulatory factors (IRFs) and the canonical NF-κB pathway, leading to the production of pro-inflammatory cytokines, IFN-I, anti-microbial proteins and chemotactic factors but also up-regulating costimulatory molecules on APCs (Mingcai *et al.*, 2009). Innate immune sensing by TLRs, the best characterized PRRs, is a field widely explored in diverse immune contexts including cell development, control and resolution of infection (Buchta & Bishop, 2014). Most of the data obtained to date is based on *in vitro* stimulation of isolated cell populations deficient in endosomal TLRs with isolated pathogen-derived material or indirectly via MyD88 signalling dependent responses. MyD88 is downstream of several TLRs but is not exclusive to TLR signalling; it can also function as an adaptor protein to the IL-1 and IL-18 receptors (Muzio *et al.*, 1997; Wesche *et al.*, 1997).

Several protozoan parasite infection models have addressed the contribution of endosomal TLRs to disease protection or exacerbation. In *Plasmodium* infection, TLR9 can be triggered by a complex of parasite-derived DNA and hemozoin (Coban *et al.*, 2010; Coban *et al.*, 2005), the by-product of hemoglobin digestion by the parasite. However, during experimental infection the contribution of TLR9 triggering is controversial. Some authors found that TLR9 deficiency increase protection against the parasite (Coban *et al.*, 2006); while others do not observe differences in

parasitemia (Lepenies *et al.*, 2008; Togbe *et al.*, 2007). In experimental toxoplasmosis, endosomal TLR triggering is required to control parasite multiplication (Andrade *et al.*, 2013). Unc93b1 deficient mice have an impaired production of protective IL-12 and IFN-γ resulting in a defective ability to control parasite burden. Endosomal TLR11 and 12 are known to sense *T. gondii* profilin. Indeed, TLR12 deficient mice are highly susceptible to the infection. (Andrade *et al.*, 2013; Koblansky *et al.*, 2013; Raetz *et al.*, 2013). Unfortunately, these TLRs are not expressed in humans. Similarly, triple TLR3-7-9 deficient mice infected with *T. cruzi* are highly susceptible to infection (Caetano *et al.*, 2011). Also, potential TLR7 ligands as guanosine- or uridine-rich single-strand RNA sequences were found by in silico analysis in the predicted parasite transcriptome (Caetano *et al.*, 2011). During *Leishmania* infection, triple TLR3-7-9 deficient mice infected with *L. major* are highly susceptible to infection (Schamber-Reis *et al.*, 2013). However, the contribution of endosomal TLRs to parasite resistance is not a general observation in *Leishmania*sis (Bankoti *et al.*, 2012; Silva-Barrios *et al.*, 2016). IL-10 derived from MZ B cells in a MyD88 dependent manner results detrimental to the host, promoting disease susceptibility (Bankoti *et al.*, 2012).

Hypergammaglobulinemia is a hallmark in most of the infectious diseases mentioned above. In this context, targeted research in B cells clarifying the role of endosomal TLRs in pathogen sensing and/or driving antibody production is limited. To date, most of the knowledge in this field was supported by data obtained from autoimmune disease models, where TLR7 and 9 in B cells contribute to antibody production (Christensen *et al.*, 2006; Han *et al.*, 2015). In addition, MyD88 in B cells was associated with IgM production and complement-mediated control of intestinal bacteria, preventing lethal dissemination of commensal bacteria during induced colonic damage (Kirkland *et al.*, 2012). T cell-dependent IgG responses in GCs have also been shown to be dependent on triggering of MyD88, TLR7 and TLR9 by virus-like particles (Hou *et al.*, 2011).

In this regard, we describe for the first time that endosomal TLRs in B cells can be directly triggered by a pathogen. In contrast to the beneficial role of pathogen sensing via endosomal TLRs in innate immune cells in various protozoan disease models, triggering of endosomal TLRs in B cells by *L. donovani* results detrimental to the host and contributes to parasite establishment. Endosomal TLR activation in B cells during VL results in cytokine secretion and contributes to hypergammaglobulinemia, promoting disease exacerbation. Interestingly, B cell-specific endosomal TLR ablation resulted in an abrogated response in antibody production (Silva-Barrios *et al.*, 2016). Various molecules from the *L. donovani* complex, including the *L. infantum* cytosolic tryparedoxin (LiTXN1), have been associated with B cell activation and enhanced expression of

IL-10 (Menezes Cabral *et al.*, 2008). However, it remains unknown which specific molecule(s) trigger endosomal TLR pathways in B cells after *L. donovani* exposure or during VL.

1.2. *L. donovani* induces cytokine expression by B cells by triggering endosomal TLRs.

In VL, one of the strategies used by *L. donovani* to promote disease exacerbation is the induction of IL-10 production. During the infection, IL-10 produced by B cells suppresses protectives Th1 responses, required for parasite control. Previous results obtained in our laboratory demonstrated that *L. donovani* induces IL-10 secretion by B cells and that IL-10 is mainly produced by MZ B cells in a MyD88-dependent manner. In this project, we found that B cells express additional cytokines including IL-1, IL-6 and IFN-I during VL. Interestingly, Fo and MZ B cells exposed to *L. donovani* showed a different cytokine expression profile (data not shown), suggesting a distinct immunoregulatory role for each B cell subset during VL. We also demonstrated that activation of endosomal TLRs and IFN-I signalling in B cells is required to induce the expression of several B cell-derived cytokines upon exposure to *L. donovani*. Indeed, B cells deficient in IFNAR or endosomal TLRs exposed to *L. donovani* and during VL failed to enhance the expression of several cytokines.

The outcome of B cell-derived cytokines can be dual, conferring protection to the host or contributing to disease exacerbation (Bankoti *et al.*, 2012; Barr *et al.*, 2010). For example, in the experimental model of *Salmonella enterica*, B cell-derived IL-6 and IFN-γ are required to enhance protective Th1 responses (Barr *et al.*, 2010). Interestingly, expression of these two cytokines was MyD88-dependant. In contrast, in experimental VL, IL-10 production by MZ B cells results detrimental to the infection (Bankoti *et al.*, 2012).

Intracellular protozoan infections require efficient Th1 responses for controlling parasite multiplication. The role of IFN-γ has been widely studied in the modulation of the cellular immune responses against protozoan infections (Kima & Soong, 2013; Schoenborn & Wilson, 2007; Sturge & Yarovinsky, 2014). However, the contribution of IFN-I to host defence against parasites is less clear. In the past few years, a growing body of literature suggests a pleiotropic function for IFN-I during protozoan infections, particularly in the innate immune response (Silva-Barrios &

Stäger, 2017). In experimental VL, we observed that production of IFN-I by B cells is dependent on endosomal TLRs and play a negative role promoting disease exacerbation and enhancing hypergammaglobulinemia. Indeed, mice with a B cell-specific deficiency in endosomal TLR or IFN-I signalling were more resistant to *L. donovani* infection than their wild-type counterpart. To date, very little is known about the function of IFN-I in VL patients. However, in agreement with our observations, microarray data obtained from PMBCs of VL patients showed a distinctive IFN-I signature and a B cell activation profile (Gardinassi *et al.*, 2016). One of the first studies exploring the role of IFN-I in experimental VL described the prophylactic treatment with synthetic dsRNA prior to *L. donovani* infection. This treatment resulted in a burst of IFN-I and in the control of the hepatic parasite burden (Herman & Baron, 1970). In CL, the contribution of endogenous IFN-I during chronic infection has been investigated using IFNAR-deficient mice (Xin *et al.*, 2010). *Ifnar*$^{-/-}$ mice infected with *L. amazonensis* developed attenuated cutaneous lesions and displayed decreased parasite levels (Xin *et al.*, 2010). The contribution of IFN-I in exacerbating protozoan infections is a fascinating subject that requires to be profoundly studied, more detailed investigation of cell-specific signalling pathways elicited by the cytokine could help to better clarify the involvement of IFN-I in the immune response. To date in experimental VL, we demonstrate that B cell-derived IFN-I plays a detrimental role, favouring parasite persistence.

Besides B cells, other immune cells might contribute to IFN-I production during *L. donovani* infection. Indeed, we observed a marked expression of IFN-I by splenocytes from infected mice during chronic experimental VL. In addition, the kinetic of IFN-I expression in the spleen during experimental VL seems to be associated with disease outcome. At early stages of the infection IFN-I appears to be beneficial, while at chronic stages it is detrimental to host immunity (Silva-Barrios & Stäger, 2019).

Also, we observed that *L. donovani* in B cells induces the expression of IL-1 and IL-6. However, the specific role of these B cell-derived cytokines in the experimental model of VL remains to be explored. IL-1α/β and IL-6 are best known to be pro-inflammatory cytokines (Apte & Voronov, 2002; Turner *et al.*, 2014). Nevertheless, they have pleiotropic characteristics (Apte & Voronov, 2002; Turner *et al.*, 2014). In experimental VL, we show that B cells express high levels of IL-1α during the infection. It was reported that IL-1α can trigger inflammatory responses by inducing IL-1β activation and production of other cytokines, such as TNF and IL-6 (Shen & Fillatreau, 2015) suggesting a possible contribution to the proinflammatory microenvironment required to establish chronic VL. Additionally, in a colorectal cancer model, IL-6 is known to influence the polarization

of macrophage towards an M2 phenotype and contributes to tumour progression (Chen *et al.*, 2018). Therefore, it is tempting to speculate that in the case of VL, IL-6 produced by B cells might contribute to M2 monocyte and macrophage polarization. M2 macrophages are more permissive to *L. donovani* infection and this is one of the niches used by the parasite to establish chronic infection (Hammami *et al.*, 2017). Also, IL-6 is known to induce regulatory capacities in T cells (Quintana, 2017) and induce Ig production by B cells (Maeda *et al.*, 2010). However, in experimental VL the role of IL-6 produced by B cells remains to be addressed.

In this research project, we identify a positive activation loop triggered in B cells by *L. donovani*. This pathway results in cytokine secretion, antibody production and involves endosomal TLRs and IFN-I signalling. However, additional mechanisms might be used by the parasite to induce cytokine secretion in B cells. Recently, research carried out in B cell-derived cytokines demonstrate that IL-10 production requires the hypoxia-associated transcription factor HIF-1α (Meng *et al.*, 2018). HIF transcription factors are enhanced by low oxygen pressures by restricting the activity of prolyl hydroxyl dioxygenase enzymes, which hydroxylate HIF-1α and HIF-2α to destabilize HIF by binding the von Hippel–Landau tumour suppressor protein (pVHL) (Kaelin Jr, 2008). In the experimental model of VL, HIF-1α upregulation and stabilization in DCs and myeloid cells exacerbates disease; nevertheless, HIF-1α is highly expressed in all splenocytes (including B cells) during the chronic stage of infection (Hammami *et al.*, 2015). It is possible that HIF-1α may modulate the expression of IL-10 and other cytokines produced by B cells together with TLR triggering by *L. donovani* in B cells. Interestingly, HIF-1α mRNA levels are upregulated in B cells at early stages of the infection (data not shown), correlating with the period in which MZ B cell-derived IL-10 suppresses T cell responses (Bankoti *et al.*, 2012). Further investigations are warranted in order to elucidate the role of HIF-1α in B cells during VL.

1.3. *L. donovani* enhances endosomal TLR expression

In addition to cytokine production, we found that *L. donovani* also upregulates TLR3, TLR7, and TLR9 expressions in B cells *in vitro* and during the infection. Interestingly, endosomal TLRs upregulation required IFN-I signalling. It is widely accepted that B cells express several TLRs and that the latter play multiple roles in B -cell differentiation and activation (Buchta & Bishop, 2014). This characteristic provides cell-intrinsic mechanisms of innate sensing that allow the regulation of adaptive immunity (Buchta & Bishop, 2014). In several infection and autoimmune models, enhanced expression of TLRs is a common feature of the innate immune response. In most of the

cases, innate immune activation contributes to the clearance of different pathogens. However, one of the strategies used by pathogens consists in hijacking TLRs signalling in order to succeed within the host. EBV, for example, activates TLR7 and TLR9 signaling and modulate the expression of the two PRRs in B cells (Martin *et al.*, 2007; Wang *et al.*, 2006), contributing to the EBV-mediated B cell transformation process that leads to viral persistence (Iskra *et al.*, 2010). Similarly, *Moraxella catarrhalis,* a common respiratory pathogen that uses B cells as a reservoir, also induces TLR9 triggering on B cells, which together with the *Moraxella* IgD binding protein induces polyclonal B cell activation. Deficient TLR9 triggering on B cells in this model leads to reduced B cell activation. Moreover, it is suggested that triggering TLR9 is a mechanism that favours bacteria internalization by B cells by enhancing BCR expression (Jendholm *et al.*, 2009; Singh *et al.*, 2012).

Activation of TLR pathways in B cells not only induces cytokine expression, but also modulates the humoral response (Buchta & Bishop, 2014). In the SLE model, for example, TRL7 and TLR9 are involved in enhancing autoantibody production (Christensen *et al.*, 2006; Han *et al.*, 2015). In this model, a positive feedback loop involving IFN-I and TLR7 occurs (Green *et al.*, 2009). We have shown for the first time that this regulatory loop also applies to chronic infections. Indeed, we observed that *L. donovani* amastigotes induced IFN-I expression in B cells in an endosomal TLR-dependent manner and that IFN-I was involved in a positive regulatory loop that upregulated endosomal TLR and IL-10 expressions, and promoted the secretion of high levels of IgG. Mice with a B cell-specific IFNAR ablation failed to develop hypergammaglobulinemia following *L. donovani* infection. Interestingly, IFN-I was also shown to promote hypergammaglobulinemia in the experimental model of chronic lymphocytic choriomeningitis virus (LCMV) (Daugan *et al.*, 2016a). However, it is still not known whether endosomal TLRs are involved or not in this model. Nevertheless, these observations suggest that high levels of IFN-I during the chronic stage of disease promote hypergammaglobulinemia and that this might be a common signature of chronic inflammatory environments.

1.4. Hypergammaglobulinemia contributes to the establishment of chronic infection

One of the most striking observations related to the biology of B cells in VL is the fact that reduced antibody levels correlate with decreased susceptibility to *L. donovani* (Silva-Barrios *et al.*, 2016),

mice with specific ablation of endosomal TLRs or IFNAR signalling in B cells failed to develop hypergammaglobulinemia and were more resistant than WT controls (Silva-Barrios *et al.*, 2016). During VL, polyclonal hypergammaglobulinemia is characterized by the presence of high levels of IgG with low-affinity against the parasite, which increasing the probability to develop autoimmunity (Louzir *et al.*, 1994). Nevertheless, high-affinity, *Leishmania*-specific antibodies do not seem to be produced at chronic stages of VL, despite the presence of hypergammaglobulinemia. In the macaque model for VL, the striking reduction in TFh cells affects the outcome of high-affinity antibodies during the chronic stage of infection (Rodrigues *et al.*, 2014). Additionally, it was also reported that IgG immune complexes promote disease exacerbation by enhancing IL-10 production in macrophages (Miles *et al.*, 2005). Unfortunately, the research exploring the role of antibodies during *L. donovani* infection is limited. To this end, we investigated the contribution of hypermutated antibodies and hypergammaglobulinemia to disease pathogenesis in the experimental model of *L. donovani* infection. As expected, we found that AID-deficient mice, a mouse model in which B cells cannot generate genetically diverse high-affinity antibodies (Muramatsu *et al.*, 2000), do not develop hypergammaglobulinemia. This was associated with a significant reduction in the splenic and hepatic parasite burden. Our results suggest that hypergammaglobulinemia, a hallmark of VL and other chronic diseases, promotes disease progression. However, how does hypergammaglobulinemia exacerbate the disease?

1.5. **Hypergammaglobulinemia modulates T cells responses in VL**

To our surprise, we found that *Aicda*$^{-/-}$ mice developed stronger IFN-γ^{+} CD4^{+} T cell responses, compared to their WT counterpart. Hence, hypergammaglobulinemia appears to inhibit protective Th1 responses during chronic infection. This observation is in agreement with our first study, in which chimeric mice with a specific ablation of endosomal TLRs failed to develop hypergammaglobulinemia and showed stronger parasite-specific Th1 responses (Silva-Barrios *et al.*, 2016). *L. donovani* parasites at chronic stages inhibit Th1 responses mostly by inducing IL-10. Tis cytokine protects tissues from inflammation-mediated damage, but at the same time it represses protective responses against the parasite. The most important source of IL-10 during chronic VL are CD4^{+} T cells that co-produce IFN and IL-10, also known as Tr1 cells (Stäger *et al.*, 2006). Extensive research on Tr1 cells has demonstrated the importance of this T cell population in maintaining immune tolerance or limiting overt inflammation in the context of infections, autoimmune disease, or transplantation (Zeng *et al.*, 2015). Interestingly, the frequency of Tr1

cells was drastically decreased in *L. donovani* -infected AID-deficient mice. The role AID has been mainly characterized in B cells. Research elucidating AID function in T cells is limited. However, it was reported that $CD4^+$ T cells co-producing IFN-γ and IL-10 have enhanced AID expression in ageing mice (Qin *et al.*, 2011). Surprisingly, we found a significant upregulation of AID expression in $CD4^+$ T cells at chronic stages of infection. Interestingly, AID expression in $CD4^+$ T cells correlated with the peak of Tr1 responses during the infection, suggesting that Tr1 cells during VL might express AID. To date, the role of AID in T cells remains unknown. Besides the enzymatic role as deaminase, AID has been shown to be involved in epigenetic modulation in B cells (Casellas *et al.*, 2016; Teater *et al.*, 2018). Consequently, it is tempting to speculate that AID might have a similar function in T cells and would modulate effector T cell plasticity. However, in our model we found that the absence of Tr1 cells in *L. donovani* infected *Aicda*$^{-/-}$ mice was not dependent on an intrinsic effect of AID in $CD4^+$ T cells, suggesting that changes in the splenic cytokine environment in infected *Aicda*$^{-/-}$ mice were responsible for this phenomenon. IL-27 and IL-10 were reported to be crucial for Tr1 differentiation (Owens *et al.*, 2012). However, we did not observe any difference in terms of IL-27 expression levels in total splenocytes between *Aicda*$^{-/-}$ deficient and wildtype mice during VL. This was not the case for IL-10, IL-6, IFN-I, and TNF. Remarkably, IL-10 expression by splenocytes of *Aicda*$^{-/-}$ is significantly decreased compared to wildtype mice at chronic stages of the infection. Differences in the kinetic of cytokine expression could explain the generation of stronger Th1 responses in knock-out mice. Therefore, hypergammaglobulinemia could shape protective T cell responses by changing the immune microenvironment.

1.6. Hypergammaglobulinemia promotes a splenic microenvironment that favour parasite persistence

The immune microenvironment can determine the effector T cell lineage fate. Indeed, effector T cell responses are shaped by a wide gradient of cytokines and chemokines that varies between the stage of the infection where the T cell priming occurs (Caza & Landas, 2015). Interestingly, the kinetic of cytokine expression differed between *L. donovani*-infected *Aicda*$^{-/-}$ and their WT counterparts. This difference could partly explain why stronger Th1 responses are observed in *Aicda*$^{-/-}$ mice. During the first two weeks of infection, *Aicda*$^{-/-}$ mice exhibited a stronger splenic pro-inflammatory microenvironment, characterized by the upregulation of IFN-I, IL-6, and TNF. Cytokine expression decreased thereafter during chronic infection, in contrast to WT mice, where

pro-inflammatory cytokines were mainly expressed at chronic stages of the disease. In experimental VL, priming of $CD4^+$ T cells occurs during the first 14 days of infection; at this time, AID-deficient but not WT mice express high levels of pro-inflammatory cytokines. It is thus possible that priming of Th1 responses is more effective in AID-deficient mice, namely in the absence of hypergammaglobulinemia.

IFN-I is known to be required for $CD8^+$ T cell priming in the context of numerous viral infections (Snell *et al.*, 2017). This cytokine also appears to be beneficial for the development of $CD4^+$ T cell responses. A recently study, performed in the chronic model of LCMV, has shown that IFN-I signalling can promote post-priming accumulation of $CD4^+$ T cells (Chang *et al.*, 2017). The splenic cytokine microenvironment in *L. donovani*-infected *Aicda*$^{-/-}$ mice is characterized by the early expression of IFN-I during the first two weeks of infection, exactly when $CD4^+$ T cell priming occurs. Hence, it is possible that this early IFN-I expression in the spleen of AID-deficient might promote the development of protectives $CD4^+$ T cell responses. In contrast to AID-deficient mice, in WT mice, the splenic cytokine profile is characterized by enhanced expression of IFN-I at chronic stages of disease. It was reported that in some models of persistent viral infections, the suppression of de novo Th1 generation is mediated by IFN-I produced al chronic stages (Osokine *et al.*, 2014). Hence, it is possible that IFN-I contributes to suppressing Th1 cells during chronic VL. We demonstrate that B cells are not a relevant source of IFN-I that drastically affect protective Th1 responses directly. Since chimeric mice with a B cell-specific IFNAR ablation infected with *L. donovani* failed to express enhanced levels of IFN-I and protective Th1 responses in this model were not greatly improved at chronic stages of VL. This observation suggest that the relevant source of IFN-I might be a different immune cell rather than B cells. A wide range of cells can produce IFN-I including plasmacytoid DCs, NK cells, T-cells, macrophages/monocytes, fibroblasts, endothelial cells among others (Silva-Barrios & Stäger, 2017). Specific cell ablation of IFN-I pathways might help to clarify relevant sources of IFN-I during VL.

Another way by which the environment may interfere with the development of $CD4^+$ T cell responses is through TNF. TNF is largely responsible for the disruption of the splenic architecture that occurs during chronic VL (Engwerda *et al.*, 2002). Interestingly, during chronic stages of VL, TNF expression by splenocytes is enhanced in wildtype mice compared to *Aicda*$^{-/-}$ mice that showed a decreased TNF expression at later stages of infection. This observation implies that splenic architecture might not be significantly affected in mice that fail to develop hypergammaglobulinemia. Recently, our laboratory demonstrated that Th1 cells undergo cell

death during the chronic stage of VL. IFN-γ^{+} CD4^{+} T cells are sensitized to cell death in VL and upregulate the death receptor 5 and caspase 8. The TLR7-IRF5- signalling axis, mostly triggered by apoptotic material, drives this phenotype. At chronic stages of VL, disruption of the splenic architecture contributes to the release of apoptotic material that then suppresses protective Th1 responses. Changes in the splenic microenvironment associated with the absence of hypergammaglobulinemia might result in reduced tissue disruption, leading to a lower amount of apoptotic material being released and ultimately to a better survival of protective Th1 responses.

Besides, splenic tissue disruption during chronic experimental VL, TNF has also been associated with the induction of IL-10 (Ato *et al.*, 2002). In agreement, we observed that *Aicda*$^{-/-}$ mice not only expressed decreased levels of splenic TNF but also of IL-10 during chronic VL, suggesting that hypergammaglobulinemia contributes in this way to promote disease exacerbation. However, it is still unknown how antibodies can affect TNF expression at chronic stages of VL; this mechanism remains to be elucidated.

Hypergammaglobulinemia promotes the formation of immunocomplexes, and human and experimental VL are no exception to this. During VL, a progressive development of high levels of antibodies that are unable to recognize and develop an effective immune response against *L. donovani* favours not only the formation but also the accumulation of Ig complexes. In *Leishmania* infection, Ig complexes are known to be detrimental for the host, mainly by reducing the leishmanicidal capacities of macrophages (Miles *et al.*, 2005). Even though hypergammaglobulinemia is absent in infected *Aicda*$^{-/-}$ mice, IgM levels were very high. In our model, parasite multiplication does not appear to be promoted by increased IgM levels.

A previous study conducted in the experimental model of intradermal-*L. infantum* infection showed that IgM promotes disease exacerbation (Deak *et al.*, 2010). In our model, *Aicda*$^{-/-}$ mice infected with *L. donovani* produce high levels of IgM at chronic stages of VL. However, we did not observe a detrimental impact caused by the enhanced levels of this Ig isotype during the infection. IgG isotypes and allotypes are known to have diverse roles in the outcome of antibody dependent immune responses**.** In VL patients, hypergammaglobulinemia is constituted mainly by IgG1 isotype; while in experimental VL, IgG1 and IgG2a are the predominant isotypes (Anam *et al.*, 1999; Honoré *et al.*, 1998). Interestingly, IgG1 have a higher affinity for the FcγRIIIa receptor compared to IgG2a; of note, classical ADCC results from the crosslinking of FcγRIIIa (Wu *et al.*, 1997). This observation suggests that antibody-dependent immune responses during VL might be

associated with predominant isotypes of antibodies produced during the infection. Also, IgG1 is known to induce highly proinflammatory immune responses (Lu *et al.*, 2018). In VL, sustained inflammation contributes to splenic disruption and promotes disease exacerbation at chronic stages of infection. In addition, TLR9 triggering is known to induce Ig class switching to IgG2a, IgG2b, and IgG3 isotypes [66, 67]. In this regard, it will be interesting to determine which Ig isotypes were mainly affected by the specific B cell ablation of endosomal TLRs and the immune consequences during VL.

Interestingly, in bacterial infections IgG1 induces the production of TNF and IL-6 in monocytes (Hussain *et al.*, 2001). In *L. mexicana* infection, IgG1 induces detrimental IL-10 secretion by macrophages. IgG1-deficient mice infected with *L. mexicana* are more resistant to infection (Chu *et al.*, 2010). Hence, it is possible that during VL IgG1 enhances TNF and IL-10 secretion. Additionally, triggering of B cell endosomal TLRs by *L. donovani* might promote the secretion of a distinctive profile of antibody isotypes. Recently, it has been observed that restriction of oxygen in lymphoid organs modulates humoral immunity. B-cell-specific depletion of pVHL leads to constitutive HIF stabilization. This results in decreased numbers of antigen-specific GC B cells and early memory cells. Also, B-cell-specific depletion of pVHL limited antibody class switching and generation of high-affinity IgG, affecting antibody responses (Cho *et al.*, 2016). This suggests a possible participation of HIF-1α in promoting hypergammaglobulinemia.

Polyclonal B cell activation is a common symptom across several protozoan parasites infections, like in the case of *Trypanosoma cruzi*, a member of the *Trypanosoma*tids family like *Leishmania*. Hypergammaglobulinemia is a hallmark of the experimental and human *T. cruzi* infection and has been associated with BAFF expression, a key cytokine in B cell survival. In experimental *T. cruzi* infection BAFF contributes in part to the development of hypergammaglobulinemia and autoantibodies (Bermejo *et al.*, 2010). Interestingly, we observed an enhanced BAFF expression by splenic B cells at chronic stages of infection (data not shown). Additionally, BAFF is known to induce SHM and antibody secretion by B cells (Hendricks *et al.*, 2018). Moreover, enhanced TLR and BCR signalling promotes AID upregulation in autoreactive B cells (Kuraoka *et al.*, 2017). In our model, mRNA AID and endosomal TLRs expression by B cells were enhanced at chronic stages of VL. However, the mechanism involving BAFF, TLRs, and AID mediating antibody production and promoting hypergammaglobulinemia remains to be evaluated in VL.

2. CONCLUSION: Hypergammaglobulinemia induced by innate B cell activation is a common strategy of various chronic diseases

An impaired B cell response leads to the development and progression of a wide range of pathologies characterized by a detrimental immune response associated with the secretion of antibodies and immunomodulatory cytokines or antibody-mediated functions. To date, the underlying mechanisms that account for B cell-dependent disease exacerbation remain poorly understood. However, the current study gives new insights into the detrimental role of B cells in a chronic infection model, underlying mechanism that promote B cell activation and contribute to disease progression. We provided evidence for the requirement of innate B cell activation during VL to promote disease exacerbation (Figure 1). Indeed, B cell triggering of innate pathways involving endosomal TLRs and IFN-I leads to cytokines secretion and hypergammaglobulinemia. Also, we observed that IFN-I signalling enhances endosomal TLRs expression and cytokine secretion during VL. In addition, we demonstrated that hypergammaglobulinemia support the establishment of an inflammatory microenvironment at chronic stages of *L. donovani* infection that support parasite multiplication. We also showed that hypergammaglobulinemia promotes suppressive Tr1 responses**.** Innate immune activation, mediated by the TLR-IFNAR axis and leading to antibody production, is a shared mechanism across different types of chronic diseases, including parasitic and autoimmune diseases (Christensen *et al.*, 2006; Green *et al.*, 2009; Han *et al.*, 2015; Silva-Barrios *et al.*, 2016). Further studies aimed at understanding how antibodies modulate the inflammatory microenvironment might provide new insights for the development of novel therapeutic interventions.

In experimental VL, the role of TLR-mediated cytokine production by B cells, besides IL-10 and IFN-I, remains to be addressed. Identification of the specific endosomal TLRs involved in driving impaired B cell responses during VL is a field that requires extended investigation. A better understanding of the impact of each endosomal TLR might bring insight for targeted therapeutic approaches. Also, it remains unknown which *L. donovani* specific molecule(s) triggers upstream endosomal TLR pathway in B cells. Characterization of interactions between B cells and *L. donovani*, and identification of *L. donovani*-derived- molecules able to activate B cells will provide new insights that will contribute to clarifying the detrimental role of B cells in the pathogenesis of VL.

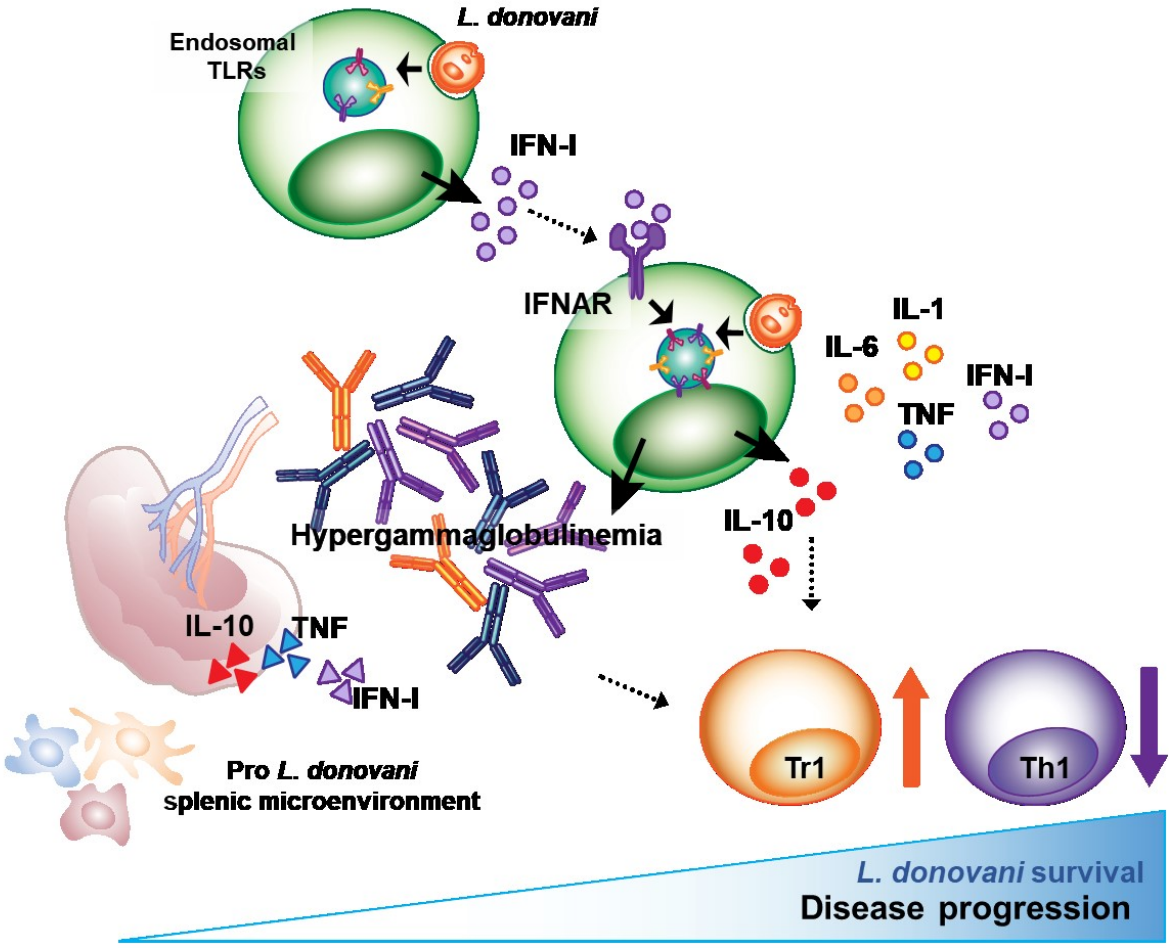


Figure 1. Detrimental B cell responses during experimental Visceral *Leishmania*sis. B cell activation by *L. donovani* results in disease exacerbation. We described a novel innate immune activation mechanism triggered by *L. donovani* in B cells leading to cytokine secretion and inducing hypergammaglobulinemia. We found that *L. donovani* triggers endosomal TLRs in B cells, inducing cytokines and endosomal TLR expression, which are further enhanced by autocrine signalling through the type I IFN receptor promoting hypergammaglobulinemia and exacerbating VL. MZ B cell-derived IL-10 is known to suppress protective Th1 responses during the infection. However, the specific role of IL-1, IL-6 and TNF produced by B cells during the infection remains unknown. Moreover, hypergammaglobulinemia promotes the establishment of a microenvironment that favours parasite persistence by enhancing suppressive Tr1 responses, therefore contributing to chronic disease progession.

CHAPTER 6:
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ANNEX

ANNEX-I: Review NO.1

PROTOZOAN PARASITES AND TYPE I IFNS

Protozoan parasites and type I IFNs

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1. ABSTRACT

For many years, the role of IFN-I has been characterized primarily in the context of viral infections. However, regulatory functions mediated by IFN-I have also been described against bacterial infections and in tumor immunology. Only recently, the interest in understanding the immune functions mediated by IFN-I has dramatically increased in the field of protozoan infections. In this review, we discuss the discrete role of IFN-I in the immune response against major protozoan infections: *Plasmodium*, *Leishmania*, *Trypanosoma*, and *Toxoplasma*.

2. INTRODUCTION

Innate and adaptive immune responses are key factors in the control of infectious and chronic diseases; the balance between these two systems is mainly orchestrated by cytokines. Interferons (IFNs) are a large family of cytokines that were first discovered in 1957 in the context of viral infections. The name interferon is due to the capacity of these anti-viral factors to interfere with viral replication in mammalian cells (Isaacs & Lindenmann, 1957). Numerous studies have been carried out since their discovery, which allowed the identification of several related molecules. Based on their structural characteristics and the restricted affinity by the receptor molecule with which they directly interact, IFN are classified into three main groups: Type I (IFN-I), type II (IFN-II) and the recently identified type III IFN (IFN-III) (Platanias, 2005).

The IFN-I family includes two main classes of related cytokines: IFN-α, which comprises 13 different subtypes encoded by 13/14 different genes; and IFN-β, a product encoded by a single gene and a group of other less studied IFNs (IFN-ε, IFNδ, IFNκ, IFNτ, IFNω)(Platanias, 2005). The ability to produce and respond to IFN-I is distributed in a wide variety of cells. This confers several autocrine and paracrine effects that have been extensively characterized mainly in viral infections. IFN-I signaling is mediated through a common cell surface receptor, the IFN-I receptor (IFNAR) (de Weerd & Nguyen, 2012) (Trinchieri, 2010).

The IFN-II family is represented by a single gene product, IFN-γ, and is mainly produced by T lymphocytes and Natural Killer (NK) cells. IFN-II responses are mediated by the binding of IFN-γ to a heterodimeric molecule, the IFN-γ receptor (IFNGR), ubiquitously expressed in a wide range of cells. IFNGR is involved in the modulation of different cell functions and is a key factor for host defence to intracellular pathogens in various infection models (Schoenborn & Wilson, 2007).

Finally, the IFN-III family, also known as IFN-λ, comprises 4 different subtypes: IFN-λ1, IFN-λ2, IFN-λ3 and IFN-λ4. The members of this novel IFN family interact through a unique receptor, the IFN-λ receptor (IFN-λR). In contrast to IFNAR and IFNGR, the expression of IFN-λR is mainly restricted to cells of epithelial origins. The role of IFN-III has yet to be better characterized; however, they appear to induce similar responses to IFN-I (Egli *et al.*, 2014).

The crosstalk between IFNs and their specific receptors elicits an intracellular signaling cascade that mainly enhances inflammatory responses. The well-characterized signaling cascades of IFN-I and IFN-II are fairly similar. In both cases Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2), associated to IFNAR and IFNGR, are activated. This results in activation and following formation of a heterodimer complex comprised by the cytoplasmic transcription factor signal transducer and activator of transcription 1 and 2 (STAT1/STAT2). STAT1/STAT2 dimers can be translocated to the nucleus and interact with the IFN regulatory factor 9 (IRF9) to form the IFN-stimulated gene factor 3 complex (ISGF3), leading to the transcription of IFN-stimulated genes (ISGs). In contrast, IFN-II signaling through IFNGR activates the JAK/STAT pathway leading to the transcription of pro-inflammatory targets downstream of γ-activated sequences (GAS) (Ivashkiv & Donlin, 2014a; Platanias, 2005).
IFN-I production is mainly induced in response to the activation of receptors on the membrane and/or cytosol, such as pattern recognition receptors (PRRs). PRRs can be activated by conserved pathogens component and endogenous molecules. In most of the cases, the production of IFN-I is related to the activation of PRRs that recognize xenogeneic or autologous nucleic acid, such as Toll-like receptors (TLRs) (Mogensen, 2009).

IFN-I are historically best known for their capacity to elicit antiviral responses; however they also play a role in bacterial infections and autoimmune diseases (Trinchieri, 2010). The role of IFN-I in regulating the immune response against pathogens is fairly complicated. IFN-I can have enhancing or suppressive effects depending on the disease, the stage of infection, and the amount produced. For instance, IFN-I enhances the antigen-presenting capacity of DCs (Ito *et al.*, 2001; Le Bon *et al.*, 2003; Montoya *et al.*, 2002), favours the development of T cell responses (Aichele *et al.*, 2006; Kolumam *et al.*, 2005; Marrack *et al.*, 1999), and promotes antibody responses (Fink *et al.*, 2006; Lund *et al.*, 2004b) during acute viral infections. In contrast, type-I IFNs play an immunosuppressive role during chronic viral infections (Ng *et al.*, 2015; Teijaro *et al.*, 2013; Wilson *et al.*, 2013), reduce IFN-γ responsiveness in macrophages (Kearney *et al.*, 2013; Rayamajhi *et al.*, 2010), block B cell functions at high concentrations (Fallet *et al.*, 2016; Moseman *et al.*, 2016),

and can promote the expression of immunosuppressive factors such as IL-10 and PDL-1 (Carrero *et al.*, 2006; Mayer-Barber *et al.*, 2011; McNab *et al.*, 2015; McNab *et al.*, 2014). This duality is also observed in the context of
autoimmune diseases, where IFN-I plays a pathogenic role in systemic lupus erythematosus and Sjogren's syndrome (Emamian *et al.*, 2009; Ko *et al.*, 2012), wheras it has therapeutic effects in multiple sclerosis (Javed & Reder, 2006).

While IFN-γ has been widely characterized in the modulation of the immune response against protozoan infections, the contribution of IFN-I to host defence against parasites is less clear. In the past few years, a growing body of literature suggests an important role for IFN-I during protozoan infections, particularly in the innate immune response.
In this review, we provide a brief overview of IFN-I mediated effects on the host response in various protozoan infection models and the possible mechanisms involved.

3. Protozoan parasites and IFN-I

IFN-I are involved in the modulation of innate immune responses promoting antigen presentation and NK cell functions. They are also known to play a role in the regulation of the adaptive immune system, promoting the development of antigen-specific T and B lymphocytes against numerous pathogens and inducing immunological memory (Ivashkiv & Donlin, 2014a). In most of the cases, these key features are important factors that limit pathogen proliferation; however, IFN-I may also lead to disease exacerbation. Protozoan parasites such as *Plasmodium*, *Leishmania*, *Trypanosoma*, and *Toxoplasma* are causing diseases that are among the most lethal and widespread around the world, primarily affecting populations of developing countries. The contribution of IFN-I in the host immune response to these pathogens will be discussed below.

3.1. Plasmodium

Plasmodium parasites are the causative agents of Malaria, one of the most widespread disease in the world. The infection presents itself in a wide range of pathologies that can degenerate into severe anemia and the high-risk cerebral malaria (CM). Members of the *Plasmodium* genus have a complex life cycle between an invertebrate (female mosquitoes of the *Anopheles* genus), in

which the sexual cycle occurs, and a mammalian host. During the mosquito blood meal, sporozoites are inoculated into the dermis of the mammalian host. In the initial phase of infection, circulating sporozoites can reach lymph nodes, where the priming of B and T cells occurs, or migrate to the liver (Chakravarty *et al.*, 2007; Obeid *et al.*, 2013). Within the liver, sporozoites transform first into schizonts within hepatocytes and then into merozoites. This phase is asymptomatic and is known as the pre-erytocytic stage (Schofield & Grau, 2005). Merozoites are then released into the blood stream. Once they reach the blood, merozoites invade red blood cells, where they undergo cyclic asexual replication initiating the typical symptomatic manifestations of blood-stage malaria, which are caused by the exponential growth of the parasite and massive destruction of erythroid cells (Stevenson & Riley, 2004).

Most of the current knowledge about the immune response to *Plasmodium* parasites has been derived from a combination of *in vitro* and *in vivo* observations in human patients (e.g. *P. falciparum, P. vivax, P. malariae, P. knowlesi,* and *P. ovale)* and murine models of infections (e.g. *P. berghei*, *P. yoelii*, *P. chabaudi* and *P. vinckei*) (Stevenson & Riley, 2004).

During the pre-erytrocytic stage, sporozoite invasion of hepatocytes and subsequent development into merozoites can be blocked by sporozoite-specific antibodies generated by previous exposure to malaria or by immunization; however, this stage is not completely efficient because sporozoites remain in circulation for a short period of time. When T cell priming takes place, infected hepatocytes can be eliminated by cytotoxic $CD8^+$ T cells (CTLs). $CD8^+$ T cells, IFNγ and TNF are critical components required for elimination of infected hepatocytes in humans and the mouse model (Bertolino & Bowen, 2015). However the immune response at this stage is insufficient and released merozoites can reach erythrocytes giving rise to blood stage malaria (Bertolino & Bowen, 2015).

In the erytrocitic stage, early interaction between merozoites and innate immune cells such as dendritic cells, monocytes, macrophages, natural killer (NK) cells, NKT cells, γδT cells are important for the control of parasite replication and the resolution of infection (Schofield & Grau, 2005). This phase is characterized by a strong pro-inflammatory response, mediated by the activation of NK, NKT, $CD8^+$ and $CD4^+$ T cells that produce large amounts of IFNγ and other pro-inflammatory cytokines. IFNγ activates phagocytic cells, such as macrophages, enhancing the secretion of pro-inflammatory cytokines and promoting phagocytosis of circulating parasites and infected red blood cells, which results in the control of parasitemia. (Riley & Stewart, 2013). Poly-reactive and specific antibodies against blood stage malaria can limit parasite propagation between erythrocytes by opsonisation and agglutination of parasites and infected erythrocytes;

however humoral responses during the infection are dependent on the presence of circulating merozoites (Urban *et al.*, 2005). Infected erythrocytes on the surface express parasitic protein which allows them to bind to vascular endothelial cells and avoid clearance. This event induces obstructions in the blood flow and is associated with a strong inflammatory response and the development of cerebral malaria (Schofield & Grau, 2005).

Although IFN-γ is the most extensively studied interferon in malaria infection, part of the attention has now been diverted to type I IFNs. IFN-I can have a host-protective or detrimental effect, depending on the stage of the infection or the species of *Plasmodium* involved.
One of the first reports involving type I IFNs demonstrated that administration of mouse serum containing high levels of IFN-I protected mice from *P. berghei* infection by reducing blood parasitemia (Jahiel *et al.*, 1970). Similar protective responses were observed after treatment with IFN-β, which prevented death related to cerebral malaria in *P. berghei*-infected mice (Morrell *et al.*, 2011). In contrast, treatment with recombinant IFN-α during the hepatic cycle in mice infected *P. yoelii* sporozoites did not alter the hepatic parasite burden. However, mice showed reduced parasitemia and decreased signs of immunopathology (Vigario *et al.*, 2007).

Plasmodium parasites were reported to induce IFN-I responses. Transcriptomic analysis carried out in mice with blood-stage infection with *P. berghei* revealed that interferon (IFN) regulatory factors were upregulated during the acute phase (Portugal *et al.*, 2011). Induction of a typical type I IFN signature was also observed in hepatocytes from mice infected *with P. berghei* and *P.chaubaudi* sporozoites, where genes such as *Mda, Irf3*, *Irf7*, and *Stat1* were upregulated (Liehl *et al.*, 2014), (Miller *et al.*, 2014), (Rocha *et al.*, 2015). Similar results were observed in humans. Patients infected with *P. vivax* and *P. falciparum* showed a predominantly IFN-I transcriptional signature during the mild and the severe phase of infection (Krupka *et al.*, 2012; Rocha *et al.*, 2015).
Recently, Liehl et al. showed that induction of IFN-I during liver stages of the infection is required for host defence against *P. berghei*. Recognition of *P. berghei* nucleic acids by Mda5 induced IFN-I and consequently the recruitment of leukocytes necessary for parasite elimination in the liver (Liehl *et al.*, 2014). In *P. yoelii* infected mice, recruitment and expansion of $CD49b^{+}CD3^{+}$NKT and $CD8^{+}$T cells to the liver were mediated by IFN-I signaling (Miller *et al.*, 2014). Migration of neutrophils to the liver is also modulated by IFN-I in mice infected with *P. chaubaudi* (Rocha *et al.*, 2015). These studies suggest that functionality of the innate immune response in the liver relies on both IFN-I and IFN-II.

In contrast to the protective effects discussed above, a pathogenic role for IFN-I in *Plasmodium* infections has also been described. For instance, impaired IFN-I signaling has been linked to a protective effect in human patients. Polymorphism in the human gene encoding for IFNAR1 are strongly associated with protection against cerebral malaria (Aucan *et al.*, 2003). This observation is in agreement with results obtained in a murine model, where the lack of IFN-I signaling led to strong resistance to cerebral malaria and reduced parasite load during *P. berghei* infection (Ball *et al.*, 2013; Haque *et al.*, 2011). Moreover, in *P. chabaudi* infected mice, IFN-I appear to suppress Th-1 responses that are crucial in the control of hyperparasitemia, by modulating dendritic cell functions (Haque *et al.*, 2014). In addition, IFN-I and MyD88 signaling are responsible for a decreased recruitment of conventional DCs to the spleen during experimental *P. berghei* or *P. yoelii* infection (Tamura *et al.*, 2015).

Perhaps a better approach for truly understanding the role and function of IFN-I during Malaria consists in the identification of modulator molecules that could act in the IFN-I signalling cascade. Recently, regulators of IFN-I response have been identified through genome-wide analysis (Trans-species expression quantitative trait locus, ts-eQLT) during *P. yoelii* infection. Eight genes (*Ak3, Fcγr1, Fosl1, Havcr2, Sipr5, Parp14, Selenbp2,* and *Helb)* had an effect on IFN-I activation. For example, *Fcγr1*$^{-/-}$ mice infected with *P. yoelii* showed significantly higher mRNA and protein levels of IFN-β than wild-type mice, suggesting a negative regulation in the IFN-β response (Wu *et al.*, 2015).

Future experiments are granted to clarify the spatio-temporal role of IFN-I during Malaria.

The role of IFN-I during *Plasmodium* infections is summarized in Table I.

Table I. Role of IFN-I in *Plasmodium* infection

Plasmodium berghei	Mouse serum containing high levels of IFN-I	Protection, ↓ blood parasitemia
	Treatment with rIFN-β	Prevents death to cerebral malaria (CM)
	Induction of IFN-1	Required to control hepatic infection
	Lack of IFN-I signaling	↑ Resistance to CM and ↓ parasite load
	IFN-I	↓ Recruitment of conventional DCs to the spleen
Plasmodium yoelii	Treatment with recombinant IFN-α	No changes in hepatic burden; ↓ parasitemia and immunopathology
	IFN-I signaling	↑ Recruitment of NKT and $CD8^+$ T cells to the liver
	IFN-I	↓ Recruitment of conventional DCs to the spleen

Plasmodium chabaudi	IFN-I	↑ Recruitment of neutrophils to the liver
	IFN-I	↓ Protective Th1 responses

3.2. Toxoplasma

Toxoplasma gondii is an obligate intracellular protozoan parasite that can infect a wide range of vertebrates and cause a zoonotic disease called Toxoplasmosis. *T gondii* could be considered one of the most successful parasites worldwide; at least 50% of the human population is infected with *Toxoplasma*. The parasite success is mainly due to its ability to invade any nucleated cell and to survive outside the mammalian host (Elmore *et al.*, 2010). *T gondii* strains are classified in three main lineages, based on the virulence of the strain in the mouse model. This virulence profile does not necessarily correlate to the degree of human infection. Type I strains of *T. gondii* are the most virulent: less than 10 parasites are able to kill a mouse at the onset of infection. In contrast, type II and III strains are less virulent and lead to the establishment of chronic infection (Darde, 2008). *T. gondii* can undergo both asexual (schizogony) and sexual (gametogony) replication. Gametogony and oocyst formation is restricted to feline species that act as a definitive host; sexual reproduction of sporozoites occurs within intestinal epithelial cells. Asexual stages of *T. gondii* are not host-specific. Many mammals and birds can act as intermediate hosts. After ingestion of *T. gondii* oocysts by an intermediate host, the parasite transforms into tachyzoites that rapidly undergo multiplication within the parasitophorous vacuole inside various cell types. If the infection is controlled, parasites are retained in tissue cysts; if not, they can cause a systemic lethal disease (Blader *et al.*, 2015; Dupont *et al.*, 2012).

Humans are considered as an accidental intermediate host for *Toxoplasma*. In immune-competent individuals, the infection with *T. gondii* is mostly clinically silent, but cause severe diseases in immune-suppressed patients in particular with an impaired T cell and IFNγ response (Dupont *et al.*, 2012). Protective immunity is typically achieved by inducing an IL-12-driven Th1 immune response (Scharton-Kersten *et al.*, 1996; Vossenkamper *et al.*, 2004).

In the mouse model, IFN-I can already be detected in the serum of *T. gondii* infected animals during the acute phase (Diez *et al.*, 1989; Omata *et al.*, 1983; Shirahata *et al.*, 1986); IFN-I levels gradually increase with the progression of the infection (Diez *et al.*, 1989). IFN-I was also detected in the brain and spleen of infected mice (Mahmoud *et al.*, 2015). These results demonstrate that *T. gondii* not only e induces IFN- γ, but also IFN-I.

Recently, inflammatory monocytes were identified as the major source of IFN-β in mesenteric lymph nodes. IFN-β production by inflammatory monocytes required three fundamental events: parasite internalization, TLR activation (mainly TLR4 and 2), and efficient MyD88 signaling. Interestingly, heat killed parasites induced higher levels of IFN-β in inflammatory monocytes (Han *et al.*, 2014), suggesting that *Toxoplasma* might limit IFN-I responses (Han *et al.*, 2014), possibly by blocking STAT1 (Rosowski *et al.*, 2014).

As for many other infection models, the first studies carried out during the 1960s on the role of IFN-I in toxoplasmosis evaluated the impact of a treatment with IFN-I on infected cells in vitro. Pre-treatment of mouse fibroblast with IFNs conferred protection to *T. gondii* infection (Remington & Merigan, 1968). In agreement with this observation, human neonatal and adult macrophages treated with IFN-I were able to control parasite multiplication, even if less effectively than IFN-γ treated cells (Wilson & Westall, 1985). Moreover, human monocyte-derived macrophages treated with human IFN-β in combination with *E. coli* lipopolysaccharides (LPS), but not with murine IFN-β (MuIFN-β) or rHuIFN-β alone (Orellana *et al.*, 1991), were more resistant to *T. gondii* infection (Schmitz *et al.*, 1989).

In the mouse model of toxoplasmosis, treatment with HuIFN- β showed a protective effect, which was enhanced by the combination of rHuIFN- β and LPS and was IFN-γ dependent (Orellana *et al.*, 1991). In agreement with these results, it was shown that *Ifnar*$^{-/-}$ mice orally infected with *T. gondii* have an increased parasite load compared to wild-type mice; higher parasite burdens correlated with a decrease in survival (Han, 2011).

These results suggest that IFN-β may be produced at the onset of infection to enhance the IFN-γ responses.

A study using human fibroblasts as host cells revealed that treatment of *T. gondii*-infected cells with IFN-I had no effect on parasite replication (Pfefferkorn & Guyre, 1984), suggesting that the protective effect of IFN-I depends on cell type and/or timing of exposure to the cytokine (prior to or after infection).

During *T. gondii* infection regulation of tryptophan metabolism is a key component for parasite survival. Indeed, tryptophan degradation inhibits parasite replication. In *T. gondii* infected mice, indoleamine 2,3-dioxygenase (IDO), a tryptophan catalyzer (Engin *et al.*, 2012; Sibley, 1993), is enhanced by IFN-II (Murray *et al.*, 1989). However, it has also been reported that IFN-I can regulate IDO in human retinal pigment epithelial cells, inhibiting therefore *T. gondii* replication (Nagineni *et al.*, 1996). Together, these results demonstrate that IFN-I also contribute to the regulation of protective immunity against *T. gondii* (s. Table II).

Table II. Role of IFN-I in *Toxoplasma* infection

Toxoplasma gondii	IFN-I treatment (in vitro infection; mouse fibroblasts; and human macrophages)	↑ Resistance to infection
	HuIFN-β treatment (in vitro)	↑ Resistance to infection
	Ifnar$^{-/-}$ mice	↑ Parasite load, ↓ survival
	IFN-I treatment, human fibroblasts	No effects on parasite replication

3.3. Leishmania

Leishmania is a complex genus of obligate intracellular protozoan parasites that cause a widespread disease collectively known as *Leishmania*sis. The life cycle of these parasites takes place between a mammalian host and a sandfly vector (genus Lutzomya and Phlebotomus). Once in the hosts, the promastigote form of the parasite preferentially infects macrophages, but can also be found in other cell types, such as dendritic cells, neutrophils, and fibroblasts. Promastigotes then transform into the non-flagellated form called amastigotes within the host's cell. The *Leishmania spp.* involved and the mammalian host immune status determine the clinical manifestation of the disease. Parasites can either reside in the skin and/or mucosal surfaces, which results in cutaneous (i.e *L.major*) or mucocutaneous (i.e *L. braziliensis*) *Leishmania*sis; or disseminate to internal organs such as liver, spleen, and bone marrow, causing visceral *Leishmania*sis (VL), the most severe form of the disease (i.e *L. donovani*) (Kaye & Scott, 2011).
Leishmania immunity is mostly mediated by T lymphocytes. In experimental models, control of infection is mediated by a polarized Th1 response, induced by an initial production of IL-12 by DCs (Engwerda *et al.*, 1998). IFN-γ secreting $CD4^+$ and $CD8^+$ T cells contribute to parasite control by enhancing the ability of phagocytic cells to kill intracellular *Leishmania* parasite (Bankoti & Stäger, 2012; Kaye & Scott, 2011).

As for many other protozoan models, IFN-II is the main mediators of the cellular immune response. However, IFN-I and IFN-I inducible genes are gradually gaining importance in the *Leishmania* field. One of the pioneer work on the role of IFN-I in *Leishmania*sis described the prophylactic treatment with synthetic double-stranded RNA (Poly I:C) prior to *L. donovani* infection. Injection of Poly I:C triggered a burst of IFN-I and led to the control of the hepatic parasite burden (Herman &

Baron, 1970). The role of endogenous IFN-I was studied for the first-time using strains causing cutaneous *Leishmania*sis. The induction of IFN-I was observed in macrophages infected *in vitro* with *L. major* promastigotes (Diefenbach *et al.*, 1998; Mattner *et al.*, 2000) and in skin macrophages from infected animals (Diefenbach *et al.*, 1998), showing that promastigotes could enhance IFN-I expression in the host cell. The combination of exogenous IFN-I with *L-major* promastigotes was shown to activate macrophages, inducing type 2 nitric oxide synthase (NOS2). NOS2 is required for parasite elimination; mice deficient in this enzyme are more susceptible to *L. major* infection (Olekhnovitch *et al.*, 2014). As for *T. gondii*, the timing of the host cell's exposure to IFN-I determines the effect on parasite control. Indeed, pre-treatment of macrophages with exogenous IFN-I failed to induce NOS2. Similar results were obtained with high doses of exogenous IFN-I while a low IFN-I dose in combination with *L. major* enhanced leishmanicidal activity (Mattner *et al.*, 2000; Mattner *et al.*, 2004). These results suggest that the design of in vitro experiments greatly influences the outcome of IFN-I treatment in infected macrophages and that the role of IFN-I should be better studied in in vivo models.

The protective role of endogenous IFN-I during infection was confirmed by neutralizing IFN-I in mice experimentally infected with *L. major.* In fact, IFN- I neutralization rendered *L. major*-infected mice more susceptible to infection and enhanced parasite multiplication. IFN-I blockade led the abolishment of NOS2 function and reduced cytotoxic activity and IFNγ production by NK cells at early stages of infection (Diefenbach *et al.*, 1998).

Opposite results were obtained in human macrophages infected *in vitro* with New World *Leishmania spp.* IFN-β treatment of *L. braziliensis* and *L. amazonensis*–infected macrophages enhanced the parasite burden through a superoxide-dependent, NO-independent mechanism (Khouri *et al.*, 2009). In this model, it was shown that IFN-β can regulate the superoxide dismutase SOD1 activity. SOD1 is responsible for catalyzing the disproportionation of superoxide to hydrogen peroxide and dioxygen and is an important constituent in apoptotic signaling and oxidative stress. It has been observed that biopsies from cutaneous *Leishmania*sis patients express high levels of SOD1 (Khouri *et al.*, 2009).

The importance of endogenous IFN-I during chronic infection has been investigated using IFNAR-deficient mice in the context of *L. amazonensis* infection. *L. amazonensis* infected *Ifnar*$^{-/-}$ mice developed attenuated cutaneous lesions and displayed a decreased parasite load. This effect appeared to be STAT1 independent, a key protein in the IFN signaling (Xin *et al.*, 2010). Furthermore, *L. amazonensis*- infected *Ifnar*$^{-/-}$mice exhibited high levels of neutrophils and lower inflammatory monocytes recruitment at early times post infection. This unique profile was also

observed in *L. major* and *L. brasiliensis* infections (Xin *et al.*, 2010). In vitro coculture of infected WT macrophages with *Ifnar*$^{-/-}$ neutrophils revealed that IFNAR deficient neutrophils promote parasite killing (Xin *et al.*, 2010). This evidence supports the pathogenic role of IFN-I signaling in cutaneous *Leishmania*sis caused by New World *Leishmania* species.

We also observed a negative role for IFN-I in an experimental model of visceral *Leishmania*sis. *L. donovani* amastigotes were shown to induce IFN-I expression in B cells in an endosomal TLR dependent manner. This cytokine was involved in a positive regulatory loop that led to the upregulation of endosomal TLRs and to IL-10 production in B cells (Silva-Barrios *et al.*, 2016). B cell-derived IL-10 was shown to suppress protective T cells responses and increase disease susceptibility (Bankoti *et al.*, 2012). B cells are known to play a detrimental role during VL (Smelt *et al.*, 2000), not only by secreting IL-10 but also for their excessive antibody production (Deak *et al.*, 2010). Indeed, hypergammaglobulinemia is a hallmark of VL. Interestingly, IFN-I seems to be regulating antibody production during VL. Specific ablation of endosomal TLRs or IFN-I signaling in B cells was shown to severely reduce the Ig titer in the serum of *L. donovani* infected mice, suggesting that parasite activation of B cells via endosomal TLRs and IFN-I are involved in the induction if hypergammaglobulinemia (Silva-Barrios *et al.*, 2016). Furthermore, mice with a B cell-specific deficiency in endosomal TLR or IFNAR were more resistant to *L. donovani* infection than their wild type counterpart.
Very little is known about the function of IFN-I in VL patients. It was reported that human mononuclear phagocytes can be activated by IFN-β, but less efficiently than IFNɣ (Passwell *et al.*, 1986). Exogenous treatments with IFN-I and IFN-II but not IL-2, failed to restore the cytotoxic activity of NK isolated from VL patients (Manna *et al.*, 1993). Also, treatment of the cutaneous lesion in patients with IFN-I did not improve healing, compared with IFN-γ treatment. (Murray *et al.*, 1989; Trau *et al.*, 1987).

Because dendritic cells can also be infected by *Leishmania*, it is important to consider the induction of IFN-I by the parasite and its possible effect in these cells as well. Transcriptomic analysis of human DCs infected in vitro with *L. major* or *L. donovani* showed a differential expression pattern for IL-12 associated genes, the NF-κB pathway, and IFN regulatory factors (Chaussabel *et al.*, 2003). IFN-β produced by *L. major* infected DCs seems to be required for IL-12 secretion by the infected DC, suggesting that protective Th1 responses, which are IL-12 depended, may also depend on IFN-I (Favila *et al.*, 2014).
A summary on the role of IFN-I during *Leishmania*sis can be found in Table III.

Table III. Role of IFN-I in *Leishmania* infection

Leishmania donovani	Treatment with Poly I:C	↓ Hepatic parasite burden
	B cell-derived IFN-I	↑ IL-10, ↑ hypergammaglobulinemia
Leishmania major	IFN-I treatment of macrophages in vitro	
	(1) At the time of infection (2) Before infection (3) High dose (4) Low dose In vivo IFN-I blockade	↑ NOS2 No effect on NOS2 No effect ↑ Leishmanicidal activity ↓ NOS2, ↓ natural killer functions
Leishmania braziliensis	IFN-β treatment of macrophages in vitro	↑ Parasite burden
Leishmania amazonensis	*Ifnar*$^{-/-}$ mice	↓ Lesions, ↓ parasite burden, ↑ neutrophils

3.4. Trypanosomes

Trypanosomes are digenetic protozoan parasites that infect domestic and wild animals, as well humans. Although many species of trypanosomes cause important veterinary disease, mainly two species cause significant human morbidity: *Trypanosoma brucei* and *Trypanosoma cruzi*. These two species are responsible for causing the sleeping sickness (African trypanosomiasis) and the Chagas disease (American trypanosomiasis), respectively.

The life cycle of these parasites takes place between the invertebrate vector and the vertebrate host. *T. brucei* and other African's trypanosomes are transmitted to the mammalian host by a tsetse fly bite. In the blood stream, metacyclic trypomastigotes differentiate into bloodstream trypomastigotes. In humans, trypanosomes proliferate in the blood and lymphatic system at early stages of the infection. This stage is associated with an anti-inflammatory response. At chronic stages, parasites can pass through the blood-brain barrier and enter the central nervous system. This stage is associated with inflammatory changes in the brain and is characterized by a neurological disturbance (MacGregor *et al.*, 2012; Namangala, 2011).

In the case *T. cruzi* (American trypanosomiasis), metacyclic trypomastigotes are released in the feces/urine of the triatomine vector after a blood meal. Trypomastigotes can successfully infect the mammalian host if they are able to reach the mucosa or injured skin areas. In contrast to

African trypanosomes, *T. cruzi* is an intracellular parasite that has the capacity to invade, differentiate into amastigotes, and replicate within a wide range of nucleated cells. This characteristic is one of the most important features of *T. cruzi* within the host. Amastigotes differentiate into infective bloodstream trypomastigotes, before being released upon cell lysis. The released parasites can then infect neighboring cells or enter the bloodstream (Cardoso *et al.*, 2015).

During the acute phase, the innate immune response against *T. cruzi* is characterized by the induction of a cell-mediated response that involves the production of IFN-γ and TNF (by NK and T cells), required for enhancing iNOS activity by phagocytic cells and for priming the adaptive immune response. iNOS activation is critical for controlling parasite growth during the infection (Cardoso *et al.*, 2015). *T. cruzi* elicits a prominent IFN-I response at early times of infection (Chessler *et al.*, 2008; Chessler *et al.*, 2009; Costales *et al.*, 2009; Vaena de Avalos *et al.*, 2002). As mentioned before for *Plasmodium*, the role of IFN-I in *T. cruzi* infection is controversial. Some studies ascribe a protective role to IFN-I; others demonstrate that IFN-I induces pathology. The effect of IFN-I mainly depends on the dose, amount of parasites, and the inoculation route used to set up the infection.

The first studies on the role of IFN-I investigated the outcome of exogenous IFN-I treatment in *T. cruzi* infected mice. The results showed that administration of IFN-I increased resistance to infection by stimulating T and NK cell activities, which are essential for protection (James *et al.*, 1982; Rottenberg *et al.*, 1988).

In an intradermal model of infection, transcriptomic analysis of excised skin from the inoculation site revealed that *T. cruzi* upregulated the expression of IFN-stimulated genes (ISGs) as early as 24h after infection. Induction of ISGs was dependent on IFN-I signalling, suggesting that IFN-I is an important component of the innate immune response to *T. cruzi* (Chessler *et al.*, 2008). In agreement with the above mentioned literature, studies carried out in *Ifnar*$^{-/-}$ mice infected with *T. cruzi* revealed that efficient IFN-I signaling was required for controlling parasites' growth during the acute phase of infection (Costa *et al.*, 2006; Une *et al.*, 2003). IFN-I was necessary for enhancing NO production in phagocytic cells (Costa *et al.*, 2006). NO is considered the major effector molecule for intracellular amastigotes elimination within infected cells, being important for the control of parasite multiplication (Cardoso *et al.*, 2015).

In contrast, another group reported a potential pathogenic role for IFN-I. In this work, a lethal dose of parasites inoculated intradermally was used to set up the infection in WT and *Ifnar*$^{-/-}$ mice.

Surprisingly, *T. cruzi* infected *Ifnar*$^{-/-}$ mice survived the challenge and were able to control parasite replication. (Chessler *et al.*, 2011). Besides the fact that splenocytes from *Ifnar*$^{-/-}$ mice produced higher levels of IFN-II, plasma cytokine profile in *T. cruzi*-infected *Ifnar*$^{-/-}$ mice were not different to control mice (Chessler *et al.*, 2011). Additionally, T cells populations were not inherently different compared with control mice (Chessler *et al.*, 2011), and IFN-γ production by $CD8^+$ T cells was not affected by impaired IFN-I signaling (Martin *et al.*, 2010), suggesting that, in this model, endogenous IFN-I is not the only relevant signal in host defense against *T. cruzi.*

Taken together, the role of IFN-I in *T. cruzii* infection differs from one experimental model to the other, depending on the dose and the route of infection (Bogdan *et al.*, 2004). This could explain the controversy about the observations on the role of IFN-I in the *T. cruzi* model of infection (s. Table IV).

The immune response to African trypanosomes is quite different than that to *T. cruzi*. First, parasites never enter the host cell at any stage of their development. The success of these parasites is mainly due to their ability to change the composition of the variant surface glycoprotein (VSG) by switching genes. This confers them the capacity to evade B- and T-cell-mediated immune responses and results in fluctuating waves of parasitemia that characterize African trypanosomiasis (Namangala, 2011). VSG is a strong antigen that induces Th1 responses and promotes autoantibody and cytokines production, in particular TNF. Other trypanosome proteins and soluble factors, such as a trypanosome-released triggering factor (TLTF), also trigger interferon (IFN)-γ production by T and NK cells, and are involved in macrophage activation towards an M1 phenotype, which is required for the control of parasite multiplication during the acute phase of infection. However, sustained activation of M1 macrophages is associated with disease exacerbation. The progression of the infection towards the development of an acute fatal disease or a prolonged chronic infection is determined by the balance between a type I and type II immune responses and the switch from the early type I immune response (dominated by M1 macrophage activation) from a type II (M2 macrophages) regulatory response that controls the inflammation (Vincendeau & Bouteille, 2006).

The literature on the role of IFN-I in African trypanosomiasis is scarce. A study involving T. *b. rhodesiense* reported a beneficial effect of IFN-I during the acute phase of infection. Indeed, *Ifnar*$^{-/-}$ mice displayed delayed control of parasite burden during the first week of infection and died earlier than wild-type controls. Moreover, mice hyperresponsive for IFN-I (*Ubp43*$^{-/-}$) exhibited a

significant defect in Th1 responses and IFN-γ production, suggesting that IFN-I play a role in the early stages of disease. Nevertheless, IFN-I contributes to the down-regulation of IFN-γ production and loss of host resistance during chronic infection (Lopez *et al.*, 2008).

No effects of IFN-I signaling were observed in *T. b. brucei*-infected *Ifnar*$^{-/-}$ mice, which showed similar levels of parasitemia to wild-type mice, suggesting that in this model parasite control is independent of IFN-I (Amin *et al.*, 2012). However, IFN-I regulates T cell infiltration to the brain parenchyma at chronic stages of the infection (Amin *et al.*, 2012).

Table IV. Role of IFN-I in *Trypanosoma* infection

Trypanosoma cruzi	IFN-I treatment in vivo	↑ Resistance to infection
		↑ T and natural killer cell activity
	Ifnar$^{-/-}$ mice	Disease exacerbation
	Ifnar$^{-/-}$, lethal dose	↑ Survival
Trypanosoma brucei rhodesiense	*Ifnar*$^{-/-}$, acute phase	↑ Control
	Ifnar$^{-/-}$, later stages	↓ Resistance, IFN-γ ↓
Trypanosoma brucei brucei	*Ifnar*$^{-/-}$	No effect on parasite control

In conclusion, the contribution of IFN-I to protective immunity against several protozoan parasites is still unclear. Variations in parasite numbers used for infections, the site of inoculation and the dose of IFN-I all seem to influence the outcome and the interpretation of the results. A spatio-temporal analysis of the role of IFN-I integrated with a more detailed investigation of cell-specific signalling pathways elicited by the cytokine could help to better dissect the involvement of IFN-I in the immune response.

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ANNEX-II: Review NO.2

THE DEADLY DANCE OF B CELLS WITH *TRYPANOSOMA*TIDS

The deadly dance of B cells with *Trypanosoma*tids

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1. Trends

B cells are overactivated during infection with *Trypanosoma*tids. This overactivation has a negative impact on disease outcome.

In the case of *Leishmania*, B cell activation results in the generation of immunosuppressive B cells that inhibit protective Th1 responses and induce detrimental Th2 cells. *Leishmania donovani* activates B cells by triggering endosomal Toll-like receptors and inducing type I interferons (IFN-I). This activation is amplified by IFN-I and ultimately results in an uncontrolled production of low-affinity antibodies.

Trypanosoma cruzi metabolic products can directly activate B cells. Besides subverting B cell functions by overactivating them, *Trypanosoma* brucei also induces an irreversible depletion of some B cell subpopulations in the spleen and hinders B cell maturation and differentiation in the bone marrow.

2. ABSTRACT

B cells are notorious actors for the host's protection against several infectious diseases. So much so that early vaccinology seated its principles upon their long-term protective antibody secretion capabilities. Indeed, there are many examples of acute infectious diseases that are combated by functional humoral responses. However, some chronic infectious diseases actively induce immune deregulations that often lead to defective, if not deleterious, humoral immune responses. In this review we summarize how *Leishmania* and *Trypanosoma* spp. directly manipulate B cell responses to induce polyclonal B cell activation, hypergammaglobulinemia, low-specificity antibodies, limited B cell survival, and regulatory B cells, contributing therefore to immunopathology and the establishment of persistent infections.

3. B Cell Subpopulations and Function

B cells are a heterogeneous lymphocyte population. They arise from hematopoietic stem cells and differentiate towards their final subtype through a series of lineage commitment and selection steps. Depending on ontogeny, location, phenotype, and function, B cells can be classified in the

following subsets: follicular B (FoB) cells, marginal zone B (MZB) cells, B1 cells, and regulatory B cells (Bregs) (Lopes-Carvalho *et al.*, 2005; Song & Cerny, 2003) **(Table 1).**

Table 1. B Cell Subpopulations

Tissue location	B cell subtype	Function	Mouse-specific markers	Human-specific markers
Secondary lymphoid organs (SLO)	Transitional B cells	Naive B cell pool renewal	IgM^{hi}IgD^{var}CD1d^{lo}CD19^{+} CD93^{+}CD21^{var}CD23^{var}	IgD^{+}CD27^{-} CD10^{var}CD24^{var}C D38^{var}
	Marginal zone B cells	Blood-borne antigen trapping and early antibody secretion	IgD^{lo}IgM^{hi}CD9^{+}CD19^{+}CD 21^{hi}CD23^{lo}CD24^{hi}CD38^{hi} CD44^{+}B220^{+}CD80^{lo}CD8 6^{+}CD1d^{hi}	IgD^{lo}IgM^{hi}CD1c^{+}C D21^{hi}CD23^{-} CD27^{+}CD1d^{hi}CXC R5^{+}
	Regulatory B cells	Immunoregulation	IgM^{hi}IgD^{var}CD19^{+}CD1d^{hi} CD21^{hi}CD23^{var}CD24^{hi}C D5^{+}CD93^{var}TIM-1^{+}IL-10^{+} (Garraud *et al.*, 2012; Mauri & Bosma, 2012)	IgM^{hi}CD19^{+}CD1d^{h} ^{i}CD5^{+}CD10^{var}CD2 7^{var}CD24^{hi}CD38^{var} CD138^{var}IL-10^{+}
	Activated B cells	Cognate antigen recognition and T cell priming	IgD/M^{var}BLA-1^{hi}CD19^{+}CD23^{var}CD38^{var} GL-7^{+}CD80^{+}CD86^{+}CD95^{+}C D24^{hi}MHC-II^{hi}	IgD/M^{hi}CD23^{var}CD 38^{+}CD44^{+} (Bohnhorst *et al.*, 2001)
	Germinal center B cells	Antigen-dependent antibody affinity maturation	IgD/M^{var}BLA-1^{var}CD19^{+}CD23^{var}CD38^{-} GL-7^{+}CD80^{+}CD86^{var}CD95^{+}C D24^{hi}MHC-II^{hi}	IgM/G/A/E^{+}IgD^{-} CD10^{+}CD20^{+}CD2 7^{+} CD23^{-} CD38^{hi}CD44^{-} CD77^{var}
	Plasmablast	High affinity antibody secretion	IgM/G/A/E^{+}IgD^{var}CD19$^{lo/-}$ CD138^{+} CD93^{+}MHC-II^{+}Ki-67^{+}	IgM/G/A/E^{+}IgD^{-} CD19^{lo}CD20^{-} CD27^{hi}CD38^{hi}CD1 38^{lo} Ki-67^{+}
	Plasma cells (short-lived)	Early antibody production	mIg^{-}sIg^{hi}B220^{lo}CD19$^{lo/-}$ CD44^{hi}CD138^{+}CD86^{-} CD93^{+}MHC-II^{var}CXCR5^{lo}CCR7^{lo}CXC R4^{hi}CXCR6^{hi}	IgM/G/A^{+}CD27^{+}C D38^{hi}CD138^{+}CXC R4^{hi}CD19^{lo}CD20^{-} CD45^{lo}MHC-II^{lo}
Peritoneal and pleural cavities	B1a B cells	Natural antibody secretion	IgM^{hi}IgD^{lo}CD5^{+}CD9^{+}CD1 1b^{+}CD19^{+}CD23^{lo}CD43^{+} B220^{lo}CD138^{-}	IgM^{var}IgD^{var}CD5^{var} CD11b^{var}CD19^{+}C D20^{+}CD27^{+}CD43^{+} B220^{+}CD86^{var}CD 70^{-}CD10^{var}
	B1b B cells	Early antibody secretion	IgM^{hi}IgD^{lo}CD5^{-} CD9^{+}CD11b^{+}CD19^{+}CD2 3^{lo}CD43^{+}CD45^{lo}CD138^{-}	

Bone marrow (BM) only	Plasma cells (long-lived)	Long term antibody production	mIg^{-} sIg^{hi}B220^{lo}CD19^{lo}MHC-II^{lo}CD138^{+}CXCR4^{+} (Manz *et al.*, 1998; McHeyzer-Williams *et al.*, 2000)	IgG/A/M^{+}CD27^{hi}CD38^{hi}CD138^{hi}CXCR4^{hi}CD19^{lo}CD20^{-}CD45^{lo}MHC-II^{lo} (Nutt *et al.*, 2015)
Alternance between SLO and BM	Follicular B cells	Adaptive immune responses	IgD^{hi}IgM^{lo}CD19^{+}CD21^{+}CD23^{hi}CD38^{+}CD43^{-}CD44^{-}B220^{+}CD80^{-}CD86^{-}CD1d^{lo}	IgD/M^{+}CD23^{var}CD27^{lo}CD38^{lo}CD44^{+}CD138^{lo}CXCR4^{lo}CD19^{+}CD20^{+}CD45^{+}MHC-II^{hi}
	Memory B cells	Immunological recall readiness	IgG/A/E^{+}IgD/M^{var}CD19^{+}CD21^{var}CD23^{var}CD38^{+}CD44^{+}B220^{+}CD80^{var}CD86^{+}	IgG/A/M^{+}IgD^{-}CD19^{+}CD20^{+}CD27^{+}CD21^{hi}CD23^{-}CD38^{lo}CD44^{+}CD138^{-}

For some time, antibody production was seen as the main function of B cells; however, only three mature B cell subtypes respond by antibody secretion on the first antigenic contact: FoB cells, B1 cells, and MZB cells (Allman & Pillai, 2008; Carsetti *et al.*, 2004). MZB cells are located in the marginal sinus of the spleen, whereas B1 cells populate pleural and peritoneal cavities. B1 and MZB cells are considered innate and innate-like B cells, respectively, and are known to produce membrane-bound and secreted polyreactive immunoglobulins with broad specificity. Most B cells will proliferate following Toll-like receptor (TLR) engagement alone, without antigen; however, only B1 or MZB cells will differentiate to antibody-secreting cells (Genestier *et al.*, 2007) and produce a first wave of antibodies to quickly limit pathogen invasion. FoB cells are the most abundant subset and are found in follicular areas of spleen and lymph nodes. These cells typically require T cell activation to differentiate into antibody-secreting **plasma cells (plasmocytes)** and generate high-affinity monoreactive antibodies(Croft *et al.*, 1997). During the initial encounter with a pathogen, plasma cells will provide protection by secreting pathogen-specific antibodies that may have neutralizing capacity.

In addition to their role in antibody production, B cells are also involved in antigenic presentation, cytokine secretion, and organogenesis of secondary lymphoid organs. This multifunctional role can either promote or protect against disease development (Lund & Randall, 2010). For instance, B cells are generally considered pathogenic in autoimmunity since they release autoantibodies and cause tissue damage. However, autoantibodies can also facilitate the removal of apoptotic cells, limiting development of autoimmune reactions (Boes *et al.*, 2000; Botto *et al.*, 1998; Kim *et al.*, 2002; Scott *et al.*, 2001).

B lymphocytes are also professional antigen-presenting cells and are involved in activation/maintenance of naïve, activated, and autoreactive T cells. Resting B cells are typically not very efficient in inducing T cell responses. However, unwanted T cell activation can sometimes occur, leading to limited clonal expansion, cell death, and immune tolerance (Croft *et al.*, 1997). Following foreign antigen recognition and cell activation, the surface expression of MHC and costimulatory molecules is increased; this promotes T cell proliferation, survival, and effector differentiation (Cassell & Schwartz, 1994; Hoehlig *et al.*, 2008; Kennedy *et al.*, 1994; Malynn *et al.*, 1985).

In addition to antibody secretion and antigen presentation, B cells can produce various proinflammatory and anti-inflammatory cytokines. Through cytokine secretion, they contribute to the maintenance and organization of tissue structure and/or regulate the immune response (Shen & Fillatreau, 2015).

Although B cells are designed for helping to resolve infections, several pathogens are able to hinder B cell functions and twist them to favour of pathogen survival and the establishment of chronic infections, as in the case of *Trypanosoma*tids. In this review we discuss various mechanisms adopted by *Trypanosoma*tids to subvert B cell function, including the induction of polyclonal B cell activation and hypergammaglobulinemia, and active depletion of B cell subpopulations in the spleen and bone marrow. Recent studies have highlighted the fatal interaction between *Trypanosoma*tids and B cells, and have shown how this deadly dance contributes to the establishment of persistent infections. Despite strong evidence that B cells exacerbate disease, the role of B cells in the immune response to *Trypanosoma*tids has often been neglected.

4. B Cells and *Leishmania*

Leishmania species are an extensive group of obligate protozoan intracellular parasites transmitted to a mammalian host by the bite of a phlebotomine sandfly. During their life cycle, *Leishmania* parasites alternate between two main morphologies: promastigotes and amastigotes. **Promastigotes (see Glossary**), known as the infective stage of *Leishmania*, are present in the proboscis of infected female sandflies. Parasites are transmitted to the mammalian host during the blood meal. In the mammalian host, promastigotes are phagocytozed mostly by professional phagocytic cells, such as macrophages. Within phagocytes, promastigotes transform into **amastigotes**, the replicative morphology in the mammalian host. Intracellular parasite

multiplication eventually causes the lysis of the infected cell, allowing amastigotes to infect adjacent phagocytic cells, remaining, or not, at the site of the lesion, depending on the *Leishmania* species involved in the infection. Phlebotomines and sandflies become infected by ingesting infected cells during blood meals. Within the invertebrate host, amastigotes transform into promastigotes (Kaye & Scott, 2011; Scott & Novais, 2016). Members of the genus *Leishmania* are known to cause a widespread array of diseases collectively known as *Leishmania*sis. The clinical manifestations are determined by the *Leishmania* species involved and the immune status of the host **(Table 2)**. Parasites can reside in the skin and/or mucosal surfaces, resulting in cutaneous *Leishmania*sis (CL) or mucocutaneous *Leishmania*sis (MCL); alternatively, parasites can disseminate to internal organs such as the liver, spleen, and bone marrow, causing visceral *Leishmania*sis (VL), the most severe form of the disease (Kaye & Scott, 2011). **Hypergammaglobulinemia** is one of the hallmarks of *Leishmania*sis. It is well documented that, during the disease, polyclonal B cell activation results predominantly in the production of low-affinity anti-*Leishmania* antibodies, as well as in an increase in the amount of circulating **immune complexes** (Carvalho *et al.*, 1989; Galvão-Castro *et al.*, 1984). In recent years, the interest in understanding the contribution of B cells to *Leishmania* immunity has increased. Research in this field suggests a dual role for B cells, promoting or controlling disease pathology that varies depending on the experimental model and/or the parasite strain used.

Table 2. Principal human and mouse disease caused by *Trypanosoma*tids

*Trypanosoma*tids	Human	Mice		References
		C57BL/6	**BALB/c**	
Leishmania major	Self-healing or chronic cutaneous *Leishmania*sis	Self-healing	Chronic	[24]
Leishmania amazonensis	Self-healing or chronic cutaneous *Leishmania*sis, and diffuse cutaneous *Leishmania*sis	Chronic	Chronic	[24]
Leishmania mexicana	Healing or chronic cutaneous *Leishmania*sis, and diffuse cutaneous *Leishmania*sis	Chronic	Chronic	[24]

Leishmania braziliensis	Healing or chronic cutaneous *Leishmania*sis, and mucosal *Leishmania*sis	Self-healing	Self-healing	[24]
Leishmania donovani	Chronic visceral *Leishmania*sis, or kala-azar (black fever)	Chronic	Chronic	[23]
Leishmania infantum	Chronic visceral *Leishmania*sis, or kala-azar (black fever)	Chronic	Chronic	[23]
Trypanosoma cruzi	American trypanosomiasis or Chagas disease, chronic trypanosomiasis	Resistant	Self-healing (partly) Relatively susceptible	[87]
Trypanosoma brucei	African trypanosomiasis or sleeping sickness, cause acute (*T. brucei rhodesiense*) and chronic (*T. brucei gambiense*) trypanosomiasis	Progressive to lethal disease (Tolerant)	Highly susceptible	[114-116,128]

4.1. The Immune Response to CL and MCL

CL is considered to be the most common form of *Leishmania*sis. The infection comprises a range of diseases from self-healing infections to chronic disfiguring disease. In humans, the response varies between individuals depending on the strength of the T cell response, characterized by delayed-type hypersensitivity (DTH) and high levels of interferon γ (IFN-γ) that enhance the killing capacity of macrophages (the main cell type infected by *Leishmania* spp.), controlling parasite replication (self-healing CL). In contrast, patients lacking a DTH response usually have higher levels of antibodies with a low affinity against *Leishmania* and fail to control parasite load, developing evident diffuse cutaneous lesions (Kaye & Scott, 2011).However, there are cases in which individuals with a marked DTH and Th1 immune response develop a severe manifestation of the disease known as MCL (Scott & Novais, 2016). MCL is characterized by gradual and progressive development of destructive lesions in the mucosal region that can develop many years after recovery from an initial lesion. Tissue disruption is thought to be a consequence of an overzealous immune system that causes **immunopathology**.

Two major mouse models are used to study the distinct types of CL. Infection of C57BL6 (Th1-dominant response) or BALB/c (Th2-dominant response) mice in combination with different *Leishmania* strains or isolates can mimic different stages of the disease, from self-healing CL to mucocutaneous pathology (Scott & Novais, 2016).

Susceptible BALB/c mice fail to control *Leishmania* major cutaneous infection due to the inappropriate and early expansion of Th2 $CD4^+$ T cells that produce interleukin-4 (IL-4) and IL-13 (Reiner & Locksley, 1995). While Th1 $CD4^+$ T cells that produce IFN-γ are generated, the presence of IL-4 affects Th1 development (Himmelrich *et al.*, 1998) and directly antagonizes IFN-γ-dependent activation of parasitized macrophages (Liew *et al.*, 1991). In contrast, resistant C57BL/6 are characterized by a Th1-polarized immune response, associated with IFN-γ, IL-2, and IL-12 production. In both models, the immune response is inhibited at the infection site by IL-10-secreting $CD4^+$ T cells (Anderson *et al.*, 2007; Belkaid *et al.*, 2002). In several experimental models of CL, it has been shown that B cells contribute to disease susceptibility (Minoprio *et al.*, 1988; Mota & Umekita, 1989; Palanivel *et al.*, 1996). Absence or depletion of B cells is associated with enhanced protection during CL. B cell depletion from newborn BALB/c mice by continuous treatment with anti-IgM serum conferred resistance to *Leishmania* tropica and *Leishmania amazonensis* infection. A sustained DTH response to *Leishmania*l antigen was observed, and they could control their cutaneous lesions (Sacks *et al.*, 1984). This observation was confirmed in B cell-deficient C57BL/6 (μMT) mice. *L. major*-infected μMT mice were less susceptible to infection (Dekrey *et al.*, 2003). However, in the CL model, the disease susceptibility associated with the absence of B cells may differ depending on the mouse strain used and the geographical area of the *Leishmania* isolate. BALB/c μMT have been shown to remain susceptible to infection with the Iranian strains of *L. major*, IR173 and IR75; however, opposite results were obtained using the Russian isolate of *L. major*, LV39 (Ronet *et al.*, 2008).

In the murine model of L. amazonensis infection, B cells play a detrimental role. B cell-deficient JHD mice infected with L. amazonensis show a significantly reduced parasite burden compared to wild-type mice, suggesting that B cells promote exacerbation of the infection, and that antibodies may contribute to immunopathology (Anonyme, !!! INVALID CITATION !!! {}). In contrast, in the case of coinfection of CH3 with both *L. major* and L. amazonensis, B cells play a protective role and are required for healing lesions (Gibson-Corley *et al.*, 2012). Peripheral B cell depletion with anti-CD20 therapy in coinfected CH3 mice enhanced the size of the lesion (Bockenstedt *et al.*, 2015).

Interferon regulatory factor 4 (IRF-4) has recently been shown to regulate B cell germinal center formation, **T follicular helper (TFh)** cell responses, and antibody secretion (Bollig *et al.*, 2012; Willis *et al.*, 2014). Interestingly, Irf4− mice are more susceptible to *L. major* infection (Willis *et al.*, 2014). Moreover, *L. major* infection of mice with IRF4 deficiency on mature B cells results in an impaired immune response (Willis *et al.*, 2014).

The mechanism by which B cells modulate the anti-*Leishmania* response is not yet completely clear. *L. major*-derived proteins, such as *L. major*-sirtuin (LmSIR2) and *L. major*-ribosomal protein (LmS3a), are known to induce polyclonal B cell activation; nevertheless, the contribution of this activation to anti-*L. major* immunity has not yet been studied (Cordeiro-da-Silva *et al.*, 2001; Silvestre *et al.*, 2006). Furthermore, antibodies were shown to have a dual role, promoting or controlling disease depending on the infection model. For instance, in some experimental conditions, antibodies play a protective role in *L. major* infection. Blocking B cells in neonatal C3H mice with an anti-mu antibody resulted in an impaired protective T cell response (Scott *et al.*, 1986). In the absence of antibodies, *L. major*-infected mice developed larger lesions, failed to control parasite growth, and showed decreased effector T cell responses (Woelbing *et al.*, 2006). Antibodies seem to favor phagocytosis of *L. major* by dendritic cells, which is required to initiate the immune response against the parasite (Woelbing *et al.*, 2006).

In contrast, other findings have shown that antibody production exacerbates infection. IgG-deficient BALB/c mice, infected with *L. major*, had smaller lesions and decreased parasite load compared to IgG-sufficient infected mice (Miles *et al.*, 2005). The contribution of B cells to susceptibility to *L. major* infection was also evidenced in resistant C.B-17 scid mice, lacking functional T and B cells. C.B-17 scid mice can restore disease susceptibility when wild-type B cells and T cells are adoptively cotransferred, but not by T cells alone. Consistent with this observation, a delayed onset of disease and development of smaller lesions was observed in *L. amazonensis*-infected mice that lacked functional B cells, and therefore antibodies (Anonyme, !!! INVALID CITATION !!! {}). In agreement with the protective role of antibodies in the experimental model of CL, similar results were obtained in Fc-mediated-phagocytosis-deficient mice. **Fc gamma receptor** (FcγR) knockout mice infected with *Leishmania* mexicana developed limited lesions compared to their wild-type counterpart (Kima *et al.*, 2000), demonstrating a key role for antibodies in enhancing Fc-mediated phagocytosis, which is required for the internalization and replication of *Leishmania* parasites. Similarly, FcγRIII-deficient mice infected with *L. mexicana* did not develop

lesions, and high levels of IFN-γ were produced, suggesting a detrimental effect of antibodies during the infection (Thomas & Buxbaum, 2008).

Besides antibody production, B cells can modulate immune responses by cytokine secretion. The immunoregulatory capacity of B cells secreting IL-10, for example, has been described in various models of infection (Candando *et al.*, 2014). In the experimental model of CL, increasing evidence indicates that B cell-derived cytokines promote susceptibility to infection. *L. major* induces IL-10 expression by B cells during the early stages of infection (Menezes Cabral *et al.*, 2008; Palanivel *et al.*, 1996). B cell-derived IL-10, mainly produced by Breg-like cells, skews the immune response towards Th2 cell development and contributes to susceptibility to infection with *L. major* LV39 (Ronet *et al.*, 2010) (**Figure 1)**. *L. amazonensis* also enhances IL-10 production by B cells from susceptible CBA/J mice *in vitro* (Veras *et al.*, 2006). These results highlight the importance of B cell-derived IL-10 in shaping the immune response to *Leishmania*.

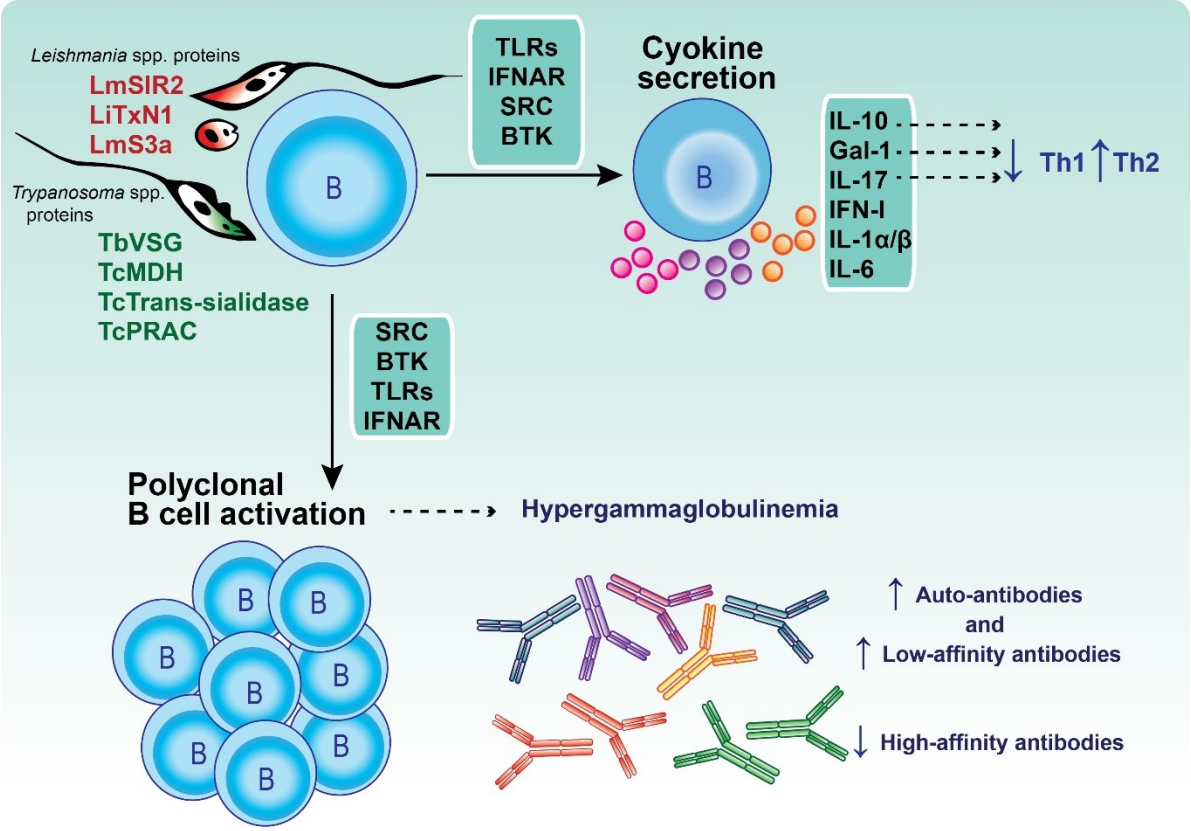

Figure 1. B Cell Activation by *Trypanosoma*tids. *Leishmania* and *Trypanosoma* spp. are known to induce polyclonal B cell activation. Parasite-derived proteins, such as L. major sirtuin (LmSIR2), L. infantum cytosolic tryparedoxin (LiTxN1), L. major-ribosomal protein (LmS3a), T. brucei variant surface glycoprotein (VSG), T. cruzi malate-dehydrogenase (MDH), T. cruzi trans-sialidase (Tc-transialidase) and TcPRAC (Tc-proline racemase), activate B cells and induce cytokine production. In recent years several

pathways of activation were proposed. In the case of *Trypanosoma* spp., Bruton's tyrosine kinase (BTK) and proto-oncogene c-Src (SRC) activation is one of the main pathways used by the parasite, while *Leishmania* spp. require endosomal Toll-like receptor (TLR) activation and type I interferon (IFN-I) signaling. Polyclonal activation is a major consequence of the interaction between *Trypanosoma*tids and B cells. This results in hypergammaglobulinemia and the high production of low-affinity antibodies against the parasite, as well as in the production of autoantibodies. IFNAR, Type I IFN receptor.

B1 cells are also capable of producing IL-10 (O'garra *et al.*, 1992). Recently it has been demonstrated that innate B1 cells can internalize L. amazonensis promastigotes and that this results in enhanced tumor necrosis factor (TNF) and IL-10 expression (Geraldo *et al.*, 2016). The contribution of B1 cells to the anti-*Leishmania* immune response is unclear. BALB/c.xid (lack B1 cells and have defective B2 cells)-infected mice are more resistant to *L. major* infection. In this model, enhanced IFN-γ production and a decrease in B cell-derived IL-10 production correlated with resistance to infection (Hoerauf *et al.*, 1994). In contrast, no differences in terms of parasite susceptibility were found in B1 cell-depleted BALB/c mice infected with *L. major* amastigotes, suggesting that B1 cells do not contribute to susceptibility in *L. major*-infected BALB/c mice (Babai *et al.*, 1999).

4.2. Visceral *Leishmania*sis

VL is caused by *L. donovani* (East Africa and the Indian subcontinent) and *Leishmania* infantum/chagasi (Europe, North Africa, and Latin America) **(Table 2).** The disease is characterized by the presence of **hepatosplenomegaly**, immunosuppression, and hypergammaglobulinemia. Secondary infections, particularly tuberculosis and pneumonia, become common and are frequent causes of death in infected individuals (van Griensven & Diro, 2012).

The mouse model of VL is characterized by an organ-specific immune response. In the liver, parasite multiplication is controlled by an efficient granulomatous response characterized by elevated levels of IFN-γ production by $CD4^+$ and $CD8^+$ T cells. In contrast, the spleen fails to control parasite growth, protective T cell responses become dysfunctional, and the organ enlarges, causing splenomegaly (Bankoti & Stäger, 2012).

In the case of VL, B cells are clearly involved in disease exacerbation. Indeed, B cell-deficient mice infected with *L. donovani* are highly resistant to infection (Smelt *et al.*, 2000). Recent studies have elucidated several mechanisms contributing to disease susceptibility. Polyclonal B cell

activation, a hallmark of VL, seems to be responsible for most of the negative effects: induction of IL-10, and hypergammaglobulinemia, characterized by the production of low-affinity antibodies against the parasite (Bankoti *et al.*, 2012; Silva-Barrios *et al.*, 2016).

Similarly, to CL, B cells also produce IL-10 and contribute to disease susceptibility during VL. In the murine model, we have shown that B cell-derived IL-10 inhibits protective Th1 responses, contributing to disease susceptibility (Bankoti *et al.*, 2012). Interestingly, most of the IL-10 was secreted by MZB cells, and this production was myeloid differentiation primary response 88 (MyD88) and endosomal TLR-dependent (Bankoti *et al.*, 2012; Silva-Barrios *et al.*, 2016). In the canine model of VL and in human VL patients, B cells also suppress T cell functions via IL-10 (Andreani *et al.*, 2015; Schaut *et al.*, 2016). IL-10 secretion was shown to depend on the spleen tyrosine kinase Syk, phosphatidylinositol-3 kinase, and p38 activation (Andreani *et al.*, 2015). IL-10 is not the only immunomodulatory cytokine produced by B cells during VL. In fact, B cells express several cytokines over the course of *L. donovani* infection, including IL-1 α and β and type I IFN (IFN-I). The role of B cell-derived IL-1 has not yet been investigated. However, we recently reported that innate B cell activation through endosomal TLRs and IFN-I promotes VL exacerbation and hypergammaglobulinemia **(Figure 1).** We demonstrated that *L. donovani* triggers endosomal TLRs in B cells, inducing cytokine and endosomal TLR expression, which are further enhanced by autocrine signaling through the type I IFN receptor (IFNAR) (Silva-Barrios *et al.*, 2016). Interestingly, mice with a B cell-specific IFNAR ablation failed to develop hypergammaglobulinemia following *L. donovani* infection (Silva-Barrios *et al.*, 2016), suggesting that IFN-I may promote the production of low-affinity antibodies during VL. In agreement with our results, a type I IFN signaling and B cell activation profile was observed in microarrays from VL-infected human PMBCs (Gardinassi *et al.*, 2016). Several proteins from the *L. donovani* complex, such as LiTXN1, have been associated with B cell activation and enhanced expression of IL-10 by B cells (Menezes Cabral *et al.*, 2008). However, how *L. donovani* triggers endosomal TLRs remains unknown.

At first glance, antibodies also appear to contribute to disease aggravation. IgM and complement activation have been associated with increased disease susceptibility [58]; IgG immune complexes enhance IL-10 production in macrophages, promoting disease (Miles *et al.*, 2005); and polyclonal B cell activation that occurs during active VL results in the production of autoreactive antibodies (Deak *et al.*, 2010). Nevertheless, high-affinity, *Leishmania*-specific antibodies do not seem to be produced during chronic infection. This lack of high-affinity antibodies might be due to the striking reduction in T follicular helper cells during the chronic stage of infection (Rodrigues *et*

al., 2014). It is thus impossible to determine whether high-affinity antibodies would play a protective role in VL. Natural antibodies are also produced during VL. A study on human VL reported that, during active disease, B1 cells are expanded and/or activated. High levels of natural autoantibodies were present in the sera of patients with active disease, which decreased after treatment (Willis *et al.*, 2014). The role of natural antibodies in VL is still unknown.

In summary, *Leishmania* can manipulate B cell responses in distinct ways (depending on the species and sometimes the strain), including the induction of immunomodulatory cytokines, low-affinity antibodies, and hypergammaglobulinemia following polyclonal B cell activation. This interaction results in lower frequencies of B cells in the spleen (Silva-Barrios *et al.*, 2016) over the course of infection, which is perhaps, in part, a consequence of cell death by overactivation. For certain *Leishmania* species and strains, it is thus important to dissect the pathways leading to polyclonal B cell activation and to be able to block them and induce the production of potentially protective high-affinity antibodies in order to promote an effective immune response against the parasite **(Figure 1, Table 3).**

5. B Cells and *Trypanosoma* spp.

Like *Leishmania*, *Trypanosoma* spp. are also known to subvert B cell responses to their own advantage. We discuss below the role of B cells and the modulation of the humoral response by the parasites *Trypanosoma* cruzi and *Trypanosoma* brucei.

5.1. Trypanosoma cruzi

The intracellular protozoan parasite *T. cruzi* causes American trypanosomiasis **(Table 2),** also known as Chagas' disease, which affects millions of people, mostly in Central and South America (Köberle, 1968). Among the *Trypanosoma*tids, *T. cruzi* presents one of the most complex life cycles, characterized by several developmental morphologies present in vertebrate and invertebrate hosts (Reduviidae family). The infective forms of the parasite are metacyclic and bloodstream trypomastigotes and amastigotes. *T. cruzi* can infect macrophages, neurons, heart muscle cells, and skeletal muscle cells among others (Melo & Brener, 1978). Within the host cell cytoplasm, metacyclic trypomastigotes change into amastigotes. Replication takes place by binary

fission. Amastigotes then differentiate into trypomastigotes, which are released into the blood stream to propagate infection. **Cardiomyopathy** and chronic gastrointestinal manifestations – as a consequence of both central nervous system and enteric nervous system impairment caused by *T. cruzi* infection – are the main symptoms of Chagas' disease in humans (de Souza *et al.*, 2010; Spinella *et al.*, 1992). During the infection, clearance of *T. cruzi* requires innate and acquired immune mechanisms, macrophages, natural killer (NK) cells, T and B lymphocytes, and the production of cytokines, which play key roles in regulating both parasite replication and immune response (Zhang & Tarleton, 1996).

T. cruzi uses several strategies to avoid the generation of an effective immune response. One of these strategies consists in modulating the B cell compartment. During early stages of infection, *T. cruzi* causes polyclonal B cell activation and low-affinity Ig secretion, leading to hypergammaglobulinemia (Minoprio *et al.*, 1989; Minoprio *et al.*, 1988; Minoprio *et al.*, 1986) **(Figure 1).** However, most of the antibodies produced are not parasite-specific and are unable to control infection (Bermejo *et al.*, 2011).

T. cruzi uses several mechanisms to manipulate B cell responses. One of these strategies consists in modulating B cell maturation. Infection by T cruzi induces a marked reduction in B cells in the bone marrow, enhancing B cell apoptosis and affecting B cell migration to the periphery (Zuniga *et al.*, 2005). Infection by *T. cruzi* induces a marked reduction in B cells in the bone marrow and spleen. In the spleen, the **Fas receptor**/Fas ligand (FasR/FasL) pathway, one of the main mechanisms involved in B cell death, participates in the elimination of mature B cells during *T. cruzi* infection (Zuñiga *et al.*, 2002). In contrast, in the bone marrow, this process occurs in an independent FasR/FasL manner. The immature B cell depletion during *T. cruzi* infection involves a secretion product from the **cyclooxygenase** pathway in myeloid cells [73], such as prostaglandins, molecules well known for promoting inflammation. B cells have been shown to play a key role in systemic protection against *T. cruzi* infection through the secretion of parasite-specific antibodies and maintenance of memory $CD8^+$ T cells (Cardillo *et al.*, 2007; Sullivan *et al.*, 2011).

In spite of the marked reduction in B cell precursors, during *T. cruzi* infection there is a notable polyclonal expansion of B cells in the spleen. Hypergammaglobulinemia, a consequence of polyclonal B cell activation, represents another mechanism by which *T. cruzi* persists in the host. *T. cruzi*-derived antigens trigger B cell activation and induce the production of low-affinity antibodies, affecting the more efficient anti-parasite humoral response. Indeed, high-affinity *T. cruzi*-specific antibodies can provide protection via complement activation and **antibody-**

dependent cellular cytotoxicity (ADCC) (Mota & Umekita, 1989; Spinella *et al.*, 1992). Moreover, despite an initial control of parasite growth, *T. cruzi*-infected μMT mice are very susceptible to infection and die during the chronic phase (Kumar & Tarleton, 1998). Disease exacerbation is associated with a decrease in proinflammatory cytokine production and defective memory $CD8^+$ T cell formation, required to mount an efficient Th1 response (Cardillo *et al.*, 2007). The polyclonal B cell activation level varies depending on the mice and the parasite strain involved in the infection (Bryan *et al.*, 2010; Spinella *et al.*, 1992). Resistant C57BL/6 mice infected with *T. cruzi* have improved parasite-specific humoral responses and decreased polyclonal B cell activation compared to susceptible BALB/c mice (Bryan *et al.*, 2010).

Polyclonal B cell activation also leads to the production of autoantibodies (Peralta *et al.*, 1981; Szarfman *et al.*, 1981). In the experimental mouse model, it has been observed **that B cell activating factor (BAFF)** signaling, a key cytokine in B cell survival (Mackay & Schneider, 2009), is required for the development of these autoantibodies. BAFF blockade also partially affects polyclonal B cell responses during the infection (Bermejo *et al.*, 2010).

Several studies have investigated the mechanisms leading to B cell activation in *T. cruzi* infection. *T cruzi*-derived metabolic proteins, such as trans-sialidase (TcTrans-sialidase), mitochondrial malate dehydrogenase (TcMDH), and *T. cruzi*-proline racemase (TcPRAC), are known to induce T-independent polyclonal B cell activation and promote the generation of low-affinity antibodies (Chamond *et al.*, 2003; Gao *et al.*, 2002; Montes *et al.*, 2002; Reina-San-Martin *et al.*, 2000). Burtons tyrosine kinase (BTK), a key molecule in B cell receptor (BCR) signaling, is required for polyclonal B cell activation by *T. cruzi* trans-sialidase (Gao & Pereira, 2001) **(Figure 1, Table 4)**.

In contrast to B2 cells, B1 cells, characterized by a **polyreactive antibody** repertoire, seem to be involved in disease progression. CD5+ B cell-deficient mice (BALB.Xid) infected with *T. cruzi* can control parasite multiplication. A marked reduction in specific and nonspecific antibodies was observed in infected BALB.Xid mice (Minoprio *et al.*, 1991). Moreover, BALB.Xid mice produce high levels of IFNγ, IL-2, and IL-4, but lower levels of IL-10; IFN-γR blockade re-establishes susceptibility and leads to an increased parasite burden and mortality (Minoprio *et al.*, 1991; Minoprio *et al.*, 1993).

B cell-derived cytokines also affect the outcome of *T. cruzi* infection by modulating protective T cell responses. Galectin-1 (Gal-1) – a member of the family of β-galactoside-binding proteins with a role in modulating cell–cell and cell–matrix interactions (Camby *et al.*, 2006) – has also been shown to mediate immunosuppression of $CD8^+$ T cell (Gandhi *et al.*, 2007). Gal-1 expression by

B cells has been shown to be enhanced by CD40 activation [93]. During *T. cruzi* infection, B cell-expressing galectin-1 triggers T cell apoptosis and reduces IFN-γ secretion (Zuñiga *et al.*, 2001). B cells also secrete the proinflammatory cytokine IL-17 and are considered the major source of IL-17 during *T. cruzi* infection (da Matta Guedes *et al.*, 2010). However, other cells, such as NK, $CD4^+$, and $CD8^+$ T cells, are known to produce this cytokine (Bermejo *et al.*, 2013). IL-17 has been associated with protection in different infectious and autoimmunity models (Korn *et al.*, 2009; Tabarkiewicz *et al.*, 2015). IL-17 exerts a protective role during the acute *T. cruzi* infection (da Matta Guedes *et al.*, 2010), limiting tissue damage by recruiting IL-10-producing neutrophils that regulate the IFN-γ response (Boari *et al.*, 2012; da Matta Guedes *et al.*, 2010). Interestingly, mice with a B cell-specific deficiency of IL-17a are more susceptible to *T. cruzi* infection. This susceptibility correlates with higher concentrations of IFN-γ and TNF. IL-17 secretion by B cells is induced by *T. cruzi* trans-sialidase, a surface membrane enzyme present on *T. cruzi* promastigotes, and requires BTK, SRC kinases, and CD45 activation in B cells (da Matta Guedes *et al.*, 2010). TcPRAC, an enzyme involved in metabolism, differentiation, and infectivity of *T. cruzi* (Chamond *et al.*, 2005), has been shown to induce IL-10 production mostly by MZB cells (Bryan & Norris, 2010). However, the contribution of MZB cell-derived IL-10 during *T. cruzi* infection remains unknown.

CCL5 **(see chemokine)**, a molecule implicated in the initiation and enhancement of antigen-specific immune responses, is another B cell-derived cytokine that has been shown to play a key role in *T. cruzi* infection (Lillard *et al.*, 2001). One of the receptor molecules that can interact with CCL5 is CCR5. During *T. cruzi* oral infection, treatment of CCR5-deficient mice with CCL5-neutralizing antibody results in decreased levels of *T. cruzi*-specific B cell responses, decreased gastric inflammation, and mucosal protection. *In vitro*, CCL5 acts in an autocrine manner to promote IgM secretion and B cell proliferation (Sullivan *et al.*, 2011). However, recently it has been shown that *T. cruzi*-specific B cells are necessary for systemic, but not mucosal, parasite challenge (Sullivan *et al.*, 2015). CCL5 levels are known to be upregulated during *T. cruzi* infection (da Matta Guedes *et al.*, 2010; Machado *et al.*, 2005). Nevertheless, it remains unknown whether *T. cruzi* can induce CCL5 secretion by B cells and what role B cell-derived CCL5 may play **(Figure 1, Table 4).**

5.2. Trypanosoma brucei

Human African trypanosomiasis (HAT), or sleeping sickness, is caused by the extracellular flagellate protozoan *Trypanosoma brucei* spp. **(Table 2).** The disease affects a wide range of mammalian species, including livestock and humans, causing delays in social, agricultural, and economic development in sub-Saharan Africa (Simarro *et al.*, 2011). Three subspecies of *T. brucei* exist: *T. brucei gambiense*, which is associated with more than 95% of reported cases and causes chronic trypanosomiasis in humans (Anonyme, 2013; Magez & Radwanska, 2009); *T. brucei* rhodesiense, which causes acute trypanosomiasis in humans; and *T. brucei brucei*, which, in normal conditions, does not infect humans but causes nagana disease in wild and domestic animals (Berriman *et al.*, 2005; Brun *et al.*, 2010; MacGregor & Matthews, 2010).

As in the case of *Leishmania*sis, trypanosomiasis is a zoonotic infection that alternates between its invertebrate and vertebrate hosts to ensure dissemination (Moran *et al.*, 2009). Upon ingestion of blood from an infected mammalian host by the tsetse fly vector, *T. brucei* trypomastigotes undergo differentiation steps going from procyclic trypomastigotes, to **epimastigotes**, and finally metacyclic **trypomastigotes** (Matthews, 2005). The last developmental stage in the vector is infectious to mammalian hosts and can be transmitted by injection to a new host during a blood meal, closing the double-host infection loop (Langousis & Hill, 2014).

Independent of the progression type, HAT disease is characterized by two clinical stages: the haemolymphatic stage, occurring in the first few weeks following infection and during which parasites replicate in the bloodstream and cause diverse clinical manifestations; and the meningoencephalitic phase, during which parasites invade extravascular spaces, including cardiac tissue and the central nervous system (Brun *et al.*, 2010; Langousis & Hill, 2014). The presence of parasites in the brain leads to progressive neurological breakdown, and clinical features are marked by general motor weakness and severe neurological changes that disrupt the sleep–wake cycle (Kennedy, 2013).

A characteristic of *T. brucei* is its capacity to escape the host's immunity. Pathogenic human *T. brucei* is a vector-borne extracellular protozoan and is thus constantly exposed to immune effectors. To prevent elimination, it undergoes antigenic variation, modifying its surface proteins (Horn, 2014). *T. brucei* is covered with densely packed variant surface glycoprotein (VSG) homodimers (Taylor & Rudenko, 2006). VSGs are highly immunogenic, and a hallmark feature of African trypanosomiasis is increased levels of circulating immunoglobulins (Radwanska *et al.*, 2000). Humoral responses are both T-dependent and T-independent, the latter being protective

against disease progression (Reinitz & Mansfield, 1990). Both human and animal trypanosome infections induce antigen-specific humoral responses, but also induce polyclonal B cell activation and secretion of nonspecific and self-reactive antibodies (Diffley, 1983; Morrison *et al.*, 2010; Oka *et al.*, 1984; Vincendeau & Bouteille, 2006) **(Figure 1, Table 4)**.

In the first week following fly bite, neutrophils, and T and B lymphocytes are recruited to the site of infection(Mwangi *et al.*, 1990). Near the initial infection site, VSG was shown to induce B cell polyclonal activation, leading to, among other effects, autoantibody secretion that can lead to tissue damage (Kazyumba *et al.*, 1986)**(Figure 1).**

T. brucei possesses the ability to quickly renew its entire protein coat and degrade defective VSGs and recycle antibody-bound VSGs or other host material (Engstler *et al.*, 2004; O'Beirne *et al.*, 1998; Wang *et al.*, 2003). To achieve this active renewal, *T. brucei* varies VSG gene expression, selecting from a pool of up to 2000 individual VSG genes and pseudogenes (Marcello & Barry, 2007a; Marcello & Barry, 2007b). This mechanism is a key virulence factor of trypanosomes and renders potential vaccine design hard to conceive. This not only allows evasion of the host's humoral responses but also enables accumulation of a growing pool of neutralizing antibodies. A possible downside for the parasites is that once critical neutralizing antibody titers are reached, predominant VSG-expressing parasites become the target of direct antibody-mediated lysis and macrophage- and antibody-dependent phagocytosis, leading to transitory decreased **parasitemia** (Cheung *et al.*, 2016; Engstler *et al.*, 2007).

Another mechanism for evading B cell responses is the irreversible MZB cell and FoB cell depletion from the spleen caused by *T. b. brucei* (Radwanska *et al.*, 2008). This infection-induced condition was recently attributed to NK cell-derived elimination of B cells through a perforin-dependent lysis mechanism (Frenkel *et al.*, 2016). Although exact mechanisms were not yet identified, results obtained by Frenkel et al. suggest that B cells acquire an NK cell-activating phenotype following trypanosome infection (Frenkel *et al.*, 2016).. It was also suggested that the densely packed VSG coat could be responsible for abnormal B cell activation and exhaustion (Dubois *et al.*, 2005). Direct cross-linking of the BCR by the parasite was suggested to cause T-independent polyclonal B cell expansion and transitional B cell apoptosis (Bockstal *et al.*, 2011). Trypanosomes are also known to release soluble VSGs, through phospholipase C (PLC) cleavage of the VSG glycosylphosphatidyl-inositol (GPI) anchor. Early after infection, soluble VSG release induces strong innate proinflammatory responses (Carrington *et al.*, 1998; Magez *et al.*, 1998; Paulnock & Coller, 2001). However, prolonged exposure to the same antigens was shown to inhibit intracellular signalling and activation (Geiger *et al.*, 2016).

Impaired humoral and T cell responses elicited against exogenous antigens were also reported during trypanosomiasis, infection-induced impairment of MHC-II presentation being the suggested mechanism (Askonas *et al.*, 1979; Namangala *et al.*, 2000a; Namangala *et al.*, 2000b; Radwanska *et al.*, 2008).

Compromised B cell responses were also shown to be a consequence of reduced bone marrow cell counts and impaired erythropoiesis and **lymphopoiesis** (Clayton *et al.*, 1980). The possible causes were partly elucidated in a recent B cell repopulation study which demonstrated that *T. brucei* abrogated bone marrow B cell lymphopoiesis and that this situation could not be recovered by splenic compensatory extramedullary B lymphopoiesis (Bockstal *et al.*, 2011). Splenic B lymphopoiesis was halted by apoptosis at the transitional B cell stage, a condition that was independent of TNF-TNF-R1-, Fas-Fas-L- and prostaglandin-mediated pathways, but was induced by direct contact between B lymphocytes and live parasites. As trypanosome lysate could not recapitulate transitional B cell apoptosis, **superantigen**-mediated death was therefore excluded. These results may explain how *T. brucei*-induced B cell exhaustion and loss of humoral immunocompetence could occur in an experimental African trypanosomiasis model (Bockstal *et al.*, 2011), possibly answering, in part, the long-time observed polyclonal B cell expansion and functional exhaustion in the spleen and lymph nodes (Corsini *et al.*, 1977; Diffley *et al.*, 1980; Mayor-Withey *et al.*, 1978). Another possible answer to the question of B cell polyclonal expansion is that *T. brucei* DNA was shown *in vitro* to induce B cell proliferation (Shoda *et al.*, 2001), a phenomenon that could be caused *in vivo* by IgM-mediated immune lysis of parasites (Reinitz & Mansfield, 1990; Stijlemans *et al.*, 2016). A possible IgM source could be from directly- or indirectly-activated B1 cells (parasite or cytokines, respectively), as these cells were shown to be overrepresented in T. congolense-infected cattle (Naessens & Williams, 1992). It would be interesting to know if *T. brucei* DNA triggers endosomal TLRs in B cells.

TNF-α overproduction by macrophages in human trypanosomiasis (Okomo-Assoumou *et al.*, 1995; Rouzer & Cerami, 1980) is also thought to be involved in hypergammaglobulinemia (Ponte-Sucre, 2016)since this cytokine is known to promote B cell activation, proliferation, and differentiation (Roldan *et al.*, 1992; Rouzer & Cerami, 1980).

To summarize, *Trypanosoma* species were shown to manipulate B cell responses through: polyclonal B cell activation that leads to hypergammaglobulinemia and increased autoreactive and low-affinity antibody production; decreased proinflammatory cytokine production that leads to reduced T cell responses; and transitional B cell apoptosis. Although high-affinity and neutralizing antibody responses can be elicited against *Trypanosoma* species, humoral

responses and new B cell development are clearly disrupted by the infection. Dissecting polyclonal B cell activation mechanisms following *Trypanosoma* infection might put forward ways to circumvent infection-related immune system deregulations and ultimately enable the development of treatments and/or prophylactic vaccines **(Figure 1, Table 4).**

6. Concluding Remarks

*Trypanosoma*tids appear to manipulate B cell responses to increase their own survival and promote the establishment of chronic infections. A common outcome of the interaction between these parasites and B cells is the induction of polyclonal activation, which often results in the inhibition of high-affinity, antigen-specific antibodies that could potentially have neutralizing effects, and eventually leads to B cell death. B cell responses are not only skewed towards the uncontrolled production of low-affinity antibodies, but they are also inhibited in the bone marrow or forced to differentiate into IL-10-producing regulatory cells. Several questions emerge, as outlined in Outstanding Questions. For instance, which parasite molecules trigger polyclonal B cell activation? Polyclonal B cell activation and hypergammaglobulinemia are fairly common features of chronic infections; it is thus possible that the environment may also play a role in this process. Are B cells only directly activated by the parasites or can the inflammatory environment also induce polyclonal B cell activation? Also, is it possible to block or reduce B cell activation? Would this be advantageous for disease resolution? TFh cells are involved in promoting the production of antigen-specific antibodies. Very few studies have investigated to date this T cell subpopulation in *Trypanosoma*tid infections. What is the role of TFh cells, and how are these responses modulated during infection with *Trypanosoma*tids? Another important point would be to analyse the specificity and affinity of antibodies in patients and also determine if high-affinity antibodies against the intracellular form of the various species of *Leishmania* and *Trypanosoma*s will be protective or not.

A major caveat in dissecting B functions and activation pathways during infection is the limitation that several mouse models may have compared to the disease in humans in terms of the size of the inoculum and chronicity of the disease. Results need therefore to be carefully interpreted and may not fully translate to human disease.

A better understanding of the molecular pathways involved in this deadly interaction, and the identification of the parasite molecules responsible for it, could help in the design of novel therapeutic or prophylactic approaches.

7. Outstanding Questions

Polyclonal B cell activation is probably the result of the activation of multiple pathways. Which is the molecular mechanism(s) involved?

Are these pathways reversible? Is it possible to block them?

Which parasite molecules trigger polyclonal B cell activation? What is the role of the inflammatory environment in the induction of polyclonal B cell activation?

Which other pathway(s) triggers hypergammaglobulinemia besides endosomal TLR recognition and IFN-I?

Does *Leishmania* interfere with lymphopoiesis in the bone marrow?

Are high-affinity-antibodies protective against *Leishmania*? How can they be induced?

How is the TFh response regulated in *Trypanosoma*tids?

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9. Glossary

Amastigote
aflagellated, intracellular replicative morphology of *Leishmania* spp. and Trypanosma spp.

Antibody-dependent cellular cytotoxicity (ADCC)
cell-mediated immune defense mechanisms whereby an effector cell actively lyses a target cell coated with antibodies.

B cell activating factor (BAFF)
potent B cell activator involved in B cell proliferation and differentiation.

Cardiomyopathy
group of diseases that affect the heart muscle.

Chemokine(C-C motif) ligand 5 (CCL5)
also known as RANTES (regulated on activation, normal T cell expressed and secreted), a chemotactic cytokine for T cells, dendritic cells, monocytes, NK cells, eosinophils, and basophils, and plays an active role in recruiting leukocytes into inflammatory sites. Also, in the presence of IL-2 and IFN-γ T cell-derived cytokines, CCL5 induces the proliferation and activation of certain natural-killer (NK) cells.

Cyclooxygenase (COX)
officially known as prostaglandin-endoperoxide synthase (PTGS), an enzyme that is responsible for formation of prostanoids, including thromboxane and prostaglandins.

Delayed-type hypersensitivity
inflammatory reactions initiated by mononuclear leukocytes, in which $CD4^+$ T cells play a central role.

Epimastigote
developmental stage of *Trypanosoma* spp. characterized by the presence of the flagellum that is located anterior of the nucleus, emerges in the middle of the cell body, and connects to the body by an undulating membrane.

FAS receptor (FasR)
also known as apoptosis antigen 1 (APO-1 or APT), cluster of differentiation 95 (CD95), or tumor necrosis factor receptor superfamily member 6 (TNFRSF6), FasR is a surface membrane protein that leads to programmed cell death. The Fas/FasL pathway has been described to play a major role in B cell apoptosis.

Fc gamma receptors
receptors that recognize the Fc region of antibodies; involved in phagocytisis of opsonized microbes.

Hepatosplenomegaly
simultaneous enlargement of the liver and the spleen.

Hypergammaglobulinemia
condition characterized by increased levels of immunoglobulins in the blood serum.

Immune complexes
complexes formed by the integral binding of an antibody to a soluble antigen.

Immunopathology
damage caused to an organism by its own immune response; disease associated.

Lymphopoiesis
generation of white blood cells occurring in the bone marrow.

Meningoencephalitis
infection or inflammation of the meninges (membranes enveloping the brain) and the brain.

Parasitemia
strictly meaning parasite content in the blood; often also used to define parasite load in a specific organ.

Plasmocyte
also called plasma cell. A B cell that secretes large volumes of antibodies.

Polyreactive antibody
a major component of the natural antibody repertoire; it binds with low affinity to a variety of structurally unrelated antigens.

Promastigote
flagellated stage of *Leishmania* spp., found in the sandfly midgut, transmitted to the mammalian host during the blood meal.

Superantigens
B cell superantigens have the potency to stimulate a substantial proportion of B cells, independently of their BCR specificity. B cells' contact with these antigens causes rapid activation that usually leads to an increased expression of activation markers and MHC-II molecules, downregulation of BCR expression, and activation-associated accelerated cell death.

T follicular helper (TFh) cells
are specialized providers of T cell help to B cells; these cells are essential for germinal center formation, affinity maturation, and the development of most high-affinity antibodies and memory B cells.

Trypomastigote

life-cycle stage of *Trypanosoma* spp., where the flagellum is posterior to the nucleus and connected to the body by a membrane.

Annex-III: Meetings and Conferences

Sasha Silva-Barrios, Mélina Smans, Salman T. Qureshi, Jörg H. Fritz, Albert Descoteaux, and Simona Stäger. "To -B or not to-B: B cells in health and disease" EMBO meeting. *"Innate immune B cell activation by Leishmania donovani exacerbates disease and mediates hypergammaglobulinemia* ". Girona, Spain. September 10-14, 2017. **Poster presentation.**

Sasha Silva-Barrios and Simona Stäger. Role of activation-induced cytidine deaminase (AID) in the context of *L. donovani* infection. Montreal Immunology Meeting - 2016. Montreal, Canada. November 7, 2016. **Poster presentation.**

Sasha Silva-Barrios and Simona Stäger. Role of activation-induced cytidine deaminase (AID) in the context of *L. donovani* infection. Annual Quebec Molecular Parasitological Symposium-CHPI. Montreal, QC, Canada. June 6-7, 2016. **Poster presentation.**

Sasha Silva-Barrios, Mélina Smans, Salman T. Qureshi, Jörg H. Fritz, Albert Descoteaux, and Simona Stäger. Canadian Society for Immunology's 2016 meeting (CSI). *"Innate immune B cell activation by Leishmania donovani exacerbates disease and mediates hypergammaglobulinemia* ". Ottawa, Canada. April 01-04, 2016. **Oral presentation.**

Sasha Silva-Barrios, Mélina Smans, Salman T. Qureshi, Jörg H. Fritz, Albert Descoteaux, and Simona Stäger. 10th World Immune Regulation Meeting. *"Innate immune B cell activation by Leishmania donovani exacerbates disease and mediates hypergammaglobulinemia* ". Davos, Switzerland.March 16-19, 2016. **Oral presentation.**

Sasha Silva-Barrios, Mélina Smans, Salman T. Qureshi, Jörg H. Fritz, Albert Descoteaux, and Simona Stäger. *"Endosomal TLRs: the pathway used by Leishmania donovani to induce B cell polyclonal activation".* Montreal Immunology Meeting - 2015. Montreal, Canada. November 23, 2015. **Oral presentation.**

Sasha Silva-Barrios, Mélina Smans, Salman T. Qureshi, Jörg H. Fritz, Albert Descoteaux, and Simona Stäger.
9th Congrès Armand-Frappier 2015. *"Endosomal TLRs: the pathway used by Leishmania donovani to induce B cell polyclonal activation".* Orford, QC, Canada November 12-14, 2015. **Oral presentation.**

Sasha Silva-Barrios, Mélina Smans, Salman T. Qureshi, Jörg H. Fritz, Albert Descoteaux, and Simona Stäger. Institut Pasteur International Network Conference. *"Leishmania donovani activates B cells and induces cytokine expression by triggering endosomal TLRs".* Paris, France. October 14-16, 2015. **Poster presentation.**

Sasha Silva-Barrios, Mélina Smans, Salman T. Qureshi, Jörg H. Fritz, Albert Descoteaux, and Simona Stäger. *"Leishmania donovani activates B cells and induces cytokine expression by triggering endosomal TLRs".* 15th Annual Quebec Molecular Parasitological Symposium-CHPI. Montreal, QC, Canada. June 9, 2015. **Oral presentation.**

Sasha Silva-Barrios, Mélina Smans, Salman T. Qureshi, Jörg H. Fritz, Albert Descoteaux, and Simona Stäger. *"Leishmania donovani activates B cells and induces cytokine expression by triggering endosomal TLRs".* 19th Annual Woods Hole Immunoparasitology Meeting-WHIP. Woods Hole, MA, USA. April 19-22, 2015. **Oral presentation.**

Sasha Silva-Barrios, Mélina Smans, Salman T. Qureshi, Albert Descoteaux, and Simona Stäger. "*Characterization of B cell activation upon exposure to Leishmania donovani".* November 24, 2014.
Poster presentation. Annual Montreal Immunology meetings-MIM. Montreal, QC, Canada. **Poster presentation**

Sasha Silva-Barrios, Mélina Smans, Salman T. Qureshi, Albert Descoteaux, and Simona Stäger." *Characterization of B cell activation upon exposure to Leishmania donovani".* Institut Pasteur International

Network Conference. Paris, France. *Work:* September 10-13, 2014. **Poster presentation**

Sasha Silva-Barrios, Mélina Smans, Salman T. Qureshi, Albert Descoteaux, and Simona Stäger. *"Characterization of B cells subpopulation responses during Leishmania donovani infection".* XIV International Congress of Bacteriology and Applied Microbiology, XIV International Congress of Mycology and Eukaryotic Microbiology XVI International Congress of Virology-IUMS. Montreal, QC, Canada. July 27 to August 1, 2014. **Oral presentation.**

Sasha Silva-Barrios, Albert Descoteaux, and Simona Stäger. *"B cell activation mechanism induced by L. donovani".* Congrès Armand-Frappier 2013. Orford, QC, Canada. *Work:* November 14-16, 2013. **Poster presentation**