Centre Armand-Frappier Santé Biotechnologie

UNTANGLING THE DYNAMICS OF B CELL-PARASITE INTERACTION IN *LEISHMANIA DONOVANI* INFECTION: IMPLICATIONS FOR POLYCLONAL B CELL ACTIVATION

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RÉSUMÉ

Bien que les cellules B et leur rôle dans l'immunité humorale soient essentiels à la défense immunitaire contre de nombreuses infections, au cours de certaines maladies, les cellules B sont activées pour produire de grandes quantités d'anticorps non protecteurs dans un état appelé hypergammaglobulinémie. L'activation des cellules B polyclonales et l'hypergammaglobulinémie qui en résulte sont une caractéristique de plusieurs infections chroniques, dont la leishmaniose viscérale (LV). Forme la plus grave de la leishmaniose, la LV est causée par les parasites *L. donovani* ou *L. infantum* et peut être fatale si elle n'est pas traitée. Au cours de la LV, il a été démontré que les cellules B jouent un rôle néfaste, car les souris déficientes en cellules B sont capables de résoudre l'infection (Smelt *et al.*, 2000). Des études ultérieures ont identifié l'hypergammaglobulinémie comme le principal mécanisme responsable de ce rôle négatif (Silva-Barrios & Stäger, 2019). Cependant, les mécanismes d'interaction entre les cellules B et le parasite qui sous-tendent l'activation des cellules B polyclonales ne sont toujours pas clairs.

En étudiant les interactions précoces entre les cellules B spléniques primaires et les amastigotes de *L. donovani in vitro*, nous avons observé la formation de longues connexions tubulaires entre les cellules B correspondant au profil des *tunnelling nanotubes* (TNT). Ces structures basées sur la F-actine sont une voie de communication intercellulaire récemment découverte, connue pour faciliter les échanges d'une variété de cargaisons, allant des protéines et des ions à des organites entiers. Cependant, un nombre croissant de preuves démontrent maintenant que ces connexions peuvent être exploitées par certains pathogènes, à savoir les virus, les bactéries et les prions, pour se propager dans des cellules non infectées. Dans ce travail, nous montrons que les amastigotes de *L. donovani* non seulement induisent la formation de TNT de cellules B de manière dépendante du récepteur 2 du complément (CR2), mais qu'ils peuvent également exploiter ces connexions pour se disséminer entre les cellules B, ce qui constitue le premier rapport de cette subversion des TNT par un parasite. Nous démontrons en outre que le contact direct entre les cellules B et le parasite est nécessaire à l'activation des cellules B, comme le montre l'augmentation de l'expression de surface du complexe majeur d'histocompatibilité de classe II (CMH-II) et du marqueur d'activation CD86. En effet, nous avons détecté *L. donovani* dans la zone des cellules B spléniques des souris infectées au 14e jour après l'infection, ce qui suggère un contact direct entre les cellules et le parasite *in vivo*. Parmi les deux principaux sous-ensembles de cellules B spléniques, les cellules B de la zone marginale (MZB) ont une plus grande capacité à capturer les parasites in vitro, ce qui est reflété par une découverte précédente

sur des cellules B isolées de souris infectées montrant que les cellules MZB étaient les principales cellules B à capturer le parasite 20 h après l'infection (Bankoti *et al.*, 2012). Ces cellules MZB sont à proximité des macrophages, la principale cible cellulaire de l'infection par *Leishmania*, dans la zone marginale de la rate et sont donc susceptibles de recevoir des parasites des macrophages. En effet, nous montrons que les parasites sont capables de se propager des macrophages dérivés de la moelle osseuse infectés par *L. donovani* vers les cellules B. Nous observons en outre la formation de connexions de type TNT entre les macrophages et les cellules B qui se colocalisent avec le parasite, ce qui indique que cette voie de communication intercellulaire participe également à cette dissémination intercellulaire.

Dans l'ensemble, nos résultats soutiennent un modèle dans lequel les amastigotes de *L. donovani* peuvent se propager des macrophages aux cellules B, ce qui peut être facilité par les TNT, et ces connexions sont ensuite exploitées par le parasite pour se disséminer parmi les cellules B, propageant ainsi l'activation des cellules B et conduisant finalement à l'activation des cellules B polyclonales.

Mots clés

Cellules B ; tunneling nanotubes; *Leishmania donovani* ; leishmaniose viscérale ; communication intercellulaire ; activation polyclonale des cellules B

ABSTRACT

While B cells and their role in humoral immunity are critical for immune defense against many infections, in the course of some diseases B cells are activated to produce large amounts of non-protective antibodies in a condition called hypergammaglobulinemia. Polyclonal B cell activation and resulting hypergammaglobulinemia is a hallmark of several chronic infections, including visceral leishmaniasis (VL). The most severe form of leishmaniasis, VL is caused by *L. donovani* or *L. infantum* parasites and can be fatal if left untreated. During VL, B cells have been shown to play a noxious role as B cell-deficient mice are able to resolve the infection (Smelt *et al.*, 2000). Later studies identified hypergammaglobulinemia as the main mechanism responsible for this negative role (Silva-Barrios & Stäger, 2019). However, the mechanisms of interaction between B cells and the parasite underlying polyclonal B cell activation are still unclear.

When studying the early interactions between primary splenic B cells and *L. donovani* amastigotes in vitro, we observed the formation of long tubular connections between B cells fitting the profile of tunneling nanotubes (TNTs). These F-actin based structures are a recently discovered route of intercellular communication known to facilitate exchanges of a variety of cargoes, ranging from proteins and ions to whole organelles; however, a growing body of evidence now also demonstrates that these connections may be exploited by some pathogens, namely viruses, bacteria, and prions, to spread to uninfected cells. In this work, we show that *L. donovani* amastigotes do not only induce the formation of B cell TNTs in a complement receptor 2 (CR2)-dependent manner, but they can also exploit these connections to disseminate between B cells, marking the first report of this subversion of TNTs by a parasite. We further demonstrate that direct contact between B cells and the parasite is required for B cell activation as evidenced by the upregulation of surface expression of the major histocompatibility complex class II (MHC-II) and activation marker CD86. Indeed, we detected *L. donovani* in the splenic B cell area of infected mice by day 14 post-infection indicating direct contact between cells and parasite *in vivo*. Of the two major splenic B cell subsets, marginal zone B (MZB) cells have a higher capacity to capture parasites in vitro, which is mirrored by a previous finding on B cells isolated from infected mice showing that MZB cells were the primary B cells to capture parasite 20 h post-infection (Bankoti *et al.*, 2012). These MZB cells are in close proximity to macrophages, the main cellular target of *Leishmania* infection, in the marginal zone of the spleen and are thus likely receive parasites from macrophages. Indeed, we show that parasites are able to spread from bone marrow-derived macrophages infected with *L. donovani* to B cells. We further observe the

formation of TNT-like connections between macrophages and B cells which colocalize with the parasite, indicating that this route of intercellular communication also participates in this intercellular spread.

Taken together, our results support a model in which *L. donovani* amastigotes may spread from macrophages to B cells which can be facilitated by TNTs, and these connections are then further exploited by the parasite to disseminate among B cells, thus propagating B cell activation and ultimately leading to polyclonal B cell activation.

Keywords

B cells; tunneling nanotubes; Leishmania donovani; visceral leishmaniasis; intercellular communication; polyclonal B cell activation

SOMMAIRE RÉCAPITULATIF

La leishmaniose est une famille de maladies parasitaires protozoaires transmises à des hôtes mammifères par la piqûre de phlébotomes femelles. Classée par l'OMS parmi les maladies tropicales négligées, elle enregistre environ 1 million de nouvelles incidences par an et touche principalement les régions confrontées à des taux de pauvreté élevés (Burza *et al.*, 2018). Chez les hôtes mammifères, les manifestations cliniques de la maladie dépendent de l'espèce du parasite et peuvent aller d'infections autogènes caractérisées par des lésions de la peau ou des muqueuses, à de graves perturbations tissulaires des organes viscéraux, qui sont fatales en l'absence de traitement. Les deux espèces de *Leishmania* connues pour causer la LV sont *L. donovani* et *L. infantum*, qui peuvent provoquer des infections chroniques potentiellement mortelles dans les organes viscéraux et la moelle osseuse, et s'accompagnent de symptômes tels que la fièvre, l'anémie, la cachexie et l'immunosuppression, contribuant encore à la létalité de la maladie en sensibilisant les hôtes aux co-infections (Chappuis *et al.*, 2007). Une caractéristique importante de la LV, partagée avec de nombreuses autres maladies et infections chroniques, est l'hypergammaglobulinémie, définie comme la présence de niveaux excessifs d'anticorps non protecteurs causés par l'activation polyclonale des cellules B (Silva-Barrios & Stäger, 2019).

En fait, les cellules B sont nocives au cours de la LV, car les souris déficientes en cellules B sont très résistantes à l'infection et peuvent contrôler l'infection dans la rate, qui est généralement chronique (Smelt *et al.*, 2000). La production de cytokines régulatrices telles que l'IL-10 est l'une des voies par lesquelles les cellules B jouent un rôle néfaste dans cette maladie. Notre laboratoire et d'autres ont déjà rapporté que les cellules B produisent de l'IL-10 pendant la LV chez l'homme, chez la souris et chez le chien (Bankoti *et al.*, 2012; Andreani *et al.*, 2015; Schaut *et al.*, 2016b). Si l'IL-10 contribue à la pathologie de la LV en supprimant les réponses protectrices des lymphocytes T $CD4^+$, cette cytokine n'explique que partiellement la susceptibilité accrue à la maladie médiée par les lymphocytes B (Deak *et al.*, 2010; Bankoti *et al.*, 2012). L'hypergammaglobulinémie a plutôt été identifiée comme la principale fonction néfaste des cellules B dans la LV. En effet, il a été démontré que l'absence d'anticorps commutés par classe ou hypermutés entraînait une amélioration de la réponse Th1 et une réduction de la gravité de la maladie, ce qui coïncidait avec une diminution marquée des cytokines telles que l'IL-10, le TNF et l'IFN-β exprimé par les splénocytes, connues pour être à l'origine de la maladie (Silva-Barrios & Stäger, 2019). Des travaux antérieurs menés dans notre laboratoire ont également démontré

que l'interaction des cellules B avec les amastigotes de *L. donovani* induit une régulation à la hausse des TLR endosomaux dépendant du récepteur de l'IFN de type I (IFNAR), qui augmente à son tour la production d'anticorps et de cytokines, y compris l'IL-10 et l'IFN-I (Silva-Barrios *et al.*, 2016). On a supposé que cette expression accrue des TLR endosomaux était due à une vague précoce d'IFN-I produite lors des interactions précoces entre les cellules B et les parasites, similaire à la boucle de rétroaction positive observée dans les cellules B après la stimulation des TLR7 et TLR9 (Green *et al.*, 2009; Thibault *et al.*, 2009). Cependant, la présence de cet IFN-I précoce et la nature de l'interaction précoce entre les cellules B et les parasites conduisant à l'activation des cellules B polyclonales et à l'hypergammaglobulinémie qui en résulte restent encore à découvrir.

Au cours d'expériences préliminaires sur les interactions précoces entre les cellules B et les amastigotes de *L. donovani* dans notre laboratoire, nous avons observé la formation de longues protubérances tubulaires membranaires s'étendant à partir des cellules B, qui étaient capables de former des connexions entre les cellules au moment du contact. Ces connexions semblent être des conduits transitoires entre deux cellules, formés par la membrane des cellules B, et ressemblent à des structures utilisées dans la communication intercellulaire, appelées les « tunneling nanotubes » (TNT). En effet, il a été rapporté que les cellules B forment des TNT pour faciliter l'échange de matériaux à longue distance entre les cellules (Osteikoetxea-Molnar *et al.*, 2016). Les TNT ont été impliquées dans le transport de nombreux types de cargaisons, allant des protéines et des acides nucléiques à des organites entiers. Cependant, ces connexions intercellulaires peuvent également être exploitées par des virus et des bactéries pour permettre la dissémination entre les cellules et la propagation de maladies (Dagar *et al.*, 2021).

Nous avons donc émis l'hypothèse que l'interaction précoce entre les cellules B et *L. donovani* induit la formation de protubérances membranaires interconnectées pour propager l'activation des cellules B, ce qui conduit finalement à une hypergammaglobulinémie nuisible et à l'exacerbation de la maladie.

L'objectif principal de ce travail était donc d'élucider la raison d'être de ces connexions intercellulaires entre les cellules B au cours de la LV. Nos objectifs spécifiques étaient (i) de caractériser les protrusions de connexion formées entre les cellules B, (ii) d'étudier l'induction des protrusions de connexion dans les cellules B, et (iii) d'évaluer la pertinence fonctionnelle de ces protrusions dans l'infection par *L. donovani*.

Pour caractériser ces protubérances, nous avons étudié les structures formées par des cellules B spléniques primaires de souris à l'aide de la microscopie confocale. Sur la base de l'apparence

et de la composition de ces connexions intercellulaires, constituées principalement de F-actine et de faibles quantités de tubuline, nous avons constaté qu'elles correspondaient au profil des nanotubes à effet tunnel. En effet, ils remplissent les trois principaux critères utilisés pour identifier les TNT dans les études : (i) ce sont principalement des structures à base d'actine qui peuvent ou non contenir de la tubuline, (ii) ils connectent deux cellules ou plus, et (iii) ils ne sont pas connectés au substrat sur lequel ils sont cultivés (McCoy-Simandle *et al.*, 2016). Notre découverte que les cellules B murines primaires forment des connexions de type TNT est en accord avec les études précédentes qui ont rapporté la capacité des cellules B à former des TNT (Osteikoetxea-Molnar *et al.*, 2016). Une caractéristique unique de ces conduits est leur rôle dans la communication intercellulaire. On a déjà constaté que les TNT formées entre les cellules B participaient au transfert de cargaisons cytosoliques telles que les vésicules et les petits organites, ainsi que de marqueurs et de colorants membranaires (Osteikoetxea-Molnar *et al.*, 2016; Halasz *et al.*, 2018). Cependant, bien qu'il ait été démontré que ces structures sont exploitées pour la propagation de cellule à cellule des virus et des agents pathogènes dans d'autres types de cellules, le contournement de cette voie de communication entre les cellules B n'a pas encore été rapporté.

Il est frappant de constater que l'exposition des cellules B à des amastigotes de *L. donovani* augmente la formation de TNT entre les cellules, alors que la présence du parasite ne modifie pas de manière significative la longueur ou l'épaisseur des connexions observées. La formation de protubérances commence après environ 1 heure de coculture et a atteint son maximum après 5 heures. Nous avons donc émis l'hypothèse qu'une interaction de surface entre les cellules B et les parasites pourrait induire la formation de TNT dans les cellules B. Comme les amastigotes de *L. donovani* purifiés à partir de souris *Rag1*$^{-/-}$ sont recouverts de complément C3 (Bankoti *et al.*, 2012), nous avons décidé d'étudier le rôle du récepteur 2 du complément (CR2 ou CD21) dans l'induction de ces connexions. Le CR2 est fortement exprimé sur les cellules B de la zone marginale (MZB), qui seraient le principal sous-ensemble de cellules B spléniques à capturer *L. donovani in vivo* (Bankoti *et al.*, 2012). En outre, nous avons constaté que les cellules MZB capturaient plus fréquemment les parasites et formaient des TNT *in vitro* que les cellules B folliculaires (FoB). Nous avons donc testé si la réticulation de CR2 ou du récepteur principal de l'antigène des cellules B (BCR) pouvait induire des TNT entre les cellules B. À cette fin, nous avons couplé des billes de latex avec des anticorps dirigés contre CR2 ou IgM, un composant du BCR, et nous avons exposé les cellules B à ces billes pendant 5 heures.

CR2 semble également jouer un rôle dans la capture des parasites par les cellules B, car le blocage par anticorps de CR2 sur les cellules B avant l'exposition au parasite a considérablement réduit le nombre de cellules B porteuses de parasites après la coculture. En outre, la recouverture de parasites congelés qui avaient perdu leur couche de C3 avec du sérum frais de souris *Rag1*$^{-/-}$ *a* restauré la capacité de ces parasites à se lier aux cellules B. Ainsi, dans ce travail, nous identifions un rôle clé pour CR2 dans la capture des amastigotes de *L. donovani* par les cellules B, ainsi que dans l'induction de TNT entre les cellules B par le parasite. Cependant, l'implication d'autres voies dans l'induction des TNT reste à étudier dans de futures études. La formation de nanotubes après un traitement à l'IFN-α a été signalée comme augmentant dans les cellules de leucémie myéloïde chronique (Omsland *et al.*, 2020). Dans ce travail, nous avons également observé une expression transitoire et précoce de l'IFN-α et de l'IFN-β dans les cellules B 30 - 45 min après l'exposition au parasite, ainsi qu'une tendance à la diminution de la formation de TNT chez les souris déficientes en IFNAR, ce qui indique une implication mineure potentielle de la signalisation de l'IFN-I dans l'induction de ces structures.

Nous rapportons également la capacité des cellules B à disséminer les amastigotes de *L. donovani* entre elles dans un processus qui est en partie médié par les TNT. En utilisant des cellules B purifiées à partir de souris C57BL/6 exprimant l'isoforme CD45.1 ou CD45.2, nous avons pu distinguer les cellules B initialement exposées aux parasites des cellules B qui n'avaient pas été exposées auparavant après la coculture, afin d'étudier si les amastigotes liés à la surface d'une cellule pouvaient être transférés à une autre. Cette propagation d'une cellule à l'autre n'était pas due au détachement du parasite d'une cellule et à son rattachement à une autre, car les surnageants des cellules B exposées n'ont donné lieu qu'à une capture négligeable d'amastigotes. Au contraire, l'inhibition des TNT à l'aide de l'inhibiteur de la polymérisation de l'actine, la cytochalasine D, a démontré qu'une partie importante de ces transferts était facilitée par ces connexions intercellulaires. Alors que les TNT ont été impliquées dans la propagation de virus, de bactéries et de prions, il s'agit du premier rapport sur l'exploitation de ces structures par un parasite pour la dissémination de cellule à cellule.

La communication par l'intermédiaire des TNT peut se faire soit par des échanges cytosoliques, soit par le transfert de molécules membranaires ou de structures liées à la surface. De même, les agents pathogènes peuvent se disséminer en passant par les conduits formés, ce qui a été documenté pour le virus T-lymphotrope humain-1 (HTLV-1) (Omsland *et al.*, 2018), les prions (Gousset *et al.*, 2009; Costanzo *et al.*, 2013) et *Chlamydia trachomatis* (Jahnke *et al.*, 2022), ou glissent le long de la membrane pour atteindre les cellules non infectées, comme cela a été

observé lors de l'infection par *Mycobacterium bovis* (Önfelt *et al.*, 2006) ou par le virus de la leucémie murine (Lehmann *et al.*, 2005). Bien que nous ayons observé la colocalisation des conduits intercellulaires B avec le parasite en microscopie confocale, ces images ne montrent pas clairement si la dissémination du parasite se produit à l'intérieur ou à l'extérieur des TNT. L'observation selon laquelle les cellules B n'internalisent pas complètement les amastigotes peut soutenir la dissémination à la surface de ces nanotubes ; cependant, il sera intéressant d'étudier plus avant la nature de ces échanges pour mieux comprendre la fonction des TNT pendant l'infection par *L. donovani* et dans le contexte des maladies parasitaires en général.

Cette étude démontre également que *L. donovani* peut être trouvé dans la zone des cellules B spléniques des souris infectées dès 14 jours après l'infection en utilisant des cryosections colorées par immunofluorescence, le nombre de parasites augmentant progressivement à 21 et 28 jours après l'infection. Ceci est en accord avec les résultats précédents de notre laboratoire qui ont démontré que les cellules B, en particulier le sous-ensemble de cellules MZB, isolées de souris 20 h après l'infection par *L. donovani*, sont porteuses de parasites (Bankoti *et al.*, 2012). Cette plus grande propension des cellules MZB à capturer les parasites s'est reflétée dans nos résultats *in vitro*. Situés à proximité des cellules MZB dans la zone marginale, les macrophages sont la cible principale de *Leishmania in vivo* (Bogdan, 2020). Nous avons donc émis l'hypothèse que les cellules MZB peuvent obtenir des amastigotes de *L. donovani* à partir des macrophages *in vivo*. À l'appui de cette notion, nous avons constaté que le parasite est capable de se disséminer à partir de macrophages infectés dérivés de la moelle osseuse vers des cellules B non exposées auparavant. Nous avons également observé la formation de structures semblables à des TNT transportant le parasite entre les macrophages et les cellules B, ce qui indique que cet échange peut être partiellement facilité par ces nanotubes. Des TNT hétérogènes similaires entre les cellules B et les macrophages ont été signalés comme étant formés au cours de l'infection par le virus de l'immunodéficience humaine-1 (VIH-1). Ces TNT ont été induits par le facteur négatif (Nef), une protéine dérivée du virus, et exploités pour sa dissémination des macrophages vers les cellules B (Xu *et al.*, 2009). Au cours de l'infection par *L. donovani*, les macrophages internalisent le parasite alors que la capture des amastigotes par les cellules B ne conduit pas à l'internalisation du parasite. Il sera donc intéressant de disséquer davantage la dynamique du transfert du parasite des macrophages aux cellules B et l'induction de ces protubérances dans les macrophages.

Cette voie de dissémination du parasite peut fonctionner parallèlement à un autre mécanisme de transport d'antigènes médié par les cellules B, appelé navette folliculaire, au cours duquel les

cellules MZB sont poussées par les chimiokines à effectuer un cycle entre la zone marginale et le follicule, transportant ainsi tout antigène lié obtenu dans la zone marginale dans les follicules (Cinamon *et al.*, 2008). À l'instar de la formation de TNT médiée par CR2 rapportée dans ce travail, il a été démontré que la navette folliculaire se produit de manière dépendante de CR1/2 (Ferguson *et al.*, 2004; Cinamon *et al.*, 2008; Zhang *et al.*, 2014a), ce qui plaide en faveur d'une action complémentaire ou simultanée des deux voies et amène les cellules MZB, dont nous avons observé qu'elles formaient préférentiellement des TNT, à proximité des FoB, favorisant ainsi potentiellement les échanges médiés par les TNT entre ces types de cellules.

Il est important de noter que nous avons identifié la nécessité d'un contact direct entre les cellules B et les amastigotes de *L. donovani* pour induire l'activation des cellules B. Des travaux antérieurs de notre groupe ont démontré que l'exposition des cellules B aux parasites induit le regroupement des cellules et des parasites *in vitro* et conduit à l'activation des cellules B, comme en témoigne la régulation à la hausse du complexe majeur d'histocompatibilité de classe II (CMH-II) et des marqueurs d'activation tels que CD86, suivie de la mort cellulaire dans les 24 à 48 heures (Bankoti *et al.*, 2012; Silva-Barrios *et al.*, 2016). En utilisant des inserts à membrane perméable pour séparer les cellules B exposées aux parasites des cellules B incapables d'établir un contact direct avec le parasite tout en permettant l'échange de messagers solubles tels que les cytokines ou les exosomes, nous avons constaté que ce contact direct entre les cellules B et les amastigotes de *L. donovani* est nécessaire pour induire l'activation des cellules B. Ces inserts membranaires perméables se sont également révélés être une méthode efficace pour inhiber physiquement la formation de TNT et la communication associée (Polak *et al.*, 2015). Nous rapportons également que l'exposition des cellules B aux amastigotes de *L. donovani* induit une expression précoce et transitoire de l'ARNm de l'IFN-α et de l'IFN-β, mesurée par RT-qPCR. Cette vague précoce d'IFN-I pourrait expliquer la régulation à la hausse des TLR endosomaux dépendante de l'IFNAR signalée par notre groupe, qui a entraîné une augmentation des titres d'anticorps et une régulation à la hausse des cytokines pathogènes IFN-I et IL-10, entre autres (Silva-Barrios *et al.*, 2016). Le rôle néfaste de la signalisation de l'IFN-I au cours de la LV active est également étayé par la découverte que les souris déficientes en IFNAR présentaient un meilleur contrôle des parasites à la fois dans la rate et le foie dans les 28 et 14 jours suivant l'infection, respectivement (Kumar *et al.*, 2020). D'autres études sont nécessaires pour mieux comprendre les voies de détection déclenchées lors des interactions précoces entre les cellules B et les amastigotes de *L. donovani,* qui aboutissent à cette induction rapide mais transitoire de l'IFN-I.

En conclusion, les résultats de ce travail soutiennent un mécanisme de transfert en série des parasites de *L. donovani* des macrophages aux cellules MZB et parmi la population de cellules B spléniques par l'intermédiaire de nanotubes à effet tunnel, ce qui étend l'activation des cellules B et peut finalement entraîner une hypergammaglobulinémie. Il s'agit du premier rapport impliquant cette voie de communication intercellulaire dans la dissémination du parasite. Dans les cellules B, nous avons constaté que le récepteur 2 du complément jouait un rôle central dans l'induction des TNT observés et dans la capture des amastigotes par les cellules. L'importance de la dissémination des parasites via les TNT est soulignée par notre découverte que le contact direct entre les cellules et les parasites est nécessaire pour cette activation des cellules B. En effet, nous avons observé que les cellules B et les parasites étaient en contact étroit dans la zone des cellules B spléniques des souris infectées dès 14 jours après l'infection. En outre, nous avons constaté que l'exposition des cellules B au parasite induisait une expression précoce et transitoire de l'IFN-I. Cet IFN-I peut ensuite être régulé à la hausse dans les cellules B de souris infectées. Cet IFN-I peut ensuite réguler à la hausse les TLR endosomaux sur les cellules B dans une boucle de rétroaction positive, ce qui entraîne une augmentation préjudiciable de la production d'anticorps et des niveaux élevés de cytokines telles que l'IL-10 et l'IFN-I (Silva-Barrios *et al.*, 2016).

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1 - INTRODUCTION

CHAPTER 3 - LEISHMANIA DONOVANI EXPLOITS TUNNELING NANOTUBES FOR DISSEMINATION AND PROPAGATION OF B CELL ACTIVATION

APPENDIX I - LITERATURE REVIEW

LIST OF TABLES

LIST OF ABBREVIATIONS

A

Aβ	amyloid β
Aicda	activation-induced cytidine deaminase
AID	activation-induced cytidine deaminase
AP-1	activator protein-1
APE	apurinic/apyrimidinic endonuclease
APRIL	a proliferation-inducing ligand
Arp2/3	actin related protein 2 and 3

B

BAFF	B-cell activating factor
BALBc/XID	X-linked immunodeficient BALB/c
BCP-ALL	B cell precursor acute lymphoblastic lymphoma cell
BCR	B cell receptor
B_{eff}	effector B cells
Blimp-1	B lymphocyte-induced maturation protein 1
BM	bone marrow
BMDC	bone marrow-derived dendritic cell
B_{reg}	regulatory B cell

C

CAD	catecholaminergic neuronal tumour
CCL	chemokine ligand
CCR	chemokine receptor
CD	cluster of differentiation
CDN	cyclic dinucleotide
cGAMP	2′ 3′-cyclic guanosine monophosphate-adenosine monophosphate
cGAS	cyclic GMP-AMP synthase
CIDR1α	cysteine-rich inter-domain region 1α
CL	cutaneous leishmaniasis
CLP	common lymphoid progenitor
CR	complement receptor
CSR	class switch recombination
CTLA-4	cytotoxic T lymphocyte antigen 4
CXCL	chemokine ligand

D

DAG	diacylglycerol

DAMP	damage-associated molecular pattern
DC	dendritic cell
DCL	diffuse cutaneous leishmaniasis
DLL-1	delta-like ligand-1
DNA	desoxyribonucleic acid
DR-5	death receptor 5
DZ	dark zone

E

EAE	experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
Eps8	epidermal growth factor receptor kinase substrate 8
ERK	extracellular signal-regulated kinase
ERp29	endoplasmic reticulum-resident chaperone protein 29

F

FasL	Fas ligand
FcεRII	Fc epsilon Receptor II
FDC	follicular dendritic cell
FLT3L	FMS-like tyrosine kinase 3 ligand
FoB	follicular B
FoxP3	forkhead box P3

G

GAS	gamma-activated sequence
GC	germinal centre
GDH	glutamate dehydrogenase
GEF	guanine nucleotide exchange factor
GM-CSF	granulocyte-macrophage colony-stimulating factor

H

HASPB-1	hydrophilic acylated surface protein B-1
HBV	hepatitis B virus
HEK	human embryonic kidney
HIF-1α	hypoxia inducible factor 1α
HIV	human immunodeficiency virus
HMG	high mobility group proteins
HSC	haematopoietic stem cell
HTLV-1	human T-cell leukaemia virus type 1
Htt	huntingtin
HUVEC	human umbilical vein endothelial cell

I

I-BAR	inverted Bin/amphiphysin/Rvs
ICAM1	intercellular adhesion molecule 1
IFN	interferon
IFNAR	type I IFN receptor
Ig	immunoglobulin
IKK	IκiB kinase
IL	interleukin
iNKT	invariant natural killer T
IP_3	inositol-1,4,5-triposphate
IRAK	IL-1 receptor–associated kinase
IRF	interferon regulatory factor
IRSp53	insulin receptor tyrosine kinase substrate protein of 53 kDa
ISG	interferon-stimulated gene
ISRE	IFN-stimulated response elements
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif

J

Jak1	Janus kinase 1

L

LAG-3	lymphocyte activation gene 3
LCL	localised cutaneous leishmaniasis
LCMV	lymphocytic choriomeningitis virus
*Li*TXN1	*L. infantum*-derived tryparedoxin
*Lm*R3arp	*L. major*-derived R3a-related protein
LPG	lipophosphoglycan
LPS	lipopolysaccheride
LZ	light zone

M

MAPK	mitogen-activated protein kinase
MASP	mannose-binding lectin-associated serine protease
MAVS	mitochondrial antiviral signaling protein
MBC	memory B cell
MBL	mannose-binding lectin
MCL	mucocutaneous cutaneous leishmaniasis
MDSC	myeloid-derived suppressor cell
MHC-II	major histocompatibility complex class II
MID	*Moraxella* IgD binding protein

MINT	MSX2-interacting nuclear target protein
mMDH	mitochondrial malate dehydrogenase
MMM	marginal zone metallophilic macrophages
MPP	multipotent progenitor cell
MSC	mesenchymal stem cells
mTORC	mammalian target of rapamycin complex
MyD88	myeloid differentiation primary response protein 88
MZ	marginal zone
MZB	marginal zone B
MZM	marginal zone macrophages

N

n-WASP	neuronal Wiskott–Aldrich Syndrome protein
Nef	negative factor
NF-κB	nuclear factor κB
NFAT	nuclear factor of activated T cells
NK	natural killer
NKT	natural killer T
NO	nitric oxide
NOS-2	nitric oxide synthase 2
Notch-2	neurogenic locus notch homologue protein-2
NTD	neglected tropical disease

P

PALS	periarteriolar lymphocyte sheath
PAMP	pathogen-associated molecular pattern
PAX5	paired box protein 5
PC	plasma cell
PD-1	programmed death protein 1
PFA	paraformaldehyde
*Pf*EMP1	*P. falciparum* erythrocyte membrane protein 1
PI3K	phosphatidylinositol-3 kinase
PIP_3	phosphatidylinositol-3,4,5-triphosphate
PKCβ	protein kinase Cβ
PKDL	post-kala-azar dermal leishmaniasis
PLC-γ2	phospholipase Cγ2
PrP	prion protein
PRR	pattern recognition receptors
PSG	promastigote secretory gel

Q

QD	Quantum dot

R

RAG	recombination-activating genes
RANKL	TNF-family member receptor activator of nuclear factor-κB ligand
RIG-I	retinoic-inducible gene-I
RIP1	receptor interacting protein 1
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
RSS	recombination signal sequences

S

S1P	sphingosine 1-phosphate
S1PR	sphingosine 1-phosphate receptor
SHM	somatic hypermutation
SHP-1	SRC homology region 2 domain-containing phosphatase-1
SIR2	Silent information regulatory 2 protein
SIRP-α	signal regulatory protein α
SLE	systemic lupus erythematosus
SLO	secondary lymphoid organ
STAT	signal transducer and activator of transcription
STING	stimulator of interferon genes
Syk	spleen tyrosine kinase

T

TBK1	TANK-binding kinase 1
TD	thymus-dependent
TdT	terminal dideoxynucleotidyl transferase
Tfh	T follicular helper
TGF-β	transforming growth factor β
Th	T helper
TI	thymus-independent
TIM	T cell immunoglobulin and mucin-domain containing protein
TLR	Toll-like receptor
TNF	tumor necrosis factor
TNFAIP2	tumour necrosis factor-α inducible protein 2
TNP	trinitrophenol
TNTs	tunnelling nanotubes
Tr1	T regulatory-1-type

TRAF	TNF receptor-associated factor
TRAIL	TNF-related apoptosis-inducing ligand
T_{reg}	regulatory T cell
TRIF	TIR-domain-containing adaptor-inducing beta interferon
Tyk2	non-receptor tyrosine kinase 2

U

UNG	uracil DNA glycosylase
UV	ultraviolet

V

VL	visceral leishmaniasis
VSV	vesicular stomatitis virus

W

WASP	Wiskott–Aldrich Syndrome protein
WAVE	WASP family verprolin-homologous 2
WHO	World Health Organization

1 INTRODUCTION

PART I: LEISHMANIASIS

Leishmaniasis is a set of diseases caused by the vector-mediated transmission of blood-borne protozoan parasites belonging to the Leishmania family. It occurs predominantly in poverty-plagued regions located in tropical and subtropical climates, and despite extensive efforts to decrease disease incidence, it has been classified as a neglected tropical disease (NTD) by the World Health Organization (WHO).

In mammalian hosts, infections with different species of *Leishmania* present in a range of clinical manifestations with varying severities, and prevention or treatment strategies are limited and often accompanied by severe side effects. Furthermore, rising temperatures due to climate change could lead to the spread of leishmaniasis to parts of the world previously uninhabitable for its transmission vector, including parts of Europe and North America (Gonzalez *et al.*, 2010; Ready, 2010). Therefore, research is required to better understand the processes underlying the pathology of these diseases. This section provides a summary of the current knowledge on the immunology and causative agents of this family of diseases.

1. Biology and transmission of leishmaniasis

Belonging to the order of Trypanosomatida, members of the genus Leishmania are obligate intracellular parasites. To date, around 53 morphologically identical species of *Leishmania* have been reported, 20 of which have been observed to be capable of infecting humans (Alvar *et al.*, 2012). This number also includes parasites of the subgenus Viannia, which are highly similar to Leishmania but differ in their colonisation of the sandfly vector (Rangel *et al.*, 1992).

Based on their geographic prevalence, *Leishmania* species are further classified into those endemic to the “Old World”, generally referring to Europe, Middle East Asia and Africa, and those present in the “New World”, meaning Central and South America. This classification is further mirrored in different genera of sandflies responsible for parasite transmission in these regions, with Old World leishmaniasis being transmitted by *Phlebotomus*, *Sergentomyia*, and *Chinius* sandflies, while the *Lutzomyia*, *Warileya*, and *Brumptomyia* genera are responsible for New World infections, making up more than 800 species of sandflies in total (Akhoundi *et al.*, 2016). These

sandflies are predominantly active at nighttime and prefer warm and humid conditions. Cycling between the sandfly vector and the mammalian host, *Leishmania* has a digenetic life cycle and exists in two main stages: the long, flagellated promastigote form present in the sandfly and the smaller, circular amastigote form that multiplies in the host and causes disease **(Figure 1)**. To lay eggs, female sandflies are required to consume blood meals, causing them to seek out warm-blooded hosts.

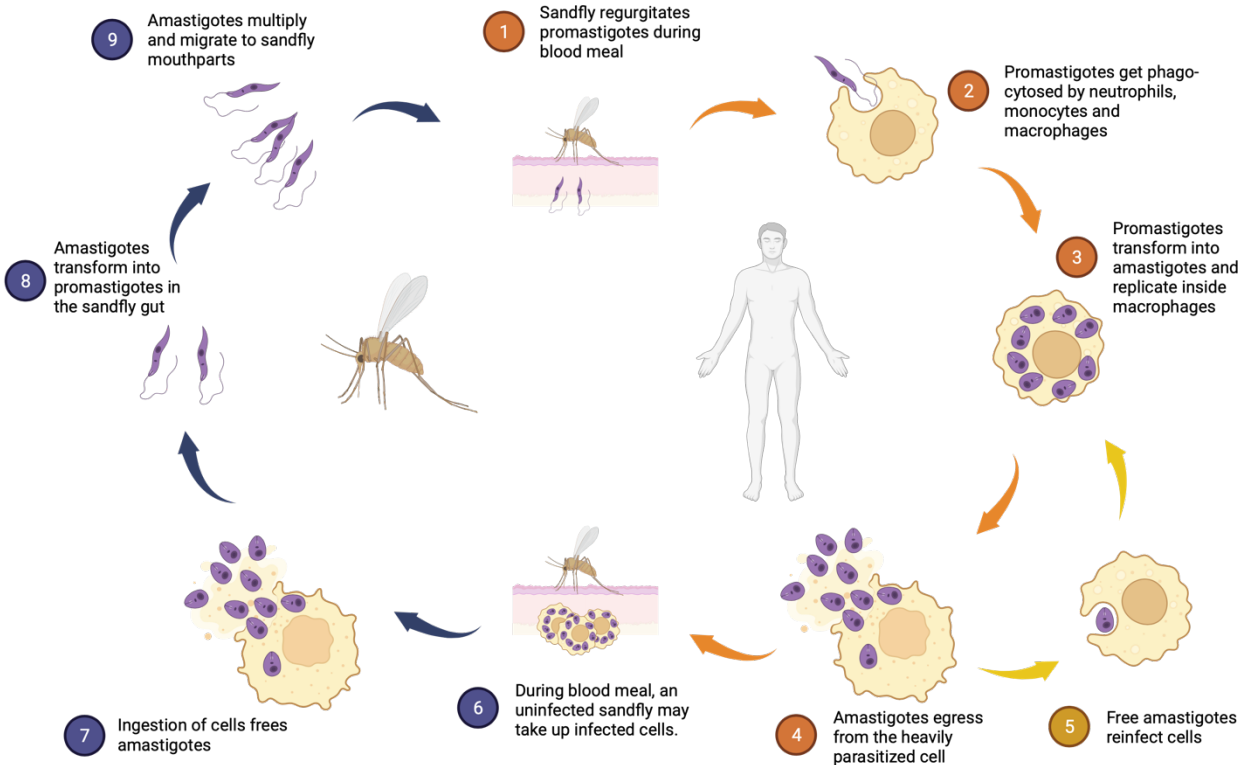

Figure 1 Life cycle of Leishmania
(adapted from *(Reithinger et al., 2007)*, created with Biorender.com)

When an infected female sandfly takes a blood meal, it regurgitates parasites in their infective metacyclic promastigote stage, mixed with a proteophosphoglycan-rich promastigote secretory gel (PSG), which has immunomodulatory properties, into the skin of the host. In the dermis, parasites are rapidly phagocytosed by neutrophils and monocytes and finally, macrophages, which represent one of the major targets of *Leishmania* infection in mammals. Inside macrophages, *Leishmania* resides in parasitophorous vacuoles, where the transition from the promastigote to the non-motile amastigote stage takes place. In the amastigote form, the parasite prolifically divides, and an increasing number of parasites may cause egress by bursting the cell. The released amastigotes are then able to reinfect more cells and spread the infection throughout the system. Upon taking a blood meal from a host with active leishmaniasis, a previously uninfected sandfly may then ingest and lyse an infected phagocytic cell, freeing the amastigotes. After turning back into their promastigote form, *Leishmania* parasites multiply in the midgut, whereas parasites of the Vianna subgenus are in the hindgut (Rangel *et al.*, 1992; Kaye & Scott, 2011). A thus newly infected sandfly can then initiate the cycle of infection anew.

Most species of leishmaniasis are thought to cause zoonotic diseases, spreading between humans and other mammals via sandfly vectors. Indeed, over 70 species of mammals and

reptiles have been reported to be able to act as reservoirs for different *Leishmania* species (Ready, 2014). Only two species, i.e. *L. tropica* and *L. donovani,* are thought to follow anthroponotic transmission; however, even among these species, rare zoonotic transmission may occur (Labony *et al.*, 2014; El Idrissi Saik *et al.*, 2022).

2. Clinical manifestations

With the large variety of *Leishmania* species, the clinical presentation of leishmaniasis can vary greatly, ranging from self-healing to potentially fatal infections. The clinical manifestations of the disease are therefore commonly grouped into two main forms: Cutaneous Leishmaniasis (CL) and Visceral Leishmaniasis (VL), both of which may be further specified according to their symptoms and severity and are dependent on the species of *Leishmania* and the immune status of the host. An overview of the diseases caused by the different parasite species is provided in **Table 1.**

Table 1 Main clinical forms caused by different Leishmania species

Main clinical presentation	Species	Subgenus	Distribution
Localized CL	*L. major*	Leishmania	Old world (North and East Africa, Central Asia, middle east)
	L. tropica	Leishmania	Old world (Central and Southeast Asia, middle east, North Africa)
	L. killicki	Leishmania	Old world (North Africa)
	L. pifanoi	Leishmania	New world (South America)
	L. venezuelensis	Leishmania	New world (Northern South America)
	L. garnhami	Leishmania	New world (South America)
	L. (V.) peruviana	Viannia	New world (Peru)
	L. (V.) guyanensis	Viannia	New world (South America)
	L. (V.) lainsoni	Viannia	New world (South America)
	L. (V.) colombiensis	Viannia	New world (South America)
Localized or Diffuse CL	*L. aethiopica*	Leishmania	Old world (Kenya, Ethiopia)
	L. amazonensis	Leishmania	New world (South America)
	L. mexicana	Leishmania	New world (South and Central America)
Localized or mucosal CL	*L. (V.) braziliensis*	Viannia	New world (South and Central America)
	L. (V.) panamensis	Viannia	New world (South and Central America)
VL or PKDL	*L. donovani*	Leishmania	Old world (Central and Southeast Asia, Africa)
	L. infantum (*L. chagasi*)	Leishmania	Old and new world (Europe, North Africa, South and Central America)

3.1. Cutaneous leishmaniasis (CL)

With an estimated number of 600,000 to 1,000,000 annual cases worldwide according to the WHO, CL and its subtypes are the most common forms of leishmaniasis. It is caused by parasites of both the Leishmania or Viannia subgenus of often overlapping geographical distribution (listed in Table 1) and as of 2021, it is classified as endemic in 87 countries by the WHO, with the great majority of cases occurring in the Middle and Southeast Asia (Afghanistan, Iran, Pakistan, Saudi Arabia, and Syria), Africa (Algeria) and South America (Brazil and Peru) (Reithinger *et al.*, 2007).

In contrast to VL, CL infection is characterised by localisation in the skin and draining lymph nodes, manifesting as skin lesions that usually appear within three months of the sandfly bite (David & Craft, 2009). The appearance, location, and distribution of these lesions vary, prompting further classification into subtypes such as localised CL (LCL), diffuse CL (DCL) and mucocutaneous CL (MCL) (Reithinger *et al.*, 2007). In LCL, a single lesion typically corresponds to an individual bite by an infected sandfly, as the lesions develop at the site of inoculation, and dissemination of parasites to other regions of the skin via the lymphatic system is rare (Thomaidou *et al.*, 2015). In contrast, an inefficient host immune response against the parasite and lower levels of protective cytokines such as IFN-γ are associated with the development of DCL (Caneda-Guzman *et al.*, 2014). This is a rare condition characterised by many lesions that progressively spread along both the lymphatic and vascular pathways, commonly populating the neck, groin, and inner edge of the shoulder blade with numerous parasite-infiltrated nodules (Convit *et al.*, 1972). The onset of the most severe form of CL, MCL, typically starts after the initial lesion has subsided and begins close to the mucosa of the nose or lips, is often accompanied by nosebleeds and nasal congestion, and can successively worsen to the point of perforation of the nasal septum and permanent disfiguration (Cincura *et al.*, 2017). Epidemiological data on the frequency at which the infection develops into MCL is limited and varies regionally, with some studies estimating that it makes up only 0.4 % of cases in South Brazil and Venezuela, but present in up to 20 % of patients in Bolivia (Goto & Lindoso, 2010). The underlying causes of progression to MCL in some patients are still incompletely understood.

While LCL is typically self-healing and may be treated predominantly to accelerate healing and minimise potentially stigmatising scarring, MCL can result in severe disfiguring and possibly life-threatening illnesses that require therapeutic intervention (Morizot *et al.*, 2013; Showler & Boggild, 2015). The choice of treatment regimen depends not only on the classification of leishmaniasis,

but also on the species of *Leishmania*, the immunocompetence of the host, the number and visibility of lesions, and the feasibility and toxicity of the treatment (Showler & Boggild, 2015). Treatments can be facilitated locally, such as via intralesional injections of antimonials; topical application of creams that often contain paromomycin as their main active ingredient; and directed cryotherapy, thermotherapy, or photodynamic therapy, or by systemic administration of drugs such as pentavalent antimonials, liposomal amphotericin B, or miltefosine are also used (Showler & Boggild, 2015; Pradhan *et al.*, 2022).

3.2. Visceral leishmaniasis (VL)

Despite the WHO estimates of annual cases being lower at 50,000 and 90,000 new infections predominantly occurring in East Africa (Ethiopia, Kenya, Somalia, South Sudan, and Sudan), Brazil and India, the severity of the disease makes VL a serious health risk in affected countries. Two *Leishmania* species predominantly cause VL, *L. donovani* and *L. infantum*, the latter of which can cause infections both in the Old World (Europe and Africa) and in the New World (South and Central America) where it is also called *L. chagasi*.

VL infection generally localises to the visceral organs and bone marrow, causing striking enlargement of the liver (hepatomegaly) and spleen (splenomegaly). Loss of appetite and weight to the point of cachexia are also commonly observed, resulting in apparent abdominal distension (Chappuis *et al.*, 2007). Anaemia and pancytopenia, as well as excessive production of non-protective antibodies in a condition called hypergammaglobulinemia, are also observed. This is accompanied by immunosuppression, which makes the host susceptible to secondary infections and greatly increases the risk of mortality. Indeed, co-infection with human immunodeficiency virus (HIV) poses a particular risk to patients because of its detrimental symbiotic effects on the host immune response, allowing for better survival and proliferation of both pathogens and leading to more efficient disease progression (Mock *et al.*, 2012). Persistent fever represents another hallmark of VL, and due to the common co-occurrence of skin hyperpigmentation during infections in Southeast Asia, VL is also sometimes called Kala-azar, meaning “Black Disease” or “Black Fever.” The onset of clinical symptoms associated with VL typically occurs between two and six months after the initial sandfly bite; however, asymptomatic infection is common in both *L. donovani* and *L. infantum* infections and is estimated to represent approximately 90 % of all cases (Srivastava *et al.*, 2013; Dos Santos Marques *et al.*, 2017).

Another presentation of the disease is a skin-related condition called post-kala-azar dermal leishmaniasis (PKDL). Although it is typically caused by *L. donovani*, immunosuppressed individuals infected with *L. infantum* may also develop this condition (Stark *et al.*, 2006). Arising from the flare-up of a previously treated infection, PKDL is characterised by numerous lesions, often originating from the face, which can cover the entire body in its most severe form. These lesions are often self-healing but are highly infiltrated with parasites that can be taken up by sandflies upon a blood meal, thus serving as reservoirs for VL, making the treatment of PKDL of interest for disease control and eradication efforts (Gedda *et al.*, 2020).

3. The immunology of VL

3.1. Experimental models of VL

As a visceralising disease, studying human VL presents many challenges, the most central of which is the invasiveness of sampling techniques. This is further complicated by the fact that VL predominantly affects poverty-plagued areas, which often have limited access to sanitation and medical care. To overcome this, experimental animal models are often used; indeed, the great majority of studies underlying our current knowledge of the disease come from animal studies.

Rodent models, such as mice and hamsters, are among the most commonly used organisms for the study of VL. Both mice and hamsters develop infections in the liver, spleen, and bone marrow; however, their disease progression varies. The skin, as the initial site of inoculation, experiences rapid initial proliferation of the parasite, leading to granuloma formation and diminution of the parasite burden within 8 weeks (Wilson *et al.*, 2005). The Syrian hamster, *Mesocricetus auratus*, is considered to be the best experimental model to study VL. Indeed, infection in the hamster model typically produces a terminal disease, during which abundant production of transforming growth factor β (TGF-β) and IL-10 counteracts the protective T helper type 1 (Th1) response and creates favourable conditions for parasite survival and replication by suppressing leishmanicidal NO generation (Melby *et al.*, 2001a). The pathology of VL in hamsters thus closely mimics the clinicopathological features of human VL, despite showing some differences in the later stages of infection, as hamsters commonly develop kidney pathology and ascites (Sartori *et al.*, 1992), which are rare complications in humans (Pearson & Sousa, 1996). Nevertheless, applications of

the hamster model in immunological studies are restricted due to the limited availability of tools such as readily available cell markers, antibodies, and cytokines (Loria-Cervera & Andrade-Narvaez, 2014).

On the other hand, mice are useful model organisms to study disease due to the wide availability of various strains, and genetic and immunological tools. Depending on their genetic background, mouse strains can be either susceptible or resistant to disease; however, even susceptible mice generally do not succumb to the disease. Mice of BALB/c, B10.D2, C57BL/6, and C57BL/10 background are considered susceptible, while the A/Jax, C3H.HeJ, CBAR and DBA/2 strains are classified as resistant (Gorczynski, 1982) and differences in genetic susceptibility to the disease are governed by mutations in the *Slc11a1* gene involved in the activation of macrophages (Vidal *et al.*, 1993). This is primarily mediated by a decrease in iNOS expression and lower generation of NO needed for the leishmanicidal activity of macrophages (Blackwell *et al.*, 2001). However, even in susceptible mice which establish a chronic infection, VL typically does not lead to death.

Experimental models of VL typically rely on inoculation of animals via the injection of parasites into the skin, peritoneum, or blood circulation. In order to mimic the progression of VL more closely and account for sandfly-specific factors during transmission, attempts have been made to develop a natural infection model using experimentally infected sandflies. While some success has been found using natural infection models to transmit *L. infantum* (Lainson *et al.*, 1977), experimental transmission of *L. donovani* via sandflies has proven difficult and is not widely established to date.

3.2. The immune response during experimental VL

Owing to the limited availability of a natural infection model in experimental VL, the early response to sandfly-mediated transmission of the causative species of visceral leishmaniasis is still unclear.

Macrophages are the main targets of *Leishmania* and can play a dual role in infection, either as a key player in the leishmanicidal immune response or as a host and site of *Leishmania* replication, and polarisation of macrophages into a pro-inflammatory M1 or anti-inflammatory M2 type is a key factor influencing the disease outcome (Bogdan, 2020). In order to initiate their leishmanicidal function, macrophages need to be activated by cytokines, the most important of which is IFN-γ, which may be produced by $CD4^+$ or $CD8^+$ T cells, Natural Killer (NK) cells, or Natural Killer T (NKT) cells (Bogdan, 2020). This cytokine can then skew them towards a pro-

inflammatory M1 type which results in the production of leishmanicidal reactive nitrogen species (RNS) and oxygen species (ROS) which contribute to parasite control (Ding *et al.*, 1988; Murray *et al.*, 2006). A key enzyme for the production of RNS, particularly highly reactive nitric oxide (NO), in response to *Leishmania* infection is nitric oxide synthase 2 (NOS-2), which is induced by IFN-γ and supported by endogenous tumor necrosis factor (TNF) (Bogdan, 2020).

T cells play a key role in directing disease outcomes in VL. The IL-12-driven development of a strong Th1 type response, characterised by a polarised $CD4^+$ T cell response centred around the production of IFN-γ, is imperative to facilitate host resistance against leishmaniasis and may be additionally aided by other cytokines such as IL-1α, IL-18, and IL-23 (Alexander & Bryson, 2005). Contrary to the previous belief that this Th1 response mediates disease clearance in contrast to an exacerbating, IL-4-driven T helper type 2 (Th2) response, this does not seem to hold true in light of our current understanding of VL. In a model using disease-susceptible BALB/c mice, IL-4/IL-4α receptor signalling has been reported to positively influence disease outcomes in *L. donovani* infection via the promotion of leishmanicidal granulomas in the liver which facilitate parasite control in this organ (Stäger *et al.*, 2003a). Rather, disease progression seems to be driven mainly by the immunosuppressive cytokine IL-10 (Murphy *et al.*, 2001), and to a lesser degree by TGF-β (Wilson *et al.*, 1998).

$CD8^+$ T cells represent another source of IFN-γ and play an important role in VL (Tsagozis *et al.*, 2003). In a mouse model of *L. infantum* infection, $CD8^+$ T cells participate in skin parasite clearance (Ahmed *et al.*, 2003). However, during *L. donovani* infection, the $CD8^+$ T cell response is dysfunctional, showing impaired expansion and increased functional exhaustion, allowing for disease progression (Joshi *et al.*, 2009). This limited clonal expansion and cell exhaustion is partly due to parasite-mediated induction of hypoxia-inducible factor 1α (HIF-1α), which skews cytokine production by DCs from IL-12 towards IL-10 and decreases $CD8^+$ T cell expansion in an interferon regulatory factor-5 (IRF-5)-dependent manner (Hammami *et al.*, 2015). T cell exhaustion, as evidenced by the increased expression of inhibitory markers, such as programmed death protein 1 (PD-1), cytotoxic T lymphocyte antigen 4 (CTLA-4), T cell immunoglobulin and mucin-domain containing protein 3 (TIM-3), and lymphocyte activation gene 3 (LAG-3), is associated with disease progression and parasite survival. The blockade of some of these exhaustion signalling pathways has been demonstrated to contribute to the recovery of cellular immune defense during VL (Murphy *et al.*, 1998; Joshi *et al.*, 2009; Schaut *et al.*, 2016a).

Cues from dendritic cells (DCs) are vital for the activation of T cells and for mounting an immune response against *Leishmania* infection. Indeed, DCs represent the main source of Th1-promoting

IL-12 in the early phase of *L. donovani* infection (Gorak *et al.*, 1998). During chronic murine VL, DC function has been shown to be impaired, resulting in decreased IL-12 production and antigen presentation, as evidenced by the reduced expression of MHC-II (Gorak *et al.*, 1998; Basu *et al.*, 2001). This may be facilitated by IRF5-mediated HIF-1α induction in DCs by *L. donovani*, which decreases IL-12 levels, thus limiting the expansion of Th1 cells (Hammami *et al.*, 2018).

IFN-γ is also produced by NK cells and its production is preceded by the recognition of *Leishmania* parasites via TLR9 (Liese *et al.*, 2007) or TLR2 (Becker *et al.*, 2003). In VL, NK cells expand and suppress protective immune responses through IL-10 production, thus contributing to disease susceptibility (Maroof *et al.*, 2008). This NK-derived IL-10 production was proposed to be due to a posttranscriptional regulation of IL-10 gene expression, leading to increased mRNA stability at later stages of infection which results in increased output of IL-10 protein by these cells (Maroof *et al.*, 2008). Similarly, NKT cell-deficient mice showed only slightly reduced parasite burden in the liver and activation of invariant NKT (iNKT) cells during *L. donovani* infection was observed to lead to disease exacerbation rather than control (Stanley *et al.*, 2008).

Remarkably, the immune response to VL appears to be organ-specific, with distinct immune responses occurring in the main target organs of the disease (Engwerda & Kaye, 2000). Hence, the next subsection discusses organ-specific responses at the main sites of infection: the liver, spleen, and bone marrow.

3.2.1. VL in the liver

While establishing a chronic disease in humans with active VL, infection of the liver is typically resolved through a robust granulomatous response within the first months in an experimental mouse model (Squires *et al.*, 1990), which has provided the basis for the majority of research on the disease.

In the early phase of infection, parasites replicate unencumbered in the liver in the presence of low levels of IFN-γ and IL-12 (Melby *et al.*, 2001b). The first target of VL infection in the liver are Kupffer cells, the tissue-resident macrophages of the liver, which rapidly take up amastigotes (Beattie *et al.*, 2013). These infected cells may then fuse with either infected or uninfected Kupffer cells to form multinucleated cells, which serve as the core for granuloma formation (Murray, 2001). The production of various cytokines and chemokines then recruits immune cells, such as

neutrophils, monocytes, B cells, iNKT cells, and $CD8^+$ and $CD4^+$ T cells, to the proximity of the infected Kupffer cells to mount the local immune response (Kaye & Beattie, 2016).

IL-17 plays a key role in the recruitment of both monocytes and neutrophils during *L. donovani* infection, both of which participate in granuloma formation and homing of inflammatory monocytes to the infected liver, and has also been shown to be CCR2 dependent (Terrazas *et al.*, 2016; Terrazas *et al.*, 2017). CCL2, CCL3, and CXCL10 are also implicated in the recruitment of monocytes and neutrophils to granulomas (Stanley & Engwerda, 2007).

Invariant NKT cells are important producers of various soluble mediators, which orchestrate early granuloma formation. The T-cell homing chemokine CXCL10 is among the most important products of iNKTs during VL and is induced through an interaction with Kupffer cells, mediated by signal regulatory protein α (SIRPα) and CD47 (Beattie *et al.*, 2010). Additionally, the production of IFN-γ by iNKT cells as well as by hepatic NK cells was shown to be required for sustained expression of *Cxcl10* (Svensson *et al.*, 2005). However, therapeutic activation of iNKT cells during *L. donovani* infection was shown to contribute to disease exacerbation, resulting in altered cytokine production and lower numbers of IFN-γ^+ $CD8^+$ T cells and leading to increased hepatic parasite growth (Stanley *et al.*, 2008).

The recruitment of T cells is vital for the formation of functional granulomas, and both $CD4^+$ and $CD8^+$ T cells play important roles in the granulomatous immune response during VL (Engwerda & Kaye, 2000). Among $CD4^+$ T cells, a robust Th1 response centred around the production of IFN-γ predominates; however, the interplay of the typically Th2 associated cytokines IL-4 and IL-13 has been shown to be important for the formation of mature and efficient granulomas (Stäger *et al.*, 2003a; McFarlane *et al.*, 2011). Vital for the polarization towards a Th1 response is the transcription factor IRF-5, as the absence of IRF-5 leads to the production of immunosuppressive cytokines such as IL-10, decreased capability to produce ROS, and impaired parasite control (Paun *et al.*, 2011). Like $CD4^+$ T cells, $CD8^+$ T cells are recruited to granulomas approximately one week after infection and are vital for the formation of these leishmanicidal structures (Stern *et al.*, 1988; Kaye *et al.*, 1992). In addition to the production of IFN-γ, the production of IL-17 by $CD4^+$ T cells and $\gamma\delta$ T cells in *L. infantum* infection has also been shown to act in a synergistic manner with IFN-γ to promote macrophage RNS production, thereby aiding in parasite killing (Nascimento *et al.*, 2015; Sheel *et al.*, 2015).

Ultimately, parasite control in the liver depends on the formation of functional granulomas. Indeed, granuloma formation has been documented in asymptomatic patients (Pampiglione *et al.*, 1974),

whereas mature granulomas are generally absent in patients with advanced VL (Kaye *et al.*, 2004). Similarly, asymptomatic dogs infected with VL display functional granuloma formation, while they are typically absent or non-functional in established canine VL (Sanchez *et al.*, 2004). Thus, despite the differences in disease progression, experimental VL models have provided valuable insights into the immune response, granuloma formation, and clearance of the parasite from the liver, which are particularly valuable for understanding asymptomatic and subclinical VL in humans.

3.2.2. VL in the bone marrow

Unlike the immune response in the liver, VL infection in the bone marrow (BM) typically remains chronic with few, if any, immature granulomas (Chandra *et al.*, 2013) and stark changes to haematopoiesis. Indeed, experimental VL causes emergency haematopoiesis and activation of haematopoietic stem cells (HSCs), giving rise to myeloid progenitor cells that predominantly differentiate into regulatory monocytes and M2-polarized macrophages, which are permissive targets for *Leishmania* infection and are ultimately detrimental to disease outcome (Abidin *et al.*, 2017; Hammami *et al.*, 2017). The increased inflammation-induced myelopoiesis and heightened susceptibility of progeny is driven by the cytokine environment which is established in the BM during chronic *L. donovani* infection (Abidin *et al.*, 2017). This is also reflected in BM aspirates from patients with active VL, which show a remarkable expansion of myeloid cells, inefficient erythropoiesis, and a decrease in megakaryocytes (Sheikha, 2004). Additionally, an influx of plasma cells has been observed, which may contribute to the observed hypergammaglobulinemia (Sheikha, 2004), and iNKT cells accumulate in the BM of patients with VL (Rai *et al.*, 2011).

Increased myelopoiesis is driven by the abundant production of granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNF by parasite-infected stromal macrophages (Cotterell *et al.*, 2000b), as well as the T cell-supported release of M-CSF, GM-CSF, and G-CSF, which further promotes the self-renewal of myeloid progenitor cells (Cotterell *et al.*, 2000a). The augmented production of myeloid lineage cells ultimately leads to exhaustion of HSCs, which is caused by TNF-driven excessive expansion of IFN-γ producing CD4+ cells (Pinto *et al.*, 2017).

Anaemia is another prominent hallmark of VL. A key mechanism underlying this condition is inefficient erythropoiesis and reduced erythrocyte survival, causing the destruction of cells prior to their exit from the bone marrow (Woodruff *et al.*, 1972). This may be facilitated by an increased

phagocytosis of erythrocytes by macrophages with downregulated SIRP-α, the receptor recognising the “don’t eat me” surface protein CD47 (Morimoto *et al.*, 2019).

3.2.3. VL in the spleen

In the spleen, VL establishes a chronic infection in both experimental models and humans. Indeed, splenomegaly and a disorganised splenic architecture caused by *L. donovani* and *L. infantum* infections have been reported in humans, dogs, and mice (Hermida *et al.*, 2018). It is generally characterised by atrophy and loss of clearly defined compartments in the white pulp, and expansion of the red pulp zone and vasculature.

In the early phase of infection, the spleen maintains its highly organised structure, with discrete areas called the red pulp, mainly comprising sinuses and blood vessels that mainly contain cells such as macrophages and erythrocytes and, to a lesser degree, plasma cells and the white pulp, which can further be divided into the periarteriolar lymphocyte sheath (PALS), marginal zone (MZ), and lymphoid follicles (Mebius & Kraal, 2005). The PALS represents the area around small splenic arteries populated predominantly by T cells, while the follicles are aggregates of B lymphocytes, named follicular B (FoB) cells, into round areas, in which germinal centre reactions can take place. Surrounding these follicles is the marginal zone, which comprises the marginal zone B (MZB) cells, dendritic cells, as well as different types of macrophages, the marginal zone macrophages (MZM) and the marginal zone metallophilic macrophages (MMM). The integrity of this structural organisation is maintained through concerted signalling via chemokines, cytokines, and adhesion molecules, such as integrins, with the help of stromal cells (den Haan *et al.*, 2012). Upon infection with blood-borne pathogens such as *Leishmania*, cells in the marginal zone, and in particular MZM and MMM, take up most amastigotes reaching the spleen, and DCs can acquire parasite antigens either directly or by phagocytosis of infected macrophages (Stanley & Engwerda, 2007). These DCs then undergo migration to the PALS mediated by the chemokines CCL19 and CCL21, where they produce protective IL-12 (Ato *et al.*, 2006). The infiltration of infected macrophages and enlargement of follicles may also be observed in the early stages of infection (Veress *et al.*, 1977). Early phase VL is also associated with the influx of additional monocytes into the spleen. Indeed, inflammatory monocytes can be seen to hone to the spleen as early as 24 h post-infection in a CCR2-dependent manner and provide additional targets for the parasite (Hammami *et al.*, 2017; Terrazas *et al.*, 2017).

During the chronic phase of the disease, which starts around 21 – 28 days post-infection in the mouse model, drastic changes take place in the infected spleen, ultimately leading to the disruption of the splenic microarchitecture. High levels of TNF produced predominantly by parasitised macrophages cause loss of MZM, and the boundaries of the marginal zone blur or may disappear entirely over the course of the infection (Engwerda *et al.*, 2002). Stromal cells, which are an important source of chemokines necessary to maintain the organised structure of the spleen, represent an additional target for the excessively produced TNF, resulting in atrophy of the stromal cell network in the PALS (Ato *et al.*, 2002). Concomitantly, an abundant production of the immunosuppressive cytokine IL-10, along with TNF, decreases the expression of the chemokine receptor CCR7 on DCs, leading to a combined decrease in signalling through chemokines CCL19 and CCL21 and causing a defect in DC migration to the PALS and thus, segregation of DCs from the T cell-abundant area of the spleen and impairment of T cell responses (Ato *et al.*, 2002).

Functional T cell responses are vital for parasite control in the spleen, as adoptive transfer of $CD8^+$ T cells with antigen specificity for *Leishmania* during the chronic phase of infection resulted in a stark decrease in splenic parasitaemia (Polley *et al.*, 2006). However, T cells were demonstrated to possess limited responsiveness to antigens derived from *Leishmania* (Nickol & Bonventre, 1985) and are more prone to undergo apoptosis during chronic VL (Kaye *et al.*, 1992). In dogs with chronic VL, Fas/FasL-mediated apoptosis has been shown to occur in both $CD4^+$ and $CD8^+$ T cells, whereas the upregulation of the TRAIL receptor was only observed in $CD8^+$ T cells (Silva *et al.*, 2013). Using a mouse model, another study found that $CD4^+$ T cell apoptosis was promoted by TLR7 signalling induced by apoptotic material which led to the IRF5-dependent upregulation of death receptor 5 (DR-5) and caspase-8 (Fabie *et al.*, 2018). Activation of hypoxia inducible factor 1α (HIF-1α) during VL was additionally shown to modulate T cell responses. Indeed, IRF5-mediated inflammation during *L. donovani* infection was shown to upregulate HIF-1α in splenic DCs, resulting in decreased $CD8^+$ T cell expansion and IL-12 production which in turn negatively affected $CD8^+$ T cell responses and led to disease exacerbation (Hammami *et al.*, 2015). This HIF-1α-mediated shift from IL-12 to IL-10 production in DCs also limits the development of protective Th1 responses, allowing for increased parasite growth in the spleen and bone marrow (Hammami *et al.*, 2018). T cell responses during VL are further inhibited by an HIF-1α-dependent increase in myeloid-derived suppressor cell (MDSC)-like cells which display a regulatory phenotype (Hammami *et al.*, 2017). The expression of IL-10 by $FoxP3^-$ $CD4^+$ T regulatory type 1 (Tr1) cells (Stäger *et al.*, 2006; Nylen *et al.*, 2007) and exhaustion of both $CD4^+$

and $CD8^+$ T cells in the later stages further leads to decreased Th1 responses and impaired activation of M1 macrophages, resulting in decreased parasite control in the spleen (Medina-Colorado *et al.*, 2017; Habib *et al.*, 2018). Additionally, IFN-I was shown to act on DCs to promote the expansion of Tr1 cells and dampen Th1 responses (Kumar *et al.*, 2020). Lastly, HIF-1α has also been shown to be involved in skewing macrophages towards a non-leishmanicidal M2-like phenotype (Hammami *et al.*, 2017).

Alternative macrophage activation, characterised by an IL-4/IL-10-mediated increase in arginase enzyme activity, Arg1 protein expression, and a decrease in the expression of RNS-producing NOS-2, has been demonstrated in experimental VL models in mice and hamsters, leading to a decreased capacity to clear parasites (Biswas *et al.*, 2011; Kong *et al.*, 2017). Additionally, the influx of mostly CCR2-expressing inflammatory monocytes with a regulatory profile can also be observed at later stages of infection (Hammami *et al.*, 2017) and extramedullary haematopoiesis in the spleen resulting in the production of monocytes has been demonstrated in a hamster model (Osorio *et al.*, 2020). These monocytes then provide additional targets for parasite infection and are associated with increased disease susceptibility and a higher parasite burden (Abidin *et al.*, 2017; Terrazas *et al.*, 2017; Varikuti *et al.*, 2019; Osorio *et al.*, 2020).

Mirroring the decrease in DC migration into the PALS, follicular dendritic cells (FDCs) are also lost during chronic stages of infection (Smelt *et al.*, 1997b). FDCs play a number of vital roles in the orchestration of B cells and germinal centre (GC) reactions, such as regulation of proliferation, somatic hypermutation, and isotype switching (Stanley & Engwerda, 2007). Thus, disruption of the FDC network leads to the disappearance of GC B cell responses, infiltration of highly parasitised macrophages, and increased splenic parasite burden (Smelt *et al.*, 1997b). Instead of functional GC responses, increased expression of B-cell activating factor (BAFF), a proliferation-inducing ligand (APRIL), and CXCL12 promotes extrafollicular B cell activation, survival, and migration of plasma cells to the red pulp and improves survival (Silva-O'Hare *et al.*, 2016). This increased abundance of plasma cells and $CD19^+$ B cells may actively contribute to splenomegaly, as BAFF deficiency reduces enlargement of the spleen during experimental VL in mice (Omachi *et al.*, 2017). A similar expansion of B cells has also been observed in the draining lymph nodes of mice infected with *L. infantum* (Deak *et al.*, 2010).

3.2.4. B cells in VL

Perhaps counterintuitively, plasma cells and B lymphocytes negatively affect disease outcomes in VL. Indeed, B cell-deficient mice demonstrated lower susceptibility to *L. donovani* infection and were able to spontaneously clear the typically chronic infiltration of parasites in the spleen (Smelt *et al.*, 2000).

B cell-deficient mice show improved $CD4^+$ and $CD8^+$ T cell responses (Bankoti *et al.*, 2012). Interestingly, during *L. major* infection, presentation of *Leishmania*-antigens of B cells to T cells has been shown to mediate susceptibility to the disease by modulating T cell responses (Ronet *et al.*, 2008). While B cells, and in particular MZB cells, which have a higher propensity to capture *L. donovani* amastigotes both *in vivo* and in vitro, were observed to be capable of antigen presentation to $CD4^+$ T cells (Bankoti *et al.*, 2012), antigen presentation does not appear to be the primary mechanism responsible for reduced parasite control in the presence of B cells (Deak *et al.*, 2010).

Instead, B cells may dampen T cell responses via the production of regulatory cytokines such as IL-10. *L. donovani* infection has been shown to induce MyD88-dependent IL-10 production in B cells in mice (Bankoti *et al.*, 2012), whereas a pathway involving Syk, phosphatidylinositol-3 kinase (PI3K), and p38 was found to underlie IL-10 induction by *L. infantum* in human B cells (Andreani *et al.*, 2015). In mice, B cell-derived IL-10 produced during experimental VL was found to predominantly stem from cells displaying an MZB cell ($CD19^+$ $CD21^+$ $CD1d^{+/-}$ $CD5^+$ $CD23^{lo}$) and regulatory B cell (B_{reg}, $CD19^+$ $CD21^+$ $CD1d^+$ $CD5^+$ $CD23^{hi}$) phenotype (Bankoti *et al.*, 2012). An experimental model using B-1 cell-deficient X-linked immunodeficient BALB/c (BALBc/XID) mice infected with *L. infantum* further revealed that the peritoneal B-1 compartment expands during infection and produces IL-10, thus contributing to disease susceptibility (Gonzaga *et al.*, 2015). Interestingly, B-1 cells also modulate other cell subsets during the infection. Indeed, splenocytes from infected BALBc/XID mice produced lower amounts of the regulatory cytokines IL-10 and TGF-β upon stimulation with parasite antigen and peritoneal macrophages from B-1 cell-deficient mice displayed a higher capacity for parasite control (Arcanjo *et al.*, 2017a). A follow-up study using the same model further implicated peritoneal B-1 cells in the development of intestinal pathology during VL, without investigating a possible underlying mechanism (Souza *et al.*, 2019). IL-10 production by B cells and B_{regs}, as well as particularities of the B-1 B cell subset, are discussed in more detail in the B cell subchapter. The thus produced IL-10 was demonstrated to negatively regulate $CD4^+$ and $CD8^+$ effector functions during *L. donovani* infection (Bankoti *et al.*, 2012). Furthermore, B cell-derived IL-10 was shown to be able to induce T cell exhaustion via

PD-1 in dogs (Schaut *et al.*, 2016b). Interestingly, the degree to which B cells adpot a regulatory profile and secrete IL-10 and other immunosuppressive cytokines appears to depend on the strain of *Leishmania* that causes VL. Indeed, a study comparing the murine immune response against antimonial-susceptible or resistant *L. donovani* strains from the Indian subcontinent found that treatment-resistant parasites induced stronger IL-6 and IL-10 production in different B cell subtypes (Mondal *et al.*, 2021). However, IL-10 only partially accounts for the contribution of B cells to the VL pathology (Deak *et al.*, 2010; Bankoti *et al.*, 2012; Andreani *et al.*, 2015). Indeed, blockade of IL-10 signalling only partially restores the secretion of protective IFN-γ and TNF by $CD4^+$ T cells (Bankoti *et al.*, 2012; Andreani *et al.*, 2015).

A central mechanism by which B cells contribute to protective immune responses is via the production of antibodies. However, during VL, starkly elevated titres of non-protective antibodies caused by polyclonal B cell activation are observed, leading to a condition called hypergammaglobulinemia. In fact, high antibody titres during VL have been proposed as an indicator of disease severity in patients (Singh *et al.*, 2019), and polyclonal B cell activation leading to the production of polyreactive antibodies, in particular IgM, was observed in mice as early as 7 days post-infection with *L. infantum* (Deak *et al.*, 2010). Similarly, high levels of parasite non-specific antibodies have been detected in the chronic phase of *L. infantum* infection in non-human primates, which coincided with the contraction of a $CD4^+$ T follicular helper subset and GCs (Rodrigues *et al.*, 2014).

Importantly, hypergammaglobulinemia has been demonstrated to be the main mechanism through which B cells exacerbate VL (Silva-Barrios *et al.*, 2016; Silva-Barrios & Stäger, 2019); however, the mechanisms underlying this polyclonal B cell activation are still not completely understood. In a mouse model, *L. donovani* was shown to trigger the upregulation of endosomal TLRs in B cells via a proposed positive feedback loop of autocrine IFN-I, which in turn augmented antibody production and negatively impacted disease outcome (Silva-Barrios *et al.*, 2016). The role of IFN-I signalling in B cells is detailed in a later subsection of this chapter. Additionally, in mice with a defect in activation-induced cytidine deaminase (*Aicda*$^{-/-}$), the absence of class-switched or hypermutated antibodies led to an improved Th1 response and reduced disease severity, coinciding with a marked decrease in disease-driving cytokines, such as IL-10, TNF, and IFN-β (Silva-Barrios & Stäger, 2019). Thus, antibody production after class switching and somatic hypermutation negatively impacts disease outcomes in VL. Hypergammaglobulinemia is also observed in humans during VL, where increased differentiation of B cells into plasma cells is

observed in recently diagnosed patients prior to treatment which appears to be supported by elevated levels of B lymphocyte-induced maturation protein 1 (Blimp-1) (Singh *et al.*, 2019).

In summary, VL infection results in increased numbers of plasma cells, polyclonal B cell activation, and resulting hypergammaglobulinemia, all of which negatively impact disease outcome. B cell-derived production of cytokines such as IL-10, IFN-β, and TNF further decreases protective T cell responses and aggravates infection.

PART II: B CELLS

B cells are a type of lymphoid immune cell. Often falsely taken to stand for **b**one marrow, their name stems from the organ in which they were first discovered and the site of haematopoiesis in birds, the **b**ursa of Fabricius (Cooper *et al.*, 1965). As these cells were initially discovered due to their central role in antibody production, it is unsurprising that their role in humoral immunity remains the best-defined function of B cells. However, they have also been shown to participate in cell-mediated immunity such as antigen presentation, cytokine production, and chemokine production (Silva-Barrios *et al.*, 2018). Thus, B cells contribute to immunity in different ways and are key players in the protection against pathogens.

Nevertheless, these mechanisms may be defective or subverted in chronic conditions, such as infections or autoimmunity, causing B cells to contribute to pathology rather than to the resolution of the disease. One such disease is VL, where polyclonal B cell activation results in the high production of non-protective antibodies, called hypergammaglobulinemia, which exacerbates the infection. Hypergammaglobulinemia is known to be a hallmark of VL for decades but the processes causing this harmful B cell activation have yet to be fully elucidated. This section summarises our understanding of B cell roles, subsets, and important signalling pathways leading up to activation.

1. B cell development and biology

Although outwardly homogenous, B cells can be divided into several subsets with distinct lineages, anatomical locations, and roles. In humans, most B cells arise from hematopoietic stem cells in the bone marrow, with the exception of the B-1 subset, which is formed during foetal development and has self-renewal capabilities. The various B cell subsets differ not only in their location and role, but also in the reactivity of their main activation receptor, the B cell receptor (BCR). While the B-1 B cells are able to react to a broad spectrum of common pathogen antigens and are thus considered to be part of innate immunity, the bone marrow-derived and sometimes called “conventional” B cells, the B-2 cells, largely undergo selection processes resulting in a highly specialised BCR necessary for targeted adaptive responses. The following subsection contrasts the different roles and ontology of the different B cell subsets.

1.1. B-1 lineage cells

1.1.1. B-1 cell development and distribution

Discovered almost 20 years after the first reports of B lymphocytes, B-1 lineage cells are a subset of B cells with distinct properties and roles which can spontaneously secrete antibodies in the absence of specific stimuli (Hayakawa *et al.*, 1983). Indeed, these so-called natural antibodies have been observed even in germ-free mice with limited exposure to dietary antigens (Hooijkaas *et al.*, 1984). Upon their initial discovery in mice, they were also called Ly-1 B cells after their defining surface antigen, which has since been named CD5. However, a subset of cells with B-1 lineage characteristics but lacking expression of CD5 has since been discovered, leading to further subclassification of these cells into $CD5^+$ B-1a and $CD5^-$ B-1b cells (Kantor, 1991).

Unlike the B2 lineage cells, B-1 cells are maintained by self-renewal and stem from a population of progenitor cells that arise during early foetal development (Hayakawa *et al.*, 1985). Hence, after the initial phase of B-1 cell production, before and shortly after birth, *de novo* development of B-1 cells is rare (Lalor *et al.*, 1989). At different stages of development, B-1 precursor cells can be found in distinct tissues, with the earliest B-1 cells stemming from the splanchnopleure region and yolk sac of embryos, followed by their differentiation from the foetal liver and lastly, bone marrow-derived B-1 cells in newborns (Baumgarth, 2017). Different tissues may preferentially produce different types of B-1 cells. Although B-1a cells frequently differentiate from the splanchnopleure region (Godin *et al.*, 1993), precursors in the bone marrow appear to preferentially produce B-1b cells (Montecino-Rodriguez *et al.*, 2006), resulting in the production of B-1a cells to typically occur earlier than those of B-1b cells. In contrast, the foetal liver appears to be a source of both B-1a and B-1b cells (Herzenberg, 2000).

The distribution and frequency of B-1 cells greatly varies between the different life stages. Owing to their developmental origin, this subset of lymphocytes is relatively abundant in neonates, accounting for approximately 30 % of the cells in the spleens of 5-day old mice, but rapidly decreases to between 1 - 2 % within 8 weeks (Hayakawa *et al.*, 1983). Similarly, B-1 cells make up 40 – 60 % of B cells in the human foetal spleen, but only comprise a small portion of B cells in the spleens of adults (Antin *et al.*, 1986). In fact, while B-2 cells are the predominant B cells found in secondary lymphoid organs, B-1 cells preferentially populate different anatomical locations, such as the peritoneal and pleural cavities, where they represent 35 – 70 % of all $CD19^+$ B cells

(Baumgarth, 2011). Additional sites of B-1 cells include circulation, bone marrow, intestinal lamina propria, and lung parenchyma, although the percentage of B-1 lineage B cells in most of these locations is very low (Baumgarth, 2011).

1.1.2. B-1 cell functions

B-1 cells located in the spleen and bone marrow, in particular, are thought to be largely responsible for the production of natural antibodies which play an important role both in the maintenance of tissue homeostasis and in the defense against pathogens (Baumgarth, 2011; Choi *et al.*, 2012). Indeed, B-1 cells substantially contribute to the overall levels of continuously produced antibodies. It has been estimated that at a steady state, up to 90 % of IgM and up to 50 % of IgA is derived from B-1 cells (Rothstein *et al.*, 2013). Their role in tissue maintenance is mediated by the production of self-reactive antibodies which can recognise apoptotic cells or low-density lipoproteins, marking them for clearance by phagocytes (Shaw *et al.*, 2000). However, their increased self-reactivity may progress to autoimmunity if their homing or activation becomes dysregulated. Indeed, B-1 cells have pathogenic roles in many autoimmune diseases, such as systemic lupus erythematosus (SLE) (Rahman *et al.*, 2016), type 1 diabetes (Diana *et al.*, 2013), and multiple sclerosis (Villar *et al.*, 2011).

Despite generally having lower affinity and specificity, constitutively produced natural antibodies are also employed to rapidly recognise a broad spectrum of pathogens and already interfere with their replication in the early stages of infection (Baumgarth, 2011). Strikingly, although B-1 cells are capable of antibody production in the absence of stimuli, the recognition of various pathogens via common signatures has been shown to further activate these cells, causing their migration from body cavities to lymphoid tissue where they differentiate into antibody-secreting cells (Smith & Baumgarth, 2019). Such molecular signatures, often termed pathogen-associated molecular patterns (PAMPs), are conserved among many different types of pathogens, including parasites, bacteria, viruses, and fungi, and prominently include Toll-like receptor (TLRs) agonists such as LPS and viral RNA. Stimulation with pathogen-derived PAMPs may also induce a long-term protective B-1 response. Indeed, vaccination of mice with LPS derived from a live vaccine strain of *Francisella tularensis* (*Ft*) resulted in the protective expansion of B-1a cells which could protect against lethal *Ft* rechallenge (Cole *et al.*, 2009). Additionally, recognition of *L. donovani*-derived hydrophilic acylated surface protein B-1 (HASPB-1) by B-1 cell-derived natural antibodies and subsequent immune complex formation and complement activation was shown to result in

protective $CD8^+$ T cell priming by inducing IL-4 secretion in $CD11b^+$ $CD11c^{lo}$ phagocytes, which in turn leads to IL-12 production by DCs (Stäger *et al.*, 2003b).

Other stimuli, such as cytokines, may also regulate the production of antibodies by B-1 lineage cells. B-1 derived antibody production can be induced by IL-5 *in vivo*, which may be due to increased Blimp-1-mediated plasma cell differentiation, similar to the mechanism observed in B-2 cells (Moon *et al.*, 2004; Emslie *et al.*, 2008). However, the secretion of IgM and IgG3 by B-1 cells is maintained in the absence of Blimp-1, indicating that terminal differentiation of B-1 cells into plasma cells is not required for the production of natural antibodies, allowing these cells to quickly adapt to changing requirements (Savage *et al.*, 2017). A feedback mechanism regulating the reconstitution and renewal of these cells has been proposed (Lalor *et al.*, 1989) and indeed, the number of B-1 cells and their proportion related to other B cell subsets appears to be inversely controlled by natural antibody levels, and in particular, IgM (Lino *et al.*, 2013).

In addition to antibody production, B-1 cells can also participate in immune regulation via the production of soluble mediators such as cytokines. Similar to B-2 cells, a small portion of B-1 cells have been shown to adopt an immunoregulatory phenotype via the constitutive expression of the immunosuppressive cytokine IL-10 of which they can produce substantial amounts (O'Garra *et al.*, 1992). This B-1 derived IL-10 production has been shown to promote intracellular parasite growth in macrophages during experimental CL caused by *L. major* (Arcanjo *et al.*, 2017b). Similarly, IL-10 produced by B-1 cells increases the susceptibility of mice to *L. infantum* infection (Gonzaga *et al.*, 2015). This indicates that B-1 derived cytokine production, particularly IL-10 expression, negatively impacts the disease outcome in leishmaniasis.

Strikingly, a small portion of B-1 cells, especially of the B-1b subtype, also possesses an increased capability for phagocytosis compared to most conventional B-2 cells. These B-1 derived phagocytes show decreased expression of IgM and lymphoid factors along with an increase in myeloid-associated genes (Popi *et al.*, 2009). They have been shown to be able to engulf bacteria, such as *Staphylococcus aureus* and *Escherichia coli* (Gao *et al.*, 2012), as well as some species of *Leishmania*, namely *L. major* (Arcanjo *et al.*, 2015) and *L. amazonensis* (Geraldo *et al.*, 2016). Interestingly, IL-10, a central cytokine that regulates susceptibility to leishmaniasis, was also shown to sensitise phagocytic B1 cells to infection by *L. major* (Arcanjo *et al.*, 2015). In a mouse model, phagocytosis of *L. amazonensis* promastigotes by B-1 cells was observed to be mediated by the mannose receptor and complement receptor 3 (CR3) (Geraldo *et al.*, 2016). However, the phagocytic capabilities of B-1 cells for other *Leishmania* species remain to be examined.

1.2. B-2 lineage cells

1.2.1. B-2 cell development and distribution

Unlike B-1 cells, B-2 lineage cells predominantly arise through several differentiation and selection steps from HSCs in the BM, which are modulated by the microenvironment, or niche, created by BM stromal cells. Some of the signals provided in the BM niche which promote B cell development are IL-7, CXC chemokine ligand 12 (CXCL12), FMS-like tyrosine kinase 3 ligand ligand (FLT3L) and the TNF-family member receptor activator of nuclear factor-κB ligand (RANKL), which provide essential signals at various stages of differentiation of B cell precursors (Nagasawa, 2006).

HSCs undergo a number of differentiation steps, subsequently losing their potential to differentiate into other types of immune cells, before acquiring the surface marker B220, which marks commitment towards a B cell lineage (Nagasawa, 2006). While the immediate progeny of HSCs, multipotent progenitor cells (MPPs), have the capacity to give rise to a large variety of immune cells, the following differentiation step, lymphoid-primed multipotent progenitor cells (LMPPs), causes the loss of potential to differentiate into erythroid cells or megakaryocytes. Further commitment of these cells towards the lymphoid lineage is then grouped under the term common lymphoid progenitor (CLPs), which serves as the precursor for B, T, or NK cells (Wang *et al.*, 2020).

Once CLPs express B220, they become B cell precursors, an irreversible lineage commitment towards differentiation into B cells which is also marked by the expression of the transcription factor paired box protein 5 (PAX5) (Nutt *et al.*, 1999). These cells then pass through several developmental stages, sometimes called fractions, in order to become mature B cells with a functional BCR. Comprised of membrane-bound immunoglobin (Ig), the BCR is the central antigen receptor of the B cell which selectively binds an individual antigen. To achieve the rich diversity in BCR specificities required to mount a response against any possible antigen, rearrangements of Ig genes pose a vital part of the B cell maturation process. This genetic rearrangement is called V(D)J recombination, as it involves the somatic reordering of the variable (V), diversity (D), and joining (J) segments on the heavy chain of immunoglobulins, whereas the light chain only undergoes V and J region recombination (Wang *et al.*, 2020). V(D)J recombination occurs in two main steps, the first of which is the double-stranded cleavage of DNA between

recombination signal sequences (RSSs) flanking the individual gene segments, which is mediated by recombination-activating genes (RAG) 1 and 2 and supported by the ubiquitous chromatin-associated high mobility group proteins (HMG) 1 and 2 (van Gent *et al.*, 1997). These double-stranded breaks in the DNA then allow for the rearrangement of genes, including insertion or deletion of segments before sequential ligation in a new configuration. V(D)J recombination first occurs on the heavy chain, where D and J segments are joined together prior to the addition of the V region, and only in later fractions does VJ recombination of the light chain take place. Introducing additional diversity in the sequence of these regions are imprecisions in the ligation of segments, which can lead to deletion or insertion of nucleotides, the latter of which can be achieved by the addition of “non-templated” N nucleotides via the enzyme terminal dideoxynucleotidyl transferase (TdT) (Wang *et al.*, 2020).

At the earliest stage of B cell lineage commitment, called pre-pro B cells, cells have not yet undergone V(D)J recombination and lack surface Ig (Hardy *et al.*, 1991). These cells express the transcription factors EBF1 and E2A, which then bind to the sequence encoding the heavy chain, thus starting off D-H recombination (Wang *et al.*, 2020). After completing this D-H recombination, the addition of the V segment marks the end of the late pro-B cell stage. This coincides with the onset of the expression of the signalling heterodimer consisting of Igα and Igβ, which, along with the completely rearranged heavy chain and a surrogate light chain, forms the pre-B cell receptor (pre-BCR). The assembly of the pre-BCR is vital as it represents a mechanism to sort cells with functionally recombined heavy chains from those with non-productive rearrangements. Only cells with a functional pre-BCR can undergo the required signalling, whereas a lack of a signal triggers receptor editing in the form of V(D)J recombination of the second *Igh* locus of the heavy chain and, in case of repeated failure to produce a functional pre-BCR, apoptosis (Wang *et al.*, 2020). Upon positive selection through successful pre-BCR signalling, late pre-B cells downregulate signalling through the pre-BCR and begin to re-express RAG1 and RAG2, which causes V-J rearrangement of the light chain. Successful rearrangement of the light chain finally leads to the assembly of the complete BCR on the cell membrane, at which stage cells are considered immature B cells (Wang *et al.*, 2020). In contrast to mature B cells, however, cross-linking of the BCR on these immature B cells does not induce proliferation but rather results in anergy or apoptosis (Norvell *et al.*, 1995).

Most of these immature B cells then travel from the bone marrow sinusoids through the bloodstream into the outer PALS of the white pulp of the spleen via the marginal zone sinus and undergo different transitional stages in order to become functionally mature B cells (Liu, 1997).

Three distinct forms of transitional B cells, called T1, T2, and T3, have been identified and each differentiation step is accompanied by a marked drop in numbers, suggesting the presence of selection processes during these transitions. At the earliest transitional stage, T1 cells express large amounts of membrane-bound IgM, while surface IgD, CD21, and CD23 are low to absent (Allman *et al.*, 2001). These T1 cells may be found either in the outer PALS of the spleen or in the bone marrow and are thought to be incapable of recirculation (Allman *et al.*, 2001). The TNF family member B cell-activating factor (BAFF) appears to be a key player in the transition from T1 to T2 cells (Schiemann *et al.*, 2001). This transition induces the honing of T2 cells into the B cell follicles and increases the expression of surface IgD, CD21, and CD23. Interestingly, T1 and T2 cells appear to not only differ in their location and expression of surface markers, but also show differential immune responses, as T1 cells, similar to immature B cells in the BM, respond to BCR stimulation with anergy or apoptosis, while BCR crosslinking could induce proliferation in T2 cells, although this may be ascribed to their increased capacity to be rescued by T cells (Chung *et al.*, 2003). Strikingly, while initially thought to be an intermediate stage of mature B cell development, T3 cells are now understood to represent an anergic subset of often self-reactive B cells, characterised by high CD23 expression similar to T2 cells but low surface IgM, which does not result in the generation of mature B cells (Teague *et al.*, 2007). Rather, the various mature B cell subsets mainly arise from precursors within the T1/T2 population.

1.2.2. Differentiation into mature B-2 subsets

Marginal zone B (MZB) and follicular B (FoB) cells represent the two major subsets of mature B2 lineage cells, and multiple factors governing the fate of differentiation into MZB or FoB have been identified to date. Similar to pre-B cells during B cell development, both MZB and FoB cells also require tonic signalling through their BCR for their maintenance (Kraus *et al.*, 2004); however, the signal strength through an individual cell's BCR may predispose a cell to differentiate into either MZB or FoB. Indeed, B cells receiving stronger signals through the BCR and downstream signalling partner Bruton's tyrosine kinase (Btk) were shown to preferentially differentiate into FoB cells, whereas weaker BCR signalling leads to maturation into MZB cells (Cariappa *et al.*, 2001).

Another key defining factor for whether B cells mature into MZB or FoB cells involves neurogenic locus notch homologue protein-2 (Notch-2) signalling. Engagement of this surface receptor on developing B cells via delta-like ligand-1 (DLL-1) is required for the development of MZB cells (Hozumi *et al.*, 2004). Increasing Notch-2 signalling by inactivating the inhibitory regulator MSX2-

interacting nuclear target protein (MINT) additionally leads to an increase in the number of MZB cells (Kuroda *et al.*, 2003). Strikingly, the fate decision after differentiation from transitional B cells may not be final, as ligation of Notch-2 has been shown to induce trans-differentiation into bona fide MZB from FoB (Lechner *et al.*, 2021). Thus, FoB cells can serve as an additional source of precursors for MZB cells.

1.2.3. Functions and characteristics of B-2 subsets

While the two major mature B-2 subsets, MZB and FoB cells, share a widely similar ontology, they differ both functionally and phenotypically. An overview of the main differences between the B-1, FoB, and MZB cells is provided in **Table 2**.

Table 2 Characteristics of mature B cell subsets

		B-2 cells	
	B-1 cells	**Marginal Zone B cells**	**Follicular B (FoB) cells**
Surface markers	IgM^{hi} IgD^{lo} $CD5^{+}$(B-1a) $CD5^{-}$(B-1b)	IgM^{hi} IgD^{lo} $CD19^{+}$ $CD21^{hi}$ $CD23^{lo}$ $CD1d^{hi}$	IgM^{lo} IgD^{hi} $CD19^{+}$ $CD21^{lo}$ $CD23^{hi}$
Primary sites	pleural & peritoneal cavities	marginal zone of the spleen	secondary lymphoid organs
Ontology	stem from fetal liver, self-renewing	from bone marrow, long-lived	from bone marrow
BCR diversity	polyreactive	polyreactive	highly specific
Require T cell help	no	variable	yes
Somatic Hypermutation	no	unclear	yes
Primary isotypes produced	mostly IgM, some IgG	mostly IgM, some IgG	high levels of IgG, other antibody classes
Memory	some	unclear	yes

On the surface, FoB cells are characterised by high expression of IgD and CD23, also known as Fc epsilon Receptor II (FcεRII), and low expression of complement receptor 2 (CR2 or CD21). In some reports, they are further separated into type I (IgM^{lo}) and type II (IgM^{hi}) FoB, based on their levels of membrane-bound IgM. In this classification, FoB I cells make up the great majority of the total B cells, while FoB II cells are less numerous and present a potential reservoir for replenishing the MZB compartment (Pillai & Cariappa, 2009).

FoB cells are primarily located in the B cell follicles of secondary lymphoid organs, i.e. the spleen and lymph nodes, but these cells are also able to enter the circulation and travel between the follicles and the bone marrow. Homing to the follicles is chiefly mediated by engaging the chemokine receptor CXCR5 on B cells via its ligand CXCL13 which is produced by FDCs (Cyster, 2005). Their localisation in the follicle also brings FoB cells in close proximity to the T cell areas of the spleen, and indeed, this B cell subset is dependent on T cell help for activation. Hence, FoB cells typically respond to thymus-dependent antigens and are able to participate in the germinal centre reaction, undergo somatic hypermutation and class switch recombination, resulting in cells with a highly specialised BCR (Punt *et al.*, 2019b).

In contrast to FoB cells, MZB cells express high levels of surface IgM and CD21, and low amounts of IgD and CD23. They are thought to be a mostly stationary cell subset that populates the outer bounds of the white pulp close to the marginal sinus. Homing to the marginal zone is mainly coordinated by signalling via the sphingosine 1-phosphate receptor (S1PR) family in response to sphingosine 1-phosphate (S1P) which is present at much higher concentrations in the blood than in the follicles and puts MZB in an ideal position to monitor for antigen entering the spleen via the circulation (Cinamon *et al.*, 2004). MZB cells typically express more polyreactive BCRs and are capable of activation by both thymus-dependent and thymus-independent antigens, resulting in their differentiation into plasma blasts capable of producing large amounts of antibodies, typically of the IgM isotype (Cerutti *et al.*, 2013).

Importantly, MZB cells also participate in the capture and transfer of antigens to the follicles of the spleen. While MZB cells do not enter circulation in mice, they are able to undergo continuous cycling between the marginal zone and B cell follicles of the spleen. Indeed, MZB cells, like FoB cells, express CXCR5 and can egress the marginal zone and migrate to the follicles where its ligand, CXCL13, is abundantly produced by FDCs, and the subsequent return of these MZB to the marginal zone is promoted by the S1PR family members $S1PR_1$ and $S1PR_3$ (Cinamon *et al.*, 2008). This cycling of MZB cells between the marginal zone and follicles of the spleen has been shown to take place even in the absence of pathogenic stimuli. During intravital two-photon laser-scanning microscopy of the spleens of naïve mice, approximately 10 % of MZB cells were found to migrate from the marginal zone to the follicle, and a similar number of these cells found in the follicle at the start of measurement migrated to the marginal zone within a 30 min time window (Arnon *et al.*, 2013).

Both their exposure to potential blood-borne pathogens in the marginal zone and their high expression of complement receptors 1 (CR1, CD35) and 2 (CR2, CD21) predispose MZB cells to

capture opsonised pathogens in the blood, and their migration between the marginal zone and follicle then allows for the deposition of these opsonised complexes on FDCs and interaction with FoB cells (Cinamon *et al.*, 2008). Indeed, this follicular shuttling of material was shown to occur in a CR1/2-dependent manner for T-independent antigens, such as trinitrophenol (TNP)–Ficoll (Cinamon *et al.*, 2008), as well as IgM- or IgG3-containing immune complexes, leading to the rapid generation of Germinal Centres (GCs) (Ferguson *et al.*, 2004; Zhang *et al.*, 2014a).

1.3. Non-lineage specific B cell phenotypes

In addition to the B-1 and B-2 lineage B cells, an additional category of B cells which are grouped based on their function rather than their origin, has garnered considerable interest in recent years. Owing to the lack of a clear phenotype, these are often identified based on their main cytokine output. Effector B cells (B_{eff}) are often defined by their expression of various pro-inflammatory cytokines, most importantly IL-6, IFN-γ and granulocyte-macrophage colony-stimulating factor (GM-CSF), while IL-10 production, among other such as IL-35 and TGF-β, is the main factor used to categorize regulatory B cells (B_{regs}) (Matsushita, 2019). Both opposing cell types are important for balancing the immunomodulation caused by B cell-derived cytokines; however, in some conditions, one group of B cell actors may become dominant and contribute to pathology (Matsushita, 2019).

1.3.1. Effector B cells

Effector B cells are a subset of B cells defined by their pro-inflammatory cytokine profile, including IL-1β, IL-2, IL-6, IL-17, IFN-γ, TNF, and GM-CSF secretion, which augments immune responses. Owed to this, they play a key role in the promotion of autoimmunity. Indeed, B cell-derived IL-6 was found to play a detrimental role in the development of experimental autoimmune encephalomyelitis, as a lack of IL-6 production by B cells attenuates disease severity (Barr *et al.*, 2012). Similarly, high numbers of B cells expressing GM-CSF and IL-6 in patients with multiple sclerosis were observed to induce a disease-promoting pro-inflammatory phenotype in myeloid cells (Li *et al.*, 2015). Additionally, mice with a B cell-specific deficiency of IFN-γ were shown to

be resistant to proteoglycan-induced arthritis which was due to a higher differentiation of $CD4^+$ T cells into regulatory T cells (T_{regs}) (Olalekan *et al.*, 2015).

B_{eff} cells also contribute to the immune response against pathogens. During infection of mice with *Listeria monocytogenes*, the expansion of an IFN-γ producing $CD11a^{hi}$ FcγRIII^{hi} B cell population was found to aid in the control of bacterial growth in macrophages, thus conferring increased resistance to infection (Bao *et al.*, 2014). A similar expansion of this population was also observed after challenging mice with *E. coli* or vesicular stomatitis virus (VSV). Furthermore, B cell-derived IL-17 was shown to be induced rapidly during *Trypanosoma cruzi* infection and was vital for an optimal immune response to the parasite (Bermejo *et al.*, 2013). In a *Heligomosomoides polygyrus* infection model, the production of IL-2 by B cells was shown to promote polarisation into a protective Th2 phenotype, whereas TNF contributed to parasite control by augmenting the humoral response (Wojciechowski *et al.*, 2009). Thus, B_{effs} appear to contribute to the resolution of some microbial infections.

1.3.2. Regulatory B cells

As key mediators of health and disease, regulatory B cells (B_{regs}) have attracted great interest in recent years. Although these cells were initially categorised by their high expression of IL-10; however, B cells producing other regulatory cytokines such as IL-35 and TGF-β are now recognised as part of the B_{reg} subset. To facilitate their study, efforts are being made to identify a marker expressed by all B_{regs} that may be used to identify them uniquely. Transcriptomic analysis has indicated that CD9 is a potential common identifier of B_{regs} in mice, but not in humans (Sun *et al.*, 2015). Similarly, TIM-1 was found to be expressed by the majority of mouse B_{regs}, irrespective of their population of origin (Ding *et al.*, 2011). However, both CD9 and TIM-1 are also found on B cells lacking a regulatory phenotype and are therefore not specific only to B_{regs} (Ding *et al.*, 2011; Sun *et al.*, 2015).

In contrast to pro-inflammatory B_{effs}, it is primarily through defects or malfunctions that B_{regs} play an important role in autoimmunity. In experimental autoimmune encephalomyelitis (EAE), recovery was found to be driven by B cell-derived IL-10 which controls the detrimental pro-inflammatory Th1 response (Fillatreau *et al.*, 2002). A later study by the same authors found that, similarly, IL-35 is a key regulator of inflammatory T cell and macrophage responses during EAE and that plasma cells are major producers of IL-10 and IL-35 (Shen *et al.*, 2014). Both IL-10 and

IL-35 were also shown to be produced by B_{regs} in an experimental autoimmune uveitis model, and IL-35 in particular alleviated the disease by limiting Th1 and Th17 responses (Wang *et al.*, 2014). An IL-10-producing population arising from the transitional marginal zone precursor B cell subset has also been shown to be protective in a collagen-induced arthritis model (Evans *et al.*, 2007). The induction of a similar population of B_{regs} in lupus-prone mice, which naturally show a strong reduction of these IL-10-expressing cells, was able to control disease progression and harmful Th1 differentiation and activation (Blair *et al.*, 2009). In addition to protection from autoimmunity, B cells with a regulatory phenotype also play a role in mediating tolerance of antigens to avoid allergy and organ transplant rejection (van de Veen *et al.*, 2016; Cherukuri *et al.*, 2019).

Whereas B_{regs} predominantly play a beneficial preventive role in the development of autoimmunity by opposing excessive inflammatory responses, this cell subset has been attributed a more noxious role in the immune defense against cancer. Indeed, B_{reg}-derived anti-inflammatory cytokines, such as IL-10 and TGF-β, can dampen protective antitumour responses facilitated by other immune cells, thus contributing to cancer immune evasion. In a lymphoma mouse model, even low numbers of IL-10-producing B_{regs} potently inhibited antibody-mediated monocyte function and subsequently impaired the clearance of lymphoma cells (Horikawa *et al.*, 2011). Similarly, a population of regulatory B cells in the blood of B-cell acute lymphocytic leukemia patients was found to hamper $CD8^+$ T cell antitumour responses via excessive IL-10 production (Wang *et al.*, 2007). These anti-inflammatory B_{regs} may be induced by tumour-derived products, such as 5-lipoxygenase metabolites, as conditioned medium from murine breast cancer cells gave rise to a population of regulatory B cells which in turn generated T_{regs} in a TGF-β-dependent manner and increased lung metastasis (Olkhanud *et al.*, 2011; Wejksza *et al.*, 2013).

Similar to the dampening of anticancer responses, B_{regs} can also attenuate protective immune cell responses against some infectious diseases, thus aiding immune evasion and permitting pathogen persistence. The induction of B_{regs} and their regulatory action has been documented in various infectious diseases caused by bacteria, viruses, and parasites. During infection with *Salmonella typhimurium*, MyD88-dependent IL-10 production by B_{regs} has been shown to hamper protective T cell, NK cell and neutrophil responses and contributes to disease-related mortality in mice (Neves *et al.*, 2010). In the same disease, B cell and in particular plasma cell-derived IL-35, has also observed to be detrimental to *Salmonella*-directed immunity (Shen *et al.*, 2014). *Helicobacter pylori* was similarly found to induce IL-10-producing B_{regs} in the intestinal and gastric mucosa which may then contribute to $FoxP3^+$ T_{reg}-driven immune evasion of the bacteria (Wei *et al.*, 2014).

Viral infection can also induce a regulatory phenotype in B cells. Infection with murine cytomegalovirus identified a key role of B_{reg}-derived IL-10 in limiting the protective $CD8^+$ response against the virus, along with a decrease in plasma cell differentiation (Madan *et al.*, 2009). Furthermore, during the early phase of untreated HIV-1 infection, higher levels of $TIM\text{-}1^+$ IL-10-producing B cells were found in the blood and sigmoid colon biopsies of infected individuals which subsequently suppressed virus-specific T cell responses and showed a positive correlation with viral load (Liu *et al.*, 2014a). Likewise, patients with chronic hepatitis B virus (HBV) infection had higher frequencies of B cells producing IL-10 and TGF-β, the former resulting in the generation of T_{regs} rather than virus-specific $CD4^+$ T cell responses, and the number of B_{regs} was positively correlated with the severity of the disease (Liu *et al.*, 2016).

During parasitic infections, regulatory B cells and B_{reg}-derived cytokines affect immunology in both protective and harmful ways. In fact, infection with helminths, such as *Schistosoma mansoni* has been proposed to help alleviate autoimmunity and allergic reactions through B_{reg}-mediated dampening of the overactive immune response (Amu *et al.*, 2010). Additionally, in severe malaria caused by *Plasmodium berghei*, IL-10 from B_{regs} was found to attenuate detrimental increases in NK and T cells in the brains of infected mice and protect these animals from cerebral malaria-related pathology and mortality; however, no difference in parasitemia was observed (Liu *et al.*, 2013). On the other hand, during infection with the protozoan parasite *Babesia microti,* which resembles malaria in its clinical presentation, the increased induction of IL-10-secreting B_{regs} contributed to heightened susceptibility to the disease in mice via the generation of T_{regs} (Jeong *et al.*, 2012).

Regulatory B cells are also induced by several *Leishmania* species. In an *L. major*-susceptible mouse model, IL-10 produced by B cells upon exposure to the parasite was found to reduce DC-derived IL-12 which can in turn inhibit protective Th1 activation and thus skew the T cell response towards a permissive Th2 profile (Ronet *et al.*, 2010). Stimulation of splenic B cells with *L. amazonensis* resulted in even higher levels of IL-10-producing B_{regs} compared to *L. major*, which may result in the expansion of the regulatory T cell subset (Veras *et al.*, 2006). Similarly, B-1 B cells, which are able to phagocytose *L. amazonensis* promastigotes, are induced to upregulate expression of both IL-10 and TNF-α in response to the infection (Geraldo *et al.*, 2016). Exposure of B cells to either *Leishmania* species primarily responsible for visceral clinical presentations, *L. infantum* and *L. donovani*, also induces B cells with a regulatory phenotype. In an *in vitro* model using human B cells, incubating the cells in the presence of *L. infantum* amastigotes resulted in B cell activation and Syk-PI3K-p38-dependent IL-10 production, the latter of which was observed

to be partly responsible for the inhibition of $CD4^+$ T cell responses by cell-free supernatants from the B cell cultures (Andreani *et al.*, 2015). Similarly, murine B cells, and in particular MZB cells, were shown to be activated to produce IL-10 in the presence of *L. donovani* amastigotes, which in turn decreased the beneficial proliferation and function of $CD4^+$ T cells, thus contributing to disease susceptibility (Bankoti *et al.*, 2012). This induction of B cell IL-10 production by *L. donovani* was found to be dependent on MyD88 and, more specifically, endosomal TLR signalling (Bankoti *et al.*, 2012; Silva-Barrios *et al.*, 2016). In dogs presenting with VL, the expansion of a population of B_{regs} which regulated T cell responses via IL-10 production and PD-1 interactions has similarly been documented (Schaut *et al.*, 2016b). Thus, regulatory B cells play a key role in modulating immune cell responses to mediate antigen tolerance which can be exploited by pathogens to enhance susceptibility to disease.

2. B cells in adaptive immunity

The main role of B cells in adaptive immunity is to produce highly specific antibodies against potentially harmful antigens, including those derived from pathogens. There are a number of processes in place to ensure a tailored humoral response, such as the formation of defined physical structures called germinal centres, which provide an ideal environment to generate diversity of the B cell antigen receptor, the BCR, as well as a mechanism to adapt the isotype of antibodies produced according to immune requirements. Recognition of antigens by highly specialised B cells and their subsequent activation can then elicit the targeted production of antibodies, which in turn are responsible for various antibody-mediated effects.

2.1. The germinal centre reaction

As a source of high-affinity antibodies via selection and mutation processes, germinal centres (GCs) are important for the development of effective immunity against pathogens via affinity maturation. These structurally defined regions form in the B cell follicles in secondary lymphoid

tissues and are formed in response to antigen recognition by B cells via their BCR, causing an upregulation of chemokine receptor 7 (CCR7) on these cells This leads to their targeted migration towards the interface of B cell and T cell zone to enlist the help of antigen-specific T cells to initiate cell division as early as 3 days after the antigen encounter (Okada *et al.*, 2005). These B cells then act as a seed for the formation of a GC which, when mature, can be separated into two distinct areas: the dark zone (DZ), where B cell division and somatic hypermutation (SHM) take place, and the light zone (LZ), which is comprised of an FDC network presenting antigens to B cells to select for high-affinity interactions (**Figure 2**) (Victora *et al.*, 2010).

In the DZ, SHM is mediated by activation-induced cytidine deaminase (AID) which targets single-stranded DNA of the switch and V(D)J regions of the heavy and light Ig chains to generate spot mutations which can induce further mutations during DNA repair processes (Pilzecker & Jacobs, 2019). The resulting functional B cells with altered antigen specificity then migrate towards the LZ, where they can interact with antigens presented on FDCs to assess their affinity with the help of T follicular helper cells (Victora *et al.*, 2010). In contrast, B cells in which mutations result in a defective BCR are marked for apoptosis and are removed by tingible body macrophages, which surround the GC and are concentrated at the interface between the LZ and DZ (Hanayama, 2004). In fact, up to 50 % of GC B cells are removed in this manner every 6 h, which is necessary to counteract the rapid proliferation of B cells in the DZ and maintain the size of GCs (Mayer *et al.*, 2017).

B cells can undergo multiple rounds of SHM and selection. Intravital two-photon laser scanning microscopy revealed that GC B cells not only move bi-directionally between the LZ and DZ but also indicated a complete turnover of cells in both areas within 18 h (Schwickert *et al.*, 2007). Additionally, while LZ B cells, sometimes called centrocytes, are often defined as having a $CXCR4^{lo}$ $CD83^{hi}$ $CD86^{hi}$ phenotype and DZ B cells, also referred to as centroblasts, are ascribed to a $CXCR4^{hi}$ $CD83^{lo}$ $CD86^{lo}$ profile (Victora *et al.*, 2010), more recent studies indicate the presence of a population comprising up to a third of all GC B cells with an intermediary phenotype, indicating cells in the process of transitioning between these states (Kennedy *et al.*, 2020).

Apart from moving back to the DZ for further division and SHM or undergoing apoptosis, a small number of GC B cells in the LZ may also differentiate into plasma cells (PCs) or memory B cells (MBCs) which then exit the germinal centres. This is initiated by positive selection during which GC B cells with higher-affinity BCRs can outcompete lower-affinity ones to more efficiently present FDC-derived antigen to T follicular helper (Tfh) cells, thus eliciting stronger co-stimulatory signals, such as CD40-CD40L interactions which further induce NF-κB signalling (Luo *et al.*, 2018), and

synergistic triggering of the IL-21 receptor (IL-21R) (Luo *et al.*, 2023). Although not essential for initiating the process, strong Tfh stimulation associated with highly specific BCRs is essential for the differentiation of centrocytes into long-lived PCs (Kräutler *et al.*, 2017).

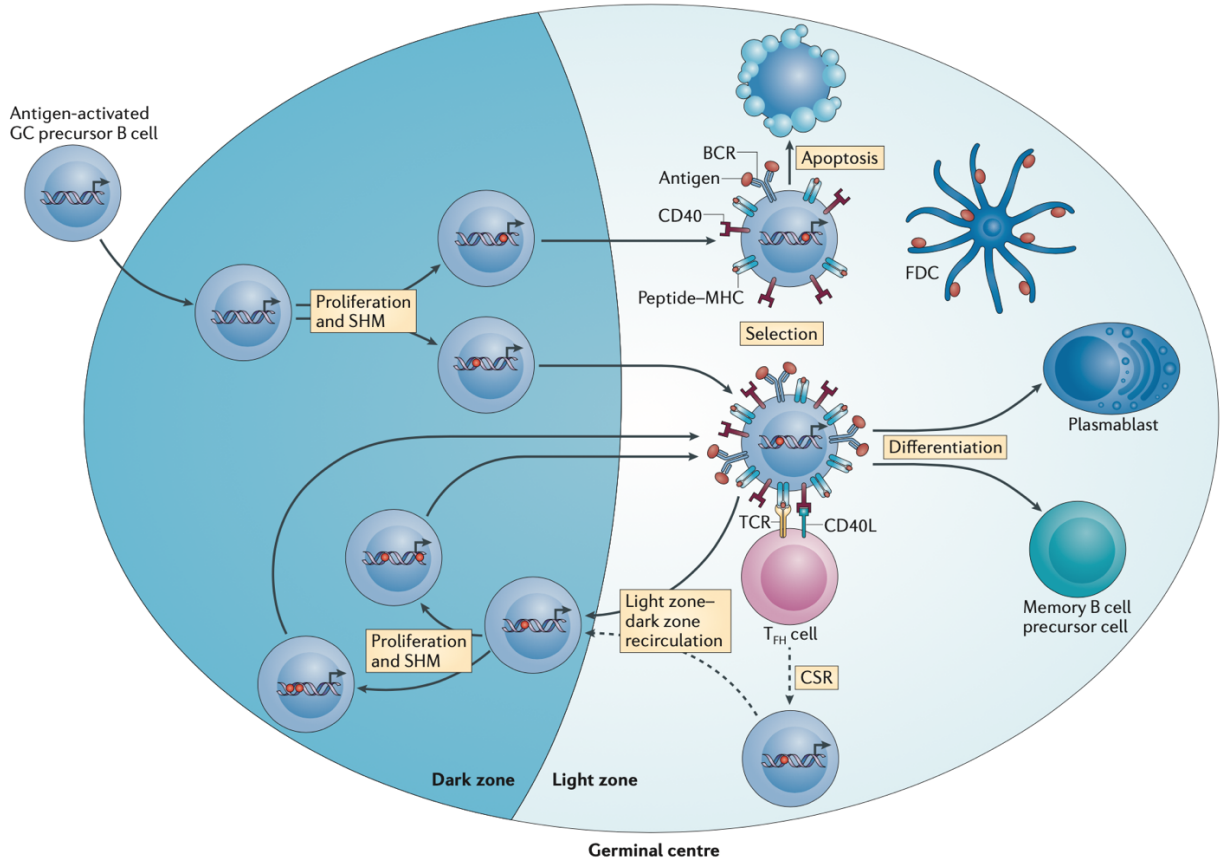

Figure 2 The Germinal Centre Reaction
(reprinted from *(De Silva & Klein, 2015)*)

B cells enter the dark zone (DZ) of a GC upon encountering cognate antigen to become rapidly proliferating centrocytes which undergo somatic hypermutation (SHM). These cells with a newly diverse repertoire of antigen receptors then migrate to the light zone (LZ) of the GC, where abundant FDCs present antigen for the B cells to test their BCRs with. Failure to engage antigen and trigger BCR signalling leads to apoptosis of the cells. On the other hand, high affinity interactions of antigen and BCR result in a more efficient transfer of antigen to the B cells to present on their MHC-II molecules and hence a stronger signal from Tfh cells. B cells unable to illicit sufficiently strong Tfh help may recirculate to the DZ of the GC to undergo more SHM and a single B cell may undergo multiple rounds of recirculation between DZ and LZ before receiving sufficient T cell help for differentiation into memory B cells or plasma cells.

In contrast, the memory B cells arising in GCs appear to develop from a population of GC B cells with lower-affinity BCRs than those differentiating into PCs. Indeed, a group of cells displaying a

$CD38^{int}$ $Bcl6^{hi/int}$ $Efnb1^{+}$ phenotype was proposed to serve as a precursor to MBCs within the LZ GC B cell pool, from which MBC differentiation is initiated through lower-level mammalian target of rapamycin complex 1 (mTORC1) activation, possibly caused by weaker T cell engagement, which is associated with a marked decrease in B-cell lymphoma protein 6 (Bcl6) expression (Inoue *et al.*, 2021).

2.2. Class switch recombination and antibody diversity

Class switch recombination (CSR) or isotype switching is a process during which the constant (C) region of the heavy chain (IgH) is rearranged to give rise to different antibody classes, or isotypes, to respond to the varying immune requirements; however, unlike V(D)J rearrangement and SHM, this process does not affect antigen specificity. The Ig heavy chain can take on one of 5 major isotypes: Igα (IgA), Igδ (IgD), Igε (IgE), Igλ (IgG), and Igμ (IgM). Additionally, IgG can be further divided into the subclasses IgG1 – IgG4 and IgA comprises the subclasses IgA1 and IgA2 (Chi *et al.*, 2020).

By default, naïve mature B cells express both IgM and IgD on their surface as part of their B cell receptors. Upon encountering antigen and triggering CSR, DNA recombination occurs via the targeted introduction of double-stranded breaks in the switch (S) region between the joining (J) region and the constant (C) region encoding the current isotype, i.e. Cμ, in tandem with a similar break in the S region ahead of the new isotype, leading to the recombination into a heavy chain of the latter isotype (Chi *et al.*, 2020). As in V(D)J recombination and SHM, AID also plays a key role in the initiation of these DNA breaks by deaminating cytosines in the S regions, which are subsequently deleted by uracil DNA glycosylase (UNG) (Schrader *et al.*, 2005). The thus created abasic sites are then further cleaved into single-stranded DNA breaks by the enzyme apurinic/apyrimidinic endonuclease (APE) and the high frequency of these breaks in the S region promotes double-stranded DNA breaks through the proximity of similar breaks on the opposite DNA strand which then allows for CSR to take place (Guikema *et al.*, 2007).

While long believed to be part of the germinal centre reaction, more recent studies have now shown that CSR rarely takes place inside GCs and the presence of class-switched B cells can be detected before GC formation (Roco *et al.*, 2019). Instead, the transition of GC from IgM to class-

switched isotypes such as IgG, which is often observed over time, appears to be due to a positive selection mechanism of switched over unswitched high-affinity GC B cells (Sundling *et al.*, 2021).

Interestingly, while interactions with antigens can induce CSR, BCR signalling is not strictly required for isotype switching. Rather, co-stimulation and secretion of various cytokines by cells in their environment may regulate B cell class switching. A central co-stimulatory molecule in this process is CD40, which is present on B cells, and its ligand, CD40L, which is highly expressed on helper T cells, as is evidenced in humans with X-linked hyper IgM syndrome (Conley *et al.*, 1994). These individuals show a defect in CD40 signalling, resulting in low levels of switching from IgM to other isotypes. Additionally, signalling through several toll-like receptors (TLRs) has been shown to induce CSR, often synergistically with other signals such as cytokines or the BCR. Indeed, stimulation with LPS, the ligand for TLR4, has been demonstrated to be sufficient to cause isotype switching (Pone *et al.*, 2012). Other TLRs like TLR9 may act synergistically with other signals such as antigenic stimulation to augment isotype switching (Hou *et al.*, 2011). Finally, during both CD40 or TLR engagement, additional signals provided by certain cytokines, such as IL-4, IL-10, and TNF-β, can further influence the resulting isotype (Litinskiy *et al.*, 2002).

Class switching of B cells is vital for an effective immune response because isotype classes vary in size and can facilitate different antibody effector functions. The form of immunoglobulin initially expressed on B cells is IgM, which predisposes this class to participate in the primary immune response. Primarily forming disulfide-bonded pentamers in its secreted form, it opsonises antigens for recognition by phagocytes and participates in the activation of the complement cascade (Schroeder & Cavacini, 2010). The second antibody class found on B cells prior to class switching is IgD, which is secreted as a monomer only at minute levels. This isotype is known mostly for its function as a membrane-bound immunoglobulin, where it participates in key signalling processes governing the homeostasis and selection of B cells (Geisberger *et al.*, 2006).

Accounting for approximately three quarters of all serum immunoglobin in healthy individuals, IgG and its four subtypes are the most well-researched of the antibody classes and within the IgG family, IgG1 represents the most abundant at two thirds of total IgG, while IgG4 makes up around 4 %. IgG subtypes share many similarities, such as their presence as monomers in the unbound secreted form; however, they differ in some key respects, including their ability to bind complement and interact with different Fc receptor classes. Similar to IgG, IgA is further separated into subclasses, namely IgA1 and IgA2. This isotype dimerises in the serum and is present at high concentrations in mucosa and bodily fluids, including breast milk and saliva. Although they

do not typically participate in the complement cascade, IgA-type antibodies are critical for the protection of mucosal areas from infection (Schroeder & Cavacini, 2010).

Lastly, IgE is the least abundant type of antibody in the serum of healthy individuals and is most frequently found in tissues where it is bound to cells such as mast cells and eosinophils via Fc epsilon receptors. It is not known to participate in the opsonisation or complement fixation of antigens; however, it is thought to play an important role in allergies, asthma, and hypersensitivity reactions. Additionally, it participates in the immune response to many parasites, such as helminths, and in the reaction to tick bites (Luker *et al.*, 2019).

However, the presence of class-switching and hypermutation in B cells may also contribute to the development of disease in some conditions. Indeed, during *L. donovani* infection, *Aicda-/-* mice which lack AID and thus cannot generate diverse high-affinity antibodies were found to be more resistant to the infection and displayed a strong protective Th1 immune response in contrast to the regulatory Tr1 response observed in wild-type C58BL/6 mice (Silva-Barrios & Stäger, 2019). This increased resistance occurred despite lower titres of Leishmania-specific antibodies and appeared to be conferred by differential regulation of the inflammatory environment. Similar to the high-affinity antibodies produced in the early stages of infection during *L. donovani* infection of mice (Silva-Barrios & Stäger, 2019), *Leishmania*-specific antibodies have also been shown to be produced during *L. infantum* infection in rhesus macaques (Rodrigues *et al.*, 2014). However, the level of these parasite-specific antibodies starkly decreased in the chronic phase, accompanied by the loss of splenic Tfh cells and the contraction of germinal centres (Rodrigues *et al.*, 2014). Despite the presence of high-affinity antibodies, the role of these Leishmania-specific antibodies is not yet understood (Silva-Barrios *et al.*, 2018).

2.3. B cell receptor signalling and activation

A central step in B cell activation is the engagement of the B cell receptor (BCR) by cognate antigen, which in turn results in the activation of several downstream signalling pathways (**Figure 3**); however, BCR engagement alone is not sufficient to sustainably activate B cells. Indeed, BCR ligation in the absence of a second signal leads to a decline in cell metabolism, followed by eventual apoptosis (Akkaya *et al.*, 2018). Consequently, B cells require a second signal in addition to BCR crosslinking for activation.

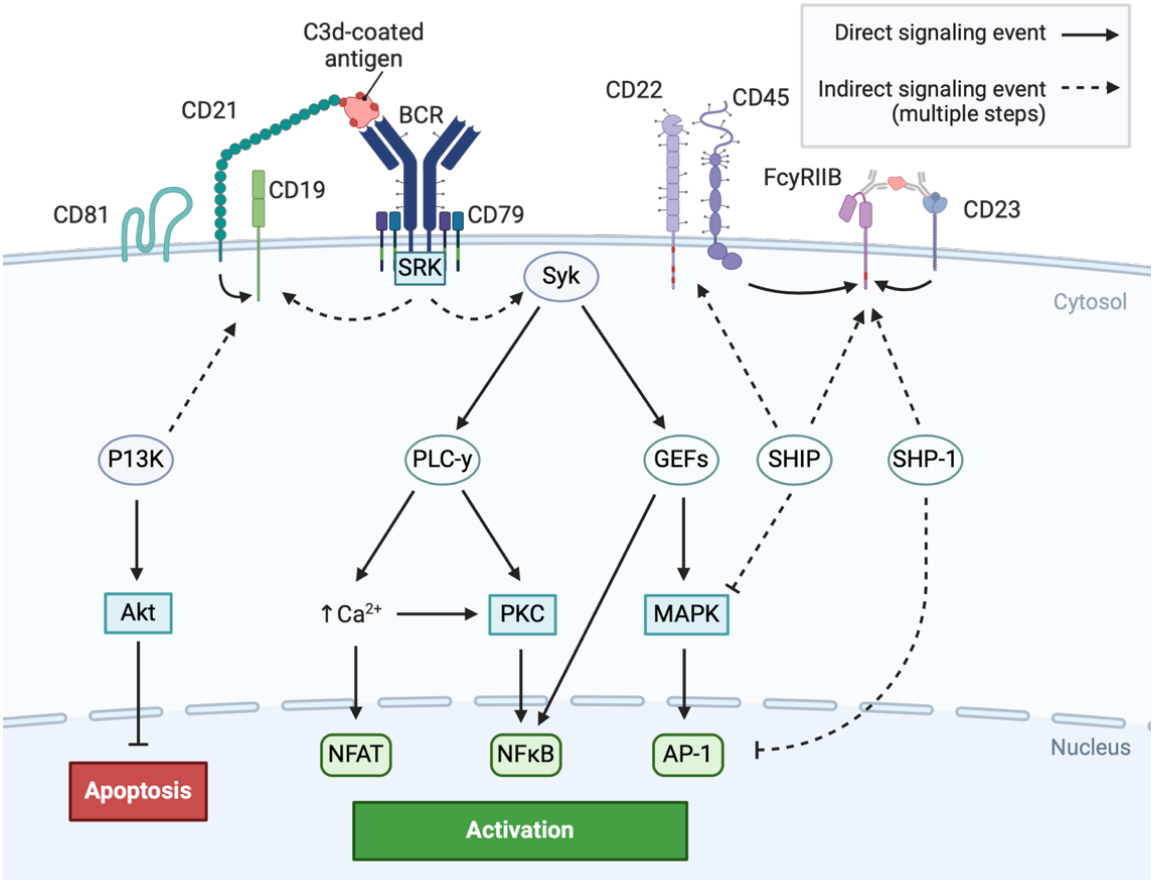

Figure 3 Signalling pathways downstream of BCR crosslinking
(Adapted from *(Sicard et al., 2020)*, Created with Biorender.com)

Upon recognition of antigen, the immunoreceptor tyrosine-based activation motifs (ITAMs) on CD79 which is covalently bound to the BCR undergo phosphorylation by SRC kinases such as LYN. This tyrosine phosphorylation in turn activates spleen tyrosine kinase (Syk), which induces the formation of a signalling complex containing phospholipase Cγ2 (PLC-γ2), leading to the production of the messenger molecule inositol-1,4,5-triposphate (IP_3). Recognition of IP_3 by its receptors stimulates influx of extracellular calcium into the cell, which then activates calcineurin to dephosphorylate nuclear factor of activated T cells (NFAT), causing its translocation into the nucleus. Concomitantly, PLC-γ2 also produces a second messenger, diacylglycerol (DAG), which engages protein kinase Cβ (PKCβ), resulting a downstream phosphorylation of IκB kinase (IKK) and ultimately NF-κB activation. Both guanine nucleotide exchange factor (GEF) and DAG modulate the activity of the small G protein Ras, which is a key regulator in the extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) pathway, resulting in the induction of the transcription factor activator protein-1 (AP-1). On the other hand, the CD19/CD21/CD81 co-receptor complex appears to be primarily responsible for regulating downstream phosphoinositide 3-kinases (PI3K) activation. LYN-mediated phosphorylation of the cytoplasmic end of CD19 unlocks a binding site for p85α, a PI3K subunit, which ultimately leads to the production of phosphatidylinositol-3,4,5-triphosphate (PIP_3) and resulting activation of Akt signalling. Counterbalancing the positive regulation of downstream BCR signalling are several inhibitory pathways. FcγRII and CD22 are surface receptors containing immunoreceptor tyrosine-based inhibition motifs (ITIMs) which, upon phosphorylation, recruit SRC homology region 2 domain-containing phosphatase-1 (SHP-1) and other tyrosine phosphatases to curb positive activation downstream of BCR (Tanaka & Baba, 2020).

Based on the nature of the second signal, antigens can typically be sorted into two categories: thymus-dependent (TD) and thymus-independent (TI) antigens. As the name suggests, the activation of B cells in response to TD antigens, typically soluble proteins, requires a secondary signal received through T cell help. To elicit this additional stimulation, B cells must present internalised antigens on their major histocompatibility complex class II (MHC-II) to cognate $CD4^+$ T cells, leading to T helper cell activation, cytokine production, and the upregulation of co-stimulatory molecules, such as CD40 ligand (CD40L), which binds to CD40 on B cells and acts as a second activation signal (Bachmann, 2009). In addition to CD40, B cells may also receive co-stimulation through the interaction of CD80 (also called B7-1, CD86, or B7-2) with CD28 or cytotoxic T-lymphocyte-associated molecule-4 (CTLA-4, CD152) (Suvas *et al.*, 2002).

On the other hand, TI antigens are often classified into two types: TI-1 antigens, exemplified by lipopolysaccharide and conserved structures eliciting a polyclonal B cell response, and TI-2 antigens, including nucleic acids, and polysaccharides (Jeurissen *et al.*, 2004). TI-1 antigens are typically recognised by pattern recognition receptors (PRRs), which will be discussed in a later section, whereas TI-2 antigens crosslink BCR on the surface of cells due to their repetitive nature (Punt *et al.*, 2019c). Although it was long believed that efficient GC reactions and the resulting PC and MBC formation take place mostly in response to TD antigens, a recent study has demonstrated that GCs formed after immunisation with TI antigens are also capable of producing PCs and MBCs, thus strengthening the evidence for B cell memory in TI activation (Liu *et al.*, 2022).

2.4. Polyclonal B cell activation and hypergammaglobulinemia

While each B cell clone carries BCRs specific for only one epitope of a given antigen, multiple clones may recognise epitopes of the same antigen, resulting in the simultaneous activation of several B cells with different specificities, called polyclonal B cell activation. This activation of multiple B cell clones results in the production of antibodies with a wider variety of binding capabilities and may even afford cross-protection between different serotypes of the same pathogen (Balakrishnan *et al.*, 2011). If this polyclonal B cell activation is dysregulated, however, it results in a harmful, excessive production of non-protective antibodies, called hypergammaglobulinemia or polyclonal gammopathy.

In humans, this hypergammaglobulinemia typically accompanies conditions such as liver diseases, autoimmune conditions, and cancer; however, hypergammaglobulinemia is also a

hallmark of numerous chronic infections (Dispenzieri *et al.*, 2001). It is observed during bacterial infections, such as infection with *Staphylococcus aureus* (Crow *et al.*, 1993). Viruses known to cause polyclonal B cell activation include lymphocytic choriomeningitis virus (LCMV) (Greczmiel *et al.*, 2020), Epstein-Barr virus (Al Tabaa *et al.*, 2011), and dengue virus (Correa *et al.*, 2015). In parasites, hypergammaglobulinemia and polyclonal B cell activation are a common mechanism of immune evasion and have been documented in chronic infections with various parasite species, such as the causative agents of VL, *L. donovani* (Silva-Barrios *et al.*, 2016; Silva-Barrios & Stäger, 2019) and *L. infantum* (Deak *et al.*, 2010), the CL-causing *L. major* (Lohoff *et al.*, 1988), as well as other parasites such as *Plasmodium falciparum* (Donati *et al.*, 2004), and *Trypanosoma cruzi* (Montes *et al.*, 2002).

Despite being shared among many different infections, the mechanisms underlying this harmful activation of B cells are not well characterised. Several cytokines have been implicated to aid in this activation, including IL-6 (Markine-Goriaynoff *et al.*, 2001), and IL-10 (Parcina *et al.*, 2013). These cytokines may be induced by direct interaction of the pathogen with B cells or dependent on the production by other cell types. Indeed, while B cells were shown to abundantly produce IL-6 during HIV-1 infection, thus contributing to humoral dysregulation in an autocrine manner (Kehrl *et al.*, 1992), predominantly macrophage-derived production of the same cytokine contributes to polyclonal B cell activation observed in response to *Toxoplasma gondii* (Markine-Goriaynoff *et al.*, 2001). Similarly, IL-10 may be produced by the B cells, as is the case during *L. donovani* infection (Silva-Barrios *et al.*, 2016), or derive from other types of immune cells such as dendritic cells in response to *S. aureus*-derived *Staphylococcal* protein A (Parcina *et al.*, 2013). Additionally, TLR activation appears to play an important part in polyclonal B cell activation. Not only is signalling through various TLRs shown to be upstream of polyclonally activating cytokines, including IFN-I (Silva-Barrios *et al.*, 2016), but it also acts directly on B cells to facilitate polyclonal B cell activation, as has been demonstrated for both TLR4 and TLR9 (Park *et al.*, 2012). This may be amplified by the fact that certain pathogens can directly augment TLR expression on the surface of B cells, which was shown for the cysteine-rich inter-domain region 1α (CIDR1α) on *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) (Simone *et al.*, 2011) and *L. donovani* amastigotes (Silva-Barrios *et al.*, 2016). TLR-mediated signalling and the role of IFN-I in B cells are discussed in the next section.

Interestingly, some pathogens known to be able to induce B cells polyclonally are classified as superantigens, which are thought to bind to conserved structures on BCRs outside of their specific antigen-binding site, making them widely polyreactive. Superantigens identified to date include

Moraxella catarrhalis-derived antigen *Moraxella* IgD binding protein (MID) (Singh *et al.*, 2012), a structure on *Mycoplasma arthritidis* and multiple antigens derived from *S. aureus* (Crow *et al.*, 1993). A similar indiscriminatory binding mechanism has been described for the cysteine-rich inter-domain region 1α (CIDR1α) on *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which binds to shared regions, such as the Fab and Fc fragments, on membrane-anchored antibodies and thus induces the proliferation and activation of B cells (Donati *et al.*, 2004).

Other parasite-derived antigens have been identified to incite polyclonal B cell activation whose mechanism of interaction with B cells remains to be clarified. A protein isolated from *T. cruzi* epimastigotes and identified as mitochondrial malate dehydrogenase (mMDH) was identified as a polyclonal B cell activator, leading to the production of mainly IgM and IgG3 class antibodies (Montes *et al.*, 2002). In the same parasites, the TI antigen trans-sialidase, and in particular its C-terminal long tandem repeat region, has been shown to induce Bruton's tyrosine kinase (Btk)-dependent B cell proliferation, non-specific activation, and splenocyte cytokines (Gao *et al.*, 2002). Similarly, *T. cruzi*-derived glutamate dehydrogenase (GDH) was found to act as a TI antigen and promote the proliferation and antibody production of polyclonal B cells (Montes *et al.*, 2006). This was accompanied by augmented production of cytokines, such as IL-6, IL-10, and TNF-family members B cell-activating factor (BAFF) and a proliferation-inducing ligand (APRIL) by $CD11b^+$ cells. Indeed, blocking BAFF activity resulted in lowered B cell proliferation and a decreased humoral response in *T. cruzi*-infected mice; however, this was accompanied by a higher parasite load in the hearts of infected animals, pointing towards a potentially protective effect of BAFF signalling in disease pathology (Bermejo *et al.*, 2010).

In *Leishmania*, both TI and TD mechanisms have been identified in polyclonal B cell activation across different parasite species. An early study found that *L. major*-specific $CD4^+$ T cell help induced multi-clone B cell activation, proliferation and antibody production (Lohoff *et al.*, 1988). Silent information regulatory 2 protein (SIR2), which was purified from *L. major* but is expressed across many *Leishmania* species, was shown to activate B cells, as evidenced by the expression of the activation marker CD69, induce proliferation, and promote the humoral response. However, the antibodies produced during this response were able to recognize amastigotes even across species to facilitate complement-mediated lysis and hamper amastigote development within macrophages (Silvestre *et al.*, 2006). Thus, the contribution of SIR2-mediated B cell activation to the observed non-specific polyclonal response remains unclear. In contrast, the *L. major*-derived homologue of the mammalian ribosomal protein S3a, R3a-related protein (*Lm*R3arp), was shown to strongly promote the activation, proliferation, and IgM secretion of non-parasite-specific B cell

clones while decreasing T cell activation (Cordeiro-Da-Silva *et al.*, 2001). This study found *that Lm*R3arp was expressed in different species, including *L. infantum*; however, its expression in *L. donovani* parasites was not tested. Another potential polyclonal activator during leishmaniasis is the cytosolic oxidoreductase *L. infantum* tryparedoxin (*Li*TXN1), which could induce B cell proliferation, secretion of cytokines such as IL-10 and augmented secretion of antibodies, mostly of the IgM, IgG1 and IgG3 subclasses (Menezes Cabral *et al.*, 2008). Similar to *T. cruzi* infection, BAFF may play a role in excessive proliferation and antibody production during leishmaniasis. Indeed, BAFF-deficient mice show decreased IgG production and splenomegaly during *L. donovani* infection (Omachi *et al.*, 2017). Notably, the lack of BAFF signalling resulted in an increased parasite burden in the liver but not in the spleens of the infected animals, indicating a dual mechanism of action.

3. Innate immune signalling in B cells

Innate immune functions are tightly interlinked with adaptive immunity, and B cells are not only able to be an accessory to innate immunity but also directly participate in innate immune processes. By producing antibodies, B cells are essential for the initiation of the classical complement pathway but B cells also express and are able to signal through various complement receptors. Furthermore, B cells are capable of responding to pattern-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), both of which are classical innate signals, through Toll-like receptors and cytosolic sensors. The pathways by which B cells respond to innate immune signals and participate in the complement system are discussed in this subsection.

3.1. B cells and the complement system

Named for its ability to “complement” the antimicrobial effects of antibodies, the complement system is an evolutionarily conserved pathway that consists of concerted serial proteolytic reactions of proteins abundantly present in the serum. These processes take place at the interface of adaptive and innate immunity and are vital for many B cell functions, including antibody production, class switching, and antigen presentation.

The complement system is usually divided into three main branches, called the classical, lectin, and alternative pathways, which differ in the initial events leading to the activation of the complement cascade but ultimately come together at the central step of generating a C3 convertase, which is defined as a complex capable of cleaving complement protein C3 into its active form (**Figure 4**) (Punt *et al.*, 2019a). C3 cleavage sets off downstream proteolytic processes resulting in the three main outcomes of complement activation: (i) the opsonisation of pathogens to tag them for recognition by immune cells such as phagocytes, (ii) the formation of the membrane attack complex set into motion by cleavage of complement C5 downstream of C3 catalysis, ultimately leading to lysis, and (iii) the release of chemoattractant anaphylatoxins which direct effector cells towards the site of infection and mediate local inflammation (Dunkelberger & Song, 2010). As the source of antibodies, B cells are important for initiating the classical pathway of the complement cascade; however, conversely, complement may also be required for an efficient humoral response. Indeed, depletion of C3 from the serum of mice significantly impaired their antibody production in response to common antigens such as sheep red blood cells and ovalbumin (Pepys, 1972), an effect mirrored in mice lacking the complement receptor 2 (CR2) which is a ligand for complement C3 fragments (Sörman *et al.*, 2014). Thus, B cells not only participate in the complement cascade by facilitating complement activation but are also able to directly interact with its products.

Of the four main types of complement receptors, B cells are known to primarily express only two: complement receptor 1 and 2 (CR1 and CR2) (Dunkelberger & Song, 2010). These transmembrane glycoproteins belong to the same family and are encoded by the same gene in mice; however, they have been ascribed different functions. CR1, also known as CD35, is expressed by most peripheral blood cells, including B cells, and binds the complement-derived fragments C3b and C4b, as well as iC3b and C3dg with lower affinity (Krych-Goldberg & Atkinson, 2001). Additionally, it has been shown to be able to interact with the cascade initiators C1q and MBL (Jacquet *et al.*, 2018). CR1 ligation activates its decay-accelerating activity on C3 and C5 convertases and is thus considered an important inhibitory receptor of the complement system, where it plays a role in the removal of immune complexes (Krych-Goldberg & Atkinson, 2001). In B cells, CR1 activation has further been shown to negatively regulate proliferation, decrease differentiation into plasma blasts and lower antibody levels (Kremlitzka *et al.*, 2013).

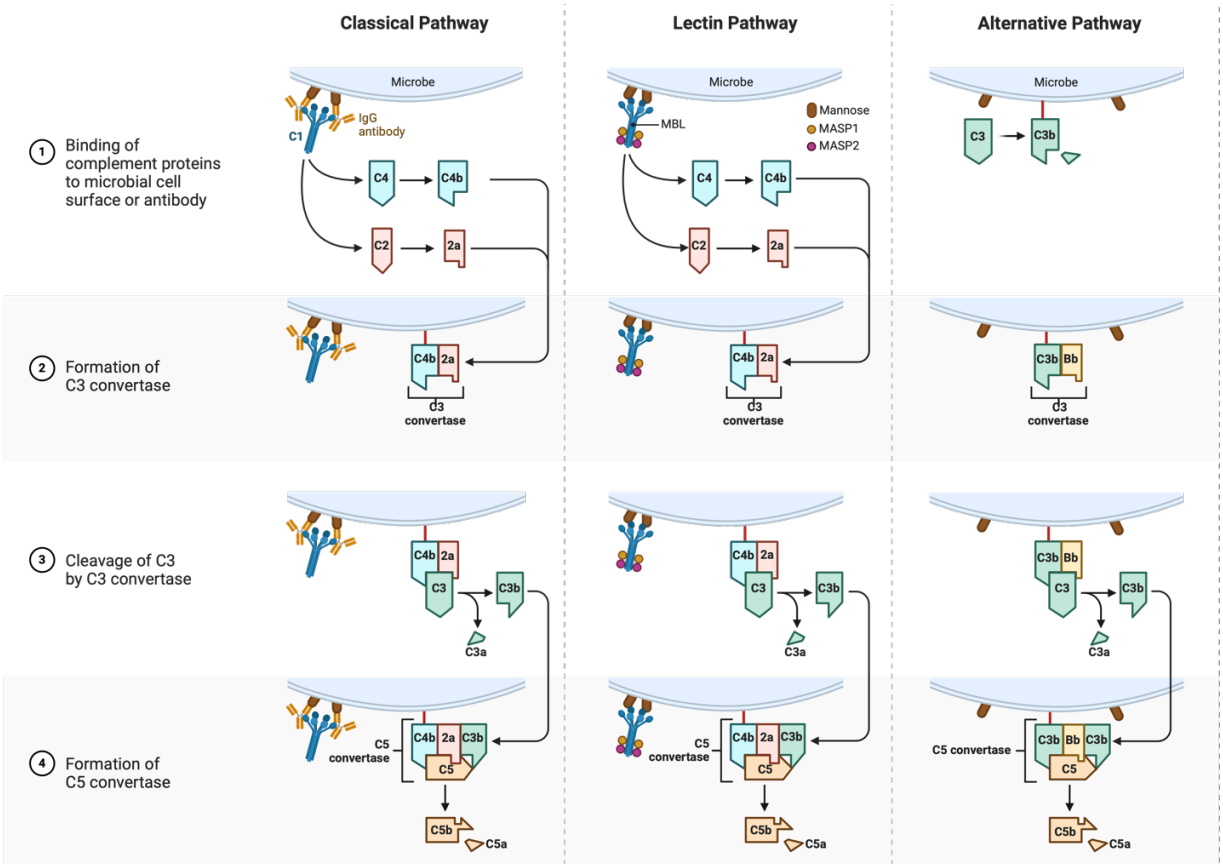

Figure 4 Complement activation pathways
(Adapted from "Three Pathways of Complement Activation", by BioRender.com (2023))

The basis for the initiation of classical pathway is the formation of immune complexes between cognate antigen and antibodies of the IgG or IgM class, which causes a conformational change in the Ig multimers. This renders binding sites accessible to the first complement complex, consisting of C1q, C1r, and C1s, which in turn activates its catalytic ability to cleave the complement proteins C2 and C4 into two fragments. The larger fragments of this reaction, C2a and C4b, can then form the C4bC2a complex, which represents the C3 convertase of the classical pathway. On the other hand, the lectin pathway relies on the recognition of pathogen-associated molecular patterns (PAMPs), such as specific carbohydrates, by certain pattern-recognition receptors (PRRs), most commonly mannose-binding lectin (MBL) and ficolins, to set off the complement cascade. Analogous to the C1 complex in the classical pathways, these PRRs are complexed with catalytic proteins such as the MBL-associated serine proteases 1 to 3 (MASP-1, -2, and -3) which similarly allow for the cleavage of C2 and C4 and the formation of the C4bC2a complex. Lastly, the alternative pathway occurs spontaneously after hydrolysis of C3, allowing to bind and subsequently cleave factor B into two fragments. The Bb fragment then binds to the hydrolysed C3 and can act as a C3 convertase and generate C3b fragments which in turn can bind Bb fragments, leading to the main C3 convertase of the alternative pathway, C3bHb. Regardless of the activation pathway, further produced C3b can ultimately associate with the C3 convertases to form the C5 convertases C4bC2aC3b or C3bHbC3b and facilitate downstream proteolytic processes (Punt *et al.*, 2019a).

Conversely, CR2, sometimes called CD21, is considered to be the main positive regulator of B cell immunity among complement receptors. Owing to structural differences, it has more restricted binding capabilities for complement proteins compared to CR1, and primarily binds C3-derived fragments, such as C3bi, C3d, and C3dg (Dunkelberger & Song, 2010). Strikingly, CR2 has also

been shown to interact with CD23, which is the Fc epsilon receptor 2 expressed by B cells, and this ligation has been shown to augment IL-4-dependent IgE production (Aubry *et al.*, 1992). This complement receptor is also central to Epstein-Barr virus (EBV) infection, where it acts as an entry receptor by interacting with the EBV envelope protein gp350 (Tanner *et al.*, 1987). CR2 is most commonly known for its participation in the CD21/CD19/CD81 complex which acts as a co-receptor and secondary signal to the BCR and reduces the activation threshold of B cells in response to opsonised antigens (Dempsey *et al.*, 1996). Furthermore, signalling through this complex was shown to enhance antigen processing and presentation by B cells (Cherukuri *et al.*, 2001). Importantly, CR2 has been described vital for the capture of opsonised antigen by both FDCs and B cells, which can be passed between the two cell types (Cinamon *et al.*, 2008; Suzuki *et al.*, 2009). This is key for the delivery of antigen to the follicles of the spleen during follicular shuttling (Cinamon *et al.*, 2008). In addition to the main CRs, B cells also express receptors for anaphylatoxins such as C3aR, which binds C3a and C3a(desArg) and causes a decrease in the production of IL-6 and TNF-α, as well as a suppression of the polyclonal B cell response (Fischer & Hugli, 1997). On the other hand, anaphylatoxin C5a, which is recognised by complement 5a receptors 1 and 2 (C5aR1 and 2), has been shown to act as a chemoattractant for some B cell subsets such as GC B cells (Kupp *et al.*, 1991).

3.2. Innate immune signalling in B cells

Reaching beyond processes acting at the interface of innate and adaptive immunity, such as the complement system, the boundaries between these two categories of immune cells long thought to be completely separate have become increasingly weaker in recent years. An increasing body of evidence has now demonstrated that B cells, as well as T cells, are able to respond to common danger signals such as pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) by triggering signalling pathways associated with innate immune sensing. These sensing pathways can be triggered by the recognition of common molecular danger patterns at the cell surface, as is the case for most TLRs, or inside the cell by endosomal TLRs or cytosolic sensors, most notably retinoic-inducible gene-I (RIG-I) and cyclic GMP-AMP synthase (cGAS) (Stögerer & Stäger, 2020). Once triggered, signals are then relayed through a series of adapter molecules and signalling complexes, resulting in the activation of various transcription factors, such as interferon regulatory factor 3 and 7 (IRF3 and IRF7) and nuclear factor κB (NF-κB) (**Figure 5**).

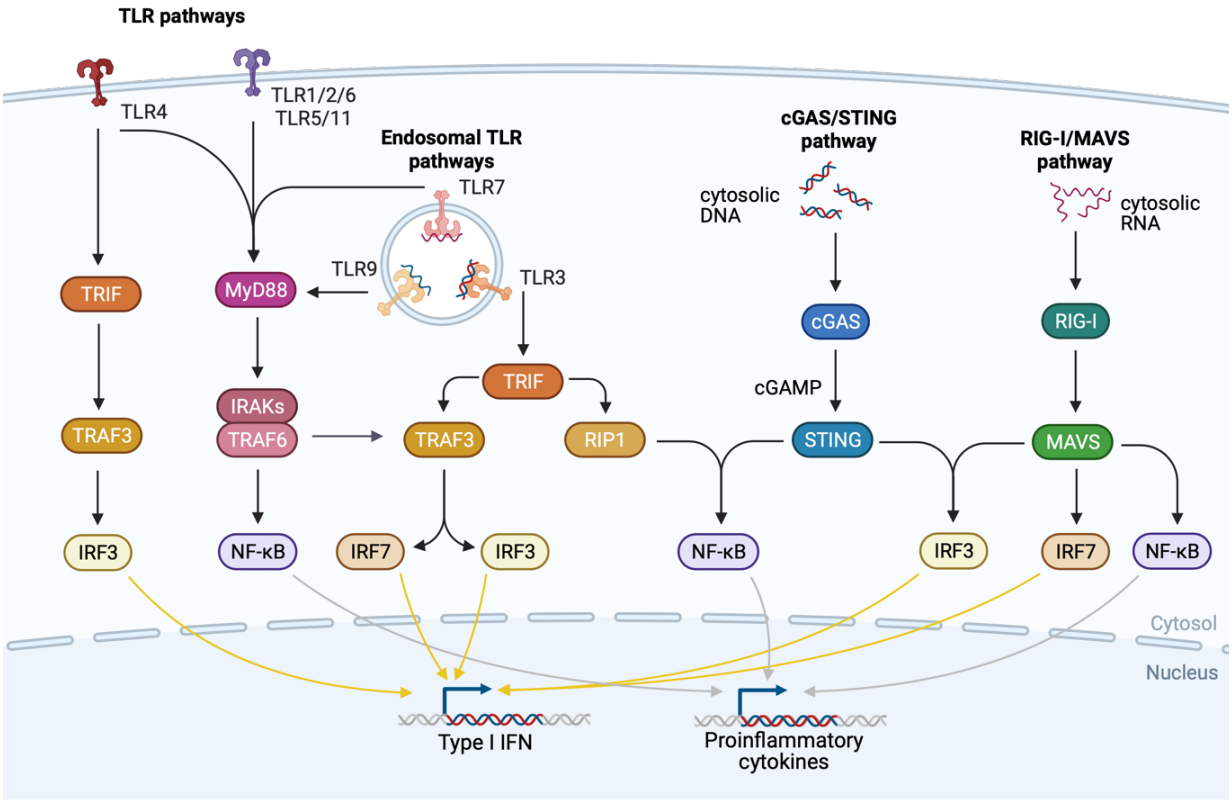

Figure 5 Innate Immune Signalling Pathways
(Created with Biorender.com)

Most TLRs located on the cell surface, such as TLR1, 2, 5, 6 and 11, signal using myeloid differentiation primary response protein 88 (MyD88) as an adaptor protein, which subsequently recruits its downstream signalling partners, the proteins of the IL-1 receptor–associated kinase (IRAK) family to activate TNF receptor-associated factor 6 (TRAF6). This in turn activates its downstream signaling partners and results in activation of the transcription factor NF-κB, which translocates to the nucleus and initiates the production of pro-inflammatory cytokines. While TLR4 can signal via MyD88, it instead engages the adaptor TIR-domain-containing adaptor-inducing beta interferon (TRIF) when internalized, which in term associates with TRAF6 or TRAF3, the latter of which can phosphorylate IRF3, causing nuclear translocation and release of type I IFN. Similarly, the endosomal TLR 3 signals via TRIF to either activate TRAF3, resulting in the activation of both IRF3 and IRF7 the production of IFN-I, or receptor interacting protein 1 (RIP1), which, together with TRAF6, can activate NF-κB. Other endosomal TLRs, such as TLR7, 8 and 9, signal through MyD88, leading to assembly of the IRAK-TRAF6 complex, which can additionally engage TRAF3, leading to activation of IRF3. In the cytoplasm, cytosolic DNA can interact with sensor proteins, most importantly cyclic GMP-AMP synthase (cGAS) which leads to the production of cyclic dinucleotides (CDNs), i.e. 2′ 3′-cyclic guanosine monophosphate-adenosine monophosphate (cGAMP). These CDNs may then act as secondary messenger molecules which activate stimulator of interferon genes (STING) which is located on the endoplasmic reticulum surface. STING may interact with TANK-binding kinase 1 (TBK1) to phosphorylate IRF3 and induce IFN-I production or associate with IκB kinase (IKK), leading to NF-κB activation. Cytosolic RNA is recognized by proteins such as retinoic acid-inducible gene I (RIG-I), causing activation of mitochondrial antiviral signaling protein (MAVS) which can signal through TRAF3, ultimately leading to IRF3 and IRF7 phosphorylation, and NF-κB activation via RIP1.

3.2.1. TLR signalling in B cells

Most studies on innate immune signalling pathways in B cells refer to TLR sensing, implicating these pathways in important homeostatic and activation processes. TLR ligation, in conjunction with cognate T helper stimulation, is considered an important second signal to BCR activation as it promotes B cell proliferation and plasma cell differentiation (Ruprecht & Lanzavecchia, 2006a); however, TLR signalling, in particular through TLR9, alone has been shown to be sufficient to induce not only proliferation but also antigen presentation in B cells (Jiang *et al.*, 2007). Similarly, the activation of TLR4 signalling using its agonist LPS was shown to promote B cell maturation (Hayashi *et al.*, 2005a) and induce isotype switching (Pone *et al.*, 2012), while TLR7 ligation was shown to initiate the formation of spontaneous GCs (Soni *et al.*, 2014).

On the other hand, TLR activation in B cells has also been linked to the development of autoimmunity. Indeed, both TLR7 and TLR9 have been implicated in the activation of autoreactive B cells in response to self-antigens, leading to the production of autoantibodies (Leadbetter *et al.*, 2002b; Lau *et al.*, 2005). Additionally, a B cell-subset associated with active disease in SLE was found to be particularly responsive to TLR7 stimulation and ligation of this TLR lead to increased autoantibody secretion and plasma cell differentiation within this subset (Jenks *et al.*, 2018).

In the context of infectious diseases, TLR signalling in B cells has been attributed important roles in the immune response to various bacteria, viruses, and parasites. Indeed, MyD88-dependent TLR signalling in B cells and its resulting cytokine production has been shown to be vital for the development of an efficient Th1 response against *Salmonella enterica* (Barr *et al.*, 2010). Activation of TLR2 on B cells by *Helicobacter*-derived ligands was also demonstrated to aid in controlling infection by inducing T regulatory-1-type (Tr1) cells which limits the associated pathology (Sayi *et al.*, 2011). During influenza virus infection, signalling via TLR7 and MyD88 was required to mediate B cell isotype switching and orchestrate an effective humoral response (Heer *et al.*, 2007). Similarly, B cell-intrinsic TLR7 triggering was vital for plasma cell differentiation and antibody production in the chronic stages of infection with LCMV (Clingan & Matloubian, 2013). More recently, TLR2 signalling via MyD88 and the resulting mTOR activation have also been identified as crucial mechanisms for B cell activation and metabolism adaptation in response to HBV, aiding in the resolution of the infection (Li *et al.*, 2021).

In parasitic diseases, literature examining the role of B cell-intrinsic TLR signalling is sparse; however, existing studies point towards an important regulatory role. Incubation of B cells with excretory/secretory products of *Echinococcus granulosus* protoscoleces was found to induce TLR2-dependent differentiation into IL-10-producing B cells with a regulatory phenotype (B_{regs}), potentially participating in the evasion of the host immune system (Pan *et al.*, 2018). Similarly, exposure of B cells to *L. donovani* amastigotes has been shown to trigger the upregulation of endosomal TLRs (TLR3, 7, and 9) and the production of pro-inflammatory cytokines, IL-10, and IFN-I, the latter of which causes excessive antibody production and results in hypergammaglobulinemia (Silva-Barrios *et al.*, 2016).

3.2.2. cGAS/STING signalling in B cells

In B cells, cytosolic DNA signalling via the adaptor protein STING has been proposed to have several regulatory effects. Direct activation of STING using CDNs has been shown to result in B cell activation, as evidenced by increased cytokine production and expression of co-stimulatory molecules (Walker *et al.*, 2018). However, a more recent study found that a B cell-targeted ablation of STING results in stronger BCR signalling, enhanced plasma cell differentiation, and increased antibody production in response to immunisation (Tang *et al.*, 2021).

While cGAS or STING expression on B cells is not required for their participation in autoreactive GCs, STING activation may contribute to autoimmunity (Green *et al.*, 2021). Excessive signalling through STING due to an accumulation of cytosolic DNA debris caused by a defect in the endonuclease DNase II was found to lead to inflammatory arthritis in mice (Pawaria *et al.*, 2015). STING expression has also been found to be significantly lower in B cells from lupus-prone mice (Dong *et al.*, 2015). Conversely, in a collagen-induced arthritis model, STING-deficient mice showed increased anti-collagen antibody titres and improved survival of B cells activated by BCR stimulation (Tansakul *et al.*, 2020). Hence, the contribution of B cell-intrinsic STING and cytosolic DNA sensing to the development of autoimmunity remains to be clarified.

A B cell-intrinsic role of STING has also been identified in the pathology of *Mycobacterium tuberculosis* infection. Indeed, pleural and splenic B cells of infected mice showed increased expression of IFN-β and, to a lesser degree, IL-6 and IL-10, which was mirrored in the pleural fluid of patients diagnosed with tuberculosis. This B cell-derived IFN-I was found to induce

macrophage polarisation towards an anti-inflammatory M2 phenotype associated with increased bacterial load in tuberculosis (Benard *et al.*, 2018).

3.2.3. RIG-I/MAVS signalling in B cells

The effects of signalling through MAVS induced via upstream RNA sensors remain largely unknown in B cells. However, promising results have been obtained using synthetic RNA as an adjuvant for influenza vaccination, showing increased antibody specificity and robust antibody production (Kulkarni *et al.*, 2014). Additionally, RIG-I and MAVS-mediated sensing of RNA was found to induce the production of cytokines such as IFN-I and IL-6 (Loetsch *et al.*, 2017) and may also regulate the expression of TLR7 and CD23 in B cells (Xu *et al.*, 2012). Another function of MAVS, along with TLR7, is the formation of spontaneous germinal centres. Indeed, in lupus-prone mice, MAVS has been shown to be vital for the formation of autoreactive GCs and the resulting production of self-reactive antibodies and autoimmune pathologies (Sun *et al.*, 2019).

Lastly, BCR signalling appears to be able to activate MAVS irrespective of IFN-I and this pathway of MAVS activation was shown to be important for normal mitochondrial appearance and metabolic function in B cells (Wang *et al.*, 2023).

3.2.4. IFN-I signalling in B cells

A common result of innate immune signalling through PRRs is the production of IFN-I. The category of type I interferons (IFNs) comprises its two most important members, IFNα, of which there are 14 isoforms, and IFNβ, as well as a number of less-described cytokines, such as IFNδ, IFNε, IFNκ, INFτ, and INFω (Pestka *et al.*, 2004). Both IFNα and IFNβ are recognised by the type I IFN receptor (IFNAR) which consists of two subunits, IFNAR1 and IFNAR2, triggering several downstream signalling processes which typically result in the transcription of a great variety of genes called interferon-stimulated genes (ISGs) (**Figure 6**).

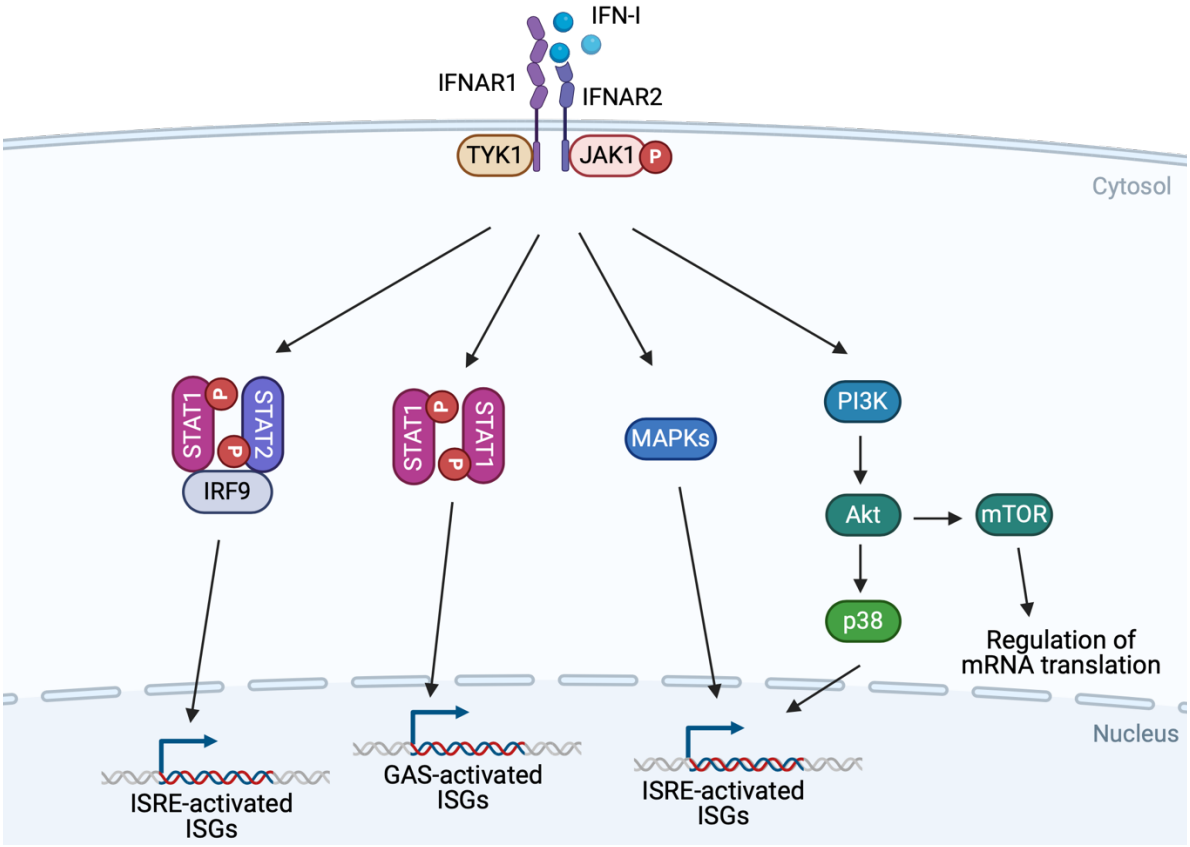

Figure 6 Downstream Signalling of IFN-I
(Created with Biorender.com)

Recognition of IFNα or IFNβ by IFNAR triggers the activation of Janus kinase 1 (JAK1) and non-receptor tyrosine kinase 2 (TYK2), which in turn phosphorylate signal transducer and activator of transcription 1 and 2 (STAT1 and STAT2). In the canonical signalling pathway, the phosphorylated STAT1 and STAT2 then form a heterodimer, allowing for interferon-regulatory factor 9 (IRF9) to bind. The thus formed STAT1-STAT2-IRF9 complex subsequently translocates to the nucleus to bind to IFN-stimulated response elements (ISRE) in the DNA, resulting in the transcription of ISGs. Alternatively, phosphorylated STAT1 can also form a homodimer, allowing it to bind to a DNA region termed gamma-activated sequence (GAS), in term promoting ISG expression. IFN-I may also trigger STAT-independent signalling pathways resulting in ISG transcription, including via mitogen activated protein kinases (MAPKs). Another downstream signalling process activated by IFNAR ligation is phosphatidylinositol 3-kinase (PI3K), which can either promote ISG transcription via p38 or modulate mRNA translation via the mammalian target of rapamycin (mTOR) pathway (Chen *et al.*, 2017).

This interferon-induced signalling plays an important role in the orchestration of various immune functions. Indeed, IFN-I has been shown to improve the survival of mature (Braun *et al.*, 2002) and T1 B cells (Hamilton *et al.*, 2017). Induction of IFN-I signalling in B cells also results in increased responsiveness to BCR stimulation by lowering the threshold for B cell activation (Braun *et al.*, 2002). Additionally, treatment of marginal zone precursor B cells with IFN-I was

demonstrated to improve their capacity for T cell co-stimulation by increasing their expression of CD86 (Wang *et al.*, 2011a). TLR signalling-derived IFN-I also modulates the humoral response to thymus-independent antigens by regulating class switching to an IgG2c isotype and improving the magnitude of antibodies produced (Swanson *et al.*, 2010). Interestingly, IFN-I production is not only a consequence of innate immune sensing through TLRs and cytosolic sensors, but also plays a role in regulating the expression of TLRs such as TLR7, resulting in a positive feedback loop of IFN-I signalling (Green *et al.*, 2009).

This positive feedback loop may have detrimental consequences in some diseases. In an experimental *L. donovani* model, amastigotes were shown to induce an IFNAR-dependent upregulation of endosomal TLRs on B cells, resulting in excessive antibody production and the production of various cytokines, including IFN-I, which in turn further drives this detrimental B cell activation (Silva-Barrios *et al.*, 2016). IFN-I is also considered a key cytokine in the antiviral response and consequently, multiple important B cell-intrinsic roles of its signalling pathways have been identified. In the early stages of influenza infection, signalling of IFN-I through IFNAR has been implicated in B cell activation, as evidenced by the upregulation of activation markers CD69 and CD86 and ISGs, resulting in an improved virus-specific antibody response (Coro *et al.*, 2006). Similarly, TLR7-dependent induction of IFN-I by vesicular stomatitis virus (VSV) was shown to be important for plasma cell differentiation and optimal antibody production (Fink *et al.*, 2006). IFN-I signalling has additionally been shown to mediate isotype switching of B cells in response to influenza (Heer *et al.*, 2007), VSV virus antigen (Bach *et al.*, 2007) and LCMV (Daugan *et al.*, 2016). However, in an LCMV infection model, B cell-intrinsic IFN-I signalling was found to impair the specific humoral response to T cell-dependent antigens, indicating a dimetric role of IFN-I in viral infections (Daugan *et al.*, 2016).

PART III: TUNNELLING NANOTUBES (TNTS)

Since the discovery of cells as building blocks of life, it has become apparent that communication routes among these individual units are necessary to coordinate processes within living organisms. It is estimated that the average human body consists of approximately 30 trillion cells and 30 trillion bacteria which are required to coordinate on a small and large scale to facilitate complex and vital processes (Sender *et al.*, 2016). It is thus unsurprising that cells have evolved a myriad of ways to exchange information and material among each other, and substantial advances in our understanding of these processes are still being made today.

According to our traditional understanding of cell-to-cell interactions, intercellular communication can occur either at a close range, by direct interactions at the cell surface, or at an intermediate range via the secretion of soluble markers, including cytokines, chemokines, and extracellular vesicles. Since their discovery in 2004 (Rustom *et al.*, 2004), tunnelling nanotubes (TNTs), also called tunnelling nanotubules or membrane nanotubes, have greatly contributed to our understanding of long-range intercellular communication over the last 20 years. Characterised by the formation of long, tubular actin-based protrusions from the cell membrane, this mode of communication has the potential to bridge the gap between traditional communication pathways and provide a more comprehensive picture of the complexities of cellular interactions. Unsurprisingly, this phenomenon has since attracted the attention of numerous researchers across a wide variety of specialisations, resulting in an ever-growing body of evidence on the context in which these structures are formed *in vivo* and *in vitro*, as well as their roles in homeostasis and pathology. Hence, the remainder of this sub-chapter provides an overview of the advances made in this topic thus far.

1. General characteristics of TNTs

1.1. Cell diversity in TNT formation *in vitro* and *in vivo*

Originally identified in the rat pheochromocytoma cell line PC12 (Rustom *et al.*, 2004), a great variety of cells has been shown capable to participate in intercellular signalling via TNTs to date, including stem cells (Sanchez *et al.*, 2017; Zhang *et al.*, 2018), macrophages (Önfelt *et al.*, 2004; Önfelt *et al.*, 2006; Hase *et al.*, 2009; Rehberg *et al.*, 2016), monocytes (Shahar *et al.*, 2021), NK

cells (Önfelt *et al.*, 2004), T cells (Sowinski *et al.*, 2008; Obermajer *et al.*, 2009; Arkwright *et al.*, 2010; Saha *et al.*, 2022) and B cells (Önfelt *et al.*, 2004; Rainy *et al.*, 2013a; Osteikoetxea-Molnar *et al.*, 2016; Toth *et al.*, 2017; Halasz *et al.*, 2018). In addition to the most frequently studied homotypic formation of TNTs between two or more cells of the same type, these protrusions have also been shown to connect different cell types, such as NK and B cells (Önfelt *et al.*, 2004) and B and T cells (Rainy *et al.*, 2013a). Immune cells have also been shown to be able to connect to several different neurons (Gousset *et al.*, 2009) and cancer cells (Saha *et al.*, 2022) via TNTs.

While most studies on TNTs to date have employed *in vitro* techniques to overcome imaging limitations such as imaging depth, there is ample evidence of the existence of these structures *in vivo* as well. In mice, functional nanotube connections have been reported to form between different cell types in the blastocyst (Salas-Vidal & Lomeli, 2004), bridging the gap between neural folds during neural tube formation in embryonal development (Pyrgaki *et al.*, 2010), dendritic cells in the corneas of adult mice (Chinnery *et al.*), and between perivascular and tissue-resident macrophages in intact cremaster muscle tissue (Rehberg *et al.*, 2016). Similar structures have also been observed in epiblast cells in developing zebrafish embryos (Caneparo *et al.*, 2011). In chicks, TNTs are known to form between cranial neural crest cells (Teddy & Kulesa, 2004) and during vertebrate tissue patterning in the limb bud (Sanders *et al.*, 2013). TNT formation also occurs in humans. In the pleural fluid of a patient with follicular B cell lymphoma, cargo-carrying tubular connections between atypical lymphocyte were observed (Scarabelli *et al.*, 2021), and in tumor specimens resected from patients with lung adenocarcinoma or malignant pleural mesothelioma, TNTs were found in all the samples studied (Lou *et al.*, 2012).

Concisely, a great number of cell types, including stem cells, immune cells, neurons, and cancer cells, can form homotypic or heterotypic tunnelling nanotubes both *in vivo* and *in vitro* to facilitate intercellular communication.

1.2. Composition of TNTs

While there is some variation in the criteria used to define membrane nanotubes, there is a clear consensus throughout the literature that these structures represent actin-based, long projections reaching out from the cell membrane that can connect two or more cells (Rustom *et al.*, 2004; Önfelt *et al.*, 2006; Sowinski *et al.*, 2008; Gousset *et al.*, 2009; Hase *et al.*, 2009; Obermajer *et*

al., 2009; Arkwright *et al.*, 2010; He *et al.*, 2010; Lokar *et al.*, 2010; Lou *et al.*, 2012; Wang & Gerdes, 2015; Kumar *et al.*, 2017; Patheja & Sahu, 2017; Sanchez *et al.*, 2017; Zhang *et al.*, 2018; Omsland *et al.*, 2020; Shahar *et al.*, 2021; Zheng *et al.*, 2021; Kato *et al.*, 2022; Lee *et al.*, 2022; Saha *et al.*, 2022). A second important distinction between TNTs and other forms of cell protrusions, such as filopodia, is the fact that nanotubes are not anchored to the substrate they are cultured on (Rustom *et al.*, 2004), but rather hover over suspended in media, setting them apart from cell projections formed for the purpose of cell migration (Sowinski *et al.*, 2008).

Interestingly, although not initially reported in the first reports of TNTs (Rustom *et al.*, 2004) and sometimes used as a criterion for defining cellular connections as TNTs (Omsland *et al.*, 2020), the presence of tubulin in these projections is now often used to further classify them into one of two types: type I protrusions, sometimes called thin TNTs, and type II protrusions, often termed thick TNTs. As the name suggests, type I TNTs typically have thinner diameters of under 0.6 nm and made of F-actin bundles in the absence of tubulin (Önfelt *et al.*, 2006; Sanchez *et al.*, 2017), while the thicker type II TNTs typically exhibit diameters over 0.6 nm and typically comprise additional structural components such as tubulin (Lokar *et al.*, 2010; Sanchez *et al.*, 2017) or cytokeratins (Lokar *et al.*, 2010). In addition to the increased thickness, type II protrusions have also been reported to form longer connections and be more stable, remaining intact for multiple hours as opposed to their actin-only counterparts, most of which appear and disappear within a span of minutes and rarely last for more than one hour (Obermajer *et al.*, 2009; Lokar *et al.*, 2010). While many reports do not further classify TNTs into two subtypes, the majority of the current literature reports the formation of protrusions that fit the description of either type I or type II TNTs; however, some studies provide evidence that the same set of cells is capable of producing both subtypes of TNTs simultaneously (Önfelt *et al.*, 2006; Sanchez *et al.*, 2017).

In those reports, thinner type I connections predominated, making up between 60 and 70 % of all TNTs formed and this balance appears to be dependent on the cell environment and culture conditions; (Önfelt *et al.*, 2006; Sanchez *et al.*, 2017). Indeed, the formation of TNTs between cells of the pancreatic cancer cell line PANC-1 was shown to be skewed from the exclusive formation of type I TNTs towards the formation of type II TNTs by the addition of macrophage-conditioned medium (Lee *et al.*, 2022). Additionally, connections formed with apoptotic or injured PC12 cells conform to the type II criteria (Wang *et al.*, 2016) which have been reported to exclusively form type I TNTs under normal culture conditions (Rustom *et al.*, 2004; Wang *et al.*, 2016). Thus, the formation of different nanotube subtypes appears to have a dynamic balance and is not simply defined by the cell types involved.

The actin cytoskeleton thus forms the basic scaffold of TNTs and is encased by the plasma membrane of the cells. The nanotube plasma membrane was found to be positive for some typical constituents of lipid rafts, such as cholesterol, glycosphingolipids and sphingomyelin (Toth *et al.*, 2017). However, other raft markers, caveolin-1, flotilin-1, and the ganglioside GM1 are not found on the membrane of TNTs (Lokar *et al.*, 2012). In fact, the lipid composition of the cell membrane may be important for TNT formation and stability. Lipids with an inverted conical shape induce high degrees of curvature of the membrane, while small lipid cholesterol can fill in gaps in highly curved parts of the membrane or organise into cholesterol-phospholipid nanodomains, thus stabilising the structures (Lokar *et al.*, 2012; Toth *et al.*, 2017). Membrane fluidity also plays an important role in the formation of connecting nanotubes, as limiting fluidity by crosslinking lipid rafts or depletion of cholesterol from the membrane suppresses the formation of TNTs (Lokar *et al.*, 2012; Toth *et al.*, 2017).

1.3. Appearance and properties of TNTs

In addition to the differences in thickness between the types of TNTs, considerable variation has been reported in the shape and appearance of the protrusions formed, both between different cell types and within TNTs formed within the same set of cells. In general, most TNTs are straight connections between cells, the lengths of which typically fall below 100 µm (He *et al.*, 2010; Saha *et al.*, 2022), although significantly longer connections have been reported in some cases (Önfelt *et al.*, 2004; Rehberg *et al.*, 2016). However, in some cases, and in particular between cells grown in a three-dimensional culture using a substrate mimicking the extracellular matrix, these connections can also take on a curved shape (Sowinski *et al.*, 2008; Kumar *et al.*, 2017). Additionally, the majority of TNTs formed by various cell types have been reported to consist of a straight connection (Önfelt *et al.*, 2006; Kalargyrou *et al.*, 2021), with simultaneous connections of more than two cells, with a single, branched nanotube being a rarer phenomenon (Rustom *et al.*, 2004; Önfelt *et al.*, 2006; Sowinski *et al.*, 2008). However, in some cases, multiple nanotubes may emanate from one cell (Önfelt *et al.*, 2006; Sowinski *et al.*, 2008; Obermajer *et al.*, 2009; He *et al.*, 2010; Kumar *et al.*, 2017; Saha *et al.*, 2022), which can lead to the simultaneous connection of multiple cells, resulting in the formation of a complex network of interconnected cells (Önfelt *et al.*, 2004; Obermajer *et al.*, 2009; Sanchez *et al.*, 2017; Saha *et al.*, 2022), or the serial connection of multiple cells with a single nanotube (Saha *et al.*, 2022).

The formed nanotubes are capable of some degree of movement and can be easily ruptured by mechanical stress and chemical treatment (Lou *et al.*, 2012). Indeed, breakage of TNTs can be induced by vigorous shaking (Sowinski *et al.*, 2008), exposure to strong laser beams (Kumar *et al.*, 2017), or harsh chemical fixation (Rustom *et al.*, 2004; Watkins & Salter, 2005). However, these connections can withstand mild paraformaldehyde fixation at concentrations at or below 2 % paraformaldehyde (PFA) (Jahnke *et al.*, 2022) and are resistant to trypsinisation for more than 60 min (Rustom *et al.*, 2004; Lou *et al.*, 2012; Kato *et al.*, 2022).

In summary, TNTs are fragile and transient connections formed between cells, vary greatly in their appearance, and can take the form of simple, straight connections between a pair of cells but also give rise to a more complex network of communication highways.

2. Formation of TNTs

Nanotubes have been shown to form between different cells in culture, both in the presence and absence of stimuli, via two different mechanisms: cell dislodgement or “de novo” formation, which also encompasses the formation via filopodial interplay (**Figure 7**). The cell dislodgement mechanism is characterised by the formation of intercellular bridges, which can occur when two cells experience prolonged contact for a minimum of 4 min before moving apart (Önfelt *et al.*, 2006; Sowinski *et al.*, 2008; Jahnke *et al.*, 2022). Similarly, connections are sometimes formed during the cell division process as the newly separated cells move apart (Sowinski *et al.*, 2008). Connections formed through this mechanism often fit type I TNT characteristics, containing only actin filaments, and have been reported to be shorter, more numerous, and more dynamic than TNTs formed through other mechanisms (Lokar *et al.*, 2010; Sanchez *et al.*, 2017).

The second mechanism of TNT formation, called filopodial interplay or “de novo” formation, occurs through the outwards extension of membrane protrusions, which gradually elongate and connect to the membrane of a distant cell (Rustom *et al.*, 2004; Hase *et al.*, 2009; Zhang *et al.*, 2021). To establish a cytosolic connection, the formed protrusions have been described to glide over the surface of the cell membrane, where they can form a transient attachment involving a multipoint cadherin system, enabling the formation of a communication junction to the cytosol of the cell (Lokar *et al.*, 2010). Similar to formation through cell dislodgment, “de novo” TNT formation

can occur within a few minutes (Rustom *et al.*, 2004). Notably, the same cells can utilise both mechanisms of TNT formation, although they might show a clear tendency towards one method (Bukoreshtliev *et al.*, 2009).

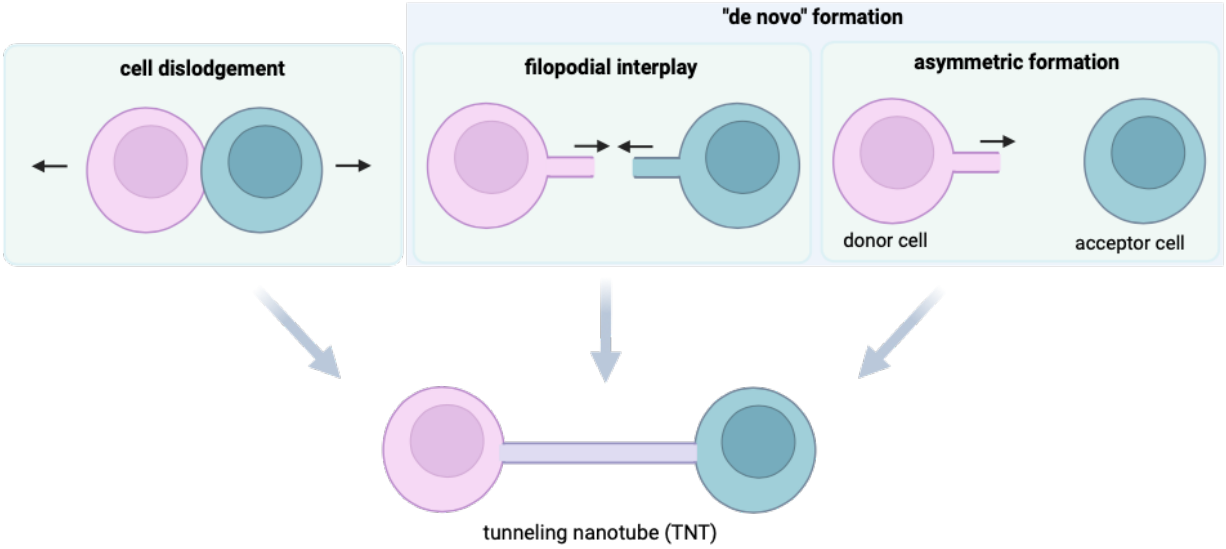

Figure 7 Mechanisms of TNT formation
(Created with Biorender.com)

TNTs may be formed either as a consequence of dislodgement of two cells who have undergone prolonged, resulting in a membrane connection being drawn between the cells, or by “de novo” formation. In the latter mechanism, TNTs may either be formed by asymmetric extension of a protrusion by one cell, which can connect with and fuse to a distant cell or result from the connection of filopodia protruding from two different cells.

While the formation of a single TNT can be quite rapid, the overall dynamics of the formation of these connections during culture appear to vary between cell types. In human mesothelioma cell line cultures, TNT formation has been reported as early as 15 min, and connections between mouse bone marrow-derived dendritic cells (BMDCs) and catecholaminergic neuronal tumour (CAD) cells appear within 30 min of co-culture (Gousset *et al.*, 2009). Interestingly, TNT formation in some cell types, including B cells and PC12 cells, peaks in the early stages of culture, 1 to 2 h after plating (Rustom *et al.*, 2004; Osteikoetxea-Molnar *et al.*, 2016), while other cell types displayed a sustained formation of these intercellular connections, such as primary pancreatic tumour-derived cells which were observed to form TNTs at 24, 48, 72, and 96 h of culture (Desir *et al.*, 2018).

Notably, the nanotubes formed were found to be very transient in some cases, with connections between the THP-1 monocyte cell line often being formed and disappearing over a period of

several minutes (Watkins & Salter, 2005), and the majority of protrusions formed between PC12 cells disappearing within one hour of formation (Bukoreshtliev *et al.*, 2009). On the other hand, protrusions formed between human embryonic kidney (HEK) 293 cells proved to be more stable, with an average lifetime of 1–5 h and remaining intact even when one of the connected cells died (Jahnke *et al.*, 2022).

2.1. Factors inducing TNT formation

2.1.1. Induction of TNTs by cellular stress

Similar to their great diversity in cell types and appearance, a large variety of factors have been reported to stimulate the formation of TNTs to date. Interestingly, stress during culture has emerged as a strong inducer of the formation of connections. Indeed, the induction of stress by culturing cells in the presence of 100 mM hydrogen peroxide (Wang *et al.*, 2011b), as well as the use of a low-serum and/or hyperglycaemic culture medium, promotes the formation of nanotubes between cells in *in vitro* studies (Wang *et al.*, 2011b; Lou *et al.*, 2012). Additionally, subjecting human umbilical vein endothelial cells (HUVEC) to ischemia/reperfusion injury not only significantly increased the formation of TNTs with uninjured mesenchymal stem cells (MSCs) but also resulted in the development of complex nanotube networks (Liu *et al.*, 2014b). Hypoxia has also been found to promote TNT formation in ovarian cancer cells (Desir *et al.*, 2016). However, more severe cellular damage by UV treatment of cells and induction of apoptosis may lead to decreased TNT formation as cell death outpaces stress-induced connection formation (Wang & Gerdes, 2015).

In addition to cellular injuries and stress caused by culture conditions, induction of cytotoxicity using chemotherapeutic agents, such as 5-fluorouracil, docetaxel, and doxorubicin, has also been shown to dramatically induce TNT formation in cancer cells (Desir *et al.*, 2018; Kato *et al.*, 2022) and since these drugs act through very different cytotoxic mechanisms, this TNT induction may be due to a more unspecific cellular stress reaction (Kato *et al.*, 2022).

2.1.2. Induction of TNTs by pathogens

Contact of cells with certain pathogens may also be a form of stress capable of inducing TNT formation, as is the case during infection with the murine Influenza A virus strain PR8 (Kumar *et al.*, 2017). The protein p8 of human T-cell leukaemia virus type 1 (HTLV-1) similarly induces nanotubes between infected cells and previously uninfected T cells and monocytes (Omsland *et al.*, 2018). Prions can also promote communication through nanotubes, as the scrapie isoform of the major prion protein (PrP^{Sc}) can induce TNT formation between CAD cells (Zhu *et al.*, 2015). Additionally, *Mycoplasma hyorhinis* infection results in a significant formation of connections among fibroblasts (Omsland *et al.*, 2020), and the common bacterial antigen and TLR4 ligand lipopolysaccharide (LPS) have been shown to lead to a dose-dependent appearance of cell-cell connections in monocytes or B cells (Osteikoetxea-Molnar *et al.*, 2016; Shahar *et al.*, 2021). Ligation of other TLRs, namely TLR7/8 and TLR2, has been reported to induce the formation of TNTs between cells, providing ample pathways via which pathogens can amplify this type of intercellular communication (Shahar *et al.*, 2021).

2.1.3. Induction of TNTs by crosstalk with the environment

TNT induction may be stimulated by other cells in the culture environment, either by direct interactions or by signalling through factors secreted by the cells. Indeed, engagement of the cell death receptors Fas and TRAIL increased the development of connecting nanotubes between T cells (Arkwright *et al.*, 2010). Additionally, the culture of MCF-7 breast cancer cells in macrophage-conditioned medium greatly increases the number of connecting protrusions (Patheja & Sahu, 2017). Although this study failed to pinpoint the mechanism underlying the observed increase, this could be due to signalling via soluble factors, such as cytokines. In fact, the treatment of chronic myeloid leukaemia cells with IFN-α has been demonstrated to significantly induce cell-cell connections, and a similar induction by other cytokines remains to be explored (Omsland *et al.*, 2020). Similarly, interactions of cells with their immediate surroundings, that is, the extracellular matrix and coated cell culture plates, may promote the formation of intercellular connections. Indeed, integrin activation and culture of cells on fibronectin- or laminin-coated culture dishes have been shown to stimulate TNT formation (Obermajer *et al.*, 2009;

Osteikoetxea-Molnar *et al.*, 2016), while inhibition of integrin signalling using a blocking peptide decreased TNT communication (Polak *et al.*, 2015).

2.1.4. Experimental induction of TNTs

In order to facilitate the study of tunnelling nanotubes *in vitro*, nanotubes between cells may also be created artificially by means of optical tweezers to model connections either produced by “de novo” formation or cell dislodgement (Pascoal *et al.*, 2010; Rainy *et al.*, 2013a). To mimic “de novo” formed TNTs, a laser trap may be focused on the surface of a cell, pinning it in place, and a membrane tether is drawn from moving the cell adhered to a support on the microscope stage (Pascoal *et al.*, 2010). A thus-created membrane tube may then be connected to another cell by forcing direct contact for a minimum of 30 s, after which a stable connection can be established. Similarly, optical tweezers may be used to create TNTs by bringing together and subsequently pulling two cells apart, thereby mimicking connections created through the cell dislodgement mechanism (Rainy *et al.*, 2013a).

2.2. Signalling pathways underlying TNT formation

Given the large variety of cells, conditions, and appearance, the signalling pathways underlying TNT formation are still incompletely understood to date. As the main structural component, the rearrangement of the actin cytoskeleton is a vital part in the process of TNT formation.

At the forefront of actin dynamics, actin related protein 2 and 3 (Arp2/3) is a multi-subunit complex responsible for the assembly of new actin filaments branching off existing actin structures (Pollard, 2007). In most cases, selective inhibition of the Arp2/3 complex using the small inhibitor CK-666 inhibits TNT formation in macrophages (Hanna *et al.*, 2017) and in human trabecular meshwork cells (Keller *et al.*, 2017).

Arp2/3 activity is governed by a group of effector proteins, including Wiskott–Aldrich Syndrome protein (WASP) and WASP family verprolin-homologous 2 (WAVE2), which directly bind to the complex. Both WASP and WAVE2 were found to be equally important for TNT formation in

macrophages (Hanna *et al.*, 2017). WASP is modulated by the binding of the small Rho GTPase Cdc42, which is found at the base of nanotubes, and WAVE2 is similarly activated by Rac1 which is located all along the TNTs in macrophages. Both GTPases were found to participate in cytoskeletal rearrangement, leading to nanotube formation (Arkwright *et al.*, 2010; Hanna *et al.*, 2017). In order to be activated and associate with the membrane, GTPases need to be prenylated by geranylgeranyl transferase and the processes leading up to this activation for TNT formation remain to be discerned; however, one study on induced T_{regs} (iT_{regs}) found adenosine sensing via the adenosine A1 receptor to induce TNTs via Cdc42-neuronal WASP (n-WASP) association (Do *et al.*, 2021).

Remarkably, in neurons, inhibition of actin polymerisation by Arp2/3 conversely induces increased TNT formation while decreasing filopodia formation (Sartori-Rupp *et al.*, 2019). This may be due to a switch of actin polymerisation from Arp2/3-mediated branched actin polymerisation in favour of the formation of longer, TNT-like protrusions via the interplay of insulin receptor tyrosine kinase substrate protein of 53 kDa (IRSp53), which is an inverted Bin/amphiphysin/Rvs (I-BAR) protein carrying a binding domain for RhoGTPases Cdc42 and Rac and effector proteins n-WASP and WAVE2,and epidermal growth factor receptor kinase substrate 8 (Eps8), which helps bundle actin and promotes TNT formation (Henderson *et al.*, 2022). Hence, TNT growth may be differentially regulated in neurons and in other cell types.

As with the actin cytoskeleton, deformation of the plasma membrane is a prerequisite for TNT formation, and not only can a favourable membrane lipid composition stabilise nanotubes, as discussed in a previous section, but processes on the plasma membrane can actively participate in TNT outgrowth. M-Sec, or sometimes called tumour necrosis factor-α inducible protein 2 (TNFAIP2) and a homologue to Sec6 of the exocyst complex was the first discovered protein to actively mediate this process in myeloid lineage cells (Hase *et al.*, 2009). It interacts with GTPases RalA or Rac1 to mediate actin remodelling and induce TNT formation (Hase *et al.*, 2009; Hanna *et al.*, 2017). The endoplasmic reticulum-resident chaperone protein 29 (ERp29) was further identified as vital for TNT formation by stabilising the protein levels of M-sec in a number of human cancer cells, including the cervical cancer cell line HeLa, epithelial breast cancer cell line MDAMB231, and the osteosarcoma cell line U2OS (Pergu *et al.*, 2019).

However, M-Sec does not appear to be required for TNT formation between other cells, such as neurons, and lymphocytes which do not typically express this protein (Ohno *et al.*, 2010; Delage *et al.*, 2016). Although this mechanism cannot account for general TNT formation in these cell types, it may still play a role in TNT formation under certain conditions. Indeed, cancerous cells

in some forms of lymphoma, including primary mediastinal large B cell and Hodgkin lymphoma, were shown to aberrantly express M-Sec (Kondratiev *et al.*, 2011) and infection with human T-lymphotropic virus-1 (HTLV-1) could similarly induce T cells to express M-Sec (Ruckes *et al.*, 2001). However, while these studies demonstrated induction of M-sec in cells which typically do not express this protein, these reports did not explore whether this expression was linked TNT formation in these cells (Ruckes *et al.*, 2001; Kondratiev *et al.*, 2011).

2.3. Experimental inhibition of TNTs

As a single, global pathway of TNT formation across all cells and conditions remains to be discovered, current studies mostly rely on physical interventions or inhibitors for their main structural components, F-actin and tubulin, in order to block nanotube-mediated communication. To physically inhibit exchange via TNTs, the nanotubes are either destroyed by vigorous shaking or their formation is prevented using permeable membrane inserts to physically separate the cells (Sowinski *et al.*, 2008; Polak *et al.*, 2015). An overview of the most common methods for TNT inhibition is presented in **Table 3**.

Table 3 Most common methods TNT inhibition

	Method	Mode of action	Range of application (cell type dependent)
Physical inhibition	permeable membrane inserts (Transwell™)	physical separation of cells	-
	physical agitation	breaking of TNTs due to shear stress	-
Chemical inhibition	Cytochalasin B	actin polymerization inhibitor	1 – 20 µM
	Cytochalasin D	actin polymerization inhibitor	350 – 500 nM
	Latrunculin A	actin polymerization inhibitor	10 µM
	Latrunculin B	actin polymerization inhibitor	100 nM – 10 µM
	Colchicine	microtubule polymerisation inhibitor	3 – 10 µM
	Colcemide	microtubule polymerisation inhibitor	3 µM
	Nocodazole	microtubule polymerisation inhibitor	15 – 30 µM

Actin polymerisation inhibitors, such as members of the cytochalasin family, are among the most commonly used compounds for inhibiting TNT formation. These compounds belong to a group of small fungal metabolites that bind to the barbed ends of actin filaments, where they inhibit polymerisation (Cooper, 1987). In particular, cytochalasin B and D have been widely used for nanotube blockade at concentrations ranging from 175 to 700 nM for cytochalasin B (Bukoreshtliev *et al.*, 2009) and from 500 nM to 20 µM for cytochalasin D (Kumar *et al.*, 2017; Whitehead *et al.*, 2020). Similarly used to block TNT formation, latrunculin A and B are compounds that originate from sponges which bind to actin filaments and prevent polymerisation (Morton *et al.*, 2000) at concentrations of 10 nM to 1 mM latrunculin A (Wang *et al.*, 2011b; Lou *et al.*, 2012) or 300 nM to 10 µM latrunculin B (Önfelt *et al.*, 2006). Instead of directly inhibiting actin polymerisation at the filaments, some studies have inhibited processes upstream of actin cytoskeleton rearrangement. Both the general inhibition of RhoGTPases, which play a key role in actin regulation, as well as specific inhibition of the small GTPases CDC42 and Rac1 decreased stimuli-induced TNT formation in Jurkat T cells (Arkwright *et al.*, 2010), macrophages (Hanna *et al.*, 2017), between breast cancer cells and endothelial cells (Dash *et al.*, 2021), and between breast cancer cells and T cells (Saha *et al.*, 2022).

Remarkably, inhibition of tubulin, the other main structural component of type II TNTs, led to contrasting results in different studies. Although the tubulin inhibitor nocodazole decreased the formation of nanotube connections between neural stem cells and brain microvascular endothelial cells (Wang *et al.*, 2016) and between lung epithelial cells (Kumar *et al.*, 2017), little to no effect of microtubule inhibition was observed using colchicine or colcemide on peripheral monocyte-derived TNTs (Shahar *et al.*, 2021), nocodazole on TNTs between astrocytes and neurons (Wang *et al.*, 2011b), TNTs between neuroblastoma cells (Zheng *et al.*, 2021), or between HEK293 cells (Lehmann *et al.*, 2005). In fact, microtubules might be more involved in the trafficking of cargo than being of structural relevance for TNT formation, as inhibition of tubulin completely abolished the transport of vesicles within type II TNTs (Önfelt *et al.*, 2006).

Other approaches to chemically inhibit TNT formation include the metabolic inhibitor metformin and the mTOR inhibitor everolimus, both of which decrease TNT formation between mesothelioma cells (Lou *et al.*, 2012) and ovarian cancer cells (Desir *et al.*, 2016), and NF-κB inhibition using cytarabine in acute myeloid leukaemia cells and adult T-cell leukaemia cells (Omsland *et al.*, 2017; Omsland *et al.*, 2018). However, their efficacy in other cell types and conditions remains to be investigated.

In comparative studies, physical interference by permeable membrane inserts was found to be more efficient in blocking TNT-mediated communication than common actin inhibitors (Sowinski *et al.*, 2008; Polak *et al.*, 2015). Actin polymerisation inhibitors used in TNT inhibition generally have short half-lives and are cytotoxic to cells at higher concentrations, limiting their applications (de Rooij *et al.*, 2017). Thus, the development of more TNT-specific and efficient inhibitors is required to accelerate advancements in intercellular communication research by connecting nanotubes and to potentially develop therapeutic applications.

In conclusion, membrane nanotubes can be formed during cell dislodgement or “de novo” formation, and many factors, including cellular stress and injury, interactions with pathogens, and signals received from the environment, for example, through integrin signalling, may induce the establishment of these connections.

3. Intercellular communication via TNTs

The formation of TNTs between two or more cells establishes intercellular bridges that can be used for the transport of various cargoes, thereby aiding in the communication between cells. This includes the exchange of materials of varying sizes, both at the membrane surface and by transport through the conduit. An overview of the exchanges detailed in the following section can be found in **Figure 8**.

3.1. Types of transfers facilitated by TNTs

3.1.1. Transfer of membrane proteins

The ability of TNTs to aid in the exchange of membrane components has been documented in various cell types since the earliest reports of TNTs (Rustom *et al.*, 2004) and has been visualized either generally, via the transfer of membrane dyes (Omsland *et al.*, 2020; Kalargyrou *et al.*, 2021), or by staining specific elements of the cell membrane.

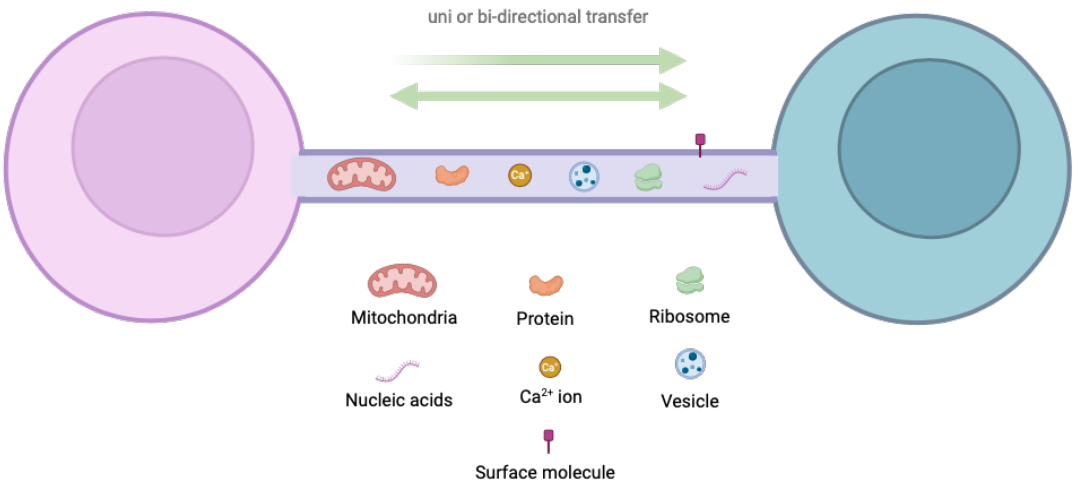

Figure 8 Transfers facilitated by TNTs
(Created with Biorender.com)

TNTs can serve in intercellular communication by allowing exchanges either at the surface or facilitating cytoplasmic exchanges. On the outside of the nanotube, membrane molecules or dyes may be transferred between cells. Cytoplasmic exchanges documented to date include organelles such as mitochondria, vesicles and ribosomes, and smaller cargo, including nucleic acids, proteins and ions (Dagar *et al.*, 2021).

In B cells, immunoregulatory proteins such as MHC-II and CD86 can be transported between cells along TNTs (Osteikoetxea-Molnar *et al.*, 2016; Halasz *et al.*, 2018), and similarly, predominantly B cell-derived connections between B cells and T cells can aid in the trafficking of CD86 and H-Ras, a small GTPase anchored to the inner leaflet of the plasma membrane, between these lymphocyte types (Rainy *et al.*, 2013a). Among T cells, the bi-directional transfer of transmembrane proteins CD81 or CD59, as well as the trafficking of the death receptor ligand FasL along nanotubes, has been demonstrated (Arkwright *et al.*, 2010), and other transmembrane proteins, such as intercellular adhesion molecule 1 (ICAM1), have been shown to be similarly transported from B-cell precursor ALL (BCP-ALL) cells to MSCs (de Rooij *et al.*, 2017). In summary, exchange of membrane components between cells using TNTs may convey immunomodulatory or death signals, as well as alter the interactions between cells and their environment.

3.1.2. Cytoplasmic exchanges of small components

On the other hand, as connections between the cytosols of two cells, TNTs can facilitate the transfer of cytosolic components inside the protrusions, which are often experimentally visualised by means of lipophilic dyes, such as DiO, DiD, and DiI (Lehmann *et al.*, 2014; Wang & Gerdes, 2015; Kumar *et al.*, 2017; Kalargyrou *et al.*, 2021), or fluorescent cytosolic proteins (Arkwright *et al.*, 2010; Lou *et al.*, 2012) in both unidirectional and bidirectional manners (Lou *et al.*, 2012; Kumar *et al.*, 2017). Similarly, genetic material and endogenous proteins have been shown to traffic between cells through nanotubes to alter gene expression or promote apoptosis. Indeed, transfer of Langerhans cell-derived mRNA to keratinocytes via cytosolic connections can impart the keratinocytes with the genetic fingerprint of the donor cells (Su & Igyarto, 2019) and active caspase 3 has been shown to move through conduits connecting T cells, thereby spreading apoptosis (Arkwright *et al.*, 2010).

Interestingly, TNTs can also serve as conduits for electrical signals via the intercellular transport of calcium ions, as has been shown for spontaneously established nanotubes between macrophages and dendritic cells (Watkins & Salter, 2005; Hase *et al.*, 2009), where electrical signals lead to phenotypic changes, such as extension and flattening of the cell membrane, which may serve as preparation for phagocytosis (Watkins & Salter, 2005). Similar Ca^{2+} fluxes have also been observed in nanotubes artificially created between HEK cells using optical tweezers, although the amplitude of the transmitted signal in these artificial nanotubes was lower than that in spontaneously established connections (Pascoal *et al.*, 2010).

3.1.3. Cytoplasmic exchanges of organelles

Perhaps the most striking feature of intercellular communication through membrane nanotubes is their ability to transport whole organelles between cells. To date, a large variety of organelles have been demonstrated to be trafficked by TNTs. These include various types of vesicles, such as microvesicles (Rustom *et al.*, 2004; Halasz *et al.*, 2018), vesicular components (Zhang *et al.*, 2021), endosomes (Wang *et al.*, 2011b), lysosomes (Rustom *et al.*, 2004; Obermajer *et al.*, 2009; Osteikoetxea-Molnar *et al.*, 2016; Kim *et al.*, 2019; Kalargyrou *et al.*, 2021; Lee *et al.*, 2022), and autophagosomes (de Rooij *et al.*, 2017). Vesicular transport can occur either unidirectionally (Rustom *et al.*, 2004) or bidirectionally (Sanchez *et al.*, 2017).

Other cellular compartments capable of passing through TNTs are ribosomes (Kumar *et al.*, 2017; Sanchez *et al.*, 2017), endoplasmic reticulum cisterns (Wang *et al.*, 2011b; Sanchez *et al.*, 2017), Golgi (Wang *et al.*, 2011b), and nuclei (Zhang *et al.*, 2021), but perhaps the most well-documented organelle transfer to date is that of whole mitochondria, which has been demonstrated between B cells (Osteikoetxea-Molnar *et al.*, 2016), T cells (Obermajer *et al.*, 2009), PC12 cells (Wang & Gerdes, 2015; Wang *et al.*, 2016), epithelial cells (Kumar *et al.*, 2017), fibroblasts (Kim *et al.*, 2019) mesenchymal stem cells (Sanchez *et al.*, 2017) and mesothelioma cell lines (Lou *et al.*, 2012), as well as between different cell types, such as from BCP-ALL to MSCs (de Rooij *et al.*, 2017) and between T_{regs} and MSCs (Do *et al.*, 2021), HUVEC and MSCs (Liu *et al.*, 2014b), iT_{regs} and MSCs (Do *et al.*, 2021) or neurons and astrocytes (Zheng *et al.*, 2021) and the small RhoGTPase Miro1 has been proposed as a vital player in this process (Saha *et al.*, 2022).

Nanotube-mediated mitochondrial transfer plays a key role in protecting cells from apoptosis or damage induced by a number of stressors. Indeed, mitochondrial transfer from healthy to apoptotic or injured cells having undergone UV treatment or ischaemia-reperfusion injury has been shown to decrease apoptosis and rescue mitochondrial respiration in damaged cells (Liu *et al.*, 2014b; Wang & Gerdes, 2015; Wang *et al.*, 2016). Similarly, the transfer of mitochondria from low-passage to senescent MSCs via TNTs has been shown to decrease the expression of senescence markers in high-passage MSCs (Whitehead *et al.*, 2020), and an increased mitochondrial exchange was shown to protect cells from cobalt nanoparticle-induced cytotoxicity (Zheng *et al.*, 2021).

In fact, mitochondrial transfer between cells may represent an explicit mechanism of cell rescue, as it can be switched from a bi-directional exchange between healthy cells (Liu *et al.*, 2014b; Sanchez *et al.*, 2017), where two mitochondria can enter the same nanotube from both cells (Lee *et al.*, 2022), to a highly directed movement of mitochondria towards the injured cells (Liu *et al.*, 2014b). However, this means of cell rescue can also be employed by cells to withstand intentionally induced cell toxicity, such as cancer cell survival during chemotherapy (Kato *et al.*, 2022). Additionally, cancer cells may unidirectionally receive mitochondria from immune cells via TNT, leading to an increase in spare respiratory capacity and basal respiration in the cancer cells and a marked reduction in spare respiratory capacity and basal respiration in the donor immune cells (Saha *et al.*, 2022). This may work in tandem with other ways of TNT signalling, as connections between BCP-ALL cells and MSC have also been shown to give rise to the secretion of pro-survival cytokines, thereby improving the survival of cancer cells (Polak *et al.*, 2015). Thus, mitochondrial transfer represents a key protective mechanism of cells against cytotoxicity, injury,

and apoptosis, and may also be employed by cancerous cells against intentionally cytotoxic treatments during chemotherapy.

3.1.4. The impact of TNT composition on transport of cargo

The diversity of cargo transported via TNTs is governed by various factors. The different compositions of TNTs may facilitate the transport of different materials, as thinner type I TNTs could predominantly aid in the transport of small cytosolic molecules and parts of the cell membrane, as well as allow for the surfing of cargo along the surface of the nanotubes (Önfelt *et al.*, 2006; Sanchez *et al.*, 2017), while the microtubule-containing type II TNTs may also participate in larger organelle transfer inside the tubes (Sanchez *et al.*, 2017). Indeed, the presence of microtubules appears to be important for the transport of some larger cytosolic cargo, as both the velocity of the cargo and the difference in diameter between the tubule and organelles, such as mitochondria, point towards an active transfer mechanism inside the TNTs (Lee *et al.*, 2022). This is supported by observations that ATP depletion can inhibit TNT-mediated trafficking (Lehmann *et al.*, 2005; Önfelt *et al.*, 2006). In fact, the microtubule motor protein kinesin was observed to co-localise with mitochondria inside pancreatic cancer cell TNTs (Lee *et al.*, 2022), whereas dynein was shown to be important for the spread of Chlamydia using these intercellular connections (Jahnke *et al.*, 2022). On the other hand, actin motor proteins, particularly those of the myosin family, have also been implicated in the transport of certain types of materials. In B cells, microvesicle transfer was shown to be efficiently mediated by non-muscle myosin 2A (Halasz *et al.*, 2018). However, myosin VI has been implicated in the transport of lysosomes between the cytoplasm of neurons (Gousset *et al.*, 2009), and myosin Va was found to participate in the trafficking of small microvesicles and endosomes via PC12 cell TNTs (Rustom *et al.*, 2004).

Interestingly, active transfer mechanisms may not only govern intratubular exchanges, but also facilitate the surfing of material on the outside of the nanotubes. Inhibition of ATPases halts viral surfing on TNTs, pointing towards an active transfer mechanism (Önfelt *et al.*, 2006), which was found to be likely myosin II-related (Lehmann *et al.*, 2005).

3.2. Exploitation of TNTs by pathogens

However, this protective mechanism may also be exploited by pathogens or pathogenic materials to spread between cells and propagate disease and to date, this type of intercellular transmission has been demonstrated for a number of viruses, bacteria, prions, and pathogenic peptides. Indeed, TNTs between epithelial cells have been shown to not only contain viral proteins, but also facilitate the spread of both viral proteins and the genome, leading to the replication of viruses in recipient cells even in the presence of therapeutic treatments, such as neutralising antibodies and antiviral drugs (Kumar *et al.*, 2017). Similarly, in T cells, HIV-1 proteins were present within nanotubes and in previously uninfected cells as early as 1h after co-culture with infected cells, indicating a rapid intercellular spread of the virus (Sowinski *et al.*, 2008), and HTLV-1 was able to not only efficiently spread between cells via TNTs, but also induce the formation of more of these intercellular connections (Omsland *et al.*, 2018). Interestingly, an additional mechanism of transmission via TNTs through viral "surfing" along the surface of the nanotubes has also been proposed for HIV-1 and murine leukaemia virus (Lehmann *et al.*, 2005). In this study, viral particles were observed to attach to the surface of the protrusions before undergoing directed movement towards the cell body, where they fuse with the cell body and enter the previously uninfected cell.

Similar surfing has also been documented for the spread of bacteria among cells. In macrophages, live attenuated *Mycobacterium bovis* was shown to attach to the surface of nanotubes before unidirectionally gliding towards the body of one connected cell and being phagocytosed by the cell (Önfelt *et al.*, 2006). In contrast, *Chlamydia trachomatis* was observed within TNTs between HEK cells which facilitated directional intercellular spread to non-infected cells even in the presence of heparin, which blocks de novo entry of Chlamydia into the cells (Jahnke *et al.*, 2022). During the TNT-mediated spread of *Mycoplasma hyrhinis*, however, bacteria were detected both inside and bound to the surface of fibroblast TNTs, indicating that some bacteria may employ both methods of transportation (Kim *et al.*, 2019).

The spread of prions and pathogenic peptides using TNTs has also been reported. The wild-type prion protein (PrP^{WT}) was observed both on the surface of TNT networks formed by neurons and inside lysosome-derived vesicles and could be transferred to uninfected cells (Gousset *et al.*, 2009). Aggregates of misfolded huntingtin (Htt), the causative agent of Huntington's disease, have also been shown to be transferred between neurons using TNTs (Costanzo *et al.*, 2013). Additionally, scrapie (PrP^{Sc}) has been shown to be efficiently transmitted through TNTs, both between neurons and heterogeneously between neuronal and dendritic cells inside endolysosomal vesicles (Gousset *et al.*, 2009; Zhu *et al.*, 2015). Lysosome-encased α-synuclein

fibrils have also been observed to move between neurons (Abounit *et al.*, 2016). Similarly, amyloid β (Aβ), a cytotoxic peptide and key player in Alzheimer's disease, can rapidly spread between rat neurons and astrocytes, causing cytotoxicity in recipient cells (Wang *et al.*, 2011b). Thus, in addition to pathogen-derived proteins, membrane nanotubes can also be exploited by prions and pathogenic peptides for the propagation of disease between cells.

3.3. Therapeutic strategies involving TNTs

The indiscriminatory nature of cargo transport through TNTs can also be exploited for therapeutic purposes. TNTs can mediate the transfer of the chemotherapeutic agent doxorubicin between connected cells and the acquisition of even small amounts of drug-induced cytotoxicity in the recipient cells (Desir *et al.*, 2018). Therapeutic nanoparticles, such as quantum dots or microparticles, can be used to efficiently deliver signals or drugs to cells. Interestingly, both passage through the nanotube conduit and a surfing mechanism on the nanotube surface may be employed for the transport of microparticles, as surface-oxidised, porous silicon microparticles were observed to disseminate via either mechanism (Ferrati *et al.*, 2012). Quantum dots (QDs) can be transported bidirectionally in vesicles of possibly endosomal origin and vesicular localization may be required for QD transport. (He *et al.*, 2010; Rehberg *et al.*, 2016). Lastly, in diseases where TNTs are exploited either for pathogen spread or mitochondrial hijacking to withstand cytotoxic treatments, such as during chemotherapy, inhibition of formation or communication through membrane nanotubes may present a promising therapeutic approach. Indeed, in metastatic breast cancer cells, inhibiting TNT-mediated mitochondrial hijacking in addition to a blockade of the Immune Checkpoint inhibitor PD1 can substantially improve the efficiency of the treatment (Saha *et al.*, 2022) and more therapeutic applications will likely emerge with our growing understanding of this mode of intercellular communication.

2 OBJECTIVES AND GENERAL HYPOTHESIS

Leishmaniasis is a family of protozoan parasite-borne diseases that present in a range of clinical pictures, the most severe of which is visceral leishmaniasis (VL). The two species of *Leishmania* known to cause VL are *L. donovani* and *L. infantum*, which can cause potentially fatal chronic infections in the visceral organs and bone marrow. They produce symptoms such as fever, anaemia, cachexia, and immunosuppression, which further sensitises hosts to co-infections (Chappuis *et al.*, 2007). An important hallmark of VL shared with numerous other chronic diseases and infections is hypergammaglobulinemia, which is defined as the presence of excessive levels of non-protective antibodies caused by polyclonal B cell activation.

In fact, B cells have been attributed a noxious role in the disease, as B cell-deficient mice are able to clear the typical chronic splenic infection (Smelt *et al.*, 2000). One way B cells play a detrimental role in VL is via the production of regulatory cytokines, such as IL-10. Our group and others have previously reported that B cells produce IL-10 during VL in humans, mice, and dogs (Bankoti *et al.*, 2012; Andreani *et al.*, 2015; Schaut *et al.*, 2016b); however, while IL-10 contributes to VL pathology by suppressing protective $CD4^+$ T cell responses, this cytokine only partially accounts for the increased disease susceptibility mediated by B cells (Deak *et al.*, 2010; Bankoti *et al.*, 2012; Andreani *et al.*, 2015). Rather, hypergammaglobulinemia was identified as the main detrimental function of B cells in VL. Indeed, the production of class-switched or hypermutated antibodies was implicated in the impaired Th1 response and disease severity, and absence of class switching and hypermutation in infected mice coincided with a marked decrease in cytokines such as IL-10, TNF, and IFN-β, which are known to drive the disease (Silva-Barrios & Stäger, 2019). Additionally, previous work in our laboratory demonstrated that the interaction of B cells with *L. donovani* amastigotes induces an IFNAR-dependent upregulation of endosomal TLRs, which in turn augments the production of antibodies and disease-driving cytokines such as IL-10 and IFN-I (Silva-Barrios *et al.*, 2016). This increased expression of endosomal TLRs was hypothesised to be due to an early wave of IFN-I produced during early B cell-parasite interactions, similar to the positive feedback loop observed in B cells after TLR7 and TLR9 stimulation (Green *et al.*, 2009; Thibault *et al.*, 2009); however, the presence of this early IFN-I and the nature of the early interaction between B cells and parasites leading to polyclonal B cell activation and resulting hypergammaglobulinemia have yet to be discovered.

When studying the early interactions between B cells and *L. donovani* amastigotes in our laboratory, we observed the formation of long tubular membrane protrusions extending from B

cells, which were able to form connections between cells upon making contact. These connections appear to be transient conduits between two cells formed by the B cell membrane which resemble structures employed in intercellular communication, called tunnelling nanotubes (TNTs). Indeed, B cells have been documented to form TNTs to facilitate long-range exchange of materials between cells (Osteikoetxea-Molnar *et al.*, 2016). TNTs have been implicated in the transport of many different types of cargo, ranging from proteins and nucleic acids to whole organelles; however, these intercellular connections may also be exploited by viruses and bacteria to allow dissemination between cells and spread of disease (Dagar *et al.*, 2021).

This led us to hypothesise that an early interaction between B cells and *L. donovani* induces the formation of interconnecting membrane protrusions to propagate B cell activation, which ultimately leads to detrimental hypergammaglobulinemia and disease exacerbation.

Hence, the main objective of this work was to elucidate the purpose of these intercellular connections between B cells during VL. To investigate our main hypothesis, the following three specific aims were addressed:

i. To characterise the connecting protrusions formed between B cells

In order to better understand their function, we first aimed to characterise the protrusions formed between B cells and establish whether these were indeed tunnelling nanotubes, which are typically defined by their appearance and actin-based nature (McCoy-Simandle *et al.*, 2016). Furthermore, we aimed to investigate the dynamics of protrusion formation in the early phase of exposure to *L. donovani* and evaluate whether this induction was specific to amastigotes or a general phenomenon also present in the presence of promastigotes or other types of parasites.

ii. To investigate the induction of connecting protrusions in B cells

B cells have been shown to be able to capture parasites and form clusters *in vitro*; however, rather than fully internalising amastigotes, B cells hold *L. donovani* tightly attached to their surface (Bankoti *et al.*, 2012; Silva-Barrios *et al.*, 2016). Thus, we hypothesised that interactions at the interface between B cells and parasites can trigger the formation of TNTs. As the main antigen receptor in B cells, the B cell receptor is a surface receptor candidate for mediating this interaction. Our group has previously reported that amastigotes purified from *Rag1*$^{-/-}$ mice are coated with

complement C3 (Bankoti *et al.*, 2012). Hence, another receptor to be investigated for its role in TNT induction is complement receptor 2 (CR2 or CD21).

iii. To evaluate the functional relevance of the protrusions in *L. donovani* infection

We aimed to investigate whether the observed connections are actually involved the dissemination of parasites between B cells, thus spreading B cell activation. TNT have been implicated in the spread of various pathogens, including bacteria, viruses, and prions (Dagar *et al.*, 2021). However, a similar mechanism is yet to be demonstrated in the context of parasitic diseases. Thus, we will investigate whether the protrusions we observe participate in the cell-to-cell spreading of the parasite *in vitro*. As macrophages are the main target of *Leishmania in vivo* and are localised in close proximity to B cells in the marginal zone of the spleen, we will also investigate the possible exchange of amastigotes between macrophages and B cells.

To answer these questions, B cells interactions will be studied in a model using primary murine B cells exposed to *L. donovani*, and the results will be detailed in Chapter 3.

3 *LEISHMANIA DONOVANI* EXPLOITS TUNNELING NANOTUBES FOR DISSEMINATION AND PROPAGATION OF B CELL ACTIVATION

Titre en français: *Leishmania donovani exploite les « tunneling nanotubes » pour la diffusion et la propagation de l'activation des cellules B*

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3.1 Abstract

Polyclonal B cell activation and the resulting hypergammaglobulinemia are a detrimental consequence of visceral leishmaniasis (VL); however, the mechanisms underlying this excessive production of non-protective antibodies are still poorly understood. Here, we show that a causative agent of VL, *Leishmania donovani*, induces CD21-dependent formation of B cells tunneling nanotubule-like protrusions in B cells. These intercellular connections are used by the parasite to disseminate among cells and propagate B cell activation, and both close contact among the cells and between B cells and parasites are required to achieve this activation. Direct contact between cells and parasites is also observed *in vivo*, as *L. donovani* can be detected in the splenic B cell area as early as 14 days post-infection. Interestingly**,** *Leishmania* parasites can also glide from macrophages to B cells via TNT-like protrusions. Taken together, our results suggest that, during *in vivo* infection, B cells may acquire *L. donovani* from macrophages via TNT-like protrusions, and these connections are subsequently exploited by the parasite to disseminate among B cells, thus propagating B cell activation and ultimately leading to polyclonal B cell activation.

3.2 Importance

Leishmania donovani is a causative agent of visceral leishmaniasis, a potentially lethal disease characterized by strong B cell activation and the subsequent excessive production of non-protective antibodies, which are known to worsen the disease. How *Leishmania* activates B cells is still unknown, particularly because this parasite mostly resides inside macrophages and would not have access to B cells during infection.

In this study, we describe for the first time how the protozoan parasite *Leishmania donovani* induces and exploit the formation of protrusions that connect B lymphocytes with each other or with macrophages, and glide on these structures from one cell to another. In this way, B cells can acquire *Leishmania* from macrophages and become activated upon contact with the parasites. This activation will then lead to antibody production. These findings provide an explanation for how the parasite may propagate B cell activation during infection.

3.3 Introduction

Leishmaniasis is a disease caused by obligate intracellular protozoan parasites of the *Leishmania* genus (Mann *et al.*, 2021). These parasites predominantly infect macrophages, where the promastigote form differentiates into the amastigote form, which proliferates inside the mammalian host (Kaye & Scott, 2011). The clinical manifestations of the disease are parasite species-dependent and can range from self-curing infections characterized by lesions in the skin or mucosal membranes to severe tissue disruptions of visceral organs, which are fatal if left untreated. The life-threatening visceral leishmaniasis (VL) is caused by *Leishmania donovani*, amongst other *Leishmania* species, and results in the often-simultaneous enlargement of the liver and spleen in a condition termed hepatosplenomegaly, along with anemia, fever, and hypergammaglobulinemia (Burza *et al.*, 2018).

Hypergammaglobulinemia has long since been identified as a characteristic symptom of VL. Characterized by abnormally elevated levels of immunoglobins in the blood serum, it presents across all species susceptible to natural VL infection, including humans (Cooper *et al.*, 1946), dogs (Silva-O'Hare *et al.*, 2016), cats (Spada *et al.*, 2020), as well as those used as experimental models, such as non-human primates (Rodrigues *et al.*, 2014), hamsters (Campos-Neto & Bunn-Moreno, 1982), and mice (Deak *et al.*, 2010; Silva-Barrios *et al.*, 2016; Silva-Barrios & Stäger, 2019). This excessive production of non-protective antibodies can closely resemble autoimmune conditions and exacerbates the disease through antibody-mediated pathology (Liberopoulos *et al.*, 2013). Indeed, high antibody titers during *L. donovani* infection was previously proposed to be predictive of disease progression in humans (Singh *et al.*, 2002). Nevertheless, our understanding of the mechanisms underlying this detrimental immune process is still limited.

Despite their central role in the production of antibodies, B cells have rarely been the focus of *Leishmania* research, and decades after drawing the link between hypergammaglobulinemia and VL, the literature on the role of B cells in this disease remains sparse (Silva-Barrios *et al.*, 2018). Their contribution to the pathology of *L. donovani* infection was first identified in a study using a mouse model of visceral leishmaniasis, where B cells were shown to play a negative role and exacerbate the disease (Smelt *et al.*, 2000). Subsequent studies by our group and others identified polyclonal B cell activation and resulting hypergammaglobulinemia as the main route through which B cells exacerbate disease (Deak *et al.*, 2010; Bankoti *et al.*, 2012; Silva-Barrios *et al.*, 2016; Silva-Barrios & Stäger, 2019). Indeed, *Aicda*$^{-/-}$ mice, which are incapable of producing hypermutated and/or class-switched immunoglobulins, are highly resistant to *L. donovani* infection (Silva-Barrios & Stäger, 2019). Interestingly, both innate immune B cell activation

through endosomal TLRs by *Leishmania* parasites and IFN-I are required to promote hypergammaglobulinemia, as B cell-specific ablation of endosomal TLR signaling or the IFN-I receptor (IFNAR) resulted in severely decreased IgG titers upon *L. donovani* infection (Silva-Barrios *et al.*, 2016). Thus, IFNAR was shown to be involved in a positive feedback loop that resulted in the upregulation of endosomal TLRs and in enhancing the expression of various cytokines upon B cell exposure to *Leishmania* amastigotes (Silva-Barrios *et al.*, 2016).

In previous work from our group studying the early stages of B cell-parasite interaction, we found the parasite to induce the formation of membrane protrusions that branch out from the B cells (Bankoti *et al.*, 2012). Rearrangements of the actin cytoskeleton leading to cell spreading and formation of short spike-like protrusions have been linked with B cell activation and, more specifically, B cell receptor signalling (Fleire *et al.*, 2006), as well as antigen extraction and internalization (Roper *et al.*, 2019); however, as the membrane protrusions observed between B cells exposed to *L. donovani* are both longer and more substantial than these described filopodia, their functional significance in the context of this infection remains to be discerned.

As *Leishmania* is an obligate intracellular parasite that mostly resides in macrophages, the question remains about how amastigotes come into direct contact with B cells during infection. We have previously reported that marginal zone B cells (MZB) were carrying the parasite few hours after intravenous infection with amastigotes and suggested that MZB may shuttle from the splenic marginal zone into the B cell follicle and thus deliver the parasite to follicular B cells (Bankoti *et al.*, 2012), but how MZB capture parasites is still unclear. Moreover, the importance of this direct interaction with the parasite for B cell activation and the dynamics underlying the communication between cells and the parasite remain to be fully elucidated.

In this study, we set out to investigate the dynamics of intercellular communication between B cells and *Leishmania donovani* amastigotes to better understand the pathways through which hypergammaglobulinemia is induced. We show that splenic naïve B cells require direct contact with the parasite to get activated and that B cells can disseminate *L. donovani* amastigotes and thus activation among themselves with the help of CD21-induced formation of tunneling nanotubule-like membrane connection between cells. This interaction also results in an early and transient IFN-I wave induced in B cells by the parasite. Moreover, we show that *L. donovani* can be found in the splenic B cell area of infected mice as early as 14 days post-infection, just before the onset of hypergammaglobulinemia. We also show that parasites or parasite components can be transferred via TNT-like structures from macrophages to B cells, suggesting that MZB may receive amastigotes from infected macrophages and will then carry them to the B cell area.

3.4 Materials and Methods

Mice and Parasites

C57BL/6 and B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ (referred to as CD45.1) congenic mice were purchased from The Jackson Laboratory and housed under specific pathogen-free conditions at the Laboratoire National de Biologie Expérimentale (LNBE) following guidelines for good animal practice provided by the Canadian Council on Animal Care. *Ifnar1*$^{-/-}$ mice were a kind gift from Dr. Alain Lamarre (INRS-CAFSB). Animals were used at 8-12 weeks of age, respecting protocols approved by the Animal Care and Use Committee of the Centre Armand-Frappier Santé Biotechnologie (Protocol numbers 1910-01 and 2002-03).

Leishmania donovani (strain LV9) was maintained by serial passage in B6.129S7-Rag1^{tm1Mom} (*Rag1*$^{-/-}$) mice and amastigotes were isolated from the spleens of infected animals. Mice were infected by intravenous injection of 2×10^{7} amastigotes via the lateral tail vein.

For *in vitro* experiments using previously frozen amastigotes, parasites were opsonized using fresh serum from *Rag1*$^{-/-}$ mice at 37 °C for 30 min prior to use. *Leishmania donovani* promastigotes (strain LV9) were grown by axenic culture of amastigotes isolated from the spleens of *Rag1*$^{-/-}$ mice at 26°C in M199 medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Wisent), hypoxanthine (Sigma), hemin (Sigma), biopterin (Sigma), biotin (Sigma) and penicillin-streptomycin (Life technologies). The resulting promastigotes were washed in DPBS, opsonized or not with serum from *Rag1*$^{-/-}$ mice as described above and finally resuspended in complete IMDM prior to use. *Toxoplasma gondii* tachyzoites (strain RH) were maintained in Vero cells by serial passage in DMEM (Gibco, Invitrogen) supplemented with 5% heat-inactivated FBS (Wisent) and supplemented with penicillin-streptomycin (Life technologies) and HEPES (Fisher) as previously published (Leroux *et al.*, 2018). Prior to experiments, the tachyzoites from Vero cells were harvested by scraping the cells, any clumps disrupted by passing through a 27G needle and cell debris pelleted and removed by centrifugation at low speed (200 x g). The egressed tachyzoites in the supernatant were pelleted by high-speed centrifugation (1,300 x g), resuspended in ice-cold phosphate-buffered saline (PBS) (pH 7.2 to 7.4), filtered from debris using a 3µm polycarbonate filter (Millipore). The resulting tachyzoites were then washed using PBS and finally resuspended in complete DMEM prior to the experiment.

Fluorescent labelling of parasites

After isolation or rapid thawing, the amastigotes to be labelled were washed in plain RPMI (Gibco, Invitrogen) and in DPBS (Mg^{2+} and Ca^{2+} free, Gibco, Invitrogen) before resuspension in 100µl Diluent C (Sigma) per 10^8 amastigotes. An equivalent volume of Diluent C containing 1:250 PKH67 Membrane linker dye (Sigma) was added to the suspension and incubated for 3 min at room temperature. The staining reaction was stopped by adding an equivalent volume to the total amount of diluent C of FBS to the parasites and incubation at room temperature for 1 min. The thus stained parasites were washed in plain RPMI and complete IMDM before use in subsequent experiments.

In vitro B Cell Culture

Naïve splenic B cells were purified using a negative selection magnetic-assisted cell sorting kit (Miltenyi Biotech) according to the manufacturer's protocol and cultured in IMDM (Gibco, Invitrogen) containing 10% heat-inactivated FBS (Wisent) and supplemented with penicillin-streptomycin (Life technologies). Purified B cells were then incubated by themselves, with the parasite at a multiplicity of infection (MOI) of 5 or 10, or 10 ng/ml mouse recombinant IFN-β (Biolegend) at 37 °C and 5 % CO_2 for the duration specified. For experiments assessing the necessity of direct contact for B cell activation, Transwell™ permeable membrane inserts (Corning) with a pore size of 0.4 µm and a PET membrane were used to separate total B cell or B cell subpopulations, and only the cells in the top compartment were exposed to PKH67-labeled parasite.

Differentiation of Bone Marrow-derived Macrophages (BMM)

To obtain macrophages, bone marrow was flushed from the femurs and tibias of C57BL/6 mice, red blood cells were lysed using a solution containing 0.17 M NH4Cl, pH 7.4, for 7 min, and cultured in adherent culture-treated Petri dishes in DMEM (Gibco, Invitrogen) supplemented with 10% heat-inactivated FBS, 10 mM HEPES, pH 7.4, and penicillin-streptomycin (Life Technologies) supplemented with 20 % L929 cell-conditioned medium (LCM) at 37°C. After one day, nonadherent cells were collected, transferred to new Petri dishes, and differentiation was re-stimulated by the addition of LCM on days 3 and 5 post-bone marrow collection. On day 7, cells

were detached from the dish surfaces using a sterile cell scraper, counted, and reseeded on sterile coverslips at a concentration of 2.5 x 10^5 macrophages per slip in DMEM containing 10% FBS for confocal microscopy experiments or in 12 or 24-well-plates at concentrations of 1 x 10^6 or 5 x 10^5, respectively, for flow cytometry experiments, and allowed to adhere 16h overnight before use.

Parasite transfer experiments

To assess parasite transfer between cells parasite-exposed and naïve cells, B cells or macrophages were isolated or differentiated from both wild-type C57BL/6 mice expressing CD45.2 and from CD45.1 congenic mice as described above. The cells were seeded in 24 wells plates at a concentration of 1 x 10^6 and the wells containing cells from one allelic variant were exposed to PKH67-labelled LV9 amastigotes for 1h for B cells, or overnight in the case of macrophages, thoroughly washed using sterile PBS for a minimum of three times until there was no presence of free parasite visible under the microscope and then further incubated with naïve cells of the other allelic variant for 2.5 or 5 h before preparation of samples for flow cytometry or confocal microscopy. For experiments using Cytochalasin D, the parasite-exposed cells were treated with 1 or 10 µM Cytochalasin D (Sigma) for 1 h before further co-incubation with the initially non-exposed cells of the other allelic variant for 2.5 or 5 h. The absence of free parasites in the supernatant was further investigated by subjecting naïve cells to the supernatants of exposed cells after washing.

Crosslinking of Surface Markers Using Antibody-Coupled Beads

Latex beads with a mean particle size of 3 µm (Sigma) were washed with sterile DPBS (Gibco), resuspended in DPBS and incubated with 1 µg per 10^7 beads of CD21/CD35 Monoclonal antibody (Clone 4E3, eBioscience), IgM Rat anti-Mouse antibody (Clone II/41, BD Biosciences) or a combination of both for 2 hours at room temperature under continuous mixing on a rotating wheel. As controls, beads were either uncoupled or coupled to the isotype control antibody Rat IgG2a kappa Isotype Control (Clone eBR2a, eBioscience). After incubation, the antibody-coupled beads were washed and resuspended in complete IMDM (Gibco, Invitrogen) before incubation with the B cells at an MOI of 7 for 5 hours.

Quantitative Real-Time PCR

RNA extraction from isolated B cells was carried out using the RNeasy mini kit (QIAGEN) following manufacturer instructions. Reverse transcription was performed using the iScript cDNA synthesis kit (Bio-Rad) per the manufacturer's protocol. Real-time PCR measurements were prepared using iTaq Universal SYBR Green Supermix (Bio-Rad) and carried out using a Stratagene mx3005p real-time PCR system. Genes for IFN-α, IFN-β, and HPRT were amplified using primers listed in Table 1. Data were normalized to HPRT and expressed as the fold increase relative to naïve controls.

The following primers were used to measure the relative gene expression using RT-qPCR:

Table 4 Primer sequences for RT-qPCR

Gene	Direction	Sequence
Hprt	Fw	5′-GTT GGA TAC AGG CCA GAC TTT GTT G-3′
	Rv	5′-GAT TCA ACC TTG CGC TCA TCT TAG GC-3′
Ifna	Fw	5′-CAT CTG CTG CTT GGG ATG GAT-3′
	Rv	5′-TTC CTG GGT CAG AGG AGG TTC-3′
Ifnb	Fw	5′-TCA GAA TGA GTG GTG GTT GC-3′
	Rv	5′-GCA CTT TCA AAT GCA GTA GAT TCA-3′

Confocal Microscopy

L. donovani amastigotes were stained with PKH67 membrane linker dye kit (Sigma) following the protocol described above prior to incubation with splenic B cells or macrophages. For experiments using only B cells, the cells were seeded at 2×10^6 cells per well in 12-well plates and incubated either alone or with PKH67-stained *L.onovani* amastigotes (at an MOI of 5:1 or 10:1) for 2.5, 5, or 8 h before fixation in a final concentration of 2% paraformaldehyde for 10 min at room temperature. To prepare cells for intracellular staining, if applicable, permeabilization was achieved by treating fixed cells using 0.1% Triton X-100 in PBS and unspecific binding of antibodies was blocked using PBS containing 5% BSA, 5% FBS, and a 1:200 dilution of FcBlock antibody (Clone 2.4G2) for 30 min at room temperature. The cells were then labeled with a combination of the following antibodies as specified: anti-IgM-AF568 (polyclonal, Invitrogen), Phalloidin-AF594 (Invitrogen), anti-Tubulin-AF647 (Clone 10D8, Biolegend), anti-CD45.1-BV421 (Clone A20, Biolegend), anti-CD45.2-AF647 (Clone 104, Biolegend), anti-CD11b-BV421 (Clone M1/70, BD Biosciences). NucBlue (Hoechst 33342, Invitrogen) was used to stain cell nuclei following the manufacturer's protocol. Labeled cells were washed in PBS and

resuspended PBS and then transferred onto coverslips coated with poly-L-lysine (Sigma-Aldrich), before being mounted on microscopy slides using Fluoromount-G mounting medium (Invitrogen).

In experiments using macrophages and B cells, sterile coverslips were treated with poly-L-lysine, and macrophages adhered directly onto the coverslips before being incubated either directly with PKH67-stained LV9 amastigotes, followed by naïve B cells, or B cells previously incubated with PKH67-stained parasite. Coverslips were incubated for 5 hours after the addition of B cells to ensure adherence of B cells to the coverslips before fixation, blocking, staining, and mounting as described above.

Immunohistochemistry

Spleens of naïve and infected C57BL/6 mice were collected on days 21, and 28 post-infection and snap-frozen in blocks of clear OCT compound (Fisher Health Care) using liquid nitrogen. Blocks were stored at -80°C until sectioning into 10 µm slices using a Microm HM525 Cryostat (Thermo Fisher) at -20°C. Tissue sections were mounted on glass slides and immediately processed by air-drying and rehydrating tissues in PBS for 30 min. Unspecific staining was blocked by treatment of tissues with PBS containing 5% BSA and 1:100 of FcBlock antibody (2.4G2) for 1 h. LV9 amastigotes were stained by incubating tissues with serum from LV9-infected hamsters at a concentration of 1:500 and then labeling bound anti-LV9 using an anti-Hamster AF488-conjugate (Biolegend). Tissue cells were labeled using CD11b-BV421 (BD Biosciences) and IgM-AF568 (Invitrogen) and subsequently fixed using 1% paraformaldehyde (PFA) before mounting coverslips on Fluoromount G mounting medium (Invitrogen) over the tissue. Images were taken on an LSM780 confocal microscope (Zeiss) using either a 40x or 63x oil immersion objective.

Western Blot

Total cell protein extracts from 1×10^7 purified B cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich) supplemented with a protease inhibitor cocktail (Complete mini, Roche) and a phosphatase inhibitor cocktail (phosSTOP, Roche). Protein concentration of lysates was determined following the instructions of a Pierce BCA Protein Assay Kit (Thermo Fisher) and equal amounts of protein (15 µg) were separated on an 8% SDS-PAGE before semi-dry transfer to a nitrocellulose membrane (Hybond, Amersham). Proteins were labeled using a Phospho-Stat1 (Tyr701, Clone 58D6) Rabbit monoclonal antibody or a Stat1 (D1K9Y) Rabbit

monoclonal antibody (both Cell Signaling Technology). Blots were cut between 76 and 52 kDa as determined by the travel of the Amersham ECL Rainbow Full-Range Molecular Weight marker (stripping buffer, Thermo Fisher) and equal loading was confirmed with a monoclonal antibody against β-actin (Santa Cruz Biotechnology).

Flow Cytometry

The presence of PKH67-stained LV9 on various B cell subsets was assessed using the following antibodies: anti-CD21-PE-CF594 (Clone 7G6, BD Biosciences), anti-CD23-APC (Clone B3B4, Biolegend), anti-CD45.1-PE (Clone A20, BD Biosciences), anti-CD45.2-APC-Cy7 (Clone 104, Biolegend), anti-CD86-BV510 (Clone GL1, BD Biosciences), and anti-MHCII-BV421 (Clone M5/114.15.2, BD Biosciences). To conserve the phosphorylation status during the measurement of type-I IFN signaling, cells were fixed in 2% PFA prior to permeabilization using cold methanol and staining using the following antibody panel: anti-CD21- PE-CF594 (Clone 7G6, BD Biosciences), anti-CD23- PE-Cy7 (Clone B3B4, BD Biosciences), anti-MHCII-FITC (Clone 2G9, BD Biosciences) and anti-pSTAT1-AF647 (Clone 4a, BD Biosciences). For each sample, 100,000 cells were acquired using an LSRFortessa cell analyzer (BD), and data were analyzed using FlowJo software (BD).

Statistical Analysis

Statistical analysis was done in GraphPad Prism using Student's t-test. Differences were considered statistically significant at a p-value < 0.05. All experiments were independently carried out at least three times.

Data Availability

All data generated during this study are included in this article.

3.5 Results

3.5.1 B cells can pass on *L. donovani* amastigotes among each other to disseminate activation via direct contact with the parasite

We have previously reported that splenic B cells can capture *L. donovani* amastigotes upon *in vitro* exposure to the parasite (Bankoti *et al.*, 2012), and that, following this interaction, the parasites sit in IgM-rich pockets on the B cell surface, leading to cell clustering, upregulation of the expression of major histocompatibility complex class-II (MHCII) and activation markers such as CD86, followed by cell death within 24-48 hours (Bankoti *et al.*, 2012; Silva-Barrios *et al.*, 2016). Thus, we first wanted to investigate whether direct contact between cells and cells and parasites is required for B cell activation or if activation can simply be achieved through soluble mediators, such as cytokines. To this end, we separated primary splenic B cells using permeable membrane inserts with a pore diameter insufficient to allow for the passage of the amastigotes and found that indeed this hindered the transport of parasites from the upper, parasite-exposed to the lower compartment to which no parasites were added initially (Figure 1 a). As *L. donovani* amastigotes isolated from mice are coated in complement C3 (Bankoti *et al.*, 2012) and this coating is lost after thawing of frozen parasites, we incubated previously frozen amastigotes with serum from *Rag1*$^{-/-}$ mice prior to their exposure to B cells to mimic their state *in vivo*. In the absence of the parasite in the lower compartment, these cells did not increase the expression of MHCII and activation markers such as CD86 at either 2.5 or 5h post-exposure, while cells in the top compartment, able to establish contact with the parasite, showed a significant increase of the proportion of MHCII^{hi} CD86^{hi} B cells at both timepoints (Figure 1 b). The lack of upregulation of these markers in the cells physically separated from the parasite and from cells carrying amastigotes indicates that contact with the parasite or with cells carrying *Leishmania* is indeed required to activate B cells and that soluble mediators alone are insufficient to propagate activation.

Next, we wanted to see if the parasite can be passed on from one cell to another as a possible explanation for how activation is disseminated among B cells. To this end, we separately exposed B cells from C57BL/6 mice (CD45.2) to *Rag1*$^{-/-}$ mouse serum-coated *L. donovani* amastigotes for 1h before thoroughly washing the cells to remove any uncaptured parasite and subsequent incubation with naïve B cells purified from CD45.1 congenic mice. Indeed, about 10% of CD45.1 B cells (receivers) were carrying parasites at 2.5 h (Figure 1 c) and 20% at 5h of co-culture (Figure 1 c).

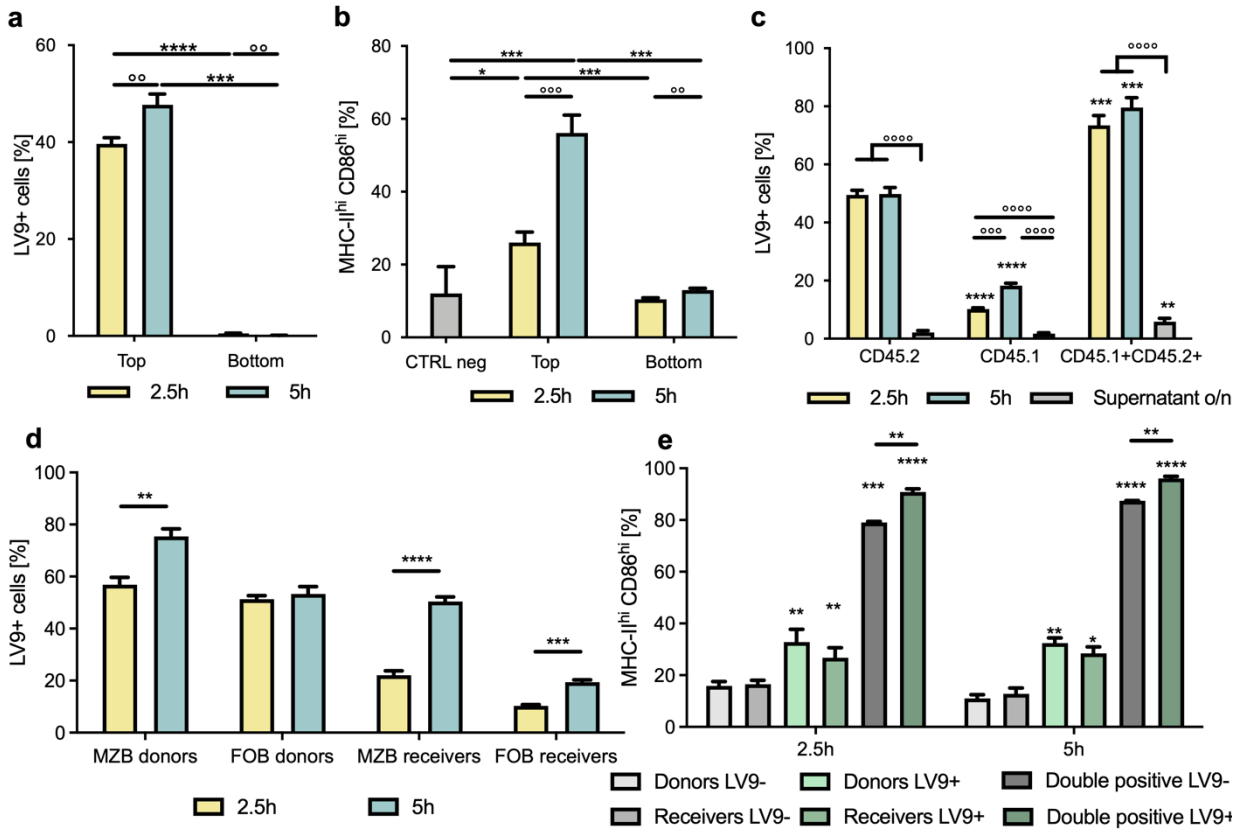

Figure 1 B cells can pass on *L. donovani* amastigotes among each other to disseminate activation via direct contact with the parasite.

(**a**, **b**) Purified B cells exposed to Rag1$^{-/-}$ mouse serum-coated *L. donovani* amastigotes (MOI 1:10) were separated from naïve B cells using permeable membrane inserts of insufficient pore size to allow for passage of parasite and exposed for 2.5h or 5h. (**a**) Percentage of cells carrying PKH67-stained amastigotes in the top and bottom compartments as assessed by FACS. (**b**) Percentage of cells expressing high levels of CD86 and MHCII in the top and bottom compartments as compared to a non-exposed control. (**c-e**) B cells purified from CD45.2 mice were exposed to Rag1$^{-/-}$ serum-coated *L. donovani* (MOI 1:10) for 1h before thorough washing to remove uncaptured parasite and exposure to naïve B cells purified from CD45.1 for 2.5h or 5h. (**c**) Percentage of cells carrying PKH67-stained positive amastigotes in cells gated on their expression of CD45.1 or CD45.2 as measured by flow cytometry. (**d**) Percentage of MZB (CD21^{hi} CD23^{lo}) and FoB (CD21^{lo} CD23^{hi}) cells carrying parasite within the CD45.2^{+} donor and CD45.1^{+} receiver groups. (e) Percentage of cells expressing high levels of CD86 and MHCII within the CD45.2^{+} donors, CD45.1^{+} receivers, CD45.2^{+}), and CD45.1^{+}CD45.2^{+} clusters carrying parasite (LV9^{+}) or not (LV9^{-}). Data represented as mean ± SD from one of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

To exclude the possibility that the parasite can simply detach from the B cells and naïve B cells can then re-capture amastigotes from the supernatant, we exposed co-cultured naïve CD45.1 and CD45.2 B cells to the supernatants collected from exposed cells and found negligible capturing of parasite by these fresh cells. This indicates that B cells can indeed pass on parasites from one cell to another by means other than a simple detachment-reattachment mechanism. Strikingly, we also observed a small population of CD45.1 CD45.2 double-positive cells, pointing towards clustering between initially exposed (donors) and non-exposed (receivers) cells, almost

all of which stained positive for the parasite. Within both the donor and receiver group, we found a higher percentage of marginal zone B cells (MZB) to carry parasite as compared to the Follicular B cells (FoB), although MZB only make up around 5 % of splenic B cells, with FoB representing the majority of B cells with about 90-95 % (Supplemental Figure 1a). This points towards a stronger involvement of MZB in the capturing or transfer of parasites (Figure 1 d), which is in agreement with our previous observations (Bankoti *et al.*, 2012).

When looking at the activation status of these cells, we found that the presence of parasites on both initially exposed and receiving cells led to an upregulation of the expression of MHCII and CD86 (Figure 1 e). Interestingly, the upregulation of the expression of these markers in the clustering cells was even more pronounced, indicating the existence of two possible levels of activation induced by the capture of parasite and clustering of the cells. Additionally, clustered cells kept their high expression of CD86 and MHCII even when no parasite was detected in the clusters, which could point towards the cells keeping their activation status even after passing on the parasite. Expression of the different CD45 isoforms did not affect the cells' capacity to capture or pass on the parasite as these observations are mirrored in experiments initially exposing CD45.1 cells and subsequent co-culture with CD45.2 B cells (Supplemental Figure 1 b-d). Taken together, our results suggest that B cells are capable of transferring *L. donovani* amastigotes among each other and that direct contact between B cells and the parasite as well as B cell clustering is required for activation.

3.5.2 Amastigotes disseminate among B cells via tunneling nanotubule-like protrusions

Subsequently, we sought to investigate the mechanism by which B cells pass on *L. donovani* amastigotes to each other. As previously reported (Bankoti *et al.*, 2012), we found B cells exposed to the parasite to form long membrane protrusions (Supplemental Video 1). Interestingly, these protrusions can be seen to establish connections between two cells. To gain a better understanding about their functional role and significance, we thus set out to characterize these structures. Interestingly, we found them to be primarily F-actin based with lower amounts of tubulin (Figure 2 a), which is in line with literature descriptions of tunneling nanotubule (TNT)-like protrusions (Mittal *et al.*, 2019; Zurzolo, 2021). Remarkably, we found the incidence, but not the length or thickness, of these connections to be significantly increased among cells exposed to *L.*

donovani amastigotes (Figure 2 b – d). Of note is that the seemingly low incidence of connecting protrusions is owed to the mode of quantification of links formed between cells in a two-dimensional plane at a fixed point in time. This likely leads to an underestimation of the number of connections, as their formation can be seen as a much more frequent and dynamic, albeit difficult to reliably quantify process in live cell microscopy videos.

Intrigued by the induction of the formation of these B cell protrusions by the parasite, we set out to investigate whether these amastigote-induced protrusions could serve any functional purpose. Previous studies have demonstrated TNTs and TNT-like protrusions formed by other cell types to be exploited for disease propagation by bacteria (Jahnke *et al.* ; Tilney & Portnoy, 1989; Önfelt *et al.*, 2006), and viruses (Hashimoto *et al.*, 2016; Ganti *et al.*, 2021; Merolli *et al.*, 2022), so we wanted to know whether these protrusions could be used by *L. donovani* to pass between cells and hence propagate activation. Indeed, we frequently observed the presence of *L. donovani* amastigotes on the protrusions formed between two B cells (Figure 2 e), indicating that they could be used to allow gliding of the parasite from one cell to another, thus aiding in the dissemination of *L. donovani* between cells, which could result in polyclonal B cell activation and ultimately hypergammaglobulinemia (Figure 2 e).

Having thus identified the induction of TNT-like protrusions by *L. donovani* amastigotes, we then wanted to investigate whether these intercellular connections could participate in the exchange of parasites among B cells. To this end, we similarly exposed B cells from CD45.1 mice to serum-coated amastigotes for 1h, then thoroughly washed until no free parasite was observed in the medium. Prior to co-culture with initially non-exposed CD45.2^{+} B cells for 2.5 and 5 h, however, we incubated the cells with the potent actin polymerization inhibitor Cytochalasin D at 1 or 10 µM, which has been previously described to inhibit the formation of nanotubules (Polak *et al.*, 2015; Osteikoetxea-Molnar *et al.*, 2016). This treatment did not negatively impact cellular viability after 5 h of incubation (Supplemental Figure 2 a), although exposure of cells to the parasite decreased the viability of the B cells as previously published (Bankoti *et al.*, 2012). Likewise, treatment with Cytochalasin D did not interfere with capture of parasites by donor B cells or with cluster formation (Figure 2 f); however, the presence of parasite on initially non-exposed (receiver) cells significantly decreased after treatment with both 1 µM and 10 µM Cytochalasin D (Figure 2 f), indicating a considerable involvement of TNT-like protrusions in the transfer of *L. donovani* amastigotes between B cells.

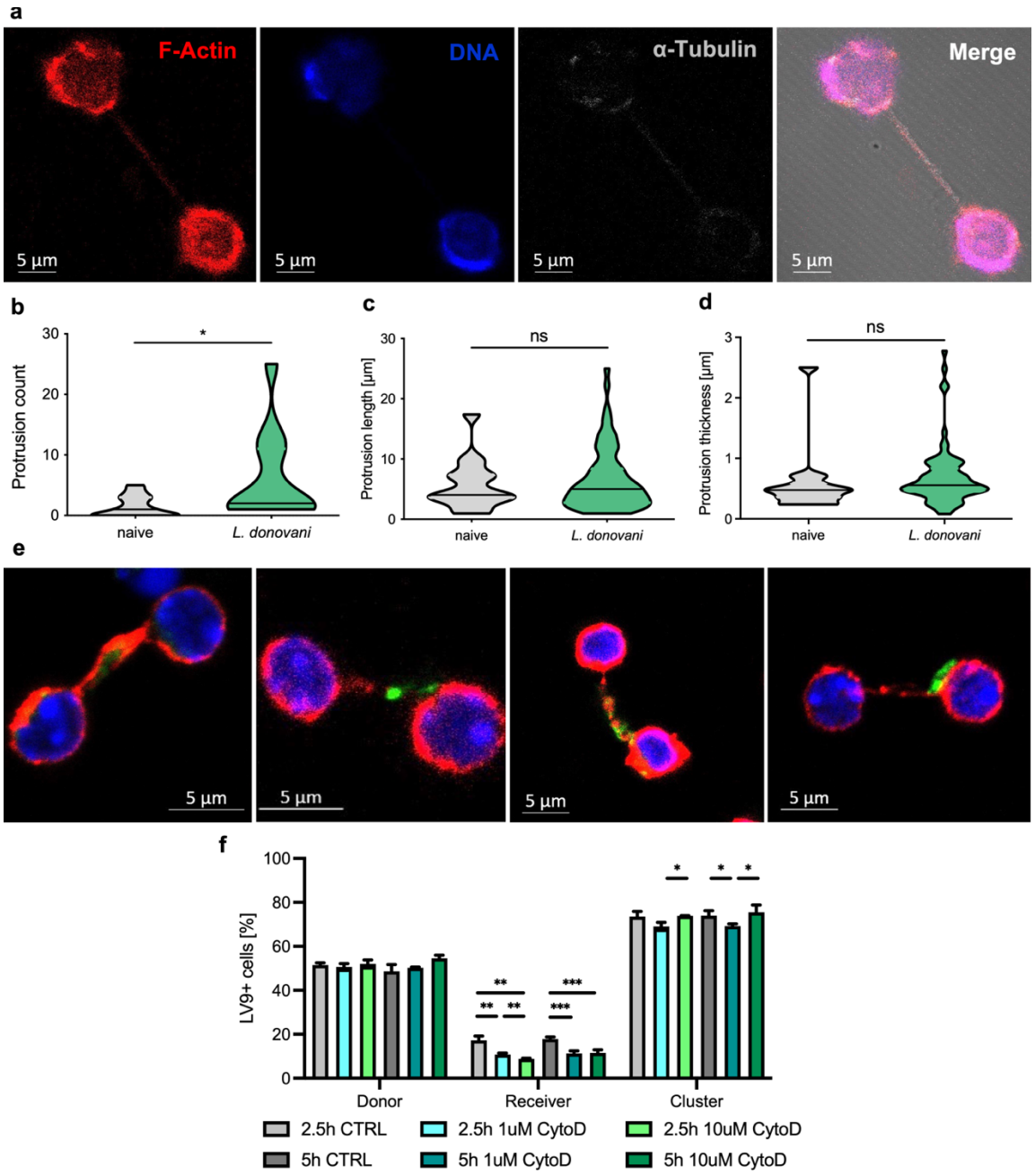

Figure 2 *L. donovani*-exposed B cells increase the formation of tunneling nanotubule-like protrusions that participate in the intercellular transfer of the parasite.

B cells were incubated for 2 - 5h with or without the presence of fluorescently labeled *L. donovani* (MOI 1:10). (**a**) Naïve B cells were labeled using phalloidin-AF594 (F-actin, red), Hoechst33342 (blue), and anti-Tubulin-AF647 (grey). (**b-d**) Protrusions between B cells with or without parasite were counted over 70 microscope fields per sample (**b**) and measured for their length (**c**) and thickness (**d**) using Zen 2011 software (Zeiss). (**e**) Representative images of TNT-like connections made of actin (Phalloidin-AF594, red) carrying PKH67-labeled *L. donovani* amastigotes (green). Nuclei stained using Hoechst33342 (blue). (**f**) B cells purified from CD45.1 mice were exposed to *L. donovani* (MOI 1:10) for 1h before thorough washing to remove uncaptured parasites and treatment with 1 or 10 µM Cytochalasin D, prior to co-incubation with CD45.2$^+$ B cells for 2.5 or 5 h. Percentage of cells carrying PKH67-stained amastigotes in cells initially exposed to parasite (donor), non-exposed (receiver), and clusters between the two types as measured by flow cytometry. Data represented as mean ± SD from three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

We next set out to investigate whether the formation of these protrusions was strictly amastigote-specific or could also be induced by different parasite forms and species. Indeed, exposure of B cells to both axenically cultured *L. donovani* promastigotes (Supplemental Video 2) and *Toxoplasma gondii* tachyzoites (Supplemental Video 3) failed to promote the formation of similar membrane connections; however, opsonizing the promastigotes with serum from *Rag1*$^{-/-}$ mice before co-incubation with B cells conferred the parasites the ability to induce clustering (Supplemental Video 4) and the formation of TNT-like structures among the B cells (Supplemental Figure 2 b, Supplemental video 5). This indicates that the serum coating on *L. donovani* amastigotes plays an important role in the induction of TNT-like protrusions in B cells.

3.5.3 Cross-linking of complement receptor 2 induces protrusion formation

MZB are the primary B cell subset seen to capture parasites *in vivo* (Bankoti *et al.*, 2012) and are also more prone to interact with *L. donovani* amastigotes than follicular B cells (FoB). Indeed, about 65% of MZB compared to 35-40% FoB were seen to carry *L. donovani* amastigotes 2.5 hours after exposure to the parasite (Figure 3 a), despite the fact that only about 5% of the B cells are MZB.

A key difference between MZB and FoB is their expression of complement receptor 2, also known as CD21, which is highly expressed in MZB and to a much lower degree in FoB (Supplemental Figure 3 a) and serves as a distinguishing marker between the two subsets. Interestingly, we have previously found the *L. donovani* amastigotes isolated from *Rag1*$^{-/-}$ mice to be coated in complement C3 (Bankoti *et al.*, 2012), whose fragments are known to be ligands for CR2 (Prodinger, 1999). Therefore, we set out to examine whether CD21 plays a role in capturing *L. donovani* amastigotes by the B cells. To this end, we incubated thawed amastigotes with serum from *Rag1*$^{-/-}$ mice to mimic the C3 coating of parasites *in vivo* (Supplemental Figure 3 b) and exposed them to cells incubated with a CD21 (CR2) blocking antibody. Indeed, a blockade of surface CR2 on B cells dramatically decreased the capacity of the cells to capture serum-coated parasite, while the blockade affected attachment of uncoated parasites to a much lower degree (Figure 3 b), solidifying the role of CD21/CR2 in the interactions at the interface between B cells and *L. donovani* amastigotes.

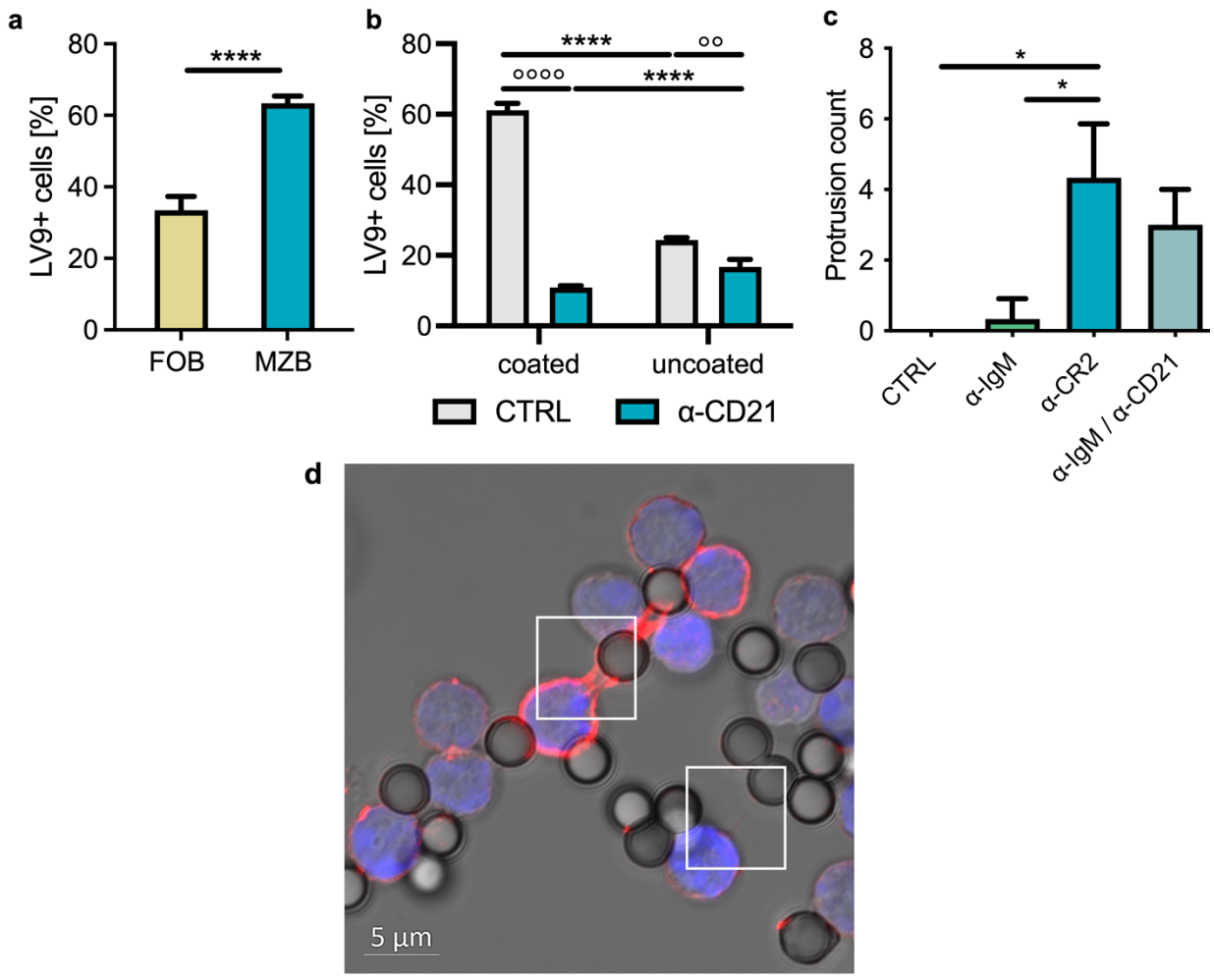

Figure 3 Complement receptor 2 mediates the capture of *L. donovani* by B cells and induces protrusion formation.

(**a**) Purified B cells were exposed to *L. donovani* (MOI 1:10) for 2.5h and the percentage of cells carrying PKH67-labeled parasite was assessed within MZB ($CD21^{hi}$ $CD23^{lo}$) and FoB ($CD21^{lo}$ $CD23^{hi}$) cells using flow cytometry. (**b**) B cells were incubated with CD21 blocking antibody 2h prior to exposure to parasite that was previously frozen in liquid nitrogen (LV9 uncoated) or treated with fresh *Rag1*$^{-/-}$ serum after thawing (LV9 coated). Percentage of cells carrying parasite was measured using flow cytometry. (**c-d**) Antibodies against IgM, CD21 or a combination of both were coupled to latex beads and exposed to B cells for 5h. (**c**) Protrusions between B cells with or without parasites were counted over 70 microscope fields per sample. (**d**) Representative image of B cells stained using phalloidin-AF594 (F-actin, red), Hoechst33342 (blue) forming protrusions with beads. Data represented as mean ± SD from three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

We then set out to elucidate which surface receptor interactions might be responsible for the induction of observed protrusions formed by the B cells. As the B cell receptor is known as a central receptor for B cell activation, we investigated crosslinking the BCR using an anti-IgM antibody in addition to the aforementioned CR2 for its ability to induce protrusions in B cells. To this end, we coupled antibodies for either the BCR (anti-IgM), CD21, or a combination of both to latex beads and incubated them with naïve B cells to examine them for any induction of TNT-like protrusions by surface receptor cross-linking. Remarkably, crosslinking CD21 on B cells gave rise

to a significant increase in protrusion formation as compared to cells exposed to uncoated beads, while crosslinking the BCR using anti-IgM coupled beads did not lead to a significant induction of TNT-like protrusions (Figure 3 c, d). Similarly, co-incubating B cells with beads coupled to a Rat IgG2a isotype control antibody did not give rise to TNT-like protrusions (Supplemental Figure 3 c). Taken together, this points towards a role of CD21 (CR2) rather than the BCR in the formation of these structures. Looking at *L. donovani*-induced protrusion formation in the two main splenic B cell subpopulations, we further identified the $CD21^{hi}$ MZB as the primary subset responsible for TNT-like protrusions (Supplemental Video 6), as opposed to the $CD21^{lo}$ FoB (Supplemental Video 7). Taken together, our results evidence an important role of CR2 in the induction of membrane protrusions by *L. donovani* amastigotes in B cells and in capturing parasites by B cells.

3.5.4 *Leishmania donovani* induces the production of an early IFN-I in B cells

In addition to the induction of TNT-like protrusions by crosslinking of CR2, other pathways may also promote the formation of these connections. In previous studies, IFNα has been identified to increase TNT formation in the human chronic myeloid leukemia cell line Kcl-22 (Omsland *et al.*, 2020) and our group has previously published an induction of IFN-I in B cells 8 hours after exposure to the parasite (Silva-Barrios *et al.*, 2016). Thus, we sought to measure whether the parasite could also induce type-I IFN at timepoints preceding the period in which we observe most connections to be formed, between 1 and 5 hours after exposure. Indeed, we found that exposure of B cells to *L. donovani* amastigotes induces a transient expression of *Ifna* and *Ifnb* mRNA at 30 min post-exposure by the B cells (Figure 4 a, b). This upregulation of *Ifna* and *Ifnb* mRNA was curtailed as it was no longer detectable at 60 min post-exposure to the parasite. Concurrently, we observed that this induced production of IFN-I leads to intracellular signaling through phosphorylation of STAT1, a key player of the JAK/STAT pathway, at 1h using Western Blot (Figure 4 c), as well as significant Y701 phosphorylation at both 45min and 60min in flow cytometry (Figure 4 d). Interestingly, although not statistically significant, the lack of type-I IFN signaling in *Ifnar1*$^{-/-}$ mice led to a lower formation of protrusions (Figure 4e), which could only be partially explained by a minimal, yet significant reduction in CD21 expression levels observed in both *Ifnar*$^{-/-}$ FoB and MZB compared with wild type cells (Supplemental Figure 4 a and b). This suggests that, in addition to inducing the upregulation of endosomal TLRs and amplifying hypergammaglobulinemia (Silva-Barrios *et al.*, 2016), IFN-1 may also be involved in the formation of TNT-like protrusions and the subsequent early parasite dissemination among B cells.

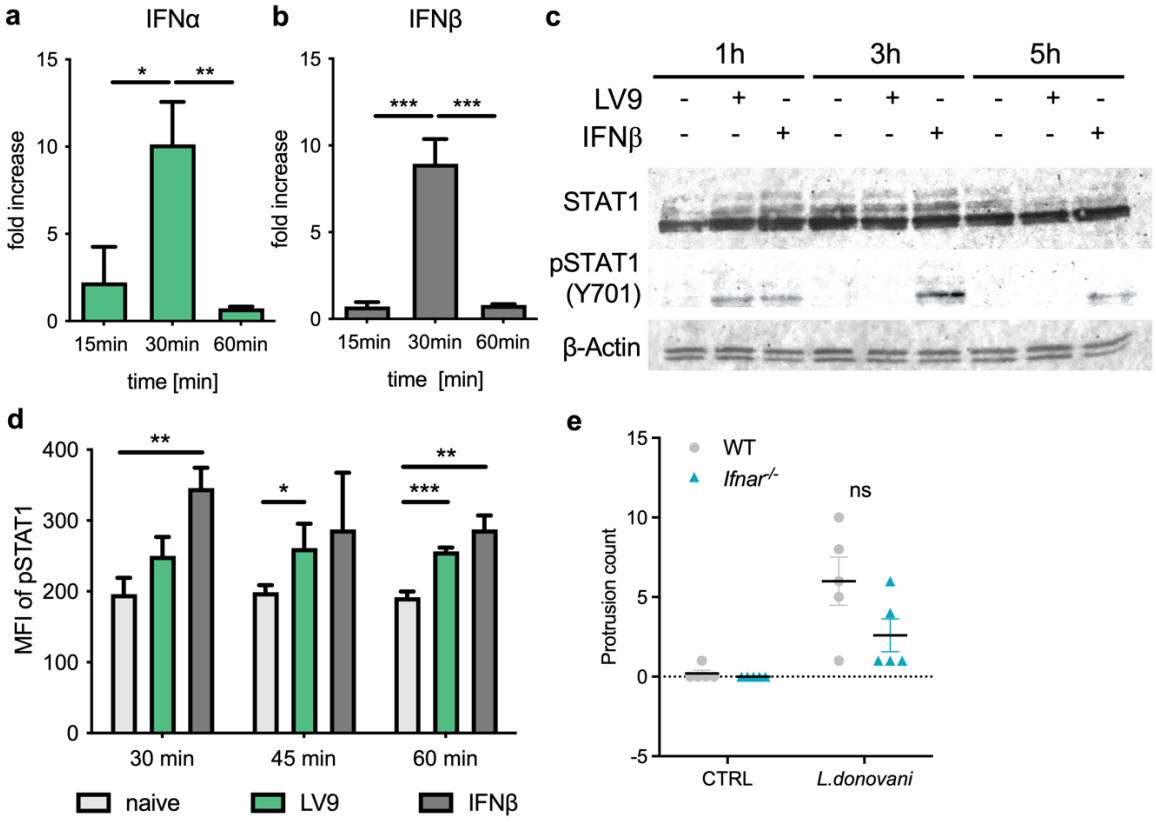

Figure 9 *L. donovani* induces a transient production of IFN-I leading to autocrine signaling in B cells.

Purified B cells were exposed to *L. donovani* amastigotes (MOI 1:10) or recombinant IFNβ as a positive control. (**a**,**b**) Fold change of IFNα (**a**) and IFNβ (**b**) mRNA expression in splenic B cells exposed to *L. donovani* relative to unexposed B cells as measured by RT-qPCR. (**c**) Protein expression of STAT1 and p-STAT1 (pY701) using β-actin as a loading control. (**d**) mean fluorescence intensity (MFI) of p-STAT1 expression (pY701) as assessed by flow cytometry. (**e**) B cells from control (C57BL/6) or *Ifnar1*$^{-/-}$mice were incubated for 2 -5h with or without the presence of fluorescently labeled *L. donovani*. Protrusions between B cells with or without parasite were counted over 70 microscope fields per sample. Data represented as mean ± SD from one of three to four independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

3.5.5 *L. donovani* amastigotes are present in the splenic B cell area of infected mice

Having now characterized the interactions of B cells and *L. donovani in vitro*, we then set out to investigate whether such interactions also take place *in vivo*. In fact, despite macrophages being regarded as the primary target of *L. donovani* infection (Kaye & Scott, 2011), previous work from

our laboratory demonstrated that B cells isolated from infected mice can be seen to carry the parasite *ex vivo* (Bankoti *et al.*, 2012). As this interaction could occur following release of parasite during tissue homogenisation, we generated cryosections of the spleens of mice infected with *L. donovani* on various timepoints post-infection, and we found *L. donovani* to be present in the B cell area as early as day 14 (Supplemental Figure 5), with the number of parasites increasing towards later stages of disease (Figure 5 a, b). These results definitively show that B cells are in direct contact with *L. donovani in vivo*, and thus could be activated through this direct interaction with the parasite.

3.5.6 *L. donovani* amastigotes can be transferred between macrophages and B cells via TNT-like protrusions

As we have now verified that *L. donovani* can indeed be found in contact with B cells *in vivo*, we sought to investigate how the parasite might come to enter the B cell area in the first place. As macrophages represent the primary infection target for *Leishmania* (Kaye & Scott, 2011) and are in close proximity to B cells in the marginal sinus of the spleen (Mebius & Kraal, 2005), we then chose to look at whether these different types of immune cells are capable of transferring *L. donovani* amastigotes among each other. Strikingly, we observed that even in the presence of macrophages, B cells still capture a significant number of amastigotes, albeit to a much lower degree than macrophages, when incubated together *in vitro* (Supplemental Figure 6 a). To see whether *L. donovani*-infected bone marrow-derived macrophages (BMM) can indeed transfer parasite to B cells, we differentiated macrophages from congenic CD45.2 mice, infected them with *L. donovani* amastigotes overnight and, after thorough washing to remove uncaptured parasite, we co-cultured these BMM with fresh B cells purified from CD45.1 mice. After both 2.5 and 5h hours of co-culture, a significant percentage of B cells stained positive for the parasite, indicating the transfer of *L. donovani* amastigotes from macrophages to B cells (Figure 6 a). The concomitant decrease of the percentage of infected macrophages after timepoint 0 (prior to the co-incubation with B cells) is likely due to both loss of parasites to B cells and clustering (Figure 6 a).

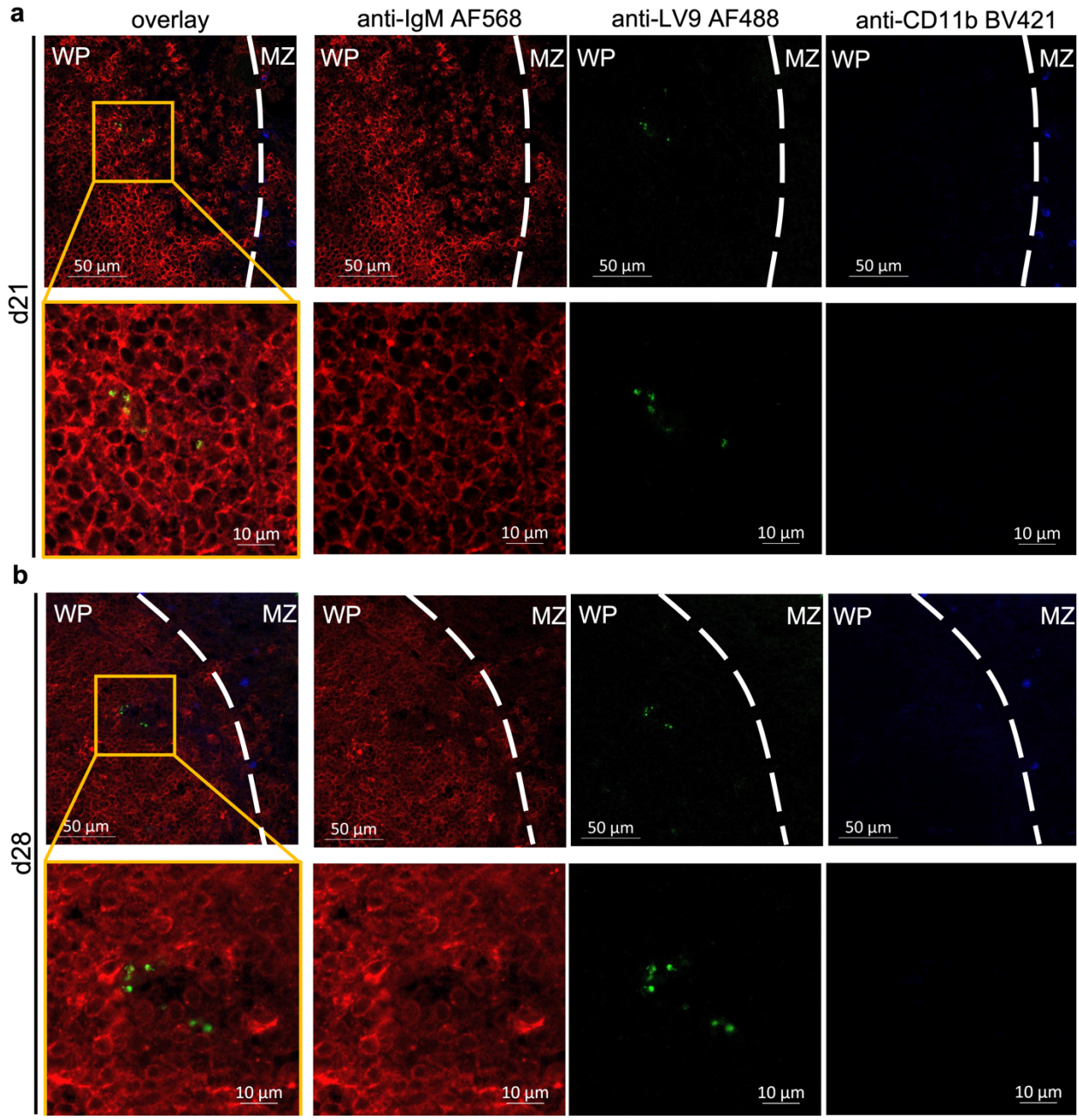

Figure 5 *L. donovani* is present in the splenic B cell area of infected mice.

Splenic cryosections *L. donovani*-infected mice at days 21 (a) and 28 (b) post-infection. The first row of each panel was taken using a 40x oil immersion objective while the second row represents an insert taken using a 63x objective at the location specified using a yellow square. Tissue sections were stained using IgM-AF568 (red) and BV421-conjugated CD11b (blue), and the parasite was stained by incubation with serum from L. donovani-infected hamsters and secondary anti-Hamster-AF488 antibody (green). Abbreviations - MZ=Marginal zone, WP=White pulp

Within the B cells staining positive for the *L. donovani*, MZB had a higher affinity towards capturing the parasite at both time points as opposed to FoB (Figure 6 b). Additionally, low levels of clusters between B cells and macrophages can be observed, which could point towards possible surface interactions between the two cell types. These heterogenous clusters carry parasite to a high degree and can also be observed during confocal microscopy of a co-culture of both immune cell types (Figures 6 d, e). Similar to transfer experiments between B cells, exposing fresh macrophages and B cells to the supernatants collected from exposed cells led to negligible capturing of parasite by these fresh cells, indicating that this transfer of cells is not due to capturing of free parasite from the medium (Figure 6 a). As expected, B cells that received *L. donovani* amastigotes from macrophages also upregulated MHCII and CD86, indicating activation (Figure 6c). Interestingly, these markers were also slightly but significantly upregulated in B cells exposed to the *L. donovani* carrying BMM which were not carrying parasite at the time of measurement, which may be due to loss of parasite after activation. One possible mechanism of loss of parasite besides the transfer of amastigotes to other naïve B cells could be the reverse transfer of parasite from the B cells to BMM. Indeed, we found that parasites could also be transferred from B cells to macrophages, as initially non-exposed macrophages can be seen to carry substantial amounts of parasite after both 2.5 and 5h of co-culture with B cells initially exposed to *L. donovani* amastigotes (Supplemental Figure 6 b).

To determine whether whole parasites or parasite components were transferred between macrophages and B cells, we performed similar experiments, where we directly seeded and infected BMM on confocal microscopy slides and after thorough washing we co-cultured them with fresh B cells. As with flow cytometry, we saw capturing of parasite by B cells, indicating that transfer indeed occurred (Figure 6d). On these slides, all parasites were observed to be bound by either B cells or macrophages, further solidifying that the measured transfer is not due to capturing of free parasite from the media. Strikingly, we also observed the formation of *L. donovani* amastigote-carrying TNT-like protrusions between B cells and macrophages (Figure 6e), which lays grounds for a possible role of this route of intercellular communication in this transfer.

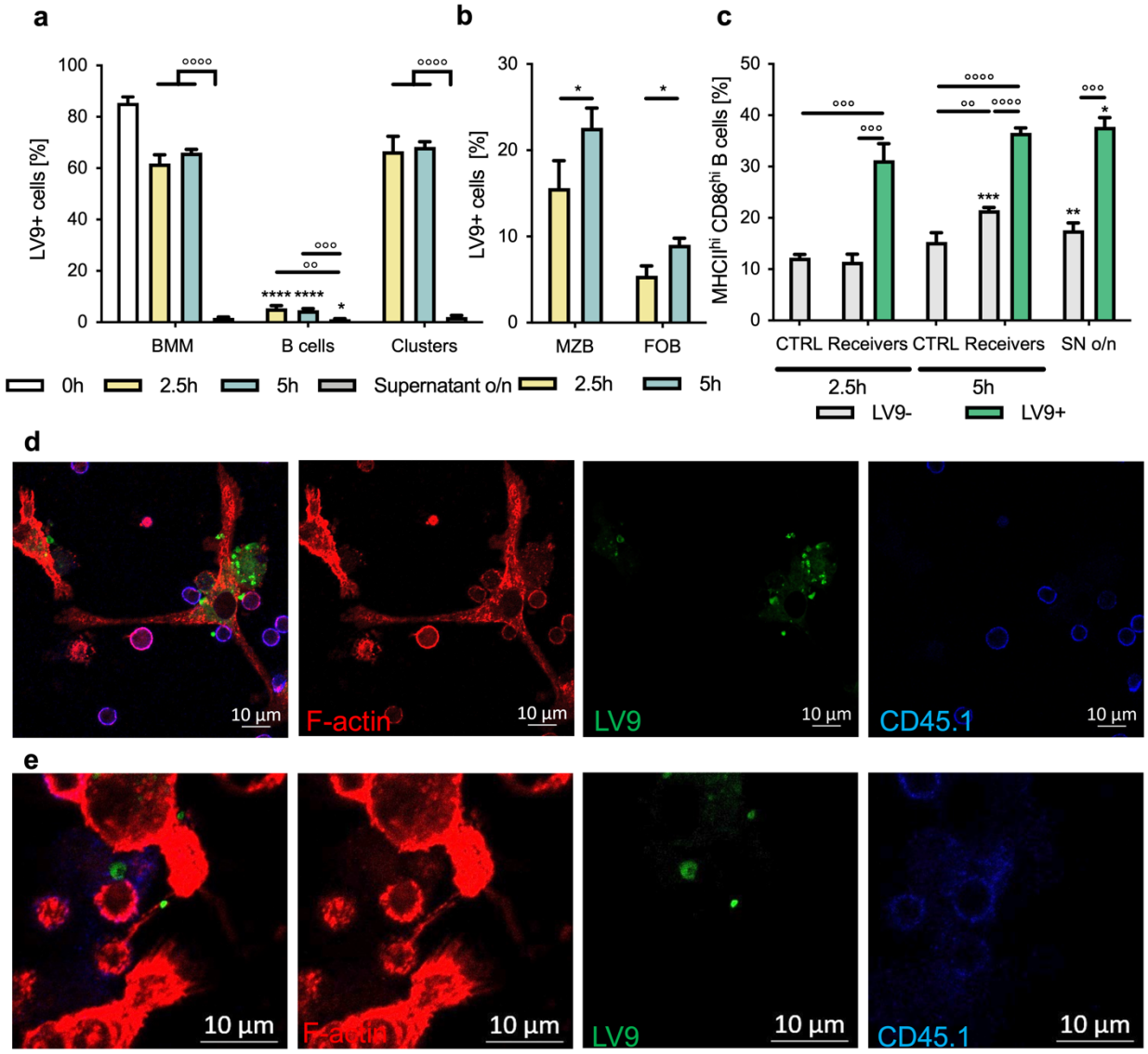

Figure 10 *L. donovani* amastigotes can be transferred between macrophages and B cells via TNT-like protrusions.

Macrophages derived from the bone marrow of CD45.2 mice (BMM) were exposed to *L. donovani* (MOI 1:10) overnight before thorough washing to remove uncaptured parasite and exposure to naïve B cells purified from CD45.1 for 2.5h or 5h, at a 1:1 ratio. (**a**) Percentage of cells carrying PKH67-stained amastigotes in cells separated into CD45.1$^+$ B cells, CD45.2$^+$ BMM, or CD45.1$^+$CD45.2$^+$ clusters as measured by flow cytometry. (**b**) Percentage of MZB (CD21^{hi} CD23^{lo}) and FoB (CD21^{lo} CD23^{hi}) cells carrying parasite within the initially non-exposed CD45.1$^+$ B cells. (**c**) Percentage of B cells expressing high levels of CD86 and MHCII separated into cells carrying parasite (LV9$^+$) and cells not in contact with the parasite (LV9$^-$). (**d-e**) Representative confocal microscopy images of BMM infected with PKH67-labeled *L. donovani* amastigotes (green) and co-cultured with naïve B cells for 5 hours. Immunofluorescence staining using Phalloidin-AF594 (F-actin, red), and CD45.1 (B cells, blue). Data represented as mean ± SD from one of four independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3.6 Discussion

To date, the mechanisms underlying the detrimental polyclonal B cell activation observed during visceral leishmaniasis caused by *Leishmania donovani* are still poorly understood. This study identifies the formation of tunneling nanotubules between B cells and the consequent cluster formation as a possible mechanism through which these cells disseminate *L. donovani* among each other, spread activation, and contribute to polyclonal B cell activation. Moreover, we propose that MZB acquire parasites or parasites' components from infected macrophages via TNT-like protrusions and then shuttle to the splenic B cell area, where FoB will then get activated by the parasite.

While we have previously shown that B cells purified from the spleens of mice infected with *L. donovani* can be seen to be in direct contact *ex vivo* (Bankoti *et al.*, 2012), the significance of this direct contact between cell and parasite for B cell activation has not been previously studied, as there could be soluble messengers, such as extracellular vesicles and cytokines, present, which might mediate the spread of activation among B cells. In fact, *Leishmania* parasites are well known to be able to spontaneously shed extracellular vesicles or exosomes, both in their amastigote, promastigote and metacyclic form (Silverman *et al.*, 2010; Castelli *et al.*, 2019; Forrest *et al.*, 2020). These vesicles are typically 50-150 nm in size and have been assigned various immunomodulatory functions in leishmaniasis, including the modulation of cytokine production and the regulation of intracellular signaling pathways (Silverman & Reiner, 2011; Castelli *et al.*, 2019). While not much about the action of *Leishmania*-derived extracellular vesicles on B cells is known to date, there is previously published evidence that exosomes spontaneously shed from *Leishmania amazonensis* promastigotes are able to activate peritoneal B-1 cells to produce IL-6 and TNF (Barbosa *et al.*, 2018). Another group of soluble mediators known to be both produced by and act on B cells are cytokines. Indeed, the production of cytokines such as IL-10, IL-6, and IFN-I by different splenic B cell subsets in response to *L. donovani* has been demonstrated in multiple studies (Deak *et al.*, 2010; Silva-Barrios *et al.*, 2016; Mondal *et al.*, 2021). Despite choosing permeable membrane inserts of a pore size sufficient to allow for the passage of cytokines, as well as vesicles substantially bigger than the average size of shed particles reported in the literature, however, we see no increase in activation in the naïve B cells that are only exposed to any soluble mediators but cannot establish direct contact with the parasite, indicating that in the case of B cell activation by *L. donovani* amastigotes, direct interaction of the parasite with naïve B cells is required for their activation.

In addition to direct contact with the parasite, we find expression of MHCII and activation markers such as CD86 to be even further increased in B cells forming clusters among each other. This could possibly be explained through interactions at the surface of B cells. Indeed, B cells have been proposed to be able to expand the number of antigen-binding B cells by transfer of BCR via close contact membrane exchange with bystander B cells, which is increased in cells undergoing BCR activation (Quah *et al.*, 2008). Hence, the increased activation in B cell clusters could be due to the transfer of BCR between B cells. Another possible mechanism of how these clusters could elevate activation above the level observed in singlet B cells in contact with the parasite could be through surface receptor interactions, as upregulation of CD86 and MHCII by B cells has been linked to increased antigen presentation [33]; however antigen presentation by B cells has been found to not greatly contribute to the exacerbation of VL (Deak *et al.*, 2010), making it unlikely that this represents a major mechanism of B cell activation in the course of this disease.

In this study, we find that direct contact with *L. donovani* amastigotes induces the formation of primarily actin-based long tubular membrane protrusions by the B cells. While the formation of short membrane protrusions and spreading of the B cells is associated with B cell activation (Fleire *et al.*, 2006), we observed the protrusions formed upon exposure to the parasite to be longer and able to connect two cells. Similar structures, called tunneling nanotubules (TNTs), have been identified in many different cell types, including immune cells, such as T cells and macrophages, and were shown to be involved in intercellular trafficking of various cargo, ranging from nucleic acids and proteins to whole organelles (reviewed in (Mittal *et al.*, 2019)). B cells were also found to be capable of forming tunneling nanotubules among themselves (Osteikoetxea-Molnar *et al.*, 2016), with T cells (Rainy *et al.*, 2013b) and macrophages (Xu *et al.*, 2009). These B cell TNTs were shown to enable the transport of plasma membrane proteins on the outside of the tubules as well as allow passage of microvesicles through the inside of the tubules (Rainy *et al.*, 2013b; Halasz *et al.*, 2018). In other cell types, previous studies have demonstrated these tubules to be exploited for disease propagation by bacteria, including *Listeria monocytogenes* (Tilney & Portnoy, 1989)*, Mycobacterium bovis* (Önfelt *et al.*, 2006), and *Chlamydia trachomatis* (Jahnke *et al.*), and viruses, such as HIV-1 (Hashimoto *et al.*, 2016), Influenza A (Ganti *et al.*, 2021) and SARS-CoV-2 (Merolli *et al.*, 2022). Thus, the involvement of these TNT-like protrusions in the dissemination of *L. donovani* among B cells represents the first report of this mechanism of intercellular communication in the context of a parasitic infection.

Interestingly, we find *L. donovani* present in the splenic B cell area as early as 14 days post-infection, evidencing the direct contact between B cells and the parasite *in vivo*, with a visible

increase in the number of parasites found at 28 days, which coincides with the loss of germinal centers in the spleen during the chronic stages of visceral leishmaniasis (Smelt *et al.*, 1997a). This is in line with previous findings of B cells from the spleens of infected mice carrying parasite *ex vivo* (Bankoti *et al.*, 2012). How these parasites come to be in the B cell area is not yet understood. One possible mechanism is the transfer of parasites from macrophages, which represent the main target for *L. donovani* and populate the marginal zone of the spleen along with the MZB, to B cells. Indeed, we find that macrophages and B cells are capable of transferring *L. donovani* bi-directionally and that this transfer can be facilitated by the formation of TNT-like connections between the two immune cell types. This is supported by a previous study in which TNTs have been shown to be formed between macrophages and B cells, where they aided in the transport of the immunosuppressive protein negative factor (Nef) involved in the pathogenesis of HIV and the development of immune dysfunction (Xu *et al.*, 2009). As MZB cells have been shown to be the predominant B cells to carry *L. donovani in vivo* (Bankoti *et al.*, 2012) and we consistently see MZB to capture the parasite to a higher degree *in vitro* in this work, marginal zone shuttling represents a plausible mechanism through which parasites could be transported into the B cell area of the spleen after parasite transfer from macrophages to MZB. A similar shuttling mechanism in which ligation of CD21 causes MZB to migrate into the white pulp has been described to deliver antigen (Cinamon *et al.*, 2008) or IgM-containing immune complexes (Ferguson *et al.*, 2004) to follicular B cells. Indeed, we have found CD21 to play a key role in capturing the parasite and inducing TNTs. This is further supported by the fact that amastigotes passaged in *Rag1*$^{-/-}$ mice are coated in C3 (Bankoti *et al.*, 2012), whose fragments are ligands for CD21, the complement receptor 2 (Prodinger, 1999). An additional consideration as to how the parasite could be exchanged between macrophages and B cells is its localization within the cells. While *Leishmania* resides in parasitophorous vacuoles within the macrophages, B cells do not fully internalize the parasite but rather carry it in actin-based cup-like structures on their surface (Bankoti *et al.*, 2012). Thus, a transfer of parasites between these cell types might require the parasite to exit the macrophage. A possible mechanism previously identified for *L. amazonesis* which may be at play here is the transfer of amastigotes enclosed in host macrophage membranes extruding from the cells (Real *et al.*, 2014). These LAMP1/LAMP2 rich structures can thus facilitate the exchange of parasites at a close range without full lysis of the macrophage. How the parasite can induce B cell activation after capture, however, remains to be fully elucidated. We have previously reported that hypergammaglobulinemia in *L. donovani* infection is exacerbated by an IFN-I-dependent upregulation of endosomal TLRs (Silva-Barrios *et al.*, 2016), and that, although the role of type I IFNs seems to depend on the strain of *Leishmania* and

the experimental used, IFN-Is play a negative role in mouse models of *L. donovani* infection (Silva-Barrios & Stager, 2017). While positive feedback loops of IFN-I signaling leading up to upregulation of TLR7 and 9 in B cells have been previously proposed (Green *et al.*, 2009; Thibault *et al.*, 2009), the presence of an early IFN-I wave that could signal on B cells to induce a detrimental upregulation of endosomal TLRs remained to be demonstrated. In this work, we show that the presence of the parasite induces a very early, transient expression of both *Ifna* and *Ifnb* mRNA, which we show to induce IFN-I signaling in the B cells within 1 hour of exposure to the parasite. Hence, this early production of IFN-I could induce the upregulation of endosomal TLRs, which in turn aggravate the disease by increased cytokine and antibody production; however, the pathway of induction of this early IFN-I remains to be elucidated, as B cell have been shown capable of IFN-I production induced via different pathways, such as (endosomal) TLRs and cytosolic sensors (Stögerer & Stäger, 2020). Furthermore, the extent to which other signaling pathways are synergistic with, or in parallel to, the enhanced endosomal TLR signaling leading up to polyclonal B cell activation remains to be elucidated in future studies.

In summary, we demonstrate the dissemination of *L. donovani* between B cells via the use of CD21-induced TNT-like protrusions, thus propagating B cell activation. Additionally, the transfer of amastigotes can occur bi-directionally and partially via TNT-like protrusions. This may lead to parasite acquisition by MZB cells from macrophages in the marginal zone and subsequent shuttling to the B cell area, ultimately leading to polyclonal B cell activation and hypergammaglobulinemia.

3.7 Acknowledgments

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3.8 Author contributions

T.S., S.S.-B., and S.S. conceived the project, designed the experimental approach, and interpreted data; T.S. and S.S.-B. performed experiments and analyzed data; L.C., L.T.M. and

S.Sw. performed experiments; A.D. provided key expertise, data interpretation, and revised the manuscript; M.J. and L.P.L. provided key reagents and revised the manuscript; T.S. and S.S. wrote the manuscript.

4 GENERAL DISCUSSION AND CONCLUSION

4.1 General discussion

In this work, we demonstrate a novel mechanism for the deleterious spread of B cell activation during VL via the formation of intercellular connections displaying all the characteristics of tunnelling nanotubes. In this first description of a role of TNTs in the context of parasite cell-to-cell spread, we report that *L. donovani* amastigotes can induce the formation of connecting protrusions among B cells via CR2 ligation and, to a lesser degree, IFNAR signalling. Exploitation of these connections formed both between macrophages and B cells and among B cells by the parasite allows for wide dissemination of the parasite and induces contact-dependent activation of B cells, resulting in polyclonal B cell activation, and ultimately, hypergammaglobulinemia.

4.1.1 Membrane connections formed between B cells are tunnelling nanotubes

While cells can spontaneously form various protrusions, many of which are primarily associated with cell motility, such as filopodia and lamellipodia, tunnelling nanotubes are structures with unique functions in intercellular communication. With their first descriptions dating back to only 20 years ago (Rustom *et al.*, 2004), however, these TNTs represent a relatively recent phenomenon and their definition has varied across studies. Due to the lack of a unique and specific marker shared across all cell types, the identification of TNTs most often relies on the fulfilment of three main criteria: (i) they are primarily actin-based structures, (ii) they connect two or more cells, and (iii) they are not connected to the substrate on which they are cultured (McCoy-Simandle *et al.*, 2016). The latter two criteria are particularly important for distinguishing them from other protrusions, such as those used for cell motility.

In this work, we report that B cells form actin-based protrusions that can connect two or more cells. These protrusions are not connected to the microscopy slides to which the cells adhere but are rather found hovering freely in the microscopy planes above the slide, all of which are well in line with the criteria set for tunnelling nanotubes (McCoy-Simandle *et al.*, 2016). Indeed, TNT formation has been previously reported between mature B cells of primary human origin and in B cell lines (Osteikoetxea-Molnar *et al.*, 2016). In the same study, B cell TNT formation was found

to commence approximately 1 h after incubation, which coincides with the protrusions observed in our model. Additionally, TNT formation between B cells was found to be restricted to mature B cells (Osteikoetxea-Molnar *et al.*, 2016). Using an immature marginal zone B cell line, X16C, we also observed that these cells lack the formation of connecting protrusions observed in mature primary B cells from mice (data not shown). Another defining characteristic of TNTs is the presence or absence of tubulin, which also governs the thickness of the observed connections (Önfelt *et al.*, 2006; Sanchez *et al.*, 2017). In our work, we primarily observed the formation of thicker tubulin-containing type II TNTs between B cells. While this could be due to the observation that thinner type I TNTs are more fragile than their tubulin-containing counterparts (Sanchez *et al.*, 2017), our findings are in line with B cell TNTs described by other groups (Osteikoetxea-Molnar *et al.*, 2016).

4.1.2 L. donovani *induces the formation of B cell tunnelling nanotubes*

Tunnelling nanotubes are a phenomenon observed between many different types of cells and they have been shown to be induced by many different stimuli, such as oxidative (Wang *et al.*, 2011b) or cytotoxic stress (Kato *et al.*, 2022). Notably, the exposure of cells to certain pathogens, including bacteria, viruses, and prions, has also been shown to induce the formation of TNTs, which could in turn be exploited for the spread of the disease.

In this work, we show that exposure to the parasite *L. donovani* can augment the number of connecting protrusions formed between B cells. While B cells and other cell types form these tunnels at a lower steady state in the absence of specific stimuli (Osteikoetxea-Molnar *et al.*, 2016; Jahnke *et al.*, 2022), the number of TNTs formed significantly increases in the presence of a pathogenic stimulus. Interestingly, rather than being triggered by BCR activation, we found that crosslinking complement receptor 2 (CR2) induced the formation of cellular connections to a level similar to that induced by exposure to the parasite. An interaction between the parasite and CR2 is in line with previously published observations from our lab, showing that freshly purified *L. donovani* amastigotes are coated with C3 which forms the ligand of CR2 (Bankoti *et al.*, 2012). *Leishmania* parasites are known to display a level of resistance to complement lysis in some life stages, including their amastigote and meta-cyclic forms (Nylen & Gautam, 2010). *L. donovani* amastigotes likely trigger CR2 crosslinking on B cells via C3 bound to their surface which in turn can induce TNT formation. This explanation is further supported by the observation that MZB

cells, which differ from FOB cells in their higher CR2 expression, are the predominant mature splenic B cell subset that forms these connections upon exposure to the parasite. While most studies on TNT formation in B cells to date have employed human and murine cell lines, tonsillar primary B cells (Osteikoetxea-Molnar *et al.*, 2016; Toth *et al.*, 2017; Halasz *et al.*, 2018), and atypical B cells (Polak *et al.*, 2015; de Rooij *et al.*, 2017; Scarabelli *et al.*, 2021), the formation of cellular connections with both MZB and FoB cells in the spleen has also been documented (Xu *et al.*, 2009; Huang *et al.*, 2021). However, these studies focused on heterogeneous TNTs and did not compare the frequency of connections between the splenic subsets.

The mechanism by which CR2 induces the formation of tunnelling nanotubes remains to be elucidated. In macrophages, complement receptors play a vital role in the recognition of *Leishmania* infection. Indeed, complement receptor 3 (CR3) which recognises C3 fragments, and more specifically, inactivated complement protein 3b (C3bi), bound to the parasite, is a key receptor mediating its phagocytosis by macrophages (Ueno *et al.*, 2009). Similarly, CR3 was shown to participate in the internalisation of *L. amazonensis* promastigotes by B-1 lineage B cells; however, in these cells, blocking the mannose receptor (MR) more efficiently inhibited the phagocytosis of parasites, indicating that the contribution of CR3-mediated parasite sensing is secondary in B-1 cells (Geraldo *et al.*, 2016). However, as descendants of the B2 lineage, both MZB and FOB cells are thought to possess limited phagocytic capabilities. Nevertheless, these cells are not only able but also required to internalise antigens to mount an optimal antibody response and this process is accompanied by extensive remodelling of the actin cytoskeleton (Martinez-Riano *et al.*, 2018). The same study found that the phagocytic capacity of B2 lineage cells appears to be limited to smaller antigen-carrying particles of less than 3 μm. It is thus possible that exposure of B cells to *Leishmania* parasites induces frustrated phagocytosis, resulting in remodelling of the cytoskeleton to form an actin-based cup-like structure around the amastigote and exerting cellular stress on the cells which in turn may induce the formation of cellular protrusions. In contrast to the integrin-family CR3, CR2 shows great structural differences and is not known to participate in phagocytosis (Vandendriessche *et al.*, 2021). In fact, in B2 cell lineage B cells, phagocytosis of antigen-coated beads was proposed to be mediated through BCR signalling rather than through CR2 (Martinez-Riano *et al.*, 2018).

In our model, we found that crosslinking BCR using anti-IgM-coated beads was ineffective in inducing connecting protrusions in B cells. Rather, when simultaneously crosslinking both CR2 and BCR, these cells displayed a trend towards decreased TNT formation compared to those observed upon CR2 ligation. This could be due to steric effects, such as the TNT-neutral BCR

crosslinking simply reducing the level of CR2 crosslinking; however, another possibility is that, conversely, BCR stimulation reduces the number of TNTs formed. Indeed, the influx of Ca^{2+} induced downstream of BCR stimulation has been shown to negatively regulate the formation of these connections through its effects on actin cytoskeleton rearrangement (Osteikoetxea-Molnar *et al.*, 2016).

Additionally, while crosslinking CR2 mimicked the induction of tunnelling nanotubes between B cells *in vitro* and contributed to their formation during *L. donovani* exposure, the interactions at the interface of the cell and parasite may be more complex and may involve other receptors and signalling processes. While initially believed to predominantly participate in signalling on cells traditionally categorized as innate immune cells, Toll-like receptors have garnered considerable interest in lymphocytes in recent years (Stögerer & Stäger, 2020). During VL, triggering of B cell TLRs, and in particular endosomal TLRs, by the parasite induces the production of cytokines, including IL-10 and IFN-I, and participates in the development of hypergammaglobulinemia which is detrimental to the disease outcome (Bankoti *et al.*, 2012; Silva-Barrios *et al.*, 2016). Interestingly, the ligation of TLRs, namely endosomal TLR 7/8 and TLR2, has been reported to induce TNT formation in human monocytes (Shahar *et al.*, 2021). In addition to the detrimental role of B cell endosomal TLRs, including TLR7, in VL, TLR2 is known to be able to recognise *Leishmania* parasites via its ligand, lipophosphoglycan (LPG), which is abundantly present on promastigotes (Jafarzadeh *et al.*, 2019). As *L. donovani* expresses undetectable or very low amounts of LPG in their amastigote form (McConville & Blackwell, 1991), however, the contribution of this TLR to TNT induction in B cells remains to be investigated.

Lastly, soluble factors, such as cytokines released in response to *L. donovani* exposure, may also contribute to the formation of tunnelling nanotubes. The induction of TNTs by soluble mediators has been documented in the breast adenocarcinoma MCF-7 cell line, which formed these cellular tunnels when cultured in conditioned media from macrophages, although the authors of this study did not further investigate the nature of these mediators (Patheja & Sahu, 2017). Cytokines represent a major group of small soluble mediators acting on immune cells. Interestingly, the polarisation of macrophages towards a pro-inflammatory cytokine profile was shown to decrease the formation of TNT-like connections between these cells, indicating a possible link between cytokines and protrusion formation (Goodman *et al.*, 2019). Additionally, IFN-α treatment has been demonstrated to increase the number of tunnels between chronic myeloid leukemia cells (Omsland *et al.*, 2020). Our group and others have previously demonstrated the induction of cytokine expression by B cells in VL models (Deak *et al.*, 2010; Bankoti *et al.*, 2012; Silva-Barrios

et al., 2016). In this work, we have additionally observed an early IFN-I expression in B cells exposed to *L. donovani* which coincides with the onset of TNT formation and a trend towards lower formation of connecting protrusions in B cells from IFNAR-deficient mice, indicating a minor contribution of cytokine signalling to the formation of B cell nanotubes in response to *L. donovani* exposure along with CR2 crosslinking.

4.1.3 L. donovani *amastigotes exploit tunnelling nanotubes for their dissemination between cells*

4.1.3.1 *Cell-to-cell spread between B cells*

Unlike other types of protrusions, the main function of tunnelling nanotubes is to transport cargo between connected cells, making them vital instruments in intercellular communication. These exchanges may occur spontaneously as part of homeostatic processes or as a rescue mechanism after cellular stress or injury. Although this transport can be beneficial for cell survival, TNTs have been shown to be exploited by various pathogens to facilitate cell-to-cell spread.

While the subversion of these conduits by pathogen is well documented for viruses, bacteria and prions, reports of TNT formation in the context of parasitic infections are rare. In fact, most descriptions of nanotube-like structures in the context of parasites reported to date appear to facilitate parasite-parasite interactions and are not formed by host cells. Upon *Plasmodium falciparum* gametocyte activation, tunnelling nanotube-like protrusions containing actin, but not tubulin, were reported to form between sexual-stage parasites and established long-distance connections (Rupp *et al.*, 2011). Similarly, the parasite *Trichomonas vaginalis* was found to form tubular protrusions resembling nanotubes which could connect to the surrounding parasites. However, these protrusions appeared to be close-ended and were proposed to have a function in parasite clumping (Salas *et al.*, 2023). These TNTs likely represent the parasite equivalent of bacterial TNTs, which are formed between bacteria, including *Escherichia coli* and *Bacillus subtilis*, as well as heterogeneously between bacteria of different species to facilitate cytoplasmic exchanges (Baidya *et al.*, 2018). However, the role of these inter-pathogen TNTs in the spread of infection between host cells remains to be explored.

In this work, we report the intercellular spread of *L. donovani* amastigotes among B cells. While MZB cells were found to capture parasites more frequently, both MZB and FoB cells were shown

to be able to receive and donate amastigotes to previously parasite-free cells. Using the actin polymerisation inhibitor Cytochalasin D to suppress TNT formation, we further attributed a substantial amount of these transfers to exchanges via these connections. Inhibitors of actin cytoskeleton remodelling, despite their various effects on other cellular processes such as BCR microcluster and immune synapse formation (Li *et al.*, 2018), are the most commonly used methods to inhibit the formation of nanotubes and study their role in the exchange of cargo as no common mechanism of protrusion formation specific to TNT formation has been identified as of yet (Lehmann *et al.*, 2005; Önfelt *et al.*, 2006; Gousset *et al.*, 2009; Kumar *et al.*, 2017). Nevertheless, while we observed that Cytochalasin D-treated B cells retained the ability to capture parasites and upregulate costimulatory and activation markers, we cannot exclude the off-target effects of actin inhibition on other actin-mediated processes, such as BCR clustering and antigen extraction.

Interestingly, a recent study also reported the presence of nanotube-like structures emanating from a rhesus macaque kidney or human foreskin fibroblast cell line in the early stages of the interaction between *Toxoplasma gondii* tachyzoites (de Souza Teles *et al.*, 2023). This finding is in contrast to our observation that *T. gondii* tachyzoites from axenic cultures do not induce TNT formation between B cells. This discrepancy may be due to several factors, the most important of which is the experimental design of the aforementioned study. In an effort to synchronise the early interactions of parasites and cells, de Souza Teles and colleagues pre-treated cells with cytochalasin D for 30 min, before washing the cells and co-incubating them with tachyzoites. Although this strategy is useful for preventing parasite entry into host cells, it also effectively inhibits the formation of new nanotubes in response to the parasite. Thus, it is likely that the structures they observed were previously formed by the cells and were independent of the presence of the parasite. Indeed, nanotubes formed between kidney cells from different organisms in the absence of stimuli have already been documented in the first report identifying TNTs as a form of intercellular communication (Rustom *et al.*, 2004). Further support for this hypothesis comes from the fact that this study did not report the formation of tunnelling nanotubes connecting two cells, but rather showed the presence of broken or incomplete structures, which could be due to the gradual disintegration of pre-existing structures after Cytochalasin D treatment. However, the authors reported the association of parasites with the nanotubes, which could indicate the presence of a capturing and gliding mechanism on the outside of the nanotubes exploiting them for intercellular spread similar to *M. bovis* (Önfelt *et al.*, 2006). However, as Cytochalasin D treatment impairs entry of the parasite into cells, the possibility of dissemination

via the observed structures and location of parasites inside or outside the nanotubes remains to be studied.

In our model, we observed the co-localisation of *L. donovani* amastigotes with actin-based nanotubes between B cells; however, these images do not allow a definitive conclusion on whether the parasites are situated inside or outside the tube. Tunnelling nanotubes may be exploited by pathogens to spread to uninfected cells by passing from one cytosol to another, as is the case for viruses such as HTLV-1 (Omsland *et al.*, 2018), prions (Gousset *et al.*, 2009; Costanzo *et al.*, 2013) and some bacteria, including *C. trachomatis* reticulate bodies (Jahnke *et al.*, 2022), or by gliding along the membrane to reach uninfected cells, which was observed for murine leukaemia virus (Lehmann *et al.*, 2005) and *M. bovis* (Önfelt *et al.*, 2006). While it is likely that *L. donovani* amastigotes "surf" these nanotubes, as the parasite does not internalise into B cells but rather tightly attaches to the cell surface (Bankoti *et al.*, 2012), future work is required to better understand the mechanism by which these conduits aid in the parasite dissemination between B cells.

4.1.3.2 Cell-to-cell spread between macrophages and B cells

While B cells have been shown to be able to capture *L. donovani* amastigotes *in vitro* resulting in activation (Bankoti *et al.*, 2012; Silva-Barrios *et al.*, 2016), it is unclear whether these interactions can also occur *in vivo* in the spleens of infected animals. Here, we demonstrate that *L. donovani* can be found in the splenic B cell area of infected mice as early as 14 days post-infection and that both major populations of B cells, MZB and FoB cells, are able to capture amastigotes *in vitro*, with MZB preferentially capturing parasites. This is in line with previous findings in our laboratory, which demonstrated that in cells isolated from the spleens of mice 20h after infection with fluorescently labelled *L. donovani*, MZB cells were the primary B cell set carrying the parasite (Bankoti *et al.*, 2012). As macrophages are the main target of *Leishmania in vivo* (Bogdan, 2020) and MZB cells are located in the marginal zone in close proximity to macrophage populations such as marginal zone macrophages and marginal-zone metallophilic macrophages (Mebius & Kraal, 2005), it is likely that parasite-carrying MZB cells obtain *L. donovani* amastigotes from macrophages.

In this work, we report the formation of heterogeneous tunnelling nanotubes between B cells and macrophages, which appeared at a similar time as homogeneously formed TNTs and could be

observed to carry *L. donovani* parasites. The formation of nanotubes between macrophages and B cells has been previously documented in the context of HIV-1 infection, during which the virus-derived protein negative factor (Nef) spread from macrophages to B cells, leading to a decreased virus-specific humoral response (Xu *et al.*, 2009). In this model, Nef was able to induce the formation of TNTs originating from macrophages and targeting B cells; however, the dynamics of the observed TNT formation between macrophages parasitised with *L. donovani* and B cells remain to be explored.

Another aspect to consider in the dissemination of parasites between macrophages and B cells is the egress of *Leishmania* from the macrophages. In fact, while phagocytosis of the parasite by macrophages is well characterised, the dynamics of the cell-to-cell spread of infection within the infected host are still a subject of controversy during *Leishmania* infection. During natural infection via the bite of an infected sandfly, the number of parasites regurgitated into the host per bite is low. Indeed, a study on *L. infantum* infection by *Lutzomyia longipalpis* sandflies found that most bites result in the transmission of less than 300 parasites (Secundino *et al.*, 2012). Thus, replication and intercellular spread within the host are required to establish infection. Extensive replication of *Leishmania* inside macrophages is a well-established fact; however, the mechanism by which parasites egress from infected cells is not yet completely understood. For many years, replication of parasites inside macrophages was believed to cause sudden egress by rupture of the parasite (Florentino *et al.*, 2014); however, this model has been challenged by research proposing a more continuous release of parasites from macrophages. Indeed, extrusion of *L. amazonensis* amastigotes was observed during the long-term culture of murine bone marrow-derived macrophages through the formation of parasitised membrane blebs in cells undergoing apoptosis, which were subsequently taken up by uninfected macrophages (Real *et al.*, 2014). Similarly, the chemical induction of apoptosis using camptothecin in THP-1 macrophages infected with *L. aethiopica* or *L. mexicana* gave rise to considerable cell-to-cell spread, with the percentage of infected cells up to doubling within 12 h (Rai *et al.*, 2017). The induction of apoptosis in these phagocytes appears to be vital for this intercellular transmission mechanism. Infection with *L. amazonensis* induced Caspase-3-mediated apoptosis and inhibition of Caspase-3 was shown to hamper the spread of amastigotes to uninfected cells (Ranatunga *et al.*, 2020). A similar activation of Caspase-3 and cell-to-cell transmission is also observed in macrophages parasitised with *L. major* (Baars *et al.*, 2023). However, different *Leishmania* species have different capacities to induce apoptosis, and thus, this apoptosis-driven egress of parasites from macrophages may vary across parasite species. Interestingly, both causative agents of VL, *L. donovani* and *L. infantum* have been found to inhibit host cell apoptosis and Caspase-3 activation in macrophages to favour

parasite persistence (Gupta *et al.*, 2016; Cianciulli *et al.*, 2018). Thus, this mechanism of intercellular spread between macrophages remains to be investigated in the context of experimental VL in order to understand the potential implications for the transfer of *L. donovani* between macrophages and B cells.

4.1.4 L. donovani *dissemination via tunnelling nanotubes contributes to polyclonal B cell activation and resulting hypergammaglobulinemia*

While polyclonal B cell activation and hypergammaglobulinemia are hallmarks of VL, the processes underlying the detrimental non-protective activation of B cells are not yet understood. Here, we propose a mechanism involving serial transfers of *L. donovani* parasites from macrophages to (marginal zone) B cells and among the splenic B cell population through tunnelling nanotubes which spreads B cell activation and may ultimately result in hypergammaglobulinemia. This route of cell-to-cell spread may work in tandem with previously documented follicular shuttling of MZB cells. Indeed, in addition to capturing *L. donovani* amastigotes and inducing TNT formation in B cells, CR2 has been demonstrated to be vital for the follicular shuttling of T-independent antigens and immune complexes into splenic B cell follicles (Ferguson *et al.*, 2004; Cinamon *et al.*, 2008; Zhang *et al.*, 2014a). This not only represents an entry route of antigen into the B cell follicle, but also puts marginal B cells, which preferentially capture parasites and can be seen to carry *L. donovani* 20h post-infection (Bankoti *et al.*, 2012), into proximity with the larger follicular B cell subset which could facilitate exchanges among the B cell populations.

Additionally, we show that *L. donovani* amastigotes are present within the splenic B cell area as early as 14 days post-infection, with the number of parasites visibly increasing by day 28. The chronic phase of visceral leishmaniasis is associated with various changes in the splenic microarchitecture, including loss of the follicular dendritic cell network and involution of germinal centres which are inversely correlated with parasite control (Smelt *et al.*, 1997b). The onset of a TNF-mediated decline in marginal zone macrophages can also be observed in the spleens of *L. donovani-infected* mice as early as 14 days post-infection, and this effect is even more striking on day 28 (Engwerda *et al.*, 2002). Thus, while initially moving from macrophages to B cells in the early stages of infection, the spread of *L. donovani* from B cell to B cell may be especially relevant in the later stages of infection.

The presence of parasites in close contact with B cells is particularly important, as our results show that direct contact between cells and amastigotes is required to induce B cell activation. Indeed, separating B cells exposed to *L. donovani* amastigotes from naïve B cells which are unable to establish direct contact with the parasite using permeable membrane inserts, has demonstrated that soluble messengers alone are insufficient to induce B cell activation. These permeable membrane inserts are also known to be the most efficient method to inhibit TNT-mediated intercellular communication (Polak *et al.*, 2015), which further strengthens the role of these exchanges in the dissemination of B cell activation. While our results identify CR2 as vital for the capture of *L. donovani* amastigotes by B cells, the interactions between cells and parasites leading to B cell activation are still incompletely understood. In our group, we have previously demonstrated that direct exposure of B cells to *L. donovani* amastigotes triggers a detrimental MyD88-dependent IL-10 production (Bankoti *et al.*, 2012). A subsequent study further showed that the parasite triggers endosomal TLR activation, leading to the production of disease-driving cytokines, including IL-10 and IFN-I, the latter of which in turn enhances excessive antibody production resulting in hypergammaglobulinemia (Silva-Barrios *et al.*, 2016). In this study, Silva-Barrios *et al.* proposed the presence of a positive feedback loop of an early wave of type I interferon, which would upregulate the expression of endosomal TLRs and in turn amplify cytokine and antibody production, based on the observation that IFNAR-deficient mice showed abolished cytokine production. A similar IFN-I-mediated control of endosomal TLR expression in B cells has been proposed for TLR7 and TLR9 (Green *et al.*, 2009; Thibault *et al.*, 2009). In addition to a possible contribution of IFN-I to the increased intercellular spread of *L. donovani* via TNTs, our results corroborate this positive feedback loop by demonstrating the presence of an early, very transient expression of both IFN-α and IFN-β within 30 to 45 min of exposure. In line with the positive feedback loop demonstrated in these studies (Green *et al.*, 2009; Thibault *et al.*, 2009), this early wave of type I interferon can then upregulate the expression of endosomal TLRs, including TLR7 and TLR9, in B cells which in turn augments both the production of disease-driving cytokines such as IL-10 and the production of non-protective antibodies, resulting in hypergammaglobulinemia (Silva-Barrios *et al.*, 2016). The capacity of B cells to produce IFN-I during VL was also corroborated in VL patients newly admitted to a specialised clinic in India, as B cells from PBMCs of infected individuals were shown to substantially upregulate IFN-α and IFN-β mRNA (Kumar *et al.*, 2020). In addition to its effect on B cell responses leading to hypergammaglobulinemia, type I IFN has been implicated in other processes governing the immune response to VL. Indeed, this family of cytokines appears to play a dual role in the disease depending on the timing of cytokine action, as an early report showed that pre-treatment of mice

with IFN-I via injection of the TLR3 agonist poly(I:C) resulted in an improved parasite control in the liver, while treatment with poly(I:C) after infection led to increased parasite burden in both the liver and spleen (Herman & Baron, 1970). This negative role of IFN-I signalling during active infection with *L. donovani* was later confirmed in a study comparing the parasite load in *Ifnar*$^{-/-}$ and wild-type C57BL/6 mice, which found that the absence of IFN-I signalling through IFNAR resulted in a lower parasite burden in the liver and spleen which was consistently noticeable as early as 14 and 28 days post-infection, respectively (Kumar *et al.*, 2020). In the same study, the authors found that IFN-I likely signalled on DCs to suppress protective antigen-specific Th1 responses and associated IFN-γ production, thus impeding parasite control; however, B cell-intrinsic IFN-I signalling does not appear to directly affect Th1 cells during VL, as a B cell-specific IFNAR knockout failed to significantly improve the Th1 response (Silva-Barrios *et al.*, 2016). Additionally, the mechanism underlying the sensing of parasite by the B cell resulting in the observed IFN-I remains to be elucidated, as B cells have been shown to have functional cytosolic, endosomal, and surface sensing pathways which could potentially be responsible for the observed IFN-I production (Stögerer & Stäger, 2020).

Apart from innate immune sensing, polyclonal B cell activation may be induced by *Leishmania* antigens. Several antigens have been identified as potential polyclonal activators in B cells, including *L. major* Silent information regulatory 2 (*Lm*SIR2) (Silvestre *et al.*, 2006), *L. infantum* cytosolic tryparedoxin peroxidase (*Lic*TXNPx) (Santarem *et al.*, 2005) and *L. infantum* tryparedoxin (*Li*TXN1) (Menezes Cabral *et al.*, 2008). These molecules are highly conserved among parasite species and antibodies against these antigens were found to be present in the sera of *L. infantum*-infected children (Santarem *et al.*, 2005). Additionally, *Li*TXN1 has been shown to be able to induce IL-10 and increase antibody production by B cells, which is associated with increased disease severity (Menezes Cabral *et al.*, 2008). However, as these antigens are located in the cytosol of parasites and are considered to be secreted by parasites (Santarem *et al.*, 2007), the role of these antigens in the observed contact-dependent polyclonal B cell activation is unclear.

While hypergammaglobulinemia and the production of class-switched antibodies was found to be the main action through which B cells contribute to disease severity in VL caused by *L. donovani* (Silva-Barrios & Stäger, 2019), IL-10 is known to play a central role in the disease pathology of VL. In a BALB/c mouse model, IL-10 was shown to increase parasite growth permissibility in the liver by inhibiting efficient early granuloma formation, decreasing IL-12 production, and dampening the Th1 response (Murray *et al.*, 2002). This finding is also reflected in human VL

caused by *L. donovani*, as IL-10 blockade during the culture of cells from splenic aspirates of patients with active VL showed improved parasite clearance and elevated levels of IFN-γ and TNF (Gautam *et al.*, 2011). B cells, and in particular B_{regs}, are among the cell types contributing to elevated IL-10 levels. Indeed, B cells have been shown to produce substantial amounts of IL-10 in experimental VL caused by both *L. donovani* (Bankoti *et al.*, 2012) and *L. infantum* (Deak *et al.*, 2010), which contributes to disease severity but is secondary to antibody-mediated noxious effects (Deak *et al.*, 2010; Silva-Barrios *et al.*, 2016). Similarly, a population of B cells producing ample amounts of IL-10 was also identified in human tonsillar B cells and peripheral blood B cells exposed to *L. infantum* (Andreani *et al.*, 2015). This B cell-derived IL-10 was demonstrated to dampen the antigen-specific $CD4^+$ and $CD8^+$ T cell effector functions and thereby contribute to disease susceptibility (Bankoti *et al.*, 2012; Andreani *et al.*, 2015). Interestingly, in mice, MZB cells, which are the main B cell subset capturing *L. donovani* amastigotes and forming tunnelling nanotubes, were found to be responsible for approximately 60 % of B cell-derived IL-10 (Bankoti *et al.*, 2012), potentially lending further support to the contact-dependency of B cell-parasite interactions. Additionally, IL-10 has been linked to TNT formation in macrophages in the context of a *M. tuberculosis* (Mtb)-HIV-1 co-infection model. Indeed, elevated levels of IL-10 present during Mtb infection were reported to promote the formation of inter-macrophage tunnelling nanotubes via a pathway involving STAT3, which in turn synergistically aided in the viral spread of HIV-1 between cells (Souriant *et al.*, 2019). Thus, the high levels of IL-10 produced by B cells and other cells during VL may not only favour parasite persistence by dampening protective T cell responses, but also potentially contribute to the cell-to-cell spread of the parasite; however, the capacity of IL-10 to induce TNTs in B cells has yet to be investigated.

4.2 Conclusion

During visceral leishmaniasis, B cells are known to contribute to disease pathology via the production of disease-driving cytokines, including IL-10 and IFN-I, and polyclonal B cell activation resulting in hypergammaglobulinemia; however, the processes underlying this harmful non-specific B cell activation are still incompletely understood.

In this work, we demonstrate the formation of intercellular connections fitting the profile of tunnelling nanotubes between B cells in response to exposure to *L. donovani* amastigotes which can be exploited by the parasite to facilitate cell-to-cell spread. These actin-based and tubulin-containing tunnels are induced by complement receptor 2 crosslinking on B cells, which is likely facilitated through complement C3 bound to the surface of the parasites; however, triggering other pathways, including TLR7/8 and TLR2 ligation and IL-10 signalling, have also been shown to induce the formation of TNTs in some models and warrant future investigation in the context of inter-B cell communication during VL. Additionally, we observed an early, transient expression of both IFN-α and IFN-β by B cells in response to exposure to the parasite, which may not only promote nanotube formation but can also contribute to hypergammaglobulinemia by an IFNAR-dependent upregulation of endosomal TLRs which are triggered by *L. donovani* and promote the expression of disease-enhancing cytokines such as IL-10 and IFN-I, the latter of which further augments the production of non-protective antibodies (Silva-Barrios *et al.*, 2016). B cell activation was found to be dependent on direct contact between cells and the parasite, indicating an important role for TNT-mediated cellular spread. Indeed, we found that *L. donovani* parasites can be observed in the B cell area of the spleens of infected mice as early as day 14 post-infection, supporting the notion that these interactions can occur *in vivo*. In agreement with the observation that marginal zone B cells are the predominant B cell subset carrying *L. donovani ex vivo* (Bankoti *et al.*, 2012), we found that macrophages, which are the main target of *Leishmania in vivo* and populate the marginal zone of the spleen, are capable of transmitting parasites to B cells via the formation of heterogeneous tunnelling nanotubes. Follicular shuttling in conjunction with inter-B cell nanotube formation then likely contributes to the dissemination of amastigotes among B cells, resulting in contact-dependent B cell activation across many different B cell clones and ultimately, hypergammaglobulinemia. However, while CR2 is a key player in capturing *L. donovani* amastigotes and inducing B cell TNTs, the interactions between B cells and the parasite leading up to endosomal TLR triggering and the resulting harmful B cell activation remain to be elucidated. Multiple *Leishmania* antigens that may act as polyclonal activators have been identified to date and may serve as a starting point for future studies. As a relatively recent field of research, the study of tunnelling nanotubes is limited by the lack of discovery of a pan-TNT marker and a conserved mechanism of formation across cell types. Hence, current methodologies rely heavily on the use of non-specific inhibitors, such as actin depolymerisation agents, to block the formation of nanotubes; however, actin cytoskeleton remodelling is central to many other cellular processes, including BCR microcluster formation (Li *et al.*, 2018). The discovery of a specific inhibitor of this

intercellular communication pathway would be invaluable to further elucidate the contribution of these structures to pathogen spread between cells.

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6 APPENDIX I – LITERATURE REVIEW

Title: Innate immune sensing by cells of the adaptive immune system

Titre en français: Détection de l'immunité innée par les cellules du système immunitaire adaptatif

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6.1 Abstract

Sensing of microbes or of danger signals has mainly been attributed to myeloid innate immune cells. However, T and B cells also express functional pattern recognition receptors (PRRs). In these cells, PRRs mediate signalling cascades that result in different functions depending on the cell's activation and/or differentiation status, on the environment, and on the ligand/agonist. Some of these functions are beneficial for the host; however, some are detrimental and are exploited by pathogens to establish persistent infections. In this review, we summarize the available literature on innate immune sensing by cells of the adaptive immune system and discuss possible implications for chronic infections.

6.2 Introduction

For many years, sensing of conserved structures called pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPS) was thought to be a prerogative of myeloid cells of the innate immune system, such as macrophages, dendritic cells or neutrophils. In the context of infectious diseases, innate immune sensing is responsible for launching a potent initial inflammatory response aimed to non-specifically eliminate invading pathogens. This initial defense mechanism is typically followed by a more specific and targeted response, which is orchestrated by cells of the adaptive immunity, namely B and T lymphocytes. It is owed to this notion that PAMP and DAMP sensing is extensively researched in innate immune cells, whereas innate immune sensing in lymphocytes has only recently been demonstrated and literature on physiological and pathological implications is still sparse. These mechanisms have perhaps been previously overlooked because lymphocyte activation typically occurs at later stages of infection, when inflammation is already established; however, recent evidence suggests that innate immune signaling can not only participate in lymphocyte maturation and improvement of B and T cell responses, but also be hijacked by pathogens such as *Leishmania donovani* to exacerbate detrimental immunosuppressive effects and induce hypergammaglobulinemia.

For the purpose of this short review, we will discuss the emerging field of innate immune sensing by cells of the adaptive immunity and its implications in *Leishmania* and other neglected tropical diseases such as Trypanosomiasis in two parts, placing individual focus on the two major actors of adaptive immunity, B and T lymphocytes.

6.3 Innate immune sensing in B cells

6.3.1 Expression and functions of innate immune sensors in B cells

Toll-Like Receptors (TLR) were the first innate immune sensors to be attributed a role in B cells. TLRs are a family lectin-rich repeats containing transmembrane proteins located on the cell surface (TLR1, TLR2, TLR4-6, and TLR10-11) or inside the endosome (TLR3, TLR7-9). Murine B cells express TLR1-4, TLR6, TLR7 and TLR9 at varying levels in different subsets (Gururajan *et al.*, 2007), while TLR expression on human B cells includes TLR1, TLR2, TLR6, TLR7, TLR9 and TLR10(Hornung *et al.*, 2002), and in the case of plasma cells also TLR3 and TLR4(Dorner *et al.*, 2009). Several beneficial roles of TLR-mediated sensing in B cell have been demonstrated, including the promotion of B cell maturation through TLR4 stimulation (Hayashi *et al.*, 2005b; Yanaba *et al.*, 2009) and enhanced antigen presentation by TLR9 ligation(Jiang *et al.*, 2007). While TLR engagement has been proposed to act as an additional signal to B cell receptor (BCR) stimulation (Ruprecht & Lanzavecchia, 2006b), B cells have been shown to produce various cytokines and chemokines solely from TLR triggering. (Agrawal & Gupta, 2011)

Recent discoveries of cytosolic innate immune sensing pathways, including sensing of cytosolic DNA involving the adaptor protein stimulator of interferon genes (STING, also termed MYPS, MITA, ERIS) or cytosolic RNA via the adaptor MAVS have greatly advanced our understanding of immunity. A variety of sensor proteins, such as cyclic GMP-AMP synthase (cGAS) and interferon-induced protein 16 (IFI16), have been proposed to directly interact with DNA, leading to enzymatic generation of a secondary messenger molecule in the form of cyclic dinucleotides (CDNs), such as 2'3'-cyclic guanosine monophosphate-adenosine monophosphate (cGAMP). These CDNs can then activate STING on surface of the endoplasmic reticulum to interact with TANK-binding kinase 1 (TBK1), resulting in phosphorylation of interferon regulatory factor 3 and subsequent IFN-I production (Chen *et al.*, 2016).

Expression and functionality of STING and its pathway have also been demonstrated in B cells, although there is a discrepancy between cells of human and murine origin. Reports unanimously confirm STING expression in murine B cells, and have demonstrated that B cells are capable of responding to STING stimulation by production of IFN-I (Jin *et al.*, 2008; Marcus *et al.*, 2018); however, conflicting literature exists on STING expression and function in human B cells. In one study on peripheral blood mononuclear cell (PBMC)-derived human B cells, the presence of STING was confirmed via both flow cytometry and qPCR (Dong *et al.*, 2015), while another study failed to detect STING via RT-qPCR in primary B cells from tonsils and PBMCs, but confirmed

the expression of upstream (cGAS, IFI16) and downstream (TBK1, IRF-3) signaling partners (Gram *et al.*, 2017). One possible explanation for the observed differences could be differences in the EBV-status of donors, as STING has been shown to be expressed in EBV-positive B cell lines, but not in EBV-negative cell lines. Both aforementioned studies did not observe IFN-I production from human B cells upon transfection of dsDNA or its synthetic homologues, which could be due to the low transfection efficacy into B cells or point towards an intrinsic defect of the STING signaling pathway in human B cells; however, Dong et al. observed a negative regulatory role of STING signaling in B cells on the JAK1-STAT1 pathway, suggesting a functional role of STING in B cells (Dong *et al.*, 2015). STING activation has also been shown to upregulate costimulatory molecules, such as CD86, across all B cell subsets, have adjuvant activity following immunization with thymus-dependent antigens, improving antigen-specific antibody responses, and mediate apoptosis both in normal and malignant B cells (Tang *et al.*, 2016; Walker *et al.*, 2018).

Another important nucleic acid sensing pathway in the cytosol is the pathway involving mitochondria antiviral-signaling protein (MAVS, also termed VISA, IPS-1 or Cardif). Several proteins have been suggested to act as cytosolic RNA sensors, including retinoic-inducible gene-I (RIG-I) and melanoma differentiation-associated gene-5 (MDA-5). These sensors can then cause aggregation of MAVS, leading to the activation of IRF-3 and IRF-7, NF-κB and production of IFN-I (Radoshevich & Dussurget, 2016).

Both MAVS and its upstream sensors, RIG-I and MDA-5, have been shown to be expressed in B cells of human and murine origin, and were demonstrated to have a functional sensing pathway. Stimulation of B cells using the synthetic RNA analogue poly(I:C) was shown to induce cytokines, predominantly IFN-β and IL-6, and to a lower degree IFN-γ, in a MAVS-dependent manner (Gram *et al.*, 2017; Loetsch *et al.*, 2017). Additionally, triggering of the RIG-I/MAVS pathway using 5'-ppp-RNA was shown to be an effective adjuvant in influenza vaccination, leading to a long-lasting antibody response of improved specificity (Kulkarni *et al.*, 2014).

6.3.2 Pathological implications of innate immune sensing in B cells

The identification of a role of innate immune sensing in the cytokine and antibody production in B cells attracted considerable attention in the field of autoimmunity research. Multiple studies have confirmed the involvement of TLR signaling, in particular MyD88-dependent TLRs and endosomal

TLR7 and TLR9, in autoreactive B cell activation and germinal center (GC) formation(Soni *et al.*, 2014), autoantibody production (Leadbetter *et al.*, 2002a; Lau *et al.*, 2005), and development of autoantibody-related pathologies such as glomerulonephritis in the context of the human disease systemic lupus erythematosus (SLE) (Nakano *et al.*, 2008; Hwang *et al.*, 2012) and in models using the lupus-prone mouse strain MRL/lpr (Christensen *et al.*, 2006).

In a mouse model of the IFN-related autoimmune condition Aicardi-Goutières Syndrome (AGS), which is mimicked by a deletion of dsDNA-degrading protein 3' repair endonuclease 1 (Trex1), B cells were shown to be responsible for the development of glomerulonephritis and greatly contributed to disease-related mortality (Gall *et al.*, 2012). A different study on lupus demonstrated a negative regulatory effect of STING on JAK1-STAT1 activation and found decreased STING expression in B cells from SLE patients and MRL/lpr lupus-prone mice (Dong *et al.*, 2015).

MAVS, on the other hand, seems to be involved in regulation of germinal center formation. The formation of spontaneous germinal centers (Spt-GCs), whose dysregulation is associated with SLE and other autoimmune diseases, was shown to be dependent on MAVS and TLR7 expression in mice, and TLR7 ligation could only partially reinstate the Spt-GC development (Schell *et al.*, 2019). Another study not only confirmed that MAVS in B cells is required for the formation of autoreactive GCs and autoantibody production in lupus-susceptible mice, but additionally linked its expression to the development of proteinuria and glomerulonephritis (Sun *et al.*, 2019).

Signaling through pattern recognition receptors (PRR) in B cells was also reported to dysregulate processes leading to antibody production. Recent evidence suggests that innate immune activation might directly contribute to detrimental antibody production, as increased TLR7 signaling has been observed to favor differentiation of lupus-associated $CD27^{-}IgD^{-}$ B cells into plasma cells excreting autoreactive antibodies, although co-stimulation by IL-21 and IFN- γ along with TLR7 is required to differentiate naïve B cells into these double negative and plasma cells. Furthermore, B cells from SLE patients were found to have increased expression of genes involved in innate RNA sensing, including TLR7, TBK1 and TRIM56, an inducer of STING (Jenks *et al.*, 2018). This is consistent with observations of a prominent IFN-I signature in SLE and other autoimmune diseases, which was shown to further upregulate TLR7 and TLR9 expression, thereby potentially amplifying detrimental autoantibody production (Thibault *et al.*, 2009).

Polyclonal B cell activation and subsequent excessive generation of antibodies, termed hypergammaglobulinemia, is not only a common feature of many autoimmune diseases in humans and in mouse models (Ishigatsubo *et al.*, 1988; He *et al.*, 2004), but is also a hallmark of

many chronic infections, including leishmaniasis and Chagas disease (Galvão-Castro *et al.*, 1984; Minoprio *et al.*, 1986). As pronounced IFN-I production has been observed in models using *L. donovani* and *T. cruzi* (Chessler *et al.*, 2009; Silva-Barrios *et al.*, 2016), similar mechanisms might be at play to exacerbate B cell activation and cause hypergammaglobulinemia in these diseases.

Not only do autoimmune diseases and chronic inflammatory diseases share many characteristics, including aberrant B cell activation and antibody production, but many pathogens have also been linked to the induction of autoimmune reactions. One of these pathogens is the intracellular protozoan parasite *Trypanosoma cruzi* which induces chronic chagasic cardiomyopathy (CCC) in 30 to 50 % of patients and accompanied by high production of inflammatory cytokines, including IL-1β, IFN-γ and TNF (Abel *et al.*, 2001). Different roles for TLRs in *T. cruzi* infection have been proposed – lack of signaling through TLR7 and TLR9 has been found to enhance susceptibility to infection and decrease parasite clearance (Bafica *et al.*, 2006; Caetano *et al.*, 2011), while TLR2 and TLR4 ligation were shown to modulate the pro-inflammatory response in the cardiac form, and in the anti-inflammatory response in the asymptomatic form of the disease (Mendes da Silva *et al.*, 2017); however, little information exists on the contribution of B cell-intrinsic TLRs in this context. Distinct clinical forms of Chagas' disease were found to have different underlying TLR expression and subsequent cytokine production in PBMCs. Elevated levels of TLR2 expression and concomitant production of pro-inflammatory cytokines TNF and IL-12 were found in patients exhibiting cardiac pathologies, while increased TLR8 and IFN-β expression was determined in the digestive forms (Pereira *et al.*, 2018). The frequency of TNF-producing B1 cells in cardiac patients was shown to be higher than in non-infected individuals and was significantly increased upon further exposure to *T. cruzi*-derived protein-enriched fraction (Passos *et al.*, 2018); however, direct studies on B cells in *T. cruzi* infection are required to elucidate the contribution of B cell TLR signaling to this cytokine production.

Like trypanosomiasis, leishmaniasis is induced by a family of protozoan parasites belonging to the group of Trypanosomatids. Contrasting roles for B cells for different parasite strains have been demonstrated both in disease protection or progression dependent on the model organism and parasite strain (reviewed in (Silva-Barrios *et al.*, 2018)); however, little is known about the contribution of innate immune sensing in B cells in the context of this disease. Using an experimental model of visceral leishmaniasis, our laboratory has previously demonstrated that *Leishmania donovani* amastigotes can induce production of pro-inflammatory cytokines, IFN-I, and IL-10, by engaging endosomal TLR3, TLR7, and TLR9. As in vitro exposure of B cells to the parasite also resulted in an IFNAR-dependent upregulation of endosomal TLR mRNA, we

proposed a positive regulatory loop of IFN-I on endosomal TLR expression, thereby enhancing the modulatory effect of endosomal TLR signaling on cytokine production and antibody production, which results in hypergammaglobulinemia and disease exacerbation (Silva-Barrios *et al.*, 2016; Silva-Barrios & Stäger, 2019). A similar feedback mechanism for IFN-β on TLR7 and TLR3 has been suggested by other groups, and investigation of the source of this IFN-I provides a link between the innate RIG-I/MAVS and TLR signaling pathways (Green *et al.*, 2009; Loetsch *et al.*, 2017): Loetsch et al. have found stimulation of the RIG-I/MAVS pathway with synthetic RNA analogue to cause upregulation of endosomal TLR expression, namely TLR3 and TLR7, in an interferon-α/β receptor (IFNAR)- and partially MAVS-dependent manner. Thus, IFN-I produced via MAVS-mediated sensing pathways could partially account for the amplification of B cell activation and hypergammaglobulinemia through upregulation of endosomal TLRs; however, the reduction of TLR upregulation in the MAVS-compromised B cells does not fully account for the reduction observed in the *Ifnar*$^{-/-}$ mice. This suggests that there might be an additional source of type-I interferon produced by a MAVS-independent pathway. In fact, DNA derived from *L. donovani* has recently been demonstrated to be able to induce IFN-I production via the cGAS/STING pathway in macrophages (Das *et al.*, 2019), and while only the B-1 lineage of B cells has been demonstrated to be able to phagocytose *Leishmania* parasites (Geraldo *et al.*, 2016), it is possible that parasite DNA is delivered to the B cell cytosol through yet unidentified pathways to trigger IFN-I production via cGAS/STING.

Finally, while innate immune signaling can thus be subverted by pathogens to exacerbate disease by dysregulating germinal center formation, antibody and cytokine production, targeted engagement of individual sensors, especially of the TLR family, has also been proposed in therapy of various inflammatory and infectious diseases (Vollmer & Krieg, 2009); however, the effect of using TLR ligands as adjuvants in therapy can differ widely even in the same model, as demonstrated by a study on established cutaneous leishmaniasis infection caused by *L. (Vianna) panamensis*. TLR9 stimulation using high doses of its ligand CpG was shown to decrease lesion size, drastically reduced parasite burden, and decreased B cell-mediated IFN-γ, while stimulation with low doses of CpG increased IFN-γ production in the same cells (Ehrlich *et al.*, 2017), highlighting the importance of further studies on innate immune signaling and its effect on adaptive immunity in B cells in order to develop safe and effective treatments for chronic infectious diseases.

The low number of studies on cytosolic nucleic acid sensors in B cells in infectious diseases limits conclusions to be drawn for its relevance in pathological settings at this point in time. The use of

a conventional knockout of cGAS in mice was shown to induce dysregulated germinal center and antibody responses and reduced parasite clearance in a non-lethal malaria model; however the effect on GC formation was found to be B cell-extrinsic (Hahn, 2018). Similarly, while MAVS$^{-/-}$ mice infected with the non-pathogenic West Nile Virus-Madagascar (WNV-MAD) strain exhibited increased GC formation, antibody-titers and plasma cell formation, this effect was found to be dependent on MAVS signaling in dendritic cells rather than B cells (Roe *et al.*, 2019). While both studies chose to focus on the most prominent role of B cells, production of antibodies, neither of them investigated the effect of cytosolic nucleic acid sensing on cytokine production. In B cells isolated from lungs and spleen of *Mycobacterium tuberculosis*-infected mice, a dramatic STING-dependent upregulation of IFN-β mRNA, along with a milder increase in IL-6 and a tendency towards upregulated IL-10 was observed. This marked increase of IFN-β production was also shown to be present in B cells purified from pleural fluid of Mtb patients as compared to healthy donors. Interestingly, a lower amount of IFN-β could also be triggered by poly(I:C), which is a ligand of TLR3 but can also be sensed via the RIG-I/MAVS pathway. Along with another interesting finding that MyD88 signaling suppressed STING-mediated IFN-β expression, this not only provides further evidence of functional cytosolic sensing in B cells in infectious diseases but also draws another connection between cytosolic and TLR sensing (Benard *et al.*, 2018). Collectively, this implies that, while cytosolic sensing in B cells might have a limited direct effect on GC formation and antibody response, it can have substantial effect on modulating cytokine production, and, through interconnections between the different innate immune sensing pathways, potentially mediated through IFN-I, a central player in many of these pathways, indirectly mediate B cell responses.

Despite studies demonstrating the functionality of intracellular innate immune sensing pathways in B cells, the question still remains as to how these mostly non-phagocytic immune cells are able to recognize pathogen-derived nucleic acids in their cytosol. One possibility has been demonstrated in the case of *Listeria monocytogenes*, which can induce nucleotide sensing in non-phagocytic cells by secretion of bacteria-derived nucleic acids (Abdullah *et al.*, 2012; Hagmann *et al.*, 2013). As many chronic infections are characterized by increased apoptosis and tissue disruption, as the case during hepatosplenomegaly in visceral leishmaniasis or cardiac damage in Chagas' disease (Chaves *et al.*, 2016; de Souza *et al.*, 2019), it is also possible that this may promote release of host nucleic acids into the tissue environment (Stroun *et al.*, 2001), which could in turn induce innate immune sensors in surrounding cells, including B cells. Nevertheless, further investigations are warranted to identify the mechanisms underlying activation of intracellular sensors in B cells by pathogens.

Functional outcomes of PRR triggering in B cells are summarized in **Table 5** and **Figure 1**.

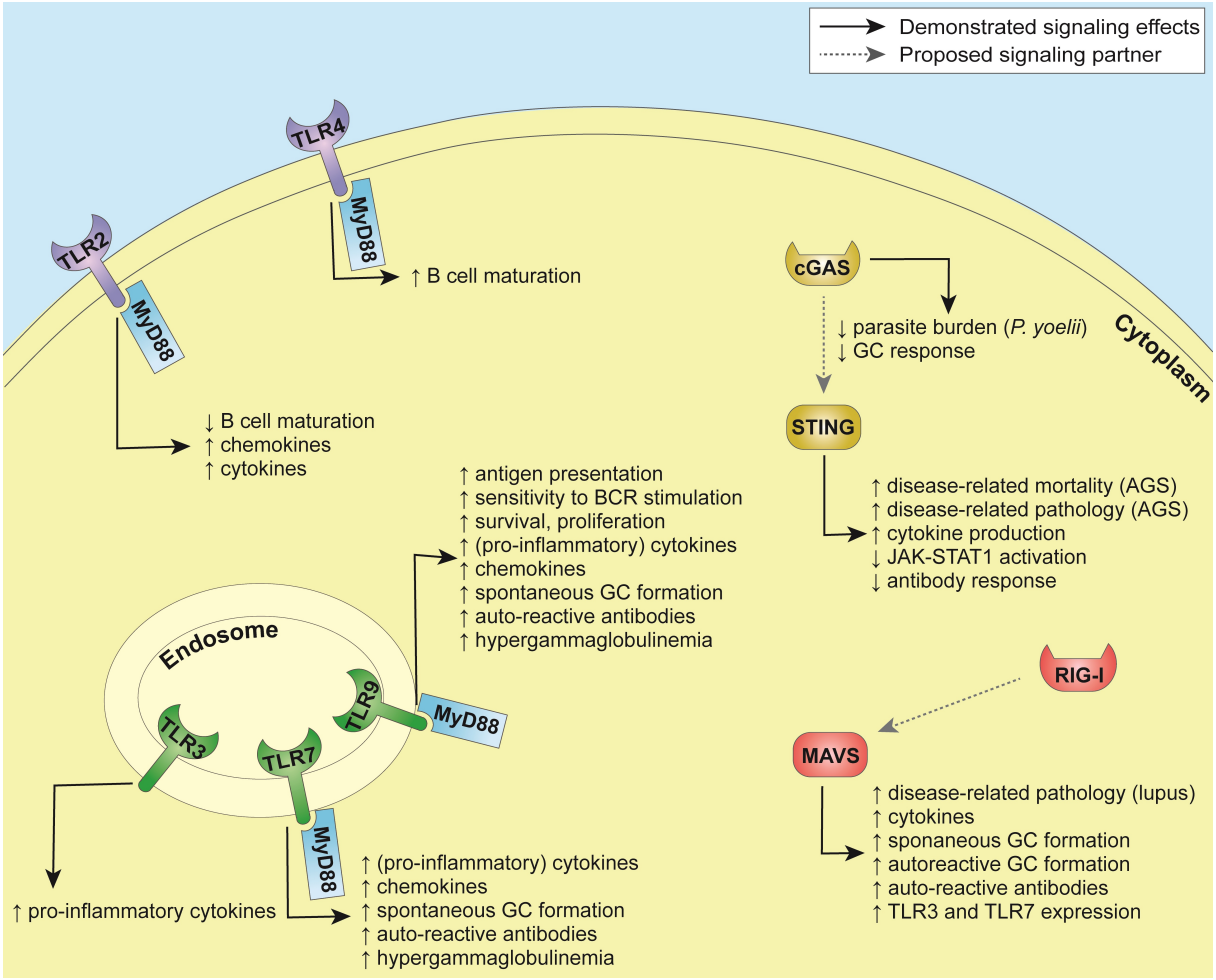

Figure 1 Summary of pathways involved in innate immune sensing in B cells.

Table 5 Innate Immune Sensing in B cells

PRR/ Adaptor	Function	Model/Agonist	Organism
TLR2	↓ B cell maturation	Pam3Cys	Mouse
	↑ cytokine and chemokine production	Pam3CSK	Human
TLR3	↑ pro-inflammatory cytokines	*L. donovani*	Mouse
TLR4	↑ B cell maturation	LPS	Mouse
TLR7	↑ cytokine and chemokine production	Imiquimod	Human
	↑ Spontaneous GC formation	Imiquimod	Mouse
	↑ Autoantibody production	Lupus-prone mice	
	↑ pro-inflammatory cytokines, ↑ hypergammaglobulinemia	*Leishmania donovani*	
TLR9	↑ proliferation, ↑ survival, ↑ costimulatory molecule expression, ↑ antigen presentation ↑ sensitivity to BCR stimulation, ↑ cytokine and chemokine production	CpG, GpG-ODN2006	Human
	↓ Spontaneous GC formation	TLR9 knockout	Mouse
	↑ Autoantibody production	Lupus-prone mice	
	↑ pro-inflammatory cytokines, ↑ hypergammaglobulinemia	*L. donovani*	
	at high CpG doses (> 1 μM): ↓ lesion size, ↓ parasite burden, ↓ IFN-γ at low CpG doses (> 40 nM) ↑ IFN-γ	CpG treatment in *Leishmania (Vianna) panamensis*	
cGAS	↓ parasite burden, ↓ GC response	*P. yoelii*	Mouse
STING	↑ disease-related mortality, ↑ glomerulonephritis	Aicardi-Goutières Syndrome	Mouse
	↓ JAK-STAT1 activation, ↓ antibody response	Systemic lupus erythematosus	Mouse/ Human
	↑ cytokine production	*Mycobacterium tuberculosis*	
MAVS	↑ Spontaneous GC formation ↑ autoreactive GC formation, ↑ autoantibody production, ↑ disease-related pathology [	Lupus-prone mice	Mouse
	↑ TLR3 and TLR7 expression, ↑ cytokine production	Poly(I:C)	

6.4 Innate immune sensing in T cells

6.4.1 Expression and functions of innate immune sensors in T cells

T lymphocytes have also been reported to express several PRRs; however, the downstream effect of PRR activation varies depending on T cell population, activation status, ligand and/or environment.

Most of the available literature on PRR expression in T lymphocytes investigates the role of TLRs in T cell differentiation and effector function. Murine and human T cells were shown to express mRNA and protein for most of the TLRs (Caramalho *et al.*, 2003; Gelman *et al.*, 2004; Komai-Koma *et al.*, 2004; Fukata *et al.*, 2008; Rahman *et al.*, 2009; Reynolds *et al.*, 2010; Dominguez-Villar *et al.*, 2015; Fabie *et al.*, 2018); however, their expression intensity depends on T cell subsets and activation status. Interestingly, TLR expression seems to be regulated by TCR-dependent activation; indeed, antigen-experienced T cells express higher TLR levels than naïve cells (Gelman *et al.*, 2004; Liu *et al.*, 2006; Fabie *et al.*, 2018). During priming, TLR activation appears to function as a sort of costimulatory signal enhancing effector function, proliferation, cell survival, and cytokine production in murine and human CD4 T cells (Komai-Koma *et al.*, 2004; Caron *et al.*, 2005; Gelman *et al.*, 2006; Reynolds *et al.*, 2010). For example, signaling through TLR9 can induce NF-κB activation in CD4 T cells via the adaptor molecule MyD88, leading to the upregulation of anti-apoptotic molecules and increasing cell survival (Gelman *et al.*, 2004). Similar anti-apoptotic functions were ascribed to TLR2 in CD8 T cells (Cottalorda *et al.*, 2006) . Moreover, signaling throughTLR9- MyD88 also promotes CD4 T cells proliferation by activating a PI3K/Akt-dependent pathway (Gelman *et al.*, 2006).

Expression of costimulatory molecules and cell trafficking are also promoted by TLR stimulation. For instance, CpG ODN (TLR9 agonist) induces expression of OX-40 and CD40L on CD4 T cells during priming; while treatment with LPS increases adhesion capacity and inhibits chemotaxis of human and murine T cells (Gramaglia *et al.*, 1998; Zanin-Zhorov *et al.*, 2007).

The importance of intrinsic MyD88-dependent signals in promoting CD8 and CD4 T cell survival and initial proliferation was also demonstrated in *in vivo* studies in various models of infection, including *Toxoplasma gondii* and Lymphocytic choriomeningitis virus (LCMV) infections (LaRosa *et al.*, 2008; Rahman *et al.*, 2008).

Moreover, MyD88 and TLR signaling appears to be essential for the differentiation of Th17. cells. The vital role of MyD88 was shown in experimental models of colitis and experimental

autoimmune encephalitis (EAE). In the colitis model, *Myd88*$^{-/-}$ CD4 T cells showed reduced survival, failed to induce severe disease, and poorly differentiated into Th17 cells (Fukata *et al.*, 2008; Tomita *et al.*, 2008). Later studies in the EAE model suggested TLR2 and TLR4 signaling as being crucial for the differentiation of Th17 cells (Reynolds *et al.*, 2010; Chang *et al.*, 2011; Reynolds *et al.*, 2012). Indeed, TLR2 activation in CD4 T cells seems to synergize with IL-23 to induce Th17 cells; additionally, TLR2-deficient CD4 T cells fail to induce EAE and to differentiate into IL-17 or IFN-γ-producing cells in adoptively transferred mice.

Taken together, the literature suggests that TLR signaling plays an important role in providing cell survival and proliferative signals during T cell priming and in enhancing effector functions and cell differentiation.

Some cytosolic nucleic acid sensors were also reported to be expressed in T cells. For instance STING expression was detected in human and murine T cells (Cerboni *et al.*, 2017; Larkin *et al.*, 2017; Imanishi *et al.*, 2019); RIG-I is expressed in human peripheral T lymphocytes (Zhang *et al.*, 2019); LGP2 is present in murine CD8 T cells (Suthar *et al.*, 2012); and the immune sensor NLRC3 was observed in murine CD4 T cells (Uchimura *et al.*, 2018). With exception of LGP2, all other pathways appear to impair T cell proliferation, function, or survival. Hence, their role in T cells will be discussed in the next section. LGP2 is a member of the RIG-I-like receptors family of cytosolic RNA helicases that includes RIG-I and MDA5. Unlike RIG-I and MDA5, which are known to initiate the activation of IRF-3 and NF-κB to induce expression of IFN-I, LGP2 can function as a negative regulator of RLR signaling inhibiting TLR-independent sensing of viral replication (Rothenfusser *et al.*, 2005) and RIG-I multimerization (Saito *et al.*, 2007), or compete with MAVS to suppress innate immune signaling (Komuro & Horvath, 2006). A positive function for LGP2 as a cofactor for RLR signaling of RIG-I and MAVS-mediated antiviral responses has also been described, but the mechanism is yet unknown (Venkataraman *et al.*, 2007; Satoh *et al.*, 2010). In CD8 T cells, LGP2 promotes cell fitness and survival by controlling sensitivity to death-receptor signaling during acute West Nile virus and LCMV infections. Indeed, LPG2-deficient CD8 T cells display enhanced activity of caspase 8, 3, and 7 and enhanced expression of death receptors TNFR-I, TRAILR2 (or DR5), and CD95 (or Fas receptor)(Suthar *et al.*, 2012).

6.4.2 Immunosuppressive effects of innate immune sensing in T cells

Despite the strong evidence that T cell-intrinsic PRR activation complements TCR and costimulatory signals to improve T cell responses during priming, a few studies have reported an inhibitory role for innate immune sensing in T cells.

Signaling via TLR2, for instance, was shown to inhibit T cell chemotaxis through upregulation of the transcription factor SOCS3 (suppressor of cytokine signaling 3) (Zanin-Zhorov *et al.*, 2005b). An additional study reported that TLR2 was also involved in downregulating the transcription factors T-bet and NF-κB (Zanin-Zhorov *et al.*, 2005a). Both studies used Heat shock protein 60 (HSP60), arguably a DAMP, to stimulate T cells. In contrast, in CD25+ CD4+ regulatory T cells (Tregs) exposed to HSP60 upon activation with anti-CD3, TLR2 was required to enhance their immunosuppressive effects via activation of PCK, PI-3 kinase, and p38 (Zanin-Zhorov *et al.*, 2006). Stimulation of Tregs with pathogen-derived TLR2 ligands induced proliferation and promoted survival (Liu *et al.*, 2006; Sutmuller *et al.*, 2006; Zanin-Zhorov *et al.*, 2006; Round *et al.*, 2011); however, whether this enhances (Zanin-Zhorov *et al.*, 2006; Round *et al.*, 2011) or curbs (Liu *et al.*, 2006; Sutmuller *et al.*, 2006) their inhibitory function is still controversial.

While TLR4 activation is essential to drive Th17 responses (Reynolds *et al.*, 2012), its effects on Th1 cells are rather inhibitory in a spontaneous model of colitis (Gonzalez-Navajas *et al.*, 2010) and in human T cells exposed to LPS (Zanin-Zhorov *et al.*, 2007), where signaling via TLR4 inhibited cell migration. Inhibitory effects were also ascribed to the TLR7 activation pathway in CD4 T cells. In a model of EAE, triggering of TLR7 suppressed Th17 cell differentiation, which resulted in reduced disease severity (Ye *et al.*, 2017). This effect was mediated by downregulation of STAT3 and induction of SOCS3 and SOCS5 (Ye *et al.*, 2017). Furthermore, in human T cells purified from the blood of HIV^+ individuals, TLR7 stimulation promoted the activation of an NFATc2-dependent anergic gene-expression program, which led to cell unresponsiveness (Dominguez-Villar *et al.*, 2015). Work from our laboratory has also revealed an inhibitory function for TLR7 activation in Th1 cells in *L. donovani* infected mice. During the chronic stage of disease, Th1 cells increasingly upregulated TLR7 expression and sensed DAMPs derived from inflammatory tissue disruption (Fabie *et al.*, 2018). Engagement of TLR7 on those cells resulted in the activation of the transcription factor interferon regulatory factor 5 (IRF-5), which induced the transcriptional activation of death receptor 5 (DR5 or TRAILR2) and caspase 8, thereby promoting cell death (Fabie *et al.*, 2018).

TLRs are not the only innate immune sensors capable of inhibiting T cell functions. The STING pathway was recently shown to be active in T cells as well. Stimulation with STING agonists not only promoted IFN-I production and interferon stimulated genes' expression, but it also led to the down-regulation of anti-apoptotic and the upregulation of pro-apoptotic genes (Larkin *et al.*, 2017). Interestingly, T cells exhibit an intensified STING pathway, which results in a different gene expression profile to innate myeloid cells and leads to cell death (Gulen *et al.*, 2017). Moreover, activation of STING was shown to have an antiproliferative effect in human and murine CD4 T cells. This antiproliferative capacity requires STING relocalization to the Golgi apparatus (Cerboni *et al.*, 2017).

The RIG-I pathway seems to also have inhibitory effects in human T cells. Zhang et al. report a positive correlation between RIG-I expression in peripheral T cells and T lymphocyte counts in patients affected by dermatomyositis. Interestingly, RIG-I induced apoptosis in these cells and inhibited their proliferative capacity (Zhang *et al.*, 2019).

Another molecule involved in innate immune sensing pathways that was recently shown to reduce T cell effector functions is NLRC3 (Zhang *et al.*, 2014b). NLRC3 belongs to the group of non inflammasome-forming NLRs (NOD-like receptors), together with NOD-1, NOD2, among others. NLRC3 is a known negative regulator of innate immunity and inflammatory responses (Schneider *et al.*, 2012; Zhang *et al.*, 2014b). This molecule is highly expressed in T cells, where it seems to fine-tune CD4 T cell activation by attenuating IFN-γ and TNF expression, decreasing proliferation of Th1 and Th17 cells, and affecting cell metabolism by reducing glycolysis and oxidative phosphorylation (Uchimura *et al.*, 2018).

It was also demonstrated that T cells are capable of sensing nucleic acids via pathways distinct of those identified so far in the innate immune system. Interestingly, higher-order structure of the nucleic acids was required for their internalization by T cells; indeed, self-DNA released from dead cells and complexes with antimicrobial peptides or histones induced costimulatory responses upon recognition by yet unidentified sensor(s), promoting the differentiation into Th2 cells (Imanishi *et al.*, 2014). Downstream effects of innate immune sensing in T cells are summarized in **Table 6** and **Figure 2**.

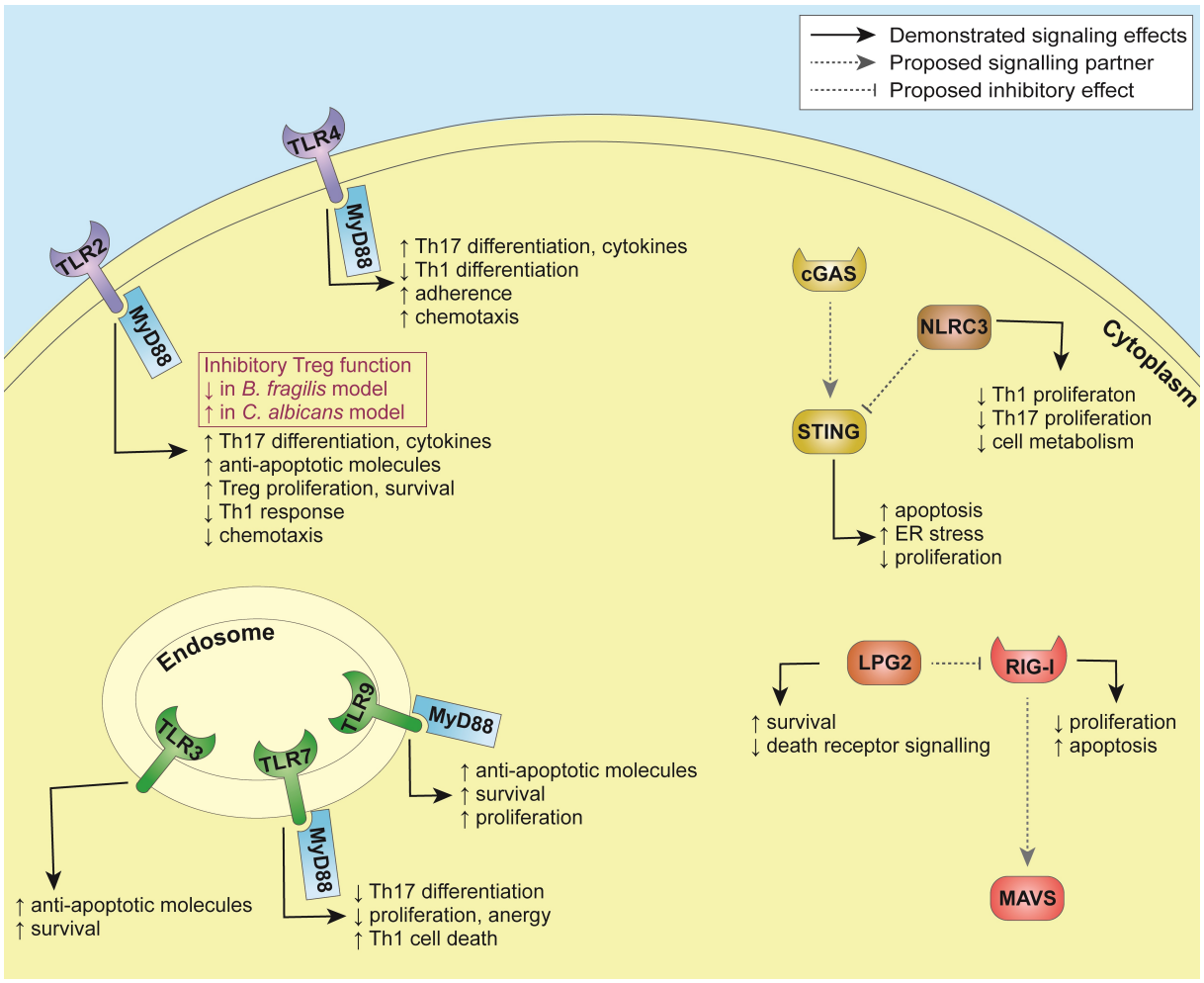

Figure 2 Summary of pathways involved in innate immune sensing in T cells.

Table 6 Innate immune sensing in T cells

PRR/ Adaptor	Function	Model/Agonist	Organism
MyD88	↑ disease susceptibility	*Toxoplasma gondii*	Mouse
	↑ clonal expansion, ↑ survival	LCMV	
	↑ survival, ↑ disease induction, ↑ Th1 and Th17 differentiation, ↑ expression of anti-apoptotic molecules	Colitis	
TLR2	↑ anti-apoptotic molecules,	Pam(3)CysSK(4)	Mouse

	↑ survival, ↑ proliferation		
	↑ Th17 differentiation, ↑ Th17 cytokine production	Experimental autoimmune encephalomyelitis	
	↑ Tregs proliferation, ↑ survival ↑ inhibitory Treg function ↓ inhibitory Treg function	Pam3CysSK4,HSP90, *Candida albicans,* Bacteroides fragilis	
	↓ chemotaxis, ↓ Th1 responses	HSP60	Human/ Mouse
TLR3	↑ anti-apoptotic molecules, ↑ survival	Ligation with poly(I:C)	Mouse
TLR4/ MyD88	↑ adherence, ↑ chemotaxis	Ligation with LPS	Human
TLR4	↑ Th17 differentiation, ↑ Th17 cytokine production	EAE	Mouse
	↓ Th1 responses	Colitis	
TLR7	↑ Th1cell death	*L. donovani*	Mouse
	↓ Th17 differentiation	EAE	
	↓ proliferation, anergy	HIV-1	Human
TLR9/ MyD88	↑ anti-apoptotic molecules, ↑ survival, ↑ proliferation	Ligation with CpG	Mouse
RIG-I	↓ proliferation, ↑ apoptosis	Dermatomyositis	Human
LPG2	↑ survival, ↓ death receptor signaling	RNA viruses	Mouse
STING	↓ proliferation	Activating STING mutations	Human/ Mouse
	↑ ER stress, ↑ apoptosis	DMXAA, CMA	Mouse
NLRC3	↓ Th1 proliferation, ↓ Th17 proliferation, ↓ cell metabolism	LCMV, EAE	Mouse
unknown Nucleic Acid Sensor	↑ co-stimulatory responses, ↑ Th2 differentiation	Ligation with TLR ligands, synthetic NA analogues, self-DNA from dead cells	Mouse

It is interesting to observe that innate sensing by T cells during the priming phase leads mainly to a positive outcome: it promotes cell survival, enhances effector function, and helps differentiation. In contrast, during chronic infections or in a chronic inflammatory environment, PRR activation in T cells results in cell death and anergy, and limits the cells' proliferative capacity. It is yet unclear why PRR signaling mediates such disparate functions depending on the cell differentiation stage and the inflammatory environment. Further studies are definitely warranted to better define the level of expression, signaling pathways and downstream targets of PRRs in various T cell populations. Also, very little information is currently available on innate immune sensing by T cells through PRRs that are upregulated during chronic infection and the importance these may have in helping pathogen persistence and inhibiting protective T cell responses. Furthermore, the nature of the ligands responsible for triggering those responses in T cells, and the stimuli required for promoting PRR expression during chronic infections have also not yet been identified. We have demonstrated that DAMPs could trigger TLR7 and induce cell death in T cells isolated from *L. donovani*-infected mice during chronic infection (Fabie *et al.*, 2018). Inflammatory tissue damage is a common characteristic of persistent infections and release of several DAMPs through this process is inevitable. It is thus possible that other PRR, such as STING and RIG-I, are also activated by DAMPS derived from tissue damage during chronic visceral leishmaniasis. While curbing of pro-inflammatory T cell responses in a chronic inflammatory environment could represent a protective mechanism to prevent tissue disruption, it may also favor pathogen persistence. Indeed, in our model, disruption of the TLR7 signaling pathway in T cells resulted in stronger Th1 responses and a lower parasite burden (Fabie *et al.*, 2018). Hence, it would be interesting to investigate the role of PRRs in T cells during other parasitic infections to identify pathways that could possibly be exploited for therapeutic purposes.

6.5 Concluding remarks

An important body of literature has now demonstrated that PRRs are expressed and functional in cells of the adaptive immune system. In these cells, PRR activation can support signaling pathways that are beneficial to host immunity or, on the contrary, promote adverse effects that favor pathogen persistence. In light of this information, it is thus important to consider the effect of TLR agonists on T and B cells, and not only on myeloid cells, when developing new vaccination strategies, particularly for therapeutic purposes. Indeed, endosomal TLR agonists' administration

during chronic stages of infection may lead to CD4 T cell death and/or exacerbate hypergammaglobulinemia. The same caveat may also be valid for immunotherapeutic interventions involving TLR agonists, which could have disease-exacerbating consequences if the wrong cells are involuntarily targeted.

Further investigations are required to better define signaling pathways and downstream targets of PRRs in T and B cells in the context of chronic infections, since these pathways could be exploited for novel therapies.

6.6 Acknowledgments

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7 APPENDIX II – OTHER PUBLICATIONS, CONFERENCES AND MEETINGS

Collaborations

Carmona-Pérez L, Dagenais-Lussier X, Mai LT, Stögerer T, Swaminathan S, Isnard S, Rice MR, Barnes B, Routy JP, van Grevenynghe J, Stäger S: The TLR7/IRF-5 axis sensitizes memory CD4+ T cells to Fas-mediated apoptosis during HIV-1 infection. *JCI Insight*. 2023

Conference presentations

1st Canadian Parasitology Network Symposium, June 27 – 28, 2023, Woods Hole, USA
Stögerer T, Silva-Barrios S, Descoteaux A, Stäger S: *Leishmania donovani* induces and exploits tunneling nanotubes for dissemination and propagation of B cell activation
Oral presentation

27th Woods Hole Immunoparasitology Meeting, April 23 – 26, 2023, Woods Hole, USA
Stögerer T, Silva-Barrios S, Descoteaux A, Stäger S: *Leishmania donovani* induces and exploits tunneling nanotubes for dissemination and propagation of B cell activation
Oral presentation

WorldLeish 7, August 1 – 6, 2022, Cartagena, Colombia
Stögerer T, Silva-Barrios S, Descoteaux A, Stäger S: Characterisation of intercellular communication in B cells upon *Leishmania donovani* exposure
Oral presentation

Annual Montreal Parasitology Meeting, June 10, 2022, St-Hyacinthe, Canada
Stögerer T, Silva-Barrios S, Descoteaux A, Stäger S: Characterisation of intercellular communication in B cells upon *Leishmania donovani* exposure
Poster presentation

12ème Congrès Armand-Frappier, November 8 – 12, 2021, Laval (virtual), Canada
Stögerer T, Silva-Barrios S, Descoteaux A, Stäger S: Characterisation of intercellular communication in B cells upon *Leishmania donovani* exposure
Poster presentation + 3 Minute Thesis

8th Annual Montreal Immunology Meeting, November 1, 2021, Montreal (virtual), Canada

Stögerer T, Silva-Barrios S., Descoteaux A., Stäger S. Characterisation of intercellular communication in B cells upon *Leishmania donovani* exposure

Poster presentation

25th Woods Hole Immunoparasitology Meeting, April 11 – 14, 2021, Woods Hole (virtual), USA

Stögerer T, Silva-Barrios S, Descoteaux A, Stäger S: Characterisation of intercellular communication in B cells upon *Leishmania donovani* exposure

Oral presentation

11ème Congrès Armand-Frappier, October 28 – 31, 2019, Saint-Sauveur, Canada

Stögerer T, Silva-Barrios S, Descoteaux A, Stäger S: Characterisation of intercellular communication in B cells upon *Leishmania donovani* exposure

Poster presentation

6th Annual Montreal Immunology Meeting, November 6, 2019, Montreal, Canada

Stögerer T, Silva-Barrios S, Descoteaux A, Stäger S: Characterisation of intercellular communication in B cells upon *Leishmania donovani* exposure

Poster presentation