Centre Armand-Frappier Santé Biotechnologie

The role of Lymphocyte Activation Gene 3 (LAG-3)-expressing CD4 T cells in chronic infection

Le rôle des lymphocytes T CD4 exprimant le Gène d'Activation Lymphocytaire 3 (LAG-3) dans l'infection chronique

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RÉSUMÉ

Le maintien des lymphocytes T CD4 est essentiel pour une réponse efficace de l'hôte aux infections. La leishmaniose viscérale (LV) est l'une de ces infections parasitaires chroniques potentiellement mortelles. Elle est causée par les parasites protozoaires *Leishmania donovani* et/ou *L. infantum/chagasi*. Dans la LV, les cytokines interféron-γ (IFNγ) et tumor necrosis factor (TNF) produites par les cellules T CD8 et CD4 (cellules T auxiliaire *(helper)* de type 1 ou Th1) sont nécessaires pour déclencher la capacité leishmanicide des macrophages. Cependant, plusieurs mécanismes inhibent la réponse des lymphocytes T CD4 en LV. Le gènes d'activation des lymphocytes 3 (LAG-3) est un récepteur inhibiteur régulé positivement après l'activation des lymphocytes T. Nos expériences préliminaires ont démontré que la fréquence et le nombre de lymphocytes T CD4 exprimant LAG-3 augmentent chez les souris infectées par *L. donovani*, incluant lors de la phase chronique de l'infection, lorsqu'il y a une mort excessive des lymphocytes T CD4 due à la détection des dommages. Dans ce travail, nous avons décidé d'examiner les sous-ensembles de lymphocytes T CD4 LAG-3$^+$ dans la LV, à la recherche de mécanismes qui soutiennent les réponses des lymphocytes T CD4 effecteurs.

Premièrement, nous avons identifié un sous-ensemble de lymphocytes T CD4 LAG-3$^+$ dans la rate des souris infectées par *L. donovani* qui coexpriment CXCR5, le récepteur de chimiokine associé à la migration des cellules folliculaires T *helper* (Tfh) vers les follicules de cellules B. La comparaison du profil transcriptomique des cellules LAG-3$^+$CXCR5$^+$PD-1$^{lo/int}$ par rapport aux cellules Tfh a révélé que cette population cellulaire possède environ 5000 gènes exprimés de manière différentielle par rapport aux cellules Tfh. De plus, le profil moléculaire et phénotypique des cellules LAG-3$^+$CXCR5$^+$PD-1$^{lo/int}$ semblait similaire à celui d'une population de cellules T CXCR5$^+$ CD8 de type progéniteur récemment décrite. Ces cellules T CXCR5$^+$ CD8 ont le potentiel de s'auto-renouveler et se différencient en lymphocytes T CD8 effecteurs au cours d'une infection chronique, agissant ainsi comme des cellules de type progénitrices qui reconstituent le compartiment décroissant des lymphocytes T CD8. Par la suite, nous avons évalué si les cellules LAG-3$^+$CXCR5$^+$PD-1$^{lo/int}$ sont les analogues CD4 des cellules T CD8 de type progéniteur CXCR5$^+$. Fait intéressant, nous avons observé que ces cellules expriment les gènes d'auto-renouvellement fortement associés aux cellules souches hématopoïétiques. De plus, lors du transfert adoptif chez des souris naïves, suivi d'une infection par L. donovani, ces cellules ont pu se différencier en différents types de cellules effectrices telles que les productrices d'IFNγ-, de TNF-, de Granzyme-B ainsi que les cellules régulatrices de type 1 (Tr1). En outre, ces cellules

étaient également présentes dans d'autres modèles d'infection chronique. Nous avons également démontré que ces cellules nécessitent en partie le facteur de transcription BCL-6 pour leur maintien.

En résumé, nous décrivons une population de lymphocytes T CD4 exprimant LAG-3 et CXCR5, qui, selon nous, supporte le poids de la différenciation en lymphocytes T CD4 effecteurs au cours des stades chroniques de l'infection.

Mots clés : lymphocytes T CD4; infections chroniques; LAG-3; CXCR5; PD-1; cellules de type progénitrices; différenciation; leishmaniose viscérale

ABSTRACT

An effective host response to chronic infections requires maintenance of CD4 T cells. One such potentially fatal chronic parasitic infection is visceral leishmaniasis (VL). It is caused by the protozoan parasites *Leishmania donovani* and/or *L. infantum/chagasi.* In VL, the cytokines interferon-γ (IFNγ)- and tumor necrosis factor (TNF) produced by CD8 and CD4 T cells (T helper type 1 or Th1 cells) are required to trigger the leishmanicidal capacity in macrophages. However, several mechanisms inhibit CD4 T cell responses in VL.

Lymphocyte activation gene 3 (LAG-3), is an inhibitory receptor upregulated following T cell activation. Our preliminary experiments showed that the frequency and number of LAG-3-expressing CD4 T cells increases in *L. donovani* infected mice, even during the chronic phase of the infection, when there is excessive CD4 T cell death due to damage-sensing. In this work, we decided to examine the LAG-3$^+$ CD4 T cell subsets in VL, in search of mechanisms that sustain the effector CD4 T cell responses.

Firstly, we identified a subset of LAG-3$^+$ splenic CD4 T cells in *L. donovani*-infected mice that coexpress CXCR5, the chemokine receptor associated with migration of T follicular helper cells (Tfh) to B cell follicles. Transcriptomic profile comparison of the LAG-3$^+$CXCR5$^+$PD-1$^{lo/int}$ cells versus Tfh cells revealed that this cell population has ~5000 differentially expressed genes compared to Tfh cells.

Moreover, the molecular and phenotypic profile of LAG-3$^+$CXCR5$^+$PD-1$^{lo/int}$ cells seemed similar to a recently described progenitor-like CXCR5$^+$ CD8 T cell population. These CXCR5$^+$ CD8 T cells have the potential to self-renew, and differentiate into effector CD8 T cell populations, during chronic infection, thereby acting as progenitor-like cells that replenish the diminishing CD8 T cell compartment. Therefore, we evaluated if the LAG-3$^+$CXCR5$^+$PD-1$^{lo/int}$ cells are the CD4-analogs of the CXCR5$^+$ progenitor-like CD8 T cells. Interestingly, we observed that these cells highly express self-renewal genes associated with hematopoietic stem cells. Moreover, upon adoptive transfer into naïve recipients followed by challenge with *L. donovani*, these cells were able to differentiate into different types of effector cells such as IFNγ-,TNF-, Granzyme-B-producers as well as Type 1 regulatory (Tr1) cells. Furthermore, these cells were also present in other models of chronic infection. We also demonstrated that these cells partially require the transcription factor BCL-6 for their maintenance.

In summary, we describe a population of CD4 T cells expressing LAG-3 and CXCR5, which we propose is bearing the weight of differentiation into CD4 T cell effectors during chronic stages of infection.

Keywords : CD4 T cell; chronic infection; LAG-3; CXCR5; PD-1; progenitor-like; differentiation; visceral leishmaniasis

SOMMAIRE RÉCAPITULATIF

Les infections chroniques constituent un problème de santé majeur dans le monde entier. Les lymphocytes T CD4 ont un rôle essentiel dans l'élimination des pathogènes lors les infections à long terme. Les infections chroniques virales, bactériennes ou parasitaires s'accompagnent également d'une hypergammaglobulinémie ou l'activation des cellules B polyclonales entraînant la surproduction d'anticorps de faible affinité. Les souris infectées par le parasite protozoaire intracellulaire *Leishmania donovani* présentent également une hypergammaglobulinémie, semblable à celle des humains souffrant de leishmaniose viscérale (LV), servant ainsi d'excellents modèles pour étudier la réponse immunitaire aux infections chroniques. La LV est répandue dans les pays d'Amérique du Sud, d'Asie et d'Afrique, causée par *L. donovani* et *L. infantum/chagasi*, et est mortelle si elle n'est pas traitée. Des mesures telles que la lutte antivectorielle, l'amélioration du diagnostic et du traitement ont entraîné une baisse du taux d'incidence de la leishmaniose au cours des 10 dernières années. Cependant, en raison de facteurs comme la résistance du parasite aux médicaments, le manque de nouveaux traitements et les lacunes dans les connaissances sur la physiopathologie de la maladie, l'Organisation mondiale de la santé classe toujours la leishmaniose parmi les maladies tropicales négligées.

Dans la LV, les lymphocytes T CD4 jouent un rôle central dans l'élimination des pathogènes. Les macrophages infectés par les parasites doivent être activés par les cytokines IFNγ et TNF, afin de présenter une capacité leishmanicide. Les lymphocytes T CD4 et CD8 sont les producteurs de ces deux cytokines. Cependant, dans la phase chronique de la LV, les lymphocytes T CD8 subissent un épuisement, et par conséquent, les lymphocytes T CD4 sont les seules sources d'IFNγ et de TNF nécessaires à la destruction des parasites. Cependant, ces cellules lymphocytes T CD4 aussi sont également supprimées par de nombreux facteurs. Ces cellules subissent un épuisement fonctionnel et la mort cellulaire (apoptose) en raison de la détection des dommages induit par l'inflammation. Leur amorçage est inhibé par l'hypoxie ainsi que par des cytokines comme les interférons de type I et l'interleukine-10. Il y a donc un déclin fonctionnel et numérique de ces cellules immunitaires protectrices (les lymphocytes T CD4), au cours de la LV, ce qui nécessite l'existence de mécanismes compensatoires pour reconstituer le compartiment des lymphocytes T CD4. Par conséquent, nous avons émis l'hypothèse qu'il existe des mécanismes d'adaptation alternatifs pour maintenir les réponses protectrices des lymphocytes T CD4 lors d'infections chroniques.

Le gène d'activation des lymphocytes 3 (LAG-3), également connu sous le nom de CD223, est un récepteur de 70 kD exprimé sur les cellules T CD4 après leur activation. Cette protéine est chargée de contrôler la prolifération des lymphocytes T. C'est la raison pour laquelle on la considère comme une molécule des points de contrôle de la réponse immunitaire (immune checkpoint). Il appartient à la superfamille des récepteurs d'immunoglobulines (IgSF). Le ligand le plus connu de LAG-3 est le MHC-II. LAG-3 est aussi connu pour se lier à quelques autres ligands non classiques, comme la Galectine-3, la LSECtine et la protéine 1 de type fibrinogène dans le contexte de certains cancers. Les mécanismes exacts par lesquels LAG-3 régule l'activation des lymphocytes T et l'expansion clonale de ces cellules sont inconnus. Cependant, un motif « KIEELE » conservé sur le domaine intracellulaire du récepteur LAG-3 joue un rôle important pour les fonctions inhibitrices. De nombreux sous-types de lymphocytes T CD4 expriment la protéine LAG-3. Ceux-ci incluent les deux types de lymphocytes T régulateurs - dépendants et indépendants de Foxp3 - ainsi les lymphocytes T épuisés co-exprimant LAG-3 avec d'autres protéines comme PD-1, TIM-3 et TIGIT. Toutefois, le rôle fonctionnel des lymphocytes T CD4 exprimant LAG-3 pendant les maladies infectieuses chroniques est inconnu. Plus encore, nous ne savons pas si ces cellules influencent ou non l'évolution des maladies infectieuses chroniques.

Nos expériences préliminaires ont montré que la fréquence et le nombre de lymphocytes T CD4 exprimant LAG-3 augmentent chez les souris infectées par *L. donovani*, incluant lors la phase chronique de l'infection, lorsqu'il y a une mort excessive apoptotique des lymphocytes T CD4 en raison de détection des dommages induits par l'inflammation. Par conséquent, nous avons décidé d'examiner les sous-types de lymphocytes T CD4 exprimant LAG-3 dans VL, pour tester notre hypothèse concernant les mécanismes alternatifs permettant la reconstitution des réponses des lymphocytes T CD4. Les objectifs spécifiques de cette étude sont (i) la caractérisation phénotypique et moléculaire et (ii) la caractérisation fonctionnelle des cellules T CD4 exprimant LAG-3 dans VL.

Tout d'abord, nous avons effectué une analyse par cytométrie en flux de cellules T CD4 spléniques de souris infectées par *L. donovani*. Nous avons observé un nouveau sous-type de lymphocytes T CD4 spléniques LAG-3^{+} qui co-expriment CXCR5, le récepteur de chimiokine associé à la migration des cellules folliculaires T auxiliaires/helper (Tfh) vers les follicules de cellules B. Il est intéressant de noter que les lymphocytes T LAG-3^{+} CXCR5^{+} expriment également d'autres marqueurs associés aux cellules Tfh, comme TCF-1, ICOS et PD-1. Cependant, ces cellules ont une expression intermédiaire de PD-1 par opposition aux cellules Tfh qui sont PD-1^{hi}

et seront donc désormais appelées cellules $T_{LCPlo/int}$. Ensuite, nous avons voulu vérifier si ces cellules sont antigène-spécifiques. Ainsi, donc nous avons utilisé des tétramères spécifiques du peptide PEPCK335-351, qui est un dérivé d'une enzyme métabolique exprimée par *Leishmania* qui s'appelle « glycosomal phosphoenolpyruvate carboxykinase (PEPCK) ». Il est important de noter qu'environ 10% de ces cellules $T_{LCPlo/int}$ étaient spécifiques de PEPCK.

Afin d'explorer les caractéristiques de ces cellules, nous avons effectué une analyse de séquençage d'ARN. À cette fin, nous avons purifié les cellules $T_{LCPlo/int}$ et les cellules Tfh du centre germinal CXCR5^{+} PD-1^{hi} (GC-Tfh), à partir de souris infectées par *L. donovani* 21 jours après l'infection. Nous avons décidé de comparer ces cellules non caractérisées aux GC-Tfh car ils exprimaient des marqueurs similaires aux cellules GC-Tfh. Nous avons choisi ce moment car la réponse des cellules GC-Tfh diminue rapidement après le jour 21 post-infection (p.i.) en VL. En revanche, à ce moment précis, la fréquence des cellules $T_{LCPlo/int}$ atteint son maximum. La comparaison du profil transcriptomique des cellules $T_{LCPlo/int}$ et GC-Tfh a révélé que cette population cellulaire possède 5,561 gènes exprimés de manière différentielle par rapport aux cellules GC-Tfh, y compris plusieurs cytokines, molécules effectrices, marqueurs de type mémoire et facteurs de transcription. Ensuite, pour tester si les lymphocytes T LAG-3^{+} CXCR5^{+} CD4 présentaient des similitudes avec l'un des types de lymphocytes T connus, nous avons effectué une analyse d'enrichissement de l'ensemble de gènes ou « gene set enrichment analysis, GSEA ». Parmi tous les sous-ensembles de lymphocytes T décrits dans la littérature, les gènes associés à la mémoire Tfh étaient les plus enrichis dans les cellules $T_{LCPlo/int}$. Néanmoins, le score d'enrichissement normalisé est relativement faible, ce qui suggère que les cellules $T_{LCPlo/int}$ constituent une population distincte et présentent peu de similitudes avec les cellules mémoire Tfh.

En examinant de plus près le profil transcriptionnel des cellules $T_{LCPlo/int}$, nous avons observé que ces cellules exprimaient des niveaux plus élevés de récepteurs co-inhibiteurs tels que *Ctla4* et *Cd244a*. Certains des récepteurs co-stimulateurs tels que *Cd40* et *Cd86* étaient également fortement exprimés par ces cellules $T_{LCPlo/int}$ par rapport aux cellules GC-Tfh, bien que d'autres tels que *Cd28* et *Cd80* ne l'étaient pas fortement exprimés. Malgré des niveaux d'expression de récepteurs co-inhibiteurs plus élevés que les cellules GC-Tfh, ces cellules $T_{LCPlo/int}$ expriment également des niveaux plus élevés de cytokines (notamment interféron-γ, IL-10, TNF), de chimiokines et de molécules effectrices (granzymes et perforines), et cela suggère qu'elles peuvent être fonctionnellement actives. Ces cellules exprimaient également des niveaux plus élevés de plusieurs facteurs de transcription caractéristiques des cellules Th1, tels que *Prdm1*,

Tbx21, *Eomes*, *Runx3* et *Id2*, par rapport aux cellules GC-Tfh. Les cellules $T_{LCPlo/int}$ exprimaient des niveaux inférieurs de facteurs de transcription canoniques liés à GC-Tfh, *Bcl6* et *Tcf7*, par rapport aux cellules GC-Tfh. Cependant, il est important de noter que ces cellules exprimaient clairement *Bcl6* et *Tcf7* car les transcrits et les valeurs TPM (Transcripts Per Kilobase Million) pour ces étaient respectivement de 19,67 ± 1,296SE et 594,9 ± 51,25SE, comme observé par l'analyse de séquençage d'ARN. Néanmoins, elles étaient significativement inférieures aux valeurs TPM observées pour les cellules GC-Tfh (*Bcl6* 74,24 ± 6,996SE et *Tcf7* 1160,782 ± 107,7SE). De la même façon, le gène codant pour le récepteur CXCR5 a été exprimé à des niveaux inférieurs dans les cellules $T_{LCPlo/int}$ par rapport aux cellules GC-Tfh (valeur TPM : 215,4 ± 14,03SE et 552,9 ± 45,87SE respectivement). Il est important de noter que de nombreuses molécules associées à la mémoire, telles que *Ccr7*, *Il2ra*, *Il7r*, *Ly6a* et *Slamf1*, étaient également fortement exprimées par les cellules $T_{LCPlo/int}$. Prises ensemble, ces données suggèrent que les cellules $T_{LCPlo/int}$ présentaient des similitudes avec la lignée des cellules Th1, bien que ces cellules expriment plusieurs marqueurs de type mémoire.

Le profil moléculaire et phénotypique des cellules $T_{LCPlo/int}$, tel que révélé par l'analyse de séquençage d'ARN, semblait similaire à une population de cellules T $CXCR5^+$ CD8 de type progénitrices récemment décrite. Ces lymphocytes T CD8 $CXCR5^+$ ont le potentiel de s'auto-renouveler et de se différencier en lymphocytes T CD8 effecteurs au cours d'une infection chronique, agissant ainsi comme des cellules de type progénitrices qui reconstituent le compartiment réduit des lymphocytes T CD8. Par conséquent, nous avons évalué si les cellules $T_{LCPlo/int}$ sont les analogues CD4 des cellules T CD8 de type progénitrices $CXCR5^+$. Fait intéressant, nous avons observé que ces cellules expriment fortement les gènes d'auto-renouvellement associés aux cellules souches hématopoïétiques. Pour confirmer que les cellules $T_{LCPlo/int}$ prolifèraient, nous avons évalué l'expression de la protéine Ki67 dans ces cellules, à partir de rates de souris infectées par *L. donovani* à 28 jours p.i. et nous avons constaté qu'elles exprimaient effectivement des niveaux significatifs de Ki67. Par conséquent, ces cellules démontrent qu'elles possèdent un potentiel d'auto-renouvellement et de prolifération. Pour évaluer la capacité d'expansion et les propriétés de type progénitrices des cellules $T_{LCPlo/int}$, nous avons conçu des expériences de transfert adoptif. Lors d'un transfert adoptif chez des souris receveuses naïves de *Rag1*$^{-/-}$ suivi d'une provocation avec *L. donovani*, ces cellules ont pu se différencier en différents types de cellules effectrices telles que les productrices d'IFNγ-, de TNF-, de Granzyme-B ainsi que les cellules régulatrices de type 1 (Tr1). Ces résultats établissent leur capacité à servir de cellules de type progénitrices, qui peuvent donner naissance à de nombreux effecteurs différents en réponse à une LV chronique.

De plus, nous avons démontré que ces lymphocytes T CD4 LAG-3^{+} CXCR5^{+} sont présents chez les souris infectées par le LCMV ainsi que chez les souris infectées par les vers helminthes *H. polygyrus bakeri*. Il convient de noter que dans chacune de ces infections chroniques, ces cellules exprimaient différents niveaux de PD-1 lors de la phase d'expansion maximale des lymphocytes T CD4. Dans l'infection par *L. donovani*, la plupart de ces cellules exprimaient des niveaux intermédiaires de PD-1 chez les souris infectées par le LCMV Cl13, la plupart de ces cellules étaient PD-1^{hi}, tandis que dans l'infection par *H. polygyrus bakeri*, la plupart étaient PD-1^{lo}. Ces résultats suggèrent que, bien que ces cellules existent dans différentes infections, le degré d'expression de PD-1 par ces cellules varie en fonction du type d'infection.

Un article publié décrivait une population similaire de lymphocytes T CD4 de type progéniteurs exprimant le facteur de transcription BCL-6. Notre analyse par cytométrie en flux a indiqué que les cellules $T_{LCPlo/int}$ étaient phénotypiquement très différentes de ces progéniteurs dépendants de BCL-6 décrits par ces autres auteurs, car elles exprimaient des molécules de surface cellulaire très différentes. Cependant, nous étions intéressés de savoir si cette protéine BCL-6 jouait un rôle significatif dans les cellules $T_{LCPlo/int}$. Par conséquent, nous avons infecté des souris possédant des lymphocytes T CD4 de type sauvage ou déficientes en BCL-6 avec *H. polygyrus bakeri*. Nous avons observé que 14 jours après l'infection, la fréquence des cellules $T_{LCPlo/int}$ a diminué significativement dans les ganglions lymphatiques mésentériques chez les souris déficientes pour *Bcl6,* spécifiquement dans les lymphocytes T CD4 par rapport aux souris dont les lymphocytes T CD4 sont *Bcl6*-suffisant. Cela suggère que BCL-6 est nécessaire au maintien de la population des cellules $T_{LCPlo/int}$. De plus, nous voulions savoir si l'absence de BCL-6 affectait l'induction des différents sous-ensembles de lymphocytes T CD4. Par conséquent, nous avons effectué des expériences de transfert adoptif en utilisant le modèle murin de VL. Nous avons observé que les souris *Rag1*$^{-/-}$ ayant reçu des lymphocytes T CD4 déficients en BCL-6 présentaient une fréquence significativement accrue de cellules IFNγ^{+}IL-10^{+} Tr1 et de Granzyme-B^{+} par rapport à celles ayant reçu des lymphocytes T CD4 suffisants en BCL6. Ainsi, l'absence de BCL-6 a favorisé la différenciation en cellules T Granzyme-B^{+} CD4 et cellules Tr1.

Les résultats compilés dans ce document de thèse pourraient être déterminants à bien des égards pour la conception de stratégies thérapeutiques à l'avenir. L'un des composants cellulaires essentiels qui contribuet à l'élimination des agents pathogènes est la réponse des lymphocytes T CD4. Cependant, dans de nombreuses infections virales, parasitaires et bactériennes chroniques, il existe un déclin général de la réponse des lymphocytes T CD4, soit en raison de l'apoptose, soit en raison d'autres mécanismes suppresseurs. Nous décrivons ici un

sous-type de lymphocytes T CD4, caractérisés par l'expression des marqueurs LAG-3 et CXCR5. Ces cellules ont le potentiel de s'auto-renouveler, peuvent proliférer et se différencier pour donner naissance à des sous-types effecteurs et régulateurs de cellules T CD4 au cours de la LV chronique. Par conséquent, nous proposons que cette population de lymphocytes T CD4 pourrait contribuer au maintien de la réponse des lymphocytes T CD4 lors d'infections chroniques.

L'une des idées centrales que nous aimerions proposer à travers ces résultats est que l'expression des récepteurs inhibiteurs par les lymphocytes T peut être une indication d'autres fonctions au-delà du rôle de marqueur de l'épuisement des lymphocytes T. En fait, les molécules de point de contrôle immunitaire (immune checkpoint molécules) LAG-3 et PD-1 sont connues pour être responsables du « réglage fin » de la réponse immunitaire aux infections rétrovirales antérieures. Le concept selon lequel les récepteurs inhibiteurs constituent des outils importants de différenciation a été le thème de quelques études ces dernières années portant sur l'immunité anti-tumorale. Il a également été récemment prouvé que l'une des fonctions principales de la molécule co-stimulatrice négative CTLA-4 est de restreindre les cellules T CD4 périphériques à des limites phénotypiques spécifiques. Il existe des études très récentes dans le domaine de l'immunologie tumorale qui démontrent que le blocage des récepteurs inhibiteurs conduit à la transgression des voies conventionnelles de différenciation des lymphocytes T CD4, entraînant l'émergence de phénotypes non canoniques. Cependant, l'importance de ces sous-ensembles et leur contribution aux réponses immunitaires anti-tumorales restent à explorer. Les cellules $T_{LCPlo/int}$ expriment également CTLA-4 et d'autres molécules co-inhibitrices. Par conséquent, il sera intéressant d'étudier si ces cellules répondent au traitement anti-CTLA-4 et si elles sont similaires ou différentes des sous-types non canoniques décrits dans les études liées à l'immunologie tumorale. Il serait également intéressant d'explorer davantage les paramètres qui sous-tendent la prolifération de ces cellules et les signaux directionnels dont elles ont besoin pour donner naissance à différents types de lymphocytes T CD4 effecteurs.

Une autre observation très intéressante dans cette étude est la découverte d'une population précurseur qui donne naissance aux cellules Tr1, nécessitant partiellement BCL-6 pour son maintien et exprimant fortement Prdm1, qui code pour BLIMP-1. Les cellules Tr1 productrices d'IL-10 jouent un double rôle complexe dans les infections chroniques. Plusieurs études montrent que l'IL-10 dérivée de Tr1 supprime les réponses Th1 dans la LV, entraînant la progression de la maladie chez l'humain. Cependant, il existe également des preuves montrant la suppression médiée par Tr1 des cellules pro-inflammatoires productrices de TNF, ce qui est essentiel pour contrôler les lésions tissulaires pathologiques dans la LV. De même, dans d'autres infections

parasitaires intracellulaires, telles que celles provoquées par des infections à Toxoplasma ou à Plasmodium, il a été démontré que les cellules Tr1 protègent l'hôte contre une immunopathologie sévère. Dans la LV, les cellules Tr1 produisent de l'IL-10 et de l'IFNγ et sont induites par l'IL-27. Dans le paludisme, l'expression de BLIMP-1 a également été suggérée comme étant essentielle au développement de ces cellules et plus récemment à l'expression de récepteurs inhibiteurs. Cependant, on sait peu de choses sur leur origine. Par conséquent, comprendre la génération périphérique de cellules Tr1 au cours d'infections chroniques et les signaux nécessaires pour déclencher ou bloquer leur différenciation est essentiel pour concevoir des outils thérapeutiques ciblant l'IL-10 et cette étude nous a fait avancer plus loin dans cet aspect. Une limite de cette étude est que nous n'avons pas démontré l'existence de ces cellules chez les patients atteints de LV. Cependant, les résultats obtenus sont très pertinents pour comprendre les mécanismes de réponses des lymphocytes T CD4 qui pourraient grandement influencer la conception de thérapies.

En conclusion, le point culminant de cette étude est la découverte d'une population de lymphocytes T CD4 de type progéniteurs qui se développe seulement dans le présence d'antigène. Il existe un déclin fonctionnel et numérique des cellules T CD4 protectrices lors d'infections chroniques, ce qui nécessite l'existence de mécanismes compensatoires pour reconstituer le compartiment des cellules T CD4. Par conséquent, nous proposons que cette population de cellules T CD4 de type progénitrices exprimant LAG-3 et CXCR5 constitue un mécanisme d'adaptation alternatif, autre que la différenciation des cellules T CD4 naïves, pour compenser la perte de cellules T CD4 lors d'infections chroniques. Nous suggérons que les cellules $T_{LCPlo/int}$ pourraient supporter le poids de la différenciation en lymphocytes T CD4 effecteurs au cours des stades chroniques de la LV. Ces cellules pourraient potentiellement répondre aux thérapies d'inhibition des points de contrôle immunitaires et pourraient donc s'avérer utiles non seulement pour traiter les maladies infectieuses, mais également les maladies potentiellement auto-immunes et les cancers.

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LIST OF ABBREVIATIONS

APC	Antigen presenting cell
BCL-6	B-cell lymphoma 6
CL	Cutaneous leishmaniasis
Cl13	Clone 13 (strain of LCMV)
DC	Dendritic cell
DNA	Deoxyribo nucleic acid
ETP	Early lymphoid progenitor
Foxp3	Forkhead box P3
GM-CSF	Granulocyte macrophage colony-stimulating factor
HSC	Hematopoietic stem cell
ICOS	Inducible T cell costimulator
IFNAR	Interferon-α/β receptor
IFNγ	Interferon-γ
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgSF	Immunoglobulin super family
IL-10	Interleukin 10
IL-12	Interleukin 12
ILC	Innate lymphoid cell

iNK-T cell	Invariant natural killer T cell
IRF	Interferon regulatory factor
JAK	Janus kinase
LAG-3	Lymphocyte activation gene 2
LAP	LAG-3-associated protein
LCMV	Lymphocytic choriomeningitis virus
LFA-1	Leukocyte function-associated factor 1
MAPK	Mitogen-activated protein kinases
MCL	Muco-cutaneous leishmaniasis
MDSC	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
MZ	Marginal zone
NK	Natural killer
NOD	Non-obese diabetic
NOS-2	Nitric oxide synthase 2
PALS	Peri-arteriolar lymphoid sheath
PAMP	Pathogen associated molecular pattern
PD-1	Programmed death 1
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C

PKDL	Post-Kala-azar dermal leishmaniasis
PRR	Pattern recognition receptor
qRT-PCR	Quantitative real-time polymerase chain reaction
RIG-I	Retinoic acid-inducible gene I
RNA	Ribo nucleic acid
SCID	Severe combined immunodeficiency
STAT	Signal transducer and activator of transcription
STH	Soil transmitted helminths
TCF-1	T cell factor 1
TCR	T cell receptor
Tfh	T follicular helper
TGF-β	Transforming growth factor β
Th1	T helper type 1
Th17	T helper type 17
Th2	T helper type 2
TH22	T helper type 22
Th25	T helper type 25
Th3	T helper type 3
Th5	T helper type 5
Th9	T helper type 9

TIM-3	T cell immunoglobulin and mucin-domain containing-3
TK	Tyrosine kinase
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TOX	Thymocyte selection-associated high-mobility group box
Tpex	T progenitor exhausted
Tr1	Type 1 regulatory
TRAIL	TNF-related apoptosis inducing ligand
Treg	T regulatory
VL	Visceral leishmaniasis
WHO	World health organization

1 INTRODUCTION

1.1 *Leishmania,* the parasites

The word “parasite” originates from *“parasitos”* in Greek, which literally means “person eating at another’s table”, and is thought to have been used in the English language since the mid-16th century. This ancient Greek definition of the word is not quite far from the modern-day definition of a parasite – “an organism that lives in or on an organism of another species (its *host*), and benefits by deriving nutrients at the other's expense”. Many other civilizations including, but not limited to the Egyptians, Chinese, Indians and Romans, were also well aware of the existence of parasites.

Leishmaniasis is a parasitic disease that is vector-borne and results in severe infection. It is caused by the unicellular, eukaryotic, flagellated, protozoan parasites of the genus *Leishmania*. There are about 22 species of *Leishmania* that is pathogenic to humans. These parasites are transmitted to mammalian hosts by the bite of female phlebotomine sand flies. These parasites are thought to have originated in the Mesozoic era (252-66 million years ago) (Steverding, 2017). However, the exact region of origin for each of the known species of *Leishmania* is a topic of continuing debate. Reports from paleoparasitological analysis have shown the existence of *Leishmania* mitochondrial DNA in Egyptian mummies from 2050–1650 BCE (Steverding, 2017).

The name “leishmaniasis” originates after the British doctor William Boog Leishman, who first reported these parasites. In British India, records from the 18th century report a disease locally known as “kala azar”, meaning black disease. As indicated by the name, the local population in India was aware of the severity of this disease, although the causative organism remained unknown. In November 1900, William Boog Leishman noticed the presence of “ovoid bodies” in the smears prepared from the spleens of English soldiers stationed in Calcutta, in the Bengal Presidency of British India, who died from splenomegaly, cachexia, high fever and anemia (Leishman, 1903). Leishman thought these ovoid bodies were degraded forms of *Trypanosoma*, and therefore, he reported this disease as a form of trypanosomiasis. Around the same period, another doctor, Charles Donovan who was stationed in the southern most Presidency of Madras, in British India, also observed similar “ovoid bodies” in patients dying from a disease causing splenomegaly and emaciation (Donovan, 1903). In 1903, Ronald Ross, who was a medical doctor stationed in British India to specifically investigate “kala azar” noticed that the “ovoid bodies” discovered by Leishman and Donovan were not only similar to each other but also matched with

the clinical case reports from “kala azar”. Therefore, he arrived at the conclusion that this disease, kala azar, is not a form of trypanosomiasis, but is caused by a previously unidentified type of parasite which he then named *Leishmania donovani* (Ross, 1903).

1.2 The life cycle of *Leishmania*

Leishmania exist in two forms – the amastigote or the promastigote form. These parasites are dependent on both mammalian hosts and sandfly vectors for the successful completion of their life cycle. Female sandflies regurgitate the *Leishmania* parasites in the promastigote form, into the dermis of the hosts while consuming a blood meal. These promastigotes are then phagocytosed mainly by neutrophils at the site of the sandfly bite (Peters *et al.*, 2008), and are later seen mostly in macrophages, dendritic cells (DCs) as well as fibroblasts. The promastigotes transform into amastigotes and multiply in number. The amastigotes are then released into the blood stream following rupture of the infected cells. Amastigotes are then transmitted to the vectors through a blood meal, and are once again transformed into promastigotes. Most of the metacyclic promastigotes reside in the anterior mid-gut of the sandflies (Rogers *et al.*, 2002), which is usually obstructed with promastigote secretory gel, and a portion of this gel, known as the filamentous proteophosphoglycan accompanies the parasites (Rogers *et al.*, 2004) as the sand-flies transmit them to the mammalian host by regurgitation. Figure 1.1 shows a schematic diagram of the life cycle of the parasite.

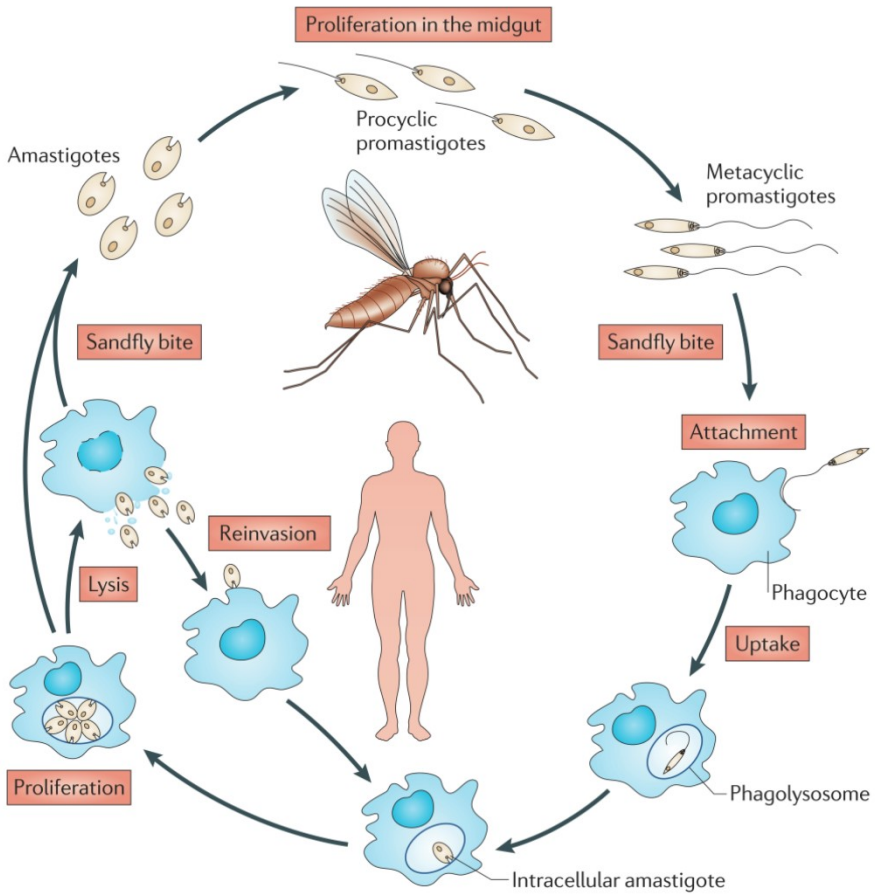

Figure 1.1 Life cycle of *Leishmania* parasites.
Reproduced from (Kaye & Scott, 2011).

1.3 Leishmaniasis and its clinical forms

As of today, leishmaniasis is known to have three different clinical forms – 1) the cutaneous form, caused by *L. major*, *L. tropica, L. amazonensis, L. aethiopica* among many others 2) the muco-cutaneous form caused by *L. braziliensis* and *L. guyanensis* and 3) and the visceral form caused by *L. donovani* and *L. infantum (chagasi)*.

1.3.1 Cutaneous leishmaniasis

CL is the most common clinical manifestation of this disease. According to the World health organization, there are about 1 million new cases of CL reported annually, worldwide. The majority of the CL cases occur in the Americas, eastern Africa, particularly Algeria where this form is endemic, the Middle East, and central Asia (World Health Organization, 12 January 2023). These skin lesions begin as nodules or papules which later progress into ulcers, and are usually self-

curing within 3-18 months (Burza *et al.*, 2018). Although these lesions heal, they leave behind severe, long lasting scars, and often lead to disfiguration and societal stigma. CL can also manifest as diffuse lesions, without ulcers, but appearing as nodules and plaques and mostly occurring in immunocompromised individuals (Mann *et al.*, 2021). Yet another form is the disseminated CL, characteristic of “non-contiguous pleomorphic lesions” in otherwise healthy hosts (Mann *et al.*, 2021).

1.3.2 Mucocutaneous leishmaniasis

MCL affects the mucous membranes in the mouth, throat, and nose. Although it mainly affects the nose and oropharynx, it can also progress to the larynx and pharynx. This manifestation of leishmaniasis is highly disfiguring, and is associated with social stigma. Bolivia, Brazil, Ethiopia and Peru are the major countries where this disease is prevalent, accounting for more than 90% of the affected population (World Health Organization, 12 January 2023).

1.3.3 Visceral leishmaniasis

The most common symptoms of VL include hepatosplenomegaly or the enlargement of the spleen and liver, cachexia, anemia, pancytopenia, and generalized immunosuppression. The major challenge with this particular form is that it can be fatal if left untreated. Even in treated individuals, the disease can reoccur, manifesting as Post-kala-azar dermal leishmaniasis (PKDL). This develops as skin rash on the face, arms and trunk of supposedly cured individuals about 8 months to one year after incidence of VL. These individuals with PKDL are considered to serve as a reservoir for *L. donovani*. The majority of VL cases occur in Brazil, east Africa, and in the Indian subcontinent. There are about 50 000 to 90 000 new cases around the world, occurring every year (World Health Organization, 12 January 2023). The most recent prevalence chart of endemic VL reported by the WHO is shown in Figure 1.2.

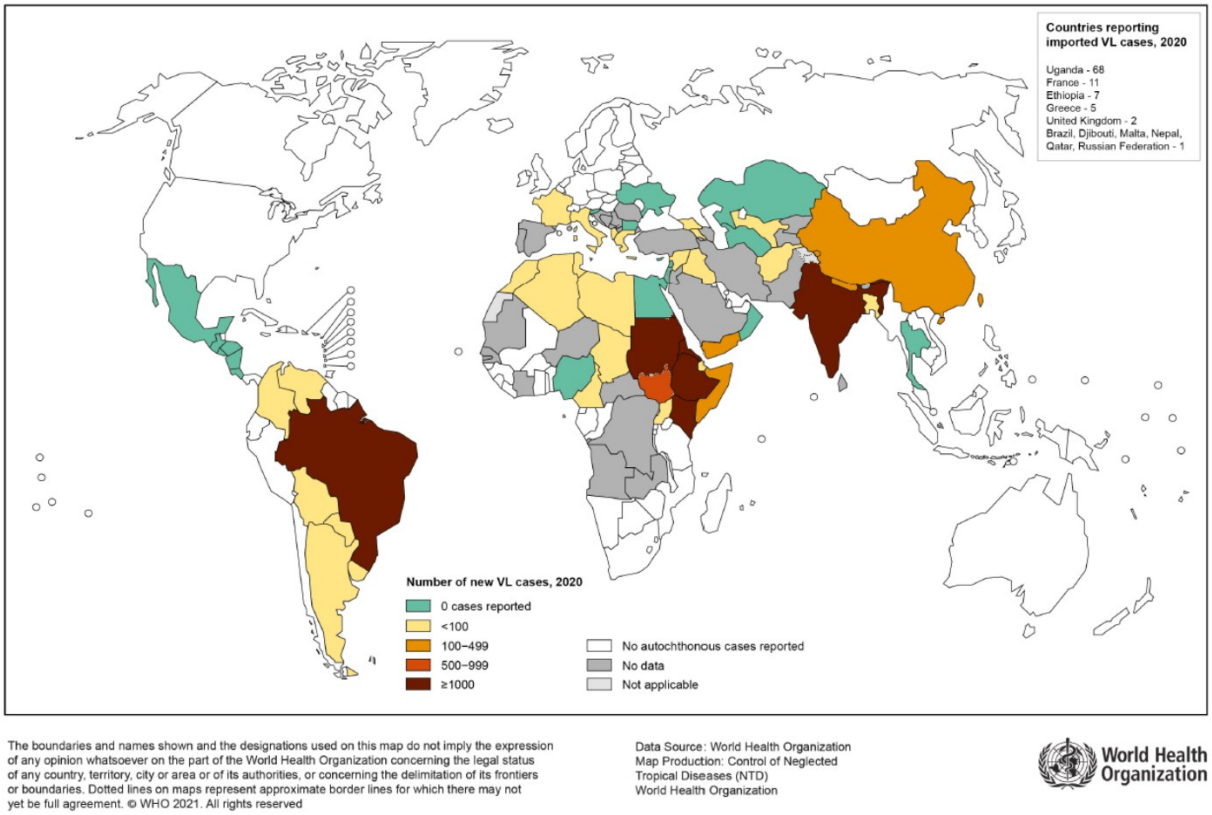

Figure 1.2 Worldwide endemicity of VL reported by WHO, as of 2020

Reproduced from the WHO website (World Health Organization, 2020).

1.4 Challenges in VL treatment

Early diagnosis of VL in the most affected areas such as those in the Indian subcontinent is also a difficult task as most affected individuals present themselves at care centers at an already advanced stage. The gold standard approach for the detection of VL is the visualization of parasites in smear samples coming from bone marrow aspirates in patients presenting with clinical symptoms (Singh & Sundar, 2015). However, there are also more specific immunological approaches to detect *Leishmania*-specific antibodies or circulating antigens in the sera of patients (Singh & Sundar, 2015). There are also molecular biology based methods such as PCR to detect *Leishmania* DNA. Quantitative real time PCR can be used to distinguish between symptomatic and asymptomatic disease – Sudarshan et al. observed that the parasite load on symptomatic individuals was 500 times more compared to asymptomatic individuals. Additionally, the asymptomatic individuals had a maximum parasite level of 5 parasite genome/ml of blood, and in

symptomatic individuals, it was always higher. Thus, the use of qPCR is an invaluable tool that could be used in early diagnosis, preventing adverse disease outcomes and possibly to prevent spread (Sudarshan & Sundar, 2014).

The current treatment strategies for VL mostly includes chemotherapy using compounds of antimony (antimonials) such as meglumine antimoniate or glucantime®, sodium stibogluconate or Pentostam® (Karunaweera & Ferreira, 2018). Some antifungal compounds such as amphotericin B, ketoconazole as well as miltefosine, which was originally developed as an anti-cancer drug, are also currently in use for treating VL (Singh *et al.*, 2016). The major drawback in the using these compounds for treatment is that none of them were developed to specifically treat this disease. Furthermore, the continued use of antimonials can be toxic and in the long run, these compounds are known to cause damaging side effects such as renal or cardiac failure (Hendrickx *et al.*, 2016; Uliana *et al.*, 2018).

The antimonials, which are the most commonly used drugs to VL, have lately been proven to be ineffective in killing *L. donovani* as the parasites have developed resistance to these drugs (Vanaerschot *et al.*, 2011). Therefore, liposomal amphotericin B, which is an antifungal agent, is now suggested as a treatment option against VL. However, there are other problems associated with its use, such as cold storage requirements, high cost and reduced availability, all of which reduces the feasibility and ease of use of this drug (Hendrickx *et al.*, 2016).

Furthermore, the molecular markers exhibited by *Leishmania* that contribute to its drug resistance capabilities are hard to discern. This is due to the ability of these parasites to modulate their gene expression or even alter their chromosomal ploidy in response to stress (Leprohon *et al.*, 2009). In addition, the parasites can exist in different stages. Therefore, the molecular characterization studies of drug-resistant clinical isolates is very hard to perform and interpret, as the resulting data might vary depending on the stage of the parasite, creating difficulties in discerning the outcomes (Hendrickx *et al.*, 2016).

To date, there are no approved prophylactic or therapeutic vaccines for any form of leishmaniasis affecting humans. This disease being prevalent among people with poor living conditions, does not offer promising financial benefits for pharmaceutical companies to spend resources and time in developing vaccines. Hence, the WHO classifies leishmaniasis as a neglected tropical disease (World Health Organization, 12 January 2023).

1.5 Mouse models of VL and the host immune response

As studying T cell responses and other aspects of host immune responses to this disease would involve very difficult and invasive techniques in VL patients, using experimental models to understand the immunopathology of this disease and host defense mechanisms, serves as a good alternative. Among all the rodent models available to study VL, the Syrian hamsters, *Mesocricetus auratus*, are the ones that closely resemble symptoms and disease progression patterns seen in humans (Hommel *et al.*, 1995). However, due to the lack of immunological tools to study these animals (such as lack of specific, monoclonal antibodies and other species-specific molecular tools), using them for immunological studies involving VL continues to be challenging. On the other hand, laboratory mice serve as good models because of several reasons. Firstly, there are many immunological tools (specific antibodies against different markers) available to study these animals. Secondly, mice infected with *L. donovani* exhibit hepatosplenomegaly, and the disease is not fatal in mice (although it is fatal in humans if left untreated). The most commonly used experimental models of VL are the C57BL/6 mice. Several factors such as mouse genetic background, dosage and strain of the parasite, mode of injection or route of entry of the parasite affect the disease outcome and influence experimental findings in mouse models of VL. There have been several studies assessing the impact of each of these factors on experimental research on VL. For instance, it is well known that the deletion of *Slc11a1* gene, which encodes for an ion transport protein NRAMP1 in macrophages, results in the increased susceptibility to VL in otherwise resistant wild type mice (Vidal *et al.*, 1995). Therefore, only mice lacking functional *Slc11a1* such as the C57BL/6 can be used for studying VL as the other strains are resistant.

Early studies aimed at understanding pathogen clearance mechanisms to *L. donovani* infection were mostly focused on the parasite control in the liver. The formation of granulomas in the liver, accompanied by IFNγ production were known to contribute immensely to pathogen clearance (Stern *et al.*, 1988). There are several early studies highlighting the role of T cells in hepatic resistance as well. Both nude mice (Murray *et al.*, 1995) and SCID mice (Kaye & Bancroft, 1992) were unable to reduce parasite load, due to the lack of IFNγ-producing NK cells, CD4 and CD8 T cells (Kaye *et al.*, 2004), which triggered the activation of infected macrophages and facilitated clearance of intracellular parasites by the production of reactive oxygen and reactive nitrogen intermediates (Murray & Nathan, 1999). These intermediates also play an important role in the early phase of the infection by recruiting inflammatory monocytes (Cervia *et al.*, 1993) and neutrophils (Murray & Nathan, 1999; Smelt *et al.*, 2000).

Although initial studies in VL were mainly focused on the infection in the liver, in the later years it became evident that the organ-specific responses in VL is varied, as in many other infections, (Engwerda & Kaye, 2000). The three main organs affected by VL are the liver, the spleen and the bone marrow. Unlike the liver, where the pathogen is cleared after 4 weeks of infection, *L. donovani* infection persists in the spleen and the bone marrow of infected mice life-long.

1.5.1 Infection in the liver

It is well-established that the *L. donovani* infection in the liver is self-healing within six to eight weeks post-infection. Rapid parasite clearance in the liver by the formation of granulomatous lesions is in fact the central feature associated with disease resistance in murine models of VL (Murray, 2001). Granulomas in VL were defined by McElrath et al., "inflammatory foci containing infected cells surrounded by mononuclear cells and granulocytes" and these authors also reported in this early study that the Kupffer cells, the liver resident macrophages, are the major cell type responsible for parasite-uptake immediately following infection (McElrath *et al.*, 1988). Granulomas consists of infected Kupffer cells along with granulocytes and monocytes initially, closely followed by infiltration of neutrophils and eosinophils as well (McElrath *et al.*, 1988). Only during the second week of infection, the recruitment of lymphocytes including T cells and B cells occurs (Moore *et al.*, 2012). Around four weeks post-infection, the size and number of granulomas reaches its maximum, accompanied by the highest parasite burden, and all of these factors subside in the following weeks as the infection and inflammatory response clear up (McElrath *et al.*, 1988). A brief outline of the granulomatous response in the liver is shown in Figure 1.3 below.

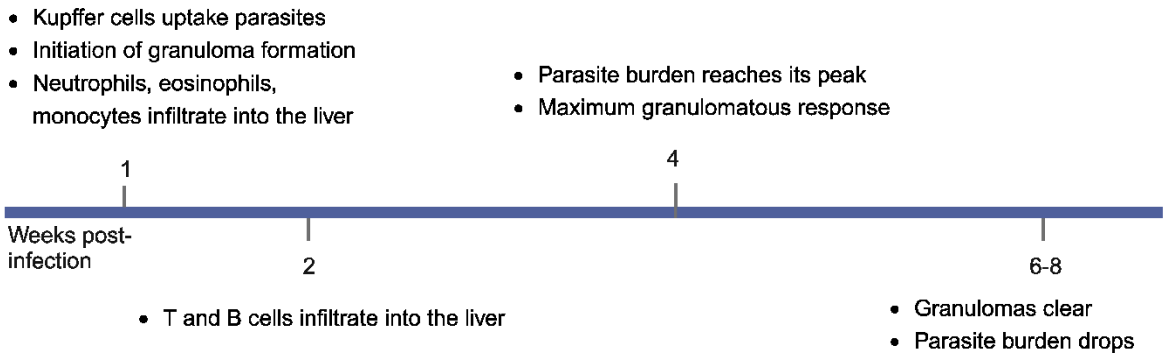

Figure 1.3 Granulomatous response in the livers of mice infected with *L. donovani*

Image created using Biorender.com

The two major IFNγ-producing cells that assist in the formation of these granulomas are the Th1 cells and the CD8 T cells (Stern *et al.*, 1988). Furthermore, IFNγ production by T cells aids in attracting monocytes to the liver during the initial weeks of the infection (Cervia *et al.*, 1993) as well as triggering the production of reactive oxygen and nitrogen intermediates, which are the principal mechanisms deployed by macrophages for killing of intracellular parasites (Murray & Nathan, 1999). The Th1 cells in the liver are triggered by IL-12 produced by the DCs, which have encountered parasites (Gorak *et al.*, 1998; Scharton-Kersten *et al.*, 1995). Indeed, treatment of mice with anti-IL-12 during initial and later stages of infection results in the suppression of IFNγ levels, and abrogation of protective T cell responses in the liver, leading to disease exacerbation (Murray, 1997), thereby establishing the central protective role for this cytokine in hepatic resistance.

1.5.2 Infection in the bone marrow

The bone marrow is the primary site of hematopoiesis or production of blood cells. The hematopoietic stem cells (HSCs) in the bone marrow give rise to the cells of the immune system. These cells are quiescent under homeostatic conditions and are only activated, and directed to migrate to other organs such as the spleen in response to stressful conditions (injuries or infections) where there is a marked loss of HSC-derived cells. This activation usually serves as a compensatory mechanism for the coping with the loss of blood cells, and is aimed at restoring homeostasis. The term “extramedullary hematopoiesis” is often used to describe such scenarios where hematopoiesis occurs in any organ other than the bone marrow, in order to compensate for loss of cells due to stress. In bacterial infections, several factors including pro-inflammatory cytokines like IFNγ and microbial sensing by TLRs, results in the triggering of hematopoiesis (Baldridge *et al.*, 2010; Burberry *et al.*, 2014). Ideally, once the infection is resolved and the associated inflammation is reduced, HSCs should revert to a dormant state for restoration of homeostasis. However, in persistent infections, there is bone marrow dysfunction, which leads to failure to cope for the loss of immune cells. Often, the invading pathogens tend to exploit these mechanisms for immune evasion, some of which are outlined below.

Indeed, symptoms of VL in humans include several hematological conditions such as anemia, pancytopenia, thrombocytopenia and many others, evidently suggesting bone marrow dysfunction (Matnani & Ganapathi, 2016; Varma & Naseem, 2010). Additionally, in hamsters infected with *L. donovani*, there is an excessive loss of erythroblasts in the bone marrow resulting in anemia. This is due to TRAIL-mediated apoptosis of these cells, triggered by the presence of excess amounts of IFNγ, due to the inflammatory response to the infection (Lafuse *et al.*, 2013).

Recently, it was shown that human HSCs derived from the bone marrow phagocytose *L. infantum* promastigotes *in vitro.* This triggered the proliferation of these human HSCs *in vitro*, and altered the expression of several markers on the resulting HSC-progeny (Carvalho-Gontijo et al., 2018).

A recent study published this year demonstrated that HSCs are infected with *L. donovani* (Karagiannis et al., 2023). Previously, it was only known that the stromal macrophages in the bone marrow are parasitized (Cotterell *et al.*, 2000b). As a consequence, there is increased production of GM-CSF and TNF, which in turn selectively enhances myelopoiesis (Cotterell *et al.*, 2000b). As early as five hours post infection, mobilization of progenitor cells arising from HSCs were detectable in the blood of *L. donovani*-infected mice, concurrent with the increase in GM-CSF and MIP-1α levels in the bone marrow (Cotterell *et al.*, 2000a). These progenitor populations derived from HSCs were detected in the spleen and the bone marrow as the parasite growth increased, along with the accumulation of growth factors such as GM-CSF, G-CSF, and M-CSF in these organs. (Cotterell *et al.*, 2000a), suggesting a direct effect of the parasite load on hematopoiesis. Additionally, *L. donovani* provokes HSC-proliferation, and hijacks HSC-differentiation to produce non-conventional precursor cells of the myeloid lineage, giving rise to more regulatory cell types that encourage parasite growth and undermine host defense (Abidin *et al.*, 2017). Specifically, as opposed to steady state conditions where myelopoiesis results in the production of both granulocyte (neutrophils) and monocytes in equal proportions, *L. donovani* infection results in an imbalance, marked by the generation of Ly6C^{hi} monocytes mostly, which showed clear signs of regulatory phenotype such as expression of Sca-1 (Abidin *et al.*, 2017) and Galectin-3 (MacKinnon *et al.*, 2008). Altogether, these studies strongly suggest that *L. donovani* deploys emergency hematopoiesis as an immune evasive mechanism to produce “safe targets” (permissive host cells) that promote parasite replication and serve to be detrimental to the host, as demonstrated in other *Leishmania* species (Hammami *et al.*, 2017; Mirkovich *et al.*, 1986).

The impact of pro-inflammatory cytokines IFNγ and TNF in shaping the bone marrow immune response to VL is complex. T cells serve as predominant cellular sources for these cytokines. In VL, TNF-dependent production of IFNγ by CD4 T cells is known to drive HSC expansion in the bone marrow, eventually leading to their exhaustion (Pinto *et al.*, 2017a). The IFNγ-producing CD4 T cells play a significant role in causing anemia by modifying the stromal microenvironment in the bone marrow, which adversely affects erythropoiesis (Preham *et al.*, 2018). The IFNγ produced by T cells also induces the activation of Ly6C^{hi} inflammatory monocytes in the bone marrow (Romano *et al.*, 2021). However, these Ly6C^{hi} monocytes promote the infection and contribute to the disease (Abidin *et al.*, 2017; Hammami *et al.*, 2017; Terrazas *et al.*, 2017). These

findings point out the differences in organ-specific responses during VL. Although IFNγ and TNF produced by T cells is critical for leishmanicidal activity in the liver, these T cells activate inflammatory Ly6C^{hi} monocytes in the bone marrow, thereby contributing to the disease. The generation of immune response in the bone marrow is multilayered, and very different from the responses in other organs and therefore, warrants further investigation.

1.5.3 Infection in the spleen

1.5.3.1 Macrophages and DCs

Due to the persistence of the parasite in the spleen, there is splenomegaly, chronic inflammation as well as changes in the splenic microarchitecture (Bankoti & Stäger, 2012). These factors contribute towards a defective host response to the parasite. The mouse spleen is segregated into different functional zones such as the marginal zone (MZ), where the DCs encounter antigens initially, the periarteriolar lymphoid sheath (PALS), which are the T cell rich zones near the arterioles, and the follicles, which contain germinal centers where B cell maturation takes place. *L. donovani* infection studies in mice indicate that although the macrophages in the MZ encounter a lot of parasites during the initial hours of infection, these cells fail to produce IL-12 necessary for triggering the protective IFNγ production by T cells (Gorak *et al.*, 1998). In contrast, the DCs in the PALS are responsible for triggering the production IFNγ in T cells, which is central for pathogen clearance (Gorak *et al.*, 1998). However, the IL-12 production by DCs declines rapidly after 24 hours following experimental infection. Furthermore, the macrophages in the splenic marginal zone start producing the pro-inflammatory cytokine TNF starting from the early periods post-infection, resulting in the gradual destruction of the splenic microarchitecture which occurs between 14 or 21 days post-infection (Engwerda *et al.*, 2002). Additionally, the DCs, which are the cellular sources of IL-12, which in turn instigate IFNγ-production, are prone to several inflammation-induced changes as well. The antigen-exposed DCs exhibit decreased expression of costimulatory molecules, increased expression of coinhibitory molecules and fail to migrate to the T cell areas or the PALS, where these two cell types interact (Ato *et al.*, 2002; Engwerda *et al.*, 2002; Joshi *et al.*, 2009a). In *L. donovani*-infected mice, the stromal cells in the spleen induce the selective generation of regulatory DCs which produce IL-10 (Svensson *et al.*, 2004). In short, during the later stages (starting from 14 and 21 days post-infection) of VL, several factors result in DC dysfunction. There is also the disruption of splenic microarchitecture during this period. These factors altogether result in defective migration of the DCs and T cells into the splenic white

pulp, leading to improper priming of the T cells by the DCs, which in turn compromises antigen-specific T cell responses.

Yet another mechanism of DC dysfunction in the chronic phase of VL is through the expression of the hypoxia inducible factor-1α (HIF-1α). HIF also induces the production of IL-10 in DCs. Most importantly, HIF-1α interferes with IL-12 expression in DCs (Hammami *et al.*, 2015b) and DC-specific deletion of this protein enhances Th1-mediated pathogen clearance in the spleen and bone marrow (Hammami *et al.*, 2018). Therefore, HIF-1α expression by DCs heavily influences the priming of protective Th1 cells. In addition, upregulation of this protein on DCs following infection-induced inflammation also results in the dysfunction of CD8 T cell responses (Hammami *et al.*, 2015b). Studies have also demonstrated that upregulation of HIF-1α in phagocytic cells, following *in vitro* infection with different species of *Leishmania*, decreases anti-microbial capacity of these cells, suggesting this as one of the mechanisms employed by the parasite to hijack host immune responses (Charpentier *et al.*, 2016; Degrossoli *et al.*, 2007; Singh *et al.*, 2012). Furthermore, in *L. donovani* infected mice, HIF-1α enhances regulatory activity of myeloid-derived cells and dampens their ability to clear the parasite (Hammami *et al.*, 2017). A short summary of critical events affecting macrophages and DCs and as a consequence, the priming of T cells during VL, is shown in through a schematic diagram in Figure 1.4.

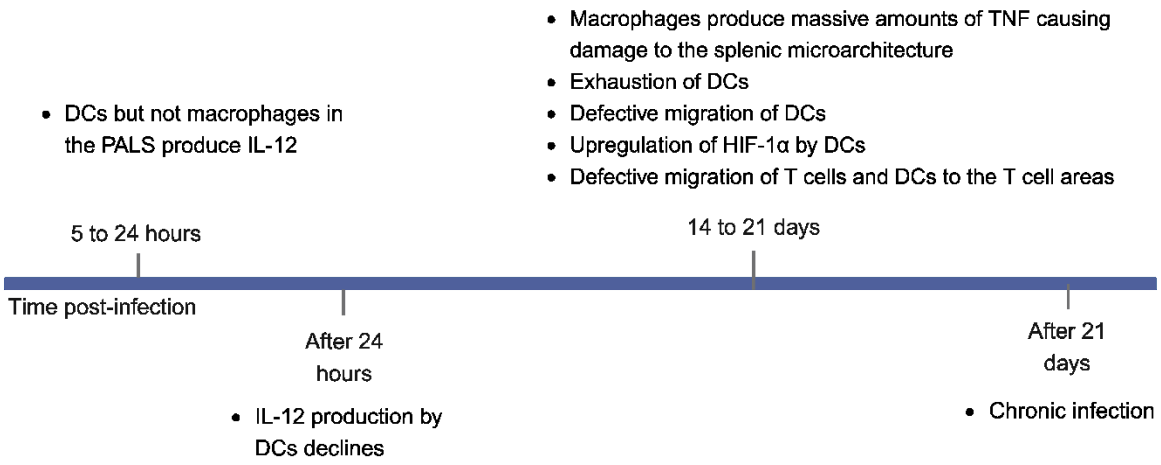

Figure 1.4 Events leading to defective T cell priming in VL

Image created using Biorender.com

1.5.3.2 B cells

One of the major cellular contributors leading to the worsening of VL are the B cells (Deak *et al.*, 2010; Silva-Barrios *et al.*, 2016; Silva-Barrios & Stäger, 2019; Smelt *et al.*, 2000; Stögerer *et al.*, 2023). Hypergammaglobulinemia is a characteristic symptom of VL, and is exhibited by natural hosts of *L. donovani* such as humans (Cooper *et al.*, 1946), dogs (Silva-O'Hare *et al.*, 2016), cats (Spada *et al.*, 2020) as well as experimental animals such as rhesus macaques (Rodrigues *et al.*, 2014b), hamsters and mice (Deak *et al.*, 2010; Silva-Barrios *et al.*, 2016; Silva-Barrios & Stäger, 2019; Smelt *et al.*, 2000; Stögerer *et al.*, 2023). It is also clearly established that the polyclonal B cell activation followed by hypergammaglobulinemia or the production of low affinity antibodies, is the main mechanism by which B cells contribute to aggravation of VL (Deak *et al.*, 2010; Silva-Barrios *et al.*, 2016; Silva-Barrios & Stäger, 2019; Smelt *et al.*, 2000; Stögerer *et al.*, 2023).

Although most of these studies are focused on the role on B cells in antibody production, these cells also have many other functions in leishmaniasis (VL and other forms) independent of antibodies, as briefly outlined below. IL-10 producing regulatory B cells induce T helper type 2 (Th2) bias in BALB/c mice infected with *L. major* (Ronet *et al.*, 2010). The B cell-deficient muMT mice display enhanced CD8 and CD4 T cell responses resulting in lowering of the parasite burden (Bankoti *et al.*, 2012), indicating that B cells play a crucial role in restraining T cell responses. In addition, Bankoti et al. also demonstrated that this restriction of T cell function by the B cells is partly mediated by MyD88-dependent IL-10 production. MyD88 is a crucial adaptor protein that is downstream of toll-like-receptors (TLRs) as well as interleuikin-1 (IL-1) receptor families (Deguine & Barton, 2014). In fact, *L. donovani* instigates the activation of innate sensing molecules such as endosomal TLRs, particularly TLR-3, TLR-7 and TLR-9, at its point of contact with B cells, resulting in the expression of cytokines such as type I interferons and IL-10, which in turn affected protective Th1 responses (Silva-Barrios *et al.*, 2016). Additionally, type I interferons are also required for the induction of TLRs on B cells, thereby contributing to a feed-forward loop (Silva-Barrios *et al.*, 2016). Therefore, the induction of endosomal TLRs on the B cells and type I interferon production by these cells compounds the major problem caused by B cells in VL, which is hypergammaglobulinemia (Silva-Barrios *et al.*, 2016). In line with these findings, the *Aicda*$^{-/-}$ mice lacking the AID enzyme, which is required for the production of hypermutated, class-switched, functional antibodies by B cells, did not exhibit hypergammaglobulinemia and were resistant to infection with *L. donovani*, when compared to wild type controls (Silva-Barrios & Stäger, 2019). Furthermore, these mice also displayed better Th1 responses and lesser frequencies of IFNγ and IL-10-expressing regulatory CD4 T cells, which enhanced protection

(Silva-Barrios & Stäger, 2019). Another recent study by our team on B cells and their interaction with *L. donovani* demonstrates that these cells may procure parasites from infected macrophages through interesting structures in the form of tubular protrusions known as tunnelling nano tubes (Stögerer *et al.*, 2023). These structures may then serve as a means to spread the parasites among B cells, activate these cells and cause polyclonal B cell activation (Stögerer *et al.*, 2023). The schematic diagram in Figure 1.5 shows the many deleterious roles of B cells in VL.

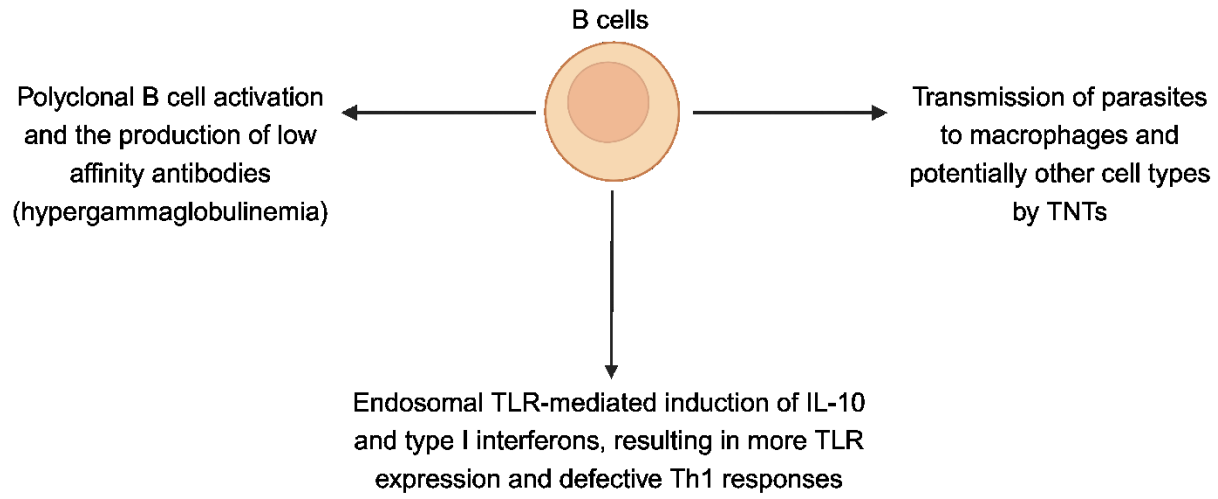

Figure 1.5 Negative roles of B cells in VL

Image created using Biorender.com

1.5.3.3 CD8 T cells

CD8 T cells recognize MHC-I-antigenic-peptide complexes and upon activation, these cells have the potential to kill target cells and produce several cytokines and chemokines. The response of CD8 T cells to different species of *Leishmania* is different and has been reviewed by Stäger & Rafati (Stäger & Rafati, 2012). Initial studies in experimental cutaneous leishmaniasis suggested that upon infection with a low parasite dose, the CD8 T cells were necessary for mounting protective Th1 responses (Belkaid *et al.*, 2002b). Later it was found that the IFNγ-producing CD8 T cells were necessary to suppress the CD4-Th2 bias in low-dose infections, and in high-dose infections, the Th1 response was independent of CD8 T cells (Uzonna *et al.*, 2004). In addition, even in the low dose infection models, the CD8 T cells also induced pathology (Belkaid *et al.*, 2002b), suggesting that these cells display dual characteristics and might prove to be protective or pathological.

Starting from the late 1980s, it is well known that CD8 T cells enable parasite clearance in the livers of *L. donovani*-infected mice, and assist in granuloma formation in the liver (Stern *et al.*, 1988). Studies in NOD-mice with transgenic I-E molecules showed significantly altered CD8 T cell responses, and these animals were not able to clear the parasite in the liver, demonstrating the crucial role of these cells in conferring resistance to infection (Kaye *et al.*, 1992). Moreover, CD8 T cells not only offer protection during primary infection but also confer resistance to reinfection (Stern *et al.*, 1988). A study involving *L. infantum*-infected mice showed the main mechanisms used by these cells to fight parasites – production of the cytokines IFNγ and TNF, via cytotoxic activity, and by the expression of Fas (CD95)/FasL interaction (Tsagozis *et al.*, 2003) This study also pointed out that apart from engaging in parasite clearance themselves, CD8 T cells also secrete chemokines such as RANTES and MIP-1α, and therefore, attract other inflammatory cells and T cells to the sites if infection. One other very interesting study showed that CD8 T cells expressing CD40 can repress regulatory T cells. The interaction between CD40/CD40L triggers granzyme and perforin expression in the CD8 T cells, eventually killing the Tregs (Martin *et al.*, 2010).

Despite all these protective roles in VL, during the later stages of this infection, CD8 T cells exhibit signs of functional exhaustion (impaired capacity to proliferate and effectively produce cytokines), eventually leading to cell death (Joshi *et al.*, 2009a). This effect can be counteracted by blocking PD-1/PD-L1 (formerly known as B7-H1) interactions, which facilitates rescue of CD8 T cells from exhaustion, and reduces parasite burden. Additionally, the adoptive transfer of functional CD8 T cells to infected mice reduces parasite burden by almost 90% (Polley *et al.*, 2006). Importantly, most experimental vaccines, either prophylactic or therapeutic, that exhibit effectivity are based on rejuvenation of CD8 T cell responses in VL (Maroof *et al.*, 2012; Stäger *et al.*, 2003b).

1.5.3.4 Type I interferons

Regulatory mechanisms that suppress pro-inflammatory responses are in place to protect the host from inflammation-mediated tissue damage. However, in many infections, particularly in parasitic diseases, such anti-inflammatory responses are often exploited by the parasites to inhibit host immunity and as a consequence, either prevent parasite-clearance or support their growth and dissemination. In VL, the majority of immunoregulatory mechanisms are regulated by the pleiotropic cytokine IL-10 as well as the family of cytokines known as type I interferons.

Type I interferons constitute several cytokines including the most common IFNα, IFNβ, and others such as IFNε, IFNτ, IFNκ, IFNω, IFNδ and IFNζ, which are not well-studied (McNab *et al.*, 2015).

There are 13 homologs of the gene encoding IFNα in humans and 14 homologs in mice, while the IFNβ is encoded by a single gene (Weissmann & Weber, 1986). Most mammalian cells can produce type I interferons in response to the activation of pattern recognition receptors such as TLRs and retinoic acid-inducible gene I (RIG-I) that sense pathogen-associated molecular patterns (PAMPs) (McNab *et al.*, 2015). IFNα, in many cases, is produced following triggering of endosomal TLRs such as TLR-7 and TLR-9 upon detection of intracellular DNA or RNA from pathogens (Yan & Chen, 2012). TLR-4 is also known to mediate IFNα expression (Richez *et al.*, 2009). The transcription factors belonging to the family of interferon regulatory factors (IRFs) play an indispensable role in controlling type I interferon induction at the transcriptional level ((Honda *et al.*, 2006).

Cytokines of the type I receptor family bind to the transmembrane receptors known as IFNARs (type I IFN receptor). The canonical downstream signaling cascade triggered by type I interferon-binding to the IFNARs proceeds as follows – the proteins Janus kinase 1 (JAK-1) and tyrosine kinase 2 (TK-2) are activated, and these kinases subsequently phosphorylate the transcription factors called signal transducer and activator of transcription (STATs). Following JAK-mediated phosphorylation, STAT proteins dimerize, forming either homo- or heterodimers, translocate to the nucleus, and can either enhance or repress gene transcription. Alternatively, type I interferons can also activate the mitogen-activated protein kinases (MAPKs) and the phosphoinositide 3-kinase (PI3K) pathways, which are considered to be more non-canonical downstream cascades, resulting in varied effects (McNab *et al.*, 2015). The biological outcome of type I interferon signalling is entirely context-dependent, as it can be influenced by several factors such as the severity of the infectious disease, type of pathogen, cell type, and the amount of cytokine produced (Ivashkiv & Donlin, 2014; Silva-Barrios & Stäger, 2017).

The role of type I interferons in VL is largely understudied. One of the earliest studies in this topic showed that the injection of synthetic double-stranded RNA (Poly I:C) resulted in the generation of excessive amounts of type I interferons resulting in resolution of hepatic parasite burden (Herman & Baron, 1970). In 2016, Silva-Barrios et al. reported on the negative effects of type I interferons in B cells during experimental VL. These authors showed that mice lacking TLR- and IFNAR-signalling in B cells, when infected with *L. donovani* exhibited lower serum Ig titers compared to wild-type mice, suggesting that these signaling pathways participate in the development of hypergammaglobulinemia. Furthermore, type I interferons trigger the expression of TLRs on B cells, thereby contributing to a positive feedback loop (Silva-Barrios *et al.*, 2016). Altogether, this study proposes that the *L. donovani* promotes B cell activation through the

expression of TLRs resulting in the induction of type I interferons and hypergammaglobulinemia, which ultimately causes worsening of the disease.

Most of the anti-parasitic activity in the chronic stage of *L. donovani* infection is carried out by IFNγ producing CD4 T cells. Silva-Barrios et al. reported that the ablation of type I interferons in B cells conferred parasite-resistance, and these mice also had increased frequencies of Th1 cells. In line with this study, Kumar et al. also showed that *Ifnar1*$^{-/-}$ mice possess higher frequencies of parasite-specific Th1 cells compared to wild-type mice (Kumar *et al.*, 2020). Additionally, this study also showed that blocking type I interferon signaling using small molecule inhibitors of JAK-1/2 confers disease resistance by improving Th1 responses in infected mice, and can prove to be useful in designing combination therapy for VL patients. Another interesting *in vitro* study published recently suggests type I interferons act in favour of *L. donovani* in making host cells such as bone marrow-derived macrophages more favourable for colonization. This study shows that type I interferons are essential for mitochondrial biogenesis in these host cells triggered by *L. donovani* leading to a metabolically suitable environment for parasite growth (Acevedo Ospina *et al.*, 2022).

1.5.3.5 IL-10

Another molecular messenger, which perhaps is the most important clinically valuable cytokine studied extensively in VL, is IL-10. Similar to type I interferons, it is also pleiotropic, and is produced by many different cell types. It confers host protection from inflammation in many scenarios although it is known to limit pathogen clearance leading to worsening of infections. Owing to these contrasting roles in mediating anti-inflammatory effects and immunosuppression, it is considered to be a two-edged knife that needs to be handled carefully while designing therapeutics.

IL-10, described as “cytokine synthesis inhibitory factor" was initially discovered as an inhibitory molecule produced by Th2 cells upon *in vitro* stimulation with concanavalin A, that limited the capacity of Th1 cells to produce IL-2, IL-3, IFNγ, TNF and GM-CSF (Fiorentino *et al.*, 1989). IL-10 was also shown to inhibit cytokine production by APCs such as macrophages and dendritic cells. This also affected their APC function and thereby indirectly resulted in limited functioning of Th1 cells (Bogdan *et al.*, 1991; Fiorentino *et al.*, 1991a; Fiorentino *et al.*, 1991b; Macatonia *et al.*, 1993). During these initial years of research exploring IL-10 functions, it was also shown that deletion of the *Il10* gene in mice resulted in chronic intestinal colitis, which conforms with other studies portraying the vital anti-inflammatory role of IL-10 in reducing inflammation-induced

pathology (Kühn *et al.*, 1993). Evidently, research over the last three decades has proven that IL-10 is an immunoregulatory molecule in many other human diseases (Wang *et al.*, 2019b).

In both clinical (Ghalib *et al.*, 1993; Karp *et al.*, 1993) and experimental VL (Murray *et al.*, 2002), elevated IL-10 levels are observed, which possibly interferes with the process of pathogen clearance. *Il10*$^{-/-}$ mice are resistant to *L. donovani* infection, and are known to harbour ten fold less parasites in the liver compared to wild type *Il10*-sufficient mice (Murphy *et al.*, 2001). Additionally, *L. donovani* infection promotes endogenous IL-10 production in murine macrophages, which modulates intracellular calcium levels, and leads to the suppression of calcium-dependent protein kinase C (PKC), a vital protein involved in pathogen clearance in the macrophages (Bhattacharyya *et al.*, 2001; St-Denis *et al.*, 1999). Concurrent with the findings in mouse models of VL, increased serum levels of IL-10 in patients suffering from VL is also known to facilitate parasite growth in human macrophages and inhibiting IL-10 reverses this effect (Nylén *et al.*, 2007). It is also well established that treatment of human VL with anti-IL-10 antibodies results in the production of more antigen-specific responses, indicating the important inhibitory role of this cytokine in suppressing *Leishmania*-specific immune responses (Carvalho *et al.*, 1994; Ghalib *et al.*, 1993).

Several cellular sources of IL-10 have been identified during infections with *Leishmania spp.* These include neutrophils (Charmoy *et al.*, 2007), NK cells (Maroof *et al.*, 2008b), B cells (Bankoti & Stäger, 2012), DCs (Bunn *et al.*, 2018; Mishra *et al.*, 2023; Svensson *et al.*, 2004), macrophages (Murray *et al.*, 2002; Nandan *et al.*, 2012), and Foxp3-dependent regulatory T cells (Belkaid *et al.*, 2002a) and most importantly, the Foxp3-independent regulatory CD4 T cells which are otherwise known as the type 1 regulatory T (Tr1) cells or the double producers of IL-10 and IFNγ (Bunn *et al.*, 2018; Nylén *et al.*, 2007; Ranatunga *et al.*, 2009b; Stäger *et al.*, 2006b). The latter are considered a clinically relevant cellular source of IL-10 in VL. In summary, IL-10 is a pleiotropic cytokine with several immunomodulatory functions which often have a deleterious effect. Some of these effects include: macrophage deactivation (Bhattacharyya *et al.*, 2001), inhibition of antigen specific-Th1 responses (Carvalho *et al.*, 1994), suppression of CD8 T cells (Bankoti *et al.*, 2012), and the defective migration of dendritic cells (Ato *et al.*, 2002).

1.6 Other chronic infections

As this work was carried out in a very collaborative environment, there were opportunities to explore the role LAG-3-expressing CD4 T cells in two other infection models as well. This section

provides a brief outline on two such infections – 1) helminth worms and 2) Lymphocytic choriomeningitis virus (LCMV). The background provided in this section, although not exhaustive, summarizes information relevant to understanding the results and discussion sections.

1.6.1 Helminth worms

1.6.1.1 Worms

Yet another parasite causing major health concerns around the world in humans and in cattle is the helminth worm. Some of the commonly known disease-causing worms in humans are *Ascaris lumbricoides*, whipworm (*Trichuris trichiura*), and hookworm (*Ancylostoma duodenale* and *Necator americanus*). These worms are often referred to as the soil-transmitted-helminths or STH, as they spread through soil contamination in regions that are damp and moist, with poor sanitary conditions (Centre for Disease Control, 2022). Although treatable and preventable, these infections still pose a major health concern to people living under economic disparity, and millions are affected worldwide each year - more than ~800 million are infected with *Ascaris*, ~700 million with whipworms, and ~500-700 million with hookworms (Centre for Disease Control, 2022). Therefore, the STH infections are also a class of neglected tropical diseases.

Mice are also prone to helminth infections. *Heligmosomoides polygyrus bakeri* (also known as *Heligmosomoides polygyrus* or *Nematospiroides dubius)* is one such extracellular macro organism belonging to the phylum of nematodes that naturally infects mice. *H. polygyrus bakeri* causes chronic infection in mice that can last for several months, and it is also very closely related to the human hookworms as it is classified under the same Order *Strongylida* (Gouÿ de Bellocq et al., 2001). Therefore, mice infected with *H. polygyrus bakeri* serve as a good model for studying immunity against helminth infections.

In the laboratory, mice are infected by oral gavage of worm larvae. Within a day following infection, the worms invade the submucosal layer of the small intestine where they reside and undergo two molts. Subsequently, about 10 days after the infection, adult worms emerge in the intestinal lumen and coil themselves around the intestinal villi, for obtaining nutrition to promote growth and reproduction. The worms mate in the lumen. The females produce eggs which are released into the environment via the feces, and these eggs hatch and give rise to larvae. Once the larvae reach L3 stage, these are ready to infect new hosts, and the lifecycle of the worm continues (Reynolds *et al.*, 2012). The life cycle of these worms is summarized in Figure 1.6 shown below.

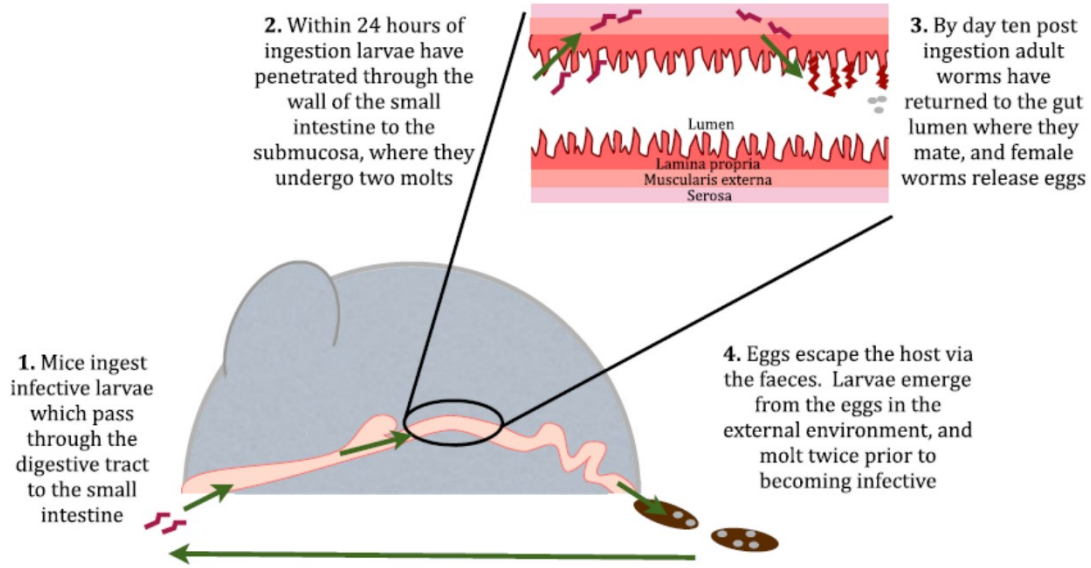

Figure 1.6 Life cycle of helminth worms infecting mice

Reproduced from the review by (Reynolds et al., 2012).

1.6.1.2 Host immune responses

One important aspect from which infection with metazoan parasites such as helminth worms is different from microbial pathogens such as viruses, bacteria and protozoan parasites is that these macroscopic pathogens induce a predominantly Th2 response. This is characterized by the presence of cytokines including IL-3, IL-4, IL-5, IL-9, IL-10, IL-13 and IL-21 (Allen & Maizels, 2011; Anthony *et al.*, 2007; Neill & McKenzie, 2011). A similar type of Th2 immunity also predominates in allergies. The mechanisms by which microbial pathogens trigger DC-activation to promote a Th1 immune response are clearly established – the PAMPs expressed on invading pathogens trigger PRRs which mainly operate through MyD88-singling to induce IL-12 production by the DCs, which then result in priming of T cells and IFNγ-production (Szabo *et al.*, 2003). However, an analogous mechanistic pathway has not been clearly established for DC-priming of CD4 Th2 responses. Although it is known that inhibition of IL-12 production in DCs is one of the essential factors, it is not the only one responsible for skewing the immune response towards the Th2 phenotype (MacDonald & Maizels 2008).

It is also established that during helminth infections, the macrophages are alternatively activated (Mylonas *et al.*, 2009). This means that they highly express the enzyme ariginase-1 rather than

its competitor NOS-2 or nitric oxide synthase 2, as the former is the one induced by Th2 cytokines in contrast to the latter that is induced by Th1 cytokines (Hesse *et al.*, 2001). These alternatively activated macrophages are critical mediators of expulsion of adult worm as well as damaging worm larvae, and they also mediate the memory Th2 response to helminth infections (Anthony *et al.*, 2006). In fact, the expulsion of adult worms is not a process solely controlled by the immune system but it is rather resulting from nervous, endocrine and immune systems acting together. For instance, the mucus production by goblet cells lining the intestinal epithelium, and the contraction of intestinal muscle walls are examples of events resulting from the complex coordination of multiple systems. In fact, the gut epithelial layers are known to participate in both sensing and removal of worms in the intestine (Bąska & Norbury, 2022). All of these processes leading to the release of worms outside the host are collectively referred to as the "weep and sweep" response.

B cells perform several vital functions in helminth infections including antibody production, antigen presentation to T cells, and production of cytokines that mediate Th2 responses (Wojciechowski *et al.*, 2009). Apart from the adaptive immune cells such as B and T cells, innate immune cells, particularly neutrophils also participate in pathogen clearance as these are present in granulomas during helminth infections (Anthony *et al.*, 2006). The role of other granulocytes such as basophils and eosinophils are less explored. An important innate immune cell type well known for participating in worm expulsion as they promote "leakiness" of the intestinal lumen, by altering the tight junction proteins that strictly regulate the permeability of the gut mucosa are the mast cells (McDermott *et al.*, 2003). Additionally, "mucosal mastocytosis" or build-up of mast cells along the intestinal epithelium is a characteristic of helminth infections in the gut (Dehlawi *et al.*, 1987). The innate lymphoid cells or ILCs, belonging to the group 2, known as ILC2s, producing cytokines similar to the Th2-type, are also involved in host protection during helminth infections. These ILC2s are thought to be triggered by the production of alarmin cytokines such as IL-25 and IL-33 released by the gut-mucosal epithelial cells in response to infections (Bouchery *et al.*, 2019). The helminth worms are not killed but rather pushed out of the body, which is referred to as worm expulsion, as a result of mucus production and muscle contractions in the gut. This host response to helminth worms, or the "weep and sweep response" is a culmination of complex interaction of different types of innate and adaptive immune cells with the microenvironment in the gut, comprising of epithelial, muscular and nervous tissues as well.

1.6.2 LCMV

1.6.2.1 The virus, its discovery and strains

LCMV is a positive single-strand RNA virus that belongs to the family of arenaviruses *(Arenaviridae).*This family of viruses is mostly spread by rodents, as these are their natural hosts as well as reservoirs. The house mouse, *Mus musculus,* serves as a natural host for LCMV. This virus was initially discovered by Charles Armstrong, and has ever since been used as a model to study the immune response to infection (Armstrong & Paul, 1935). As far as human infections are concerned, only those exposed to urine, feces, saliva, or blood of wild mice are posed with the risk of infection (Centre for Disease Control, 2014). However, it is a zoonotic pathogen that is present worldwide, and poses risk of lethality in severely immune-deficient individuals. It is also known to cause congenital birth defects.

There are several strains of LCMV including WE, Docile, Pasteur, UBC, Traub, Armstrong and Clone-13 (Dutko & Oldstone, 1983); and the genomic variation in these strains are also known to cause different disease outcomes, particularly in the degree of persistence of the infection (Hotchin, 1962). Therefore, comparing infections from different strains of LCMV provides excellent models for studying acute versus chronic infections in great detail. In fact, the clone 13 (Cl13) strain and Armstrong strain of LCMV differ only in one amino acid yet produce different types of infections. The Cl13 strain causes persistent infection due to suppression of CD8 T cell responses while the Armstrong strain causes acute infection that is cleared within a few days (Ahmed *et al.*, 1988; Salvato *et al.*, 1991).

1.6.2.2 Mouse models and host immune responses

A vast majority of research related to mechanisms of T cell mediated immune responses have been studied using LCMV infection. It was using LCMV infections models that the role of MHC-restricted, antigen-specific cytotoxic CD8 T lymphocytes in clearing virus-infected cells was demonstrated (Zinkernagel & Doherty, 1974). This discovery of specific interaction between MHC-TCR complexes resulted later to the development of tetramers, which are tools used until today for the determination of epitope-specific T cells (Altman *et al.*, 1996). Multitudes of studies in immunology have been conducted using LCMV models of infection and therefore it is out of scope to discuss the vast literature on host immune responses to this infection. Therefore, we will be limiting our discussion only to the relevant literature concerning exhaustion of CD8 T cells during

LCMV infection in this section. The CD4 T cell responses, along with the bias in Tfh cell development during this infection, are discussed briefly later in the section 1.8.4.

The functional exhaustion or ineffectiveness of CD8 T cells during persistent LCMV infection was first demonstrated in 1998 (Gallimore *et al.*, 1998). A few years later, another group also reported that the CD8 T cells progressively lose the capacity to produce cytokines such as IFNγ, IL-2 and TNF. These cells are unable to kill infected cells effectively and stop proliferating. These effects correlate with an increasing viral load, and, therefore, are antigen-driven (Wherry *et al.*, 2003). Currently, there are many other additional parameters used for defining CD8 T cell exhaustion in a more comprehensive and rather exhaustive manner. These include (1) the expression of several co-inhibitory receptors such as PD-1, LAG-3 TIM-3, 2B4 (Blackburn *et al.*, 2009; Richter *et al.*, 2009; Wherry *et al.*, 2007), (2) the expression of TOX, the master transcription factor recently described to control CD8 T cell exhaustion (Khan *et al.*, 2019; Seo *et al.*, 2019; Yao *et al.*, 2019), (3) metabolic reprogramming involving increased lipolysis and fatty acid oxidation (Zheng *et al.*, 2022).

The exhausted CD8 T cells develop as a result of sustained TCR-stimulation by persisting antigens (Mueller & Ahmed, 2009). Therefore, exhaustion is not a drastic instantaneous process but a gradual one that develops due to antigen persistence. These aspects led to the speculation that the pool of exhausted CD8 T cells is rather likely to be heterogeneous. Indeed, recent research shows that the pool of exhausted CD8 T cells in chronic LCMV infections constitutes many different subpopulations which are phenotypically and functionally heterogeneous (He *et al.*, 2016; Im *et al.*, 2016b; Leong *et al.*, 2016a; Paley *et al.*, 2012; Utzschneider *et al.*, 2016; Wu *et al.*, 2016b). Although there seem to be many different subsets of exhausted CD8 T cells (Sandu *et al.*, 2020), broadly, these can be classified into two main categories – the $CXCR5^{+}PD\text{-}1^{int}$ $TCF1^{hi}$, which have stem- like properties, and are capable of undergoing proliferative burst after anti-PD-1 therapy; and the terminally exhausted $PD\text{-}1^{hi}TIM\text{-}3^{hi}$ cells, that eventually undergo Fas-mediated cell death. The former subset of CD8 T cells, which are “progenitor-like” or “stem-like” have been described by several authors to have the capacity to differentiate into effector, memory and exhausted CD8 T cell subsets (He *et al.*, 2016; Im *et al.*, 2016b; Leong *et al.*, 2016a; Utzschneider *et al.*, 2016; Wu *et al.*, 2016b). However, as their differentiation capacity is restricted only to CD8 T cell subpopulations, referring to them as “multipotent” or “progenitor-like” or “stem-like” are terms to be used with caution as clearly pointed out in a review article (Kallies *et al.*, 2020). As suggested in this review, these cells will henceforth be referred as the “T-progenitor-exhausted” cells or Tpex cells. These cells have gained much attention in the last few years, as

these cells respond to immunotherapeutic strategies involving checkpoint inhibition (Im *et al.*, 2016b). This has not only been shown using mouse models, but has been proven to hold true in human studies as well (Miller *et al.*, 2019; Sade-Feldman *et al.*, 2018). This has also challenged the manner in which T cell exhaustion is perceived. The studies on Tpex cells postulate that immune checkpoint inhibition therapy results in increased numbers of exhausted cells, as a result of the selective expansion of the Tpex subset following treatment, in contrast to the belief that immune checkpoint therapy restores functions of T cells. This idea is depicted in the following figure.

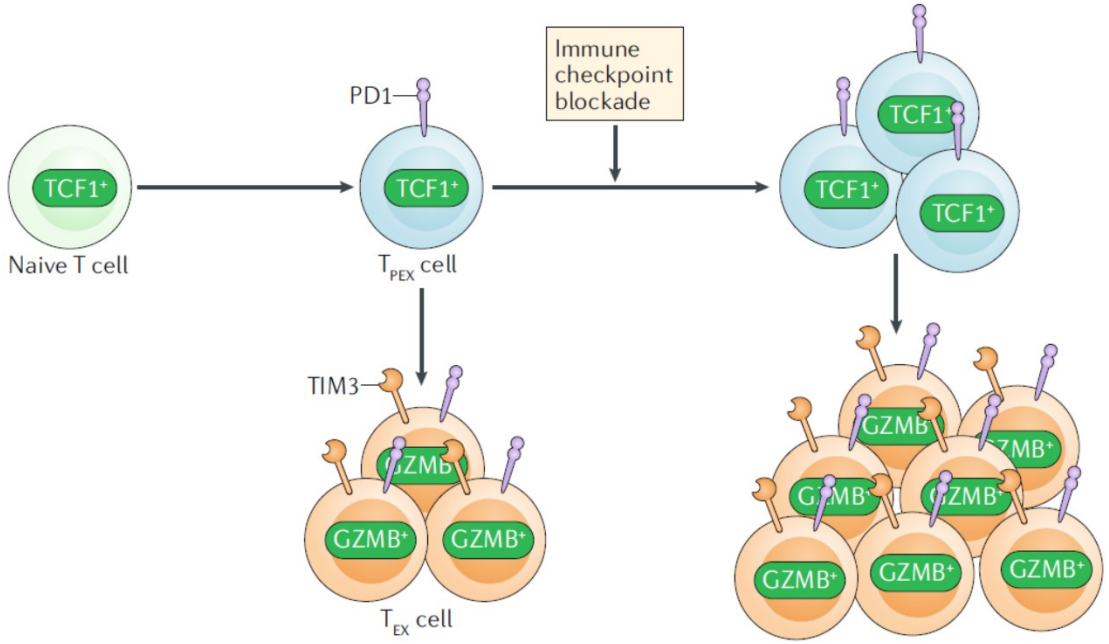

Figure 1.7 Proposed mechanism of sustaining CD8 T cell responses by Tpex cells in response to immune checkpoint blockade

Reproduced from (Kallies et al., 2020).

1.7 CD4 T cells

1.7.1 CD4 T cell development and their role in infections

T lymphocytes or T cells mostly constitute CD4 and CD8 T cells. These T cells are derived from the lymphoid progenitors in the bone marrow, which must exit the marrow, circulate in blood and following directional cues enter into the thymus (Petrie, 2003). The term “early lymphoid

progenitors" (ETPs) is used to refer to lymphoid progenitors settled in the thymus that undergo further development. ETPs do not express any of the cell surface receptors such as CD3, CD4 or CD8, nor do they possess a rearranged TCR. From this triple negative state, these cells undergo somatic DNA rearrangement, a process involving reshuffling of genes in three regions of the TCR gene locus, namely the variable (V) region, diversity (D) and joining (J) (Rezuke *et al.*, 1997). Following αβTCR rearrangement, these $CD4^{+}CD8^{+}$ double positive thymocytes then undergo positive and negative selection

Positive selection refers to the mechanism by which double positive T cells with a successfully rearranged MHC-I- or MHC-II-restricted TCR, are selected. These cells are "selected" as they are provided with long term survival signals as well as signals to enable their migration to the thymic medulla. The double positive thymocytes then become either CD4-single positive or CD8-single positive cells (Germain, 2002). Negative selection refers to the process of elimination of highly-self-reactive thymocytes in order to prevent autoimmunity otherwise referred to as the process of induction of central tolerance (Spits, 2002). However, the process of inducing self-tolerance is not only a thymus-related event. The peripheral lymphoid organs also play a major role in regulation of autoimmunity and in contributing to tolerance, as reviewed earlier (Hoyne *et al.*, 2000).

CD4 T cells are a major cellular component of the adaptive immune response to infections. These cells perform several functions such as helping with B cell maturation and antibody production, assisting cytotoxic CD8 T cells in pathogen clearance, activating innate immune cells such as macrophages and enabling them to kill intracellular pathogens as well as inducing immunosuppression to calm down inflammation. During infections, naïve CD4 T cells interact through their TCRs with antigenic-peptide-MHC-complex presented by an APC, following which these cells are activated. The T cell activation process requires three signals as shown in figure 1.8. Signal 1 involves the interaction of TCR with MHC II + antigenic peptide presented on APCs. The second step involves the engagement of co-stimulatory molecules such as CD28 and CD40 (Bretscher, 1999). The third signal constitutes instructive cytokines such as IL-12 or type I interferons. Following activation, these cells can then undergo differentiation into many different types of effector or regulatory subpopulations. Although the CD4 T cells were initially divided into the T-helper type 1 (Th1) and T-helper type 2 cells, there are also other subdivisions known today such as T-hepler 3 (Th3), T-helper 5 (Th5), T-helper 17 (Th17), T-helper 22 (Th22), T-helper 25 (Th25), T-helper 9 (Th9), T-follicular helper (Tfh) cells, Foxp3-dependent T regulatory (Treg) cells, type-1 regulatory (Tr1) cells which are the Foxp3-indenpent $IFN\gamma^{+}$-$IL\text{-}10^{+}$ T cells (Luckheeram *et al.*, 2012; Tuzlak *et al.*, 2021). However, lately this classification system based only on the

production of one or two cytokines is considered insufficient to adequately describe the broad range of functions that these cells perform, and, therefore, other means of describing these helper cells based on their functional role in assisting other immune cell types has been proposed (Tuzlak *et al.*, 2021). The role of CD4 T cells in fighting three different infections - VL, *H. polygyrus bakeri* infection and LCMV infection – are discussed in detail in the following sections.

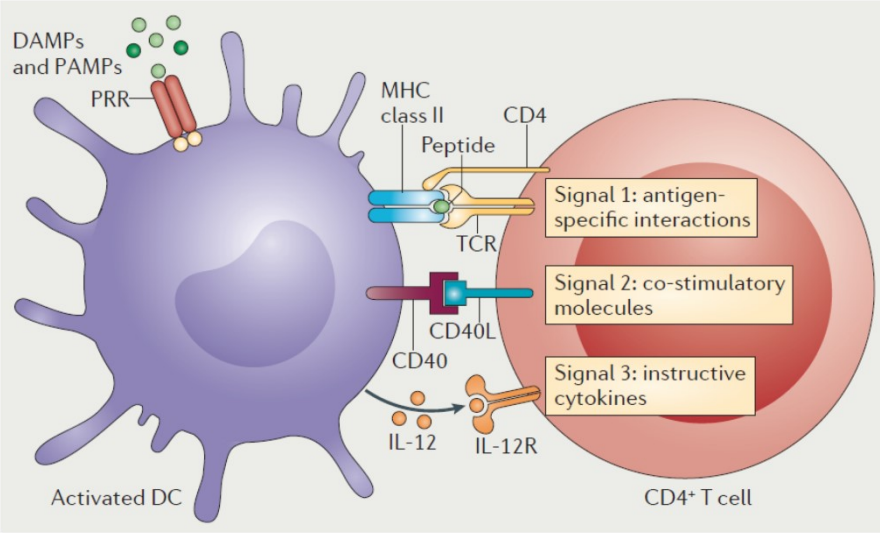

Figure 1.8 The three signals required for CD4 T cell activation

Reproduced from (Kambayashi & Laufer, 2014).

1.7.2 CD4 T cells in VL

In VL, CD4 T cells play an indispensable role in pathogen clearance as these cells are one of the major sources of IFN-γ and TNF required for the activation of leishmanicidal activity of infected macrophages (Murray & Delph-Etienne, 2000). CD8 T cells also produce these cytokines required for parasite killing. However, during the chronic phase of VL, CD8 T cells are exhausted, and hence dysfunctional (Hammami *et al.*, 2015b; Joshi *et al.*, 2009a), which makes CD4 T cells the central players in parasite clearance. During the early stages of infection in the spleen, the splenic dendritic cells start producing IL-12, following MyD88-dependent sensing of the parasites (Gorak *et al.*, 1998). This IL-12 in turn acts on CD4 T cells, and triggers the activation of Signal Transduction and Activator of Transcription-4 (STAT-1) and T-bet, the major transcription factor associated with IFNγ production (Rosas *et al.*, 2006). Although the Th1 cells are vital for anti-leishmanial activity, they also induce inflammation and tissue damage, which is detrimental to host responses. These pathological effects are seen clearly in the spleen and the bone marrow. In fact this pathological role of the pro-inflammatory cytokines IFNγ and TNF produced by CD4 T

cells in the bone marrow during VL, promotes HSC-exhaustion and establishment of non-resolving persistent infection in the bone marrow (Pinto *et al.*, 2017b).

In the 1980s and 1990s, the subtypes of CD4 T cells were limited to Th1 and Th2 cells. Th2 cells, expressing GATA-3, the major transcription factor controlling IL-4 production, were thought to be the polar opposite of Th1 cells (Ouyang *et al.*, 1998; Zheng & Flavell, 1997). Although in cutaneous leishmaniasis models IL-4 was demonstrated to induce alternative activation of macrophages resulting in disease progression and parasite persistence, in VL, it was shown that IL-4 does not necessarily favour parasite growth (Kaye *et al.*, 1991; Stäger *et al.*, 2003a). A review from 2012 also summarizes how the Th1/Th2 paradigm no longer holds relevant in the case VL (Alexander & Brombacher, 2012). There are many different subsets of CD4 T cells in VL. Therefore, it is of relevance to consider the many types of CD4 T cell responses to VL instead of limiting the discussion to either Th1 or Th2 phenotypes. Additionally, it is important to note that these differentiation programs are often not a permanently occurring phenomenon, and there is some degree of plasticity exhibited by CD4 T cells subsets such as Treg cells (Wohlfert & Belkaid, 2010) and Th17 cells (Stockinger & Omenetti, 2017) or the ability to switch phenotype and functionality. A multitude of factors such as types of pathogens, cytokines in the microenvironment, and interaction with other cells can potentially play a role in shaping the CD4 T cell response to infections.

Th17 cells were initially described as a pathogenic T cell subset that expands in response to IL-23 and promotes autoimmunity, in contrast to the protective Th1 cells which are not induced by IL-23 but rather by IL-12 (Langrish *et al.*, 2005). Later it was discovered that these cells are essential for maintaining immunity at the gut mucosal barrier, and the transcription factors RORγt and STAT-3 serve as lineage determining factors (Yang *et al.*, 2007). Additionally, IL-23 is required for the stabilization of the lineage, and the cytokines TGF-β and IL-6 also play an important role in inducing Th17 differentiation (Bettelli *et al.*, 2007). It is also known that these cells share a common differentiation pathway with peripheral Treg cells (Bettelli *et al.*, 2007). Maintaining a balance between Th17 cells and Treg cells is essential in effective host immune responses. Alteration of this balance has profound effects on the disease outcome in the case of infections (Valverde-Villegas *et al.*, 2015), autoimmunity (Eisenstein & Williams, 2009), and metabolic disorders (Zhang *et al.*, 2021). Studies in patients with mucocutaneous leishmaniasis show that Th17 cells contribute to the immunopathology as elevated levels of IL-17 and IFNγ leads to infiltration of neutrophils in the sites of tissue damage (Boaventura *et al.*, 2010). On the contrary, in VL patients, Th17 cells were associated with better recovery. The higher levels of IL-

17 and IL-22 produced by PBMCs were associated with patients who cleared the infection effectively and recovered well (Pitta *et al.*, 2009). In C57BL/6 mice infected with *L. infantum*, IL-17 assisted in overall parasite clearance, and acted in concert with IFNγ, to activate anti-leishmanial activity of infected macrophages. Mice lacking IL-17R were more prone to infection, and showed decreased frequencies of Th1 cells. These mice also possessed increased frequencies of regulatory subsets such as Foxp3+ Treg cells and Tr1 cells (Nascimento *et al.*, 2015).

In the context of VL, the subset of immunosuppressive T cells that has been studied extensively is the Foxp3-independent IFNγ^{+}-IL-10^{+} T cells. In experimental and human VL, these Tr1 cells were identified as the major IL-10-producing cell population, and their expansion is regulated by the cytokines IL-6 and Il-12p40 produced by DCs (Nylén *et al.*, 2007; Stäger *et al.*, 2006b). The Foxp3-expressing Tregs do not play a major role in *L. donovani* infection, as the depletion of these cells does not substantially affect the immune response, pathogen load or outcome of the disease (Bunn *et al.*, 2018). Foxp3-expressing Treg cells with a suppressive function on other cells has been identified in BALB/c mice infected with *L. infantum.* However, this study only identified the existence of these cells and their capacity to produce suppressive cytokines; it did not demonstrate the mechanisms by which these could affect the disease outcome (Rodrigues *et al.*, 2009).

The role of T follicular helper cells or Tfh cells in VL remains vastly unexplored, except for one study performed in macaques. These authors showed that although there is an initial proliferation of Tfh cells during the acute phase of VL, this response crashes during the chronic phase. This defect in Tfh response eliminates the possibility for the sustained production of parasite-specific IgG during the chronic phase of the infection, thereby hampering effective humoral responses (Rodrigues *et al.*, 2014b). Figure 1.9 quickly summarizes the different subtypes of CD4 T cells in VL discussed in this section and their role in VL.

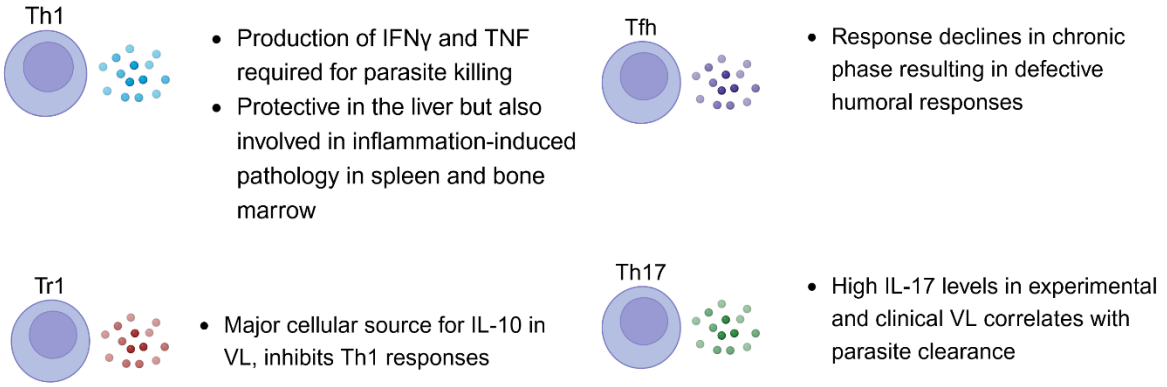

Figure 1.9 Short summary of T cell responses to VL

Image created using Biorender.com

The Th1 cells in VL are also prone to exhaustion (Esch *et al.*, 2013b). This phenomenon of T cell exhaustion is a characteristic of chronic infections and occurs due to pathogen persistence. It is defined as the inability to produce a recall response, accompanied by high expression of inhibitory receptors, and eventually leading to cell death. As seen in CD8 T cells in VL (Joshi *et al.*, 2009a), CD4 T cells also upregulate PD-1, and the blockade of this molecule is known to improve pathogen clearance (Esch *et al.*, 2013b). Th1 cells are also suppressed by myeloid-derived suppressor cells (MDSCs) in the spleen, and this is enhanced by HIF-1α upregulated on these cells (Hammami *et al.*, 2017). Furthermore, in VL, DCs lose their ability to migrate to T cell areas and express HIF-1α, which interferes with their capacity to produce IL-12 and effectively induce protective Th1 responses (Hammami *et al.*, 2018). Additionally, in VL, the functions of DCs are severely impaired by type I interferons, which results in reduced Th1 priming (Kumar *et al.*, 2020).

Yet another protein that is involved in the complex regulation CD4 T cell responses during VL is Interferon regulatory factor 5 (IRF-5). Although this protein was studied in different viral infections, its role in regulating T cell responses was first described in experimental models of VL (Paun *et al.*, 2011). Later, it was also shown that IRF-5 induces HIF-1α expression on DCs, and thereby interfered with their capacity to produce IL-12, and help in the generation of protective Th1 responses (Hammami *et al.*, 2015b; Paun *et al.*, 2011). In addition to this indirect effect, IRF-5 is known to be upregulated in Th1 cells in VL and drive these cells towards apoptotic cell death due to sensing of tissue damage through TLR-7 (Fabié *et al.*, 2018). In summary, several mechanisms such as exhaustion, cell death, hypoxia and immunosuppressive cytokines contribute to the suppression of protective Th1 responses during VL.

1.7.3 CD4 T cells in helminth infections

In *H. polygyrus bakeri* infection of laboratory mice, Th2 and Tfh cells mediate most of the T cell responses causing worm expulsion (Reynolds *et al.*, 2012). The Th2 cells, which stimulate other intestinal epithelial and goblet cells to accelerate worm expulsion, are primed in the mesenteric lymph nodes draining the gut following activation of DCs by the worm larvae (Urban *et al.*, 1995). Mainly, the CD4 T cells in helminth infections were proven to be essential for IgE production and prevention of secondary infections (Urban *et al.*, 1991). In addition to the IgE responses, potent IgG1 responses are also central players in conferring protection (Pritchard *et al.*, 1983). As in the case of allergic responses, in helminth infections as well, it was later discovered that the Tfh cells are major sources of IL-4 and IL-21, that are required for class-switching of antibodies in the gut draining lymph nodes (Gowthaman *et al.*, 2019; King & Mohrs, 2009; Meli *et al.*, 2016).

One factor that seems to be leading towards a predominant Th2 response as opposed to a Th1 response in helminth infections is the strength of TCR stimulation (Paul & Zhu, 2010). A low avidity signal during T cell-DC interaction results in Th2 differentiation while a high avidity interaction results in Th1 or Th17 differentiation (Bouchery *et al.*, 2014). Additionally, it is known that Tfh differentiation requires a stronger TCR stimulation compared to Th1 differentiation (Tubo *et al.*, 2013). In addition to TCR signaling, other costimulatory molecules are important determining factors for Tfh differentiation. For instance, a cell adhesion molecule named LFA-1 or leukocyte function-associated factor 1, governs the process of Tfh differentiation (Meli *et al.*, 2016). Tfh cells express high levels of LFA-1 in its active form, and the blockade of LFA-1 signaling leads to compromised Tfh differentiation and, as a consequence, defective humoral immune responses during helminth infection (Meli *et al.*, 2016).

As it is the case in many parasitic infections, helminths also modulate host immune responses by deploying regulatory T cells. *H. polygyrus bakeri* produces a protein that mimics TGF-β and is capable of binding to its receptors and selectively inducing Treg expansion and promote immunosuppression (Johnston *et al.*, 2017). Mice infected with *H. polygyrus bakeri* induce a strong Treg response (Smith *et al.*, 2016). However, mice lacking Tregs possess a higher worm burden, along with increased Th1 and not Th2 responses (Smith *et al.*, 2016), indicating that Treg responses are indeed required for effective immunity and are their role is not entirely negatively impacting the host. Indeed, Treg-mediated immunosuppression, under moderation is required for control of gut pathology from excessive infection-induced inflammation (D'Elia *et al.*, 2009).

IL-10 is another anti-inflammatory cytokine whose role in contributing to a balanced and effective immune response is often ignored. In helminth infections, IL-10 singling through the IL-10R is

required for limiting gut pathology, maintenance of the integrity of gut epithelial barrier, and preventing opportunistic infections (Duque-Correa *et al.*, 2019). The transcription factor Blimp-1 promotes the expression of IL-10 by many T helper subsets (Neumann *et al.*, 2014). In addition, we recently demonstrated that the transcriptional repressor protein BCL-6, an antagonist of Blimp-1, is an active player in controlling IL-10 expression by CD4 T cells (Meli *et al.*, 2023). The findings presented in this article conform to the findings in this thesis.

1.7.4 CD4 T cells in LCMV infection

Evidently, as it is in the case of most infections, CD4 T cells assist in the generation of virus-specific CD8 T cell responses, and in the process of B cell maturation as well as the production of virus-specific antibody responses. As the main focus of this work is in understanding the mechanisms regulating CD4 T cell responses to chronic infections, this section will mostly focus on discussing the differentiation process of these cells during Cl13 LCMV infection, and its implications on the functionality of these cells.

During chronic LCMV infections, there is a rapid and accelerated loss of protective Th1 responses, as observed in most persistent infections (Brooks *et al.*, 2005). Indeed, much of this loss is attributed to CD4 T cell exhaustion. However, there is also evidence suggesting that during chronic infections, the high strength TCR-stimulation caused by the persistence of the antigen drives the CD4 T cell differentiation more towards the Tfh program, rather than the Th1 program (Fahey *et al.*, 2011; Fazilleau *et al.*, 2009). This continuous TCR-stimulation leading to Tfh differentiation is a process independent of B cells (Deenick *et al.*, 2010). The infection of mice with either Armstrong or Cl13 strains of LCMV resulted in increased percentages of CXCR5+ CD4 T cells (Fahey *et al.*, 2011). However, in the Cl13 infection, this proportion increased to about two-thirds of the total CD4 T cells at 30 days post-infection (Fahey *et al.*, 2011). The major role of Tfh cells in viral infections is to promote B-cell maturation and production of neutralizing virus-specific antibodies. However, the Tfh-B cell interaction in chronic LCMV infection is still dysfunctional, as an effective neutralizing antibody response does not occur until several months following infection (Battegay *et al.*, 1993). Hypergammaglobulinemia and glomerulonephritis are also common symptoms of Cl13-infected mice, indicative of ineffective Tfh-B cell interactions (Hunziker *et al.*, 2003). One proposed reason behind the skewed Tfh-response is that this further helps in sustaining the B-cell-driven antibody response, and thereby ultimately proves to be beneficial in combating chronic infection (Fahey *et al.*, 2011).

The transcriptional profiling of exhausted CD4 T cells in chronic LCMV infections revealed that this population is very heterogeneous, as these cells were not particularly enriched for the expression of any one particular lineage determining protein such as Blimp-1, T-bet, Eomes, and BCL-6 but rather expressed many of them at moderate levels when compared to effector or memory subsets (Crawford *et al.*, 2014). As explained in the section 1.7.2, CD8 T cells in LCMV infections are also heterogeneous – however, there are clearly distinguishable subsets of exhausted CD8 T cells, such as the Tpex, and terminally exhausted CD8 T cells, with distinct transcription factor expression and responsiveness to anti-PD-1 therapy. In contrast, the exhausted CD4 T cells in LCMV infection possess a more complicated transcriptional profile. The blockade of PD-1:PD-L1 signalling using antibodies did not prove to be very effective in CD4 T cells, unlike CD8 T cells, where following immune checkpoint inhibition therapy, there was selective expansion of Tpex CD8 T cell populations. Blockade of PD-1:PD-L1 signalling impacted CD4 T cell responses differently – it did not improve Th1 responses as it also triggered the induction of Tregs (Snell *et al.*, 2021). Clearly, the programming of CD4 T cells during chronic LCMV infection is complicated, and the mechanisms governing differentiation and exhaustion pathways of these cells warrant further investigation.

1.8 Lymphocyte activation gene 3 (LAG-3)

1.8.1 LAG-3 protein structure and properties

LAG-3, also known as CD223, is a 70-kD receptor protein (Baixeras *et al.*, 1992) located on the cell membrane, and it is an immune molecule belonging to the superfamily of immunoglobulin receptors (IgSF). LAG-3 was first identified to be expressed only on activated human T cells and NK cells, although not expressed by resting peripheral blood lymphocytes (Triebel *et al.*, 1990). As described by Triebel et al. in 1990, the human LAG-3 is a protein with 498 amino acids consisting of four extracellular IgSF domains, which is a characteristic feature of type I membrane proteins in the IgSF (Triebel et al., 1990). The LAG-3 protein is closely related to the CD4 molecule; the genes encoding these two proteins are located very close to each other, on the same chromosome (in humans, chromosome 12 and in mice, chromosome 6). Additionally, there are also protein sequence homologies between the four IgSF domains of LAG-3 and CD4, suggesting that these genes might be the products of a gene duplication event, and probably arise from a common ancestral gene (Triebel *et al.*, 1990). However, there are also several important differences in the structure of these two molecules, which reflects on their varied functions.

Although LAG-3 interacts with MHC-II like CD4, it binds MHC-II with greater affinity, and the interaction motif in the LAG-3 protein consists of a 30-amino acid loop structure, which is absent in the CD4 protein (Huard *et al.*, 1997). In addition to MHC-II binding, LAG-3 also interacts with other ligands such as Galectin-3 (Kouo *et al.*, 2015), liver and lymph node sinusoidal endothelial cell C-type lectin LSECtin (Xu *et al.*, 2014), and Fibrinogen-like protein 1 (Wang *et al.*, 2019a), mostly in the context of cancer. LAG-3 is also known to bind to α-synuclein pre-formed fibrils, which are recombinant proteins used as a model to study Parkinson's disease (Mao *et al.*, 2016). This study also highlighted the pathological role of LAG-3 in this disease as it mediated the passing of pathological forms of α-synuclein from one neuron to another (Mao *et al.*, 2016). Recently, LAG-3 has been shown to limit T cell activation by associating with the CD3/TCR complex independent of MHC-II (Guy *et al.*, 2022).

Despite its similarity to CD4, the peptide that connects the extracellular IgSF domains in LAG-3 to the transmembrane domain is much longer compared to CD4, and it is prone to cleavage by metalloproteases such as ADAM10 and ADAM17, resulting in the production of soluble LAG-3 (Li *et al.*, 2007a). The cytoplasmic domain of CD4 is known to be associated with protein tyrosine kinases such as p56lck, which are involved in signal transduction and mediate T cell activation (Granja *et al.*, 1994). However, the cytoplasmic tail of LAG-3 is not involved with any tyrosine kinases but rather has a conserved "KIEELE" motif which plays a role in the downstream signaling events, although the exact pathways involved are still unclear (Workman *et al.*, 2002a; Workman & Vignali, 2003). There is also an "EP" motif (glutamic acid and proline rich motif) which binds the protein LAP, or LAG-3-assoicated protein, that is suggested to be triggering inhibitory pathways affecting T cell activation, although these mechanisms are uncertain (Iouzalen *et al.*, 2001). A recent study also showed that the role of "KIEELE" motif in inhibitory activities of LAG-3 which were reported earlier are not reproducible and, in fact, there are two other motifs, described as "the F*XX*L motif in the membrane-proximal region and the C-terminal E*X* repeat", which are implicated in transducing the inhibitory signals downstream of LAG-3 (Maeda *et al.*, 2019). Further studies are required to delineate the complex signalling events following engagement of LAG-3 to its ligand(s).

1.8.2 Regulation of LAG-3 expression in T cells

The expression of LAG-3 is triggered by the cytokines IL-2, IL-7 and IL-12, but not IL-4, IL-6, IL-10, TNF-γ, TNF-β, or IFN-γ (Bruniquel *et al.*, 1998). It is mainly expressed on the surface of CD4 T cells producing IFNγ and not Th2 cells (Annunziato *et al.*, 1996). The regulatory activity of LAG-

3 is strictly regulated as it is prone to cleavage by metalloproteases ADAM-10 and ADAM-17 on the cell surface, and following cleavage, it is released from the cell membrane in its soluble form (Li *et al.*, 2007b). The localization of CD4 and LAG-3 proteins appear to be very different. In resting T cells, CD4 is mostly expressed on the cell surface while LAG-3 is present on microtubule organizing center, early/recycling endosomes and secretory lysosomes prior to T cell activation. It translocates to the cell surface very rapidly compared to CD4 post-activation (Woo *et al.*, 2010). One of the main factors dictating the release of vesicles containing LAG-3 post T cell activation is protein kinase C (PKC) as inhibitors of PKC singling significantly block membrane localization of LAG-3 following T cell activation (Bae *et al.*, 2014). Indeed, CD4 is downregulated upon T cell activation, and this is due to phosphorylation of serine residues in the cytoplasmic domain of CD4 molecule by PKC (Sleckman *et al.*, 1992), the same molecule that promotes LAG-3 localization to the cell membrane. However, PKC does not induce conformational changes in LAG-3 as it does in CD4 (Bae *et al.*, 2014), as the cytoplasmic tails of these two proteins are significantly different, and the exact mechanisms by which PKC initiates LAG-3 trafficking remains elusive.

1.8.3 Physiological and pathological functions of LAG-3 in T cells

Although initially discovered as a protein expressed by activated but not resting T cells and NK cells, it is now established that LAG-3 is expressed by several other cell types. These include γδ-T cells (Fahrer *et al.*, 2001; Workman *et al.*, 2002b), B cells (Kisielow *et al.*, 2005), regulatory T cells (Huang *et al.*, 2004b; Li *et al.*, 2004), Foxp3-independent Tr1 cells (Gagliani *et al.*, 2013), exhausted CD8 T cells (Blackburn *et al.*, 2009) and plasmacytoid dendritic cells (Workman *et al.*, 2009). Although it is known that all these cell types express LAG-3, its function and the factors affecting its regulation are not clearly understood in all of these cell populations. Some of the known functions of LAG-3 in T cells are discussed below.

1.8.3.1 Role of LAG-3 in activated T cells

LAG-3-MHC-II interaction stops antigen-dependent proliferation and clonal expansion of CD4 T cells (Huard *et al.*, 1996; Huard *et al.*, 1994b). In activated T cells, LAG-3 interferes with CD3/TCR signaling, and prevents T cell proliferation and cytokine secretion (Hannier *et al.*, 1998). LAG-3 clusters together with CD3/TCR complexes in the cell membrane, and this is suggested to be essential for its inhibitory effect on activation following engagement with peptide-MHC-complexes (Hannier & Triebel, 1999). This clustering of LAG-3 with CD3/TCR and CD4 or CD8 molecules on the cell surface occurs in human T cells on “rafts” on the membrane rich in cholesterol (Maçon-

Lemaître & Triebel, 2005). LAG-3, irrespective of the cell type expressing it, does not generically recognize MHC-II, and only specifically recognizes stable peptide-MHC-II complexes (Maruhashi *et al.*, 2018). This finding suggests that LAG-3 function can be influenced by properties of the APCs such as the expression level of MHC-II, their capability to phagocytose pathogens and process antigenic peptides, as well as the nature (sequence) of antigenic peptide to form stable complexes with MHCs (Maruhashi *et al.*, 2018). Therefore, LAG-3 function appears to be very context-specific – it serves as a fine-tuning tool to tailor the immune response rather than a generic coarse adjustment mechanism broadly affecting T cell activation and proliferation.

The initial studies exploring LAG-3's function in T cells revealed a role for this receptor in controlling the size of the memory T cell pool during infections, as *Lag3*$^{-/-}$ mice produced a greater number of memory T cells compared to wild type mice during experimental viral infections (Workman *et al.*, 2004). However, the exact molecular mechanisms regulated by LAG-3 in memory T cell development still remain elusive. In addition to its intrinsic effects on the T cells expressing it, LAG-3 can influence the APCs expressing its canonical ligand, MHC-II, which in turn can reduce APC functions and lead to negative regulation of T cell activation. For instance, *in vitro* experiments have shown that soluble LAG-3 prevents the differentiation of monocytes into macrophages. Instead, monocytes when cultured *in vitro* with soluble LAG-3, gave rise to DCs with significantly reduced antigen-presentation capacity, which reflected on their inability to induce T cell proliferation (Buisson & Triebel, 2005). Although these *in vitro* studies provide an insight into the possible functions of soluble LAG-3 and its impact on inhibition of T cell functions, these mechanisms are yet to be demonstrated *in vivo* in the context of infectious diseases. There has been evidence supporting the role of LAG-3 in tweaking the T cell response to retroviral infections. In concert with the transcription factors BCL-6 and TCF-1, and the checkpoint inhibitor PD-1, LAG-3 is known to restrict differentiation towards the cytotoxic CD4 T cell phenotype and promote differentiation towards the Tfh-lineage (Donnarumma *et al.*, 2016).

1.8.3.2 LAG-3-expressing regulatory T cells in cancer and autoimmunity

$CD4^+$ $CD25^+$ $Foxp3^+$Tregs are an important subpopulation of CD4 T cells expressing LAG-3. Their ability to suppress effector cells is significantly diminished both *in vitro* and *in vivo*, when treated with anti-LAG-3 antibodies, clearly indicating that LAG-3 mediates their suppressive function (Huang *et al.*, 2004b). Furthermore, Tregs from *Lag3*$^{-/-}$ mice exhibited decreased suppressive functions on effector cells and ectopic expression of LAG-3 on CD4 T cells conferred on them,

the ability to suppress other effector cells (Huang *et al.*, 2004b). One of the molecular mechanisms utilized by regulatory T cells to inhibit DC functions is via LAG-3-MHC-II interactions (Liang *et al.*, 2008). The impact of LAG-3-expressing Treg-mediated suppression has been greatly explored in the context of tumor immunology. Numerous studies have demonstrated the deleterious effects of tumor-infiltrating Tregs expressing LAG-3 (Camisaschi *et al.*, 2010; Gandhi *et al.*, 2006; Hemon *et al.*, 2011). These LAG-3-expressing Tregs in tumors inhibit tumor-specific CD8 cytotoxic T cell responses, and contribute towards immune escape (Gandhi *et al.*, 2006). These LAG-3-expressing Treg populations infiltrating tumors also exhibit high proliferative capacities and release extensive amounts of immunosuppressive cytokine such as IL-10 and TGF-β (Camisaschi *et al.*, 2010). Yet another mechanism by which these suppressor cells add on their undesirable effects in melanomas is by inducing constitutive anti-apoptotic signaling initiated by LAG-3-MHC-II interaction, leading to evasion of tumor cell death (Hemon *et al.*, 2011). In addition to these mechanisms, LAG-3 can also interact with other non-canonical ligands in tumors, as previously discussed in the section 1.9.1. Moreover, in tumors, autoimmune diseases and infections (in the case of exhausted T cells), LAG-3 is known to act in concert with other immune checkpoints such as PD-1, TIM-3, CTLA-4 and TIGIT (Anderson *et al.*, 2016; Cai *et al.*, 2023). Although anti-LAG-3 treatments alone do not significantly lower tumor burden, combination therapy using anit-PD-1 and anti-LAG-3 antibodies has proven to be useful in eliminating tumors (Woo *et al.*, 2012). Several anti-LAG-3 antibodies are currently under clinical trials for the treatment of different tumor types – the prospects of these treatment strategies have been discussed and reviewed extensively in the recent years (Andrews *et al.*, 2017; Huo *et al.*, 2022; Wei & Li, 2022).

Another class of T cell expressing LAG-3 include the IL-10-producing $Foxp3^-$ Tr1 cells. In fact, a decade ago, coexpression of the cell surface markers LAG-3 and CD49b was proposed as a method of defining Tr1 cells (Gagliani *et al.*, 2013). However, a few years ago, this proposition was challenged by another study which showed that $Foxp3^+$ conventional Tregs as well as CD8 T cells coexpress these surface markers and produce IL-10 as well (Huang *et al.*, 2018). Therefore, the coexpression of LAG-3 and CD49b is to be used more broadly to define IL-10-producing T cell populations rather than the Tr1 cells specifically. In VL, the expression of LAG-3 and other coinhibitory receptors such as PD-1, TIM-3, TIGIT and CCR5 have been proposed as molecular signatures characteristic to these Tr1 cells (Edwards *et al.*, 2023a).

Additionally, the topic of LAG-3 expression by different regulatory T cell subsets has to be dealt with caution as the nomenclature of Tregs *per se* in the literature is not consistent, and there have

been attempts in the past to rectify this problem (Abbas *et al.*, 2013). Initially, Sakaguchi *et al* described a population of $CD4^+$ $CD25^+$ T cells in mice with immunosuppressive capacity (Sakaguchi *et al.*, 1995). Later, the existence of these cells was also confirmed in humans (Baecher-Allan *et al.*, 2001). In the following years, the role of Foxp3 as a master transcription factor of Tregs was discovered in humans and mice (Fontenot *et al.*, 2005; Yagi *et al.*, 2004). Some authors emphasize the importance of adding low expression of the IL-7 receptor, CD127 in $Foxp3^+$ Tregs as an additional marker for these cells (Liu *et al.*, 2006). Furthermore, the Foxp3-dependent Treg subsets are classified based on their origin. The Tregs that develop in the thymus, are in some older articles referred to as “natural” Tregs, although the accepted convention nowadays is the term “thymic” Tregs or tTregs (Abbas *et al.*, 2013). The peripheral Tregs or pTregs are derived from naïve CD4 T cells in the peripheral organs under inflammatory conditions (Curotto de Lafaille & Lafaille, 2009). The iTregs or the induced Tregs are generated *ex vivo* by culturing CD4 T cells in the presence of TGF-β which resulting in upregulation of Foxp3 expression (Chen *et al.*, 2003). There are also other classes of Treg cells that are independent of Foxp3-expression, which include the type 1 regulatory T cells or Tr1 cells that mainly produce IL-10 (Gregori *et al.*, 2012) and the IL-35-producing regulatory cells (Collison *et al.*, 2010). Clearly, each of these studies describe Tregs with many different markers and it could be misleading to state that all these types of Tregs express LAG-3. Therefore, to avoid discrepancies and confusion, throughout this text, care has been taken to mention the phenotypic markers pertaining to the subset being referred to, as it was described in the original articles and not just broadly mentioned as Tregs.

The LAG-3 expression in IL-10-producing Tr1 cells is primarily controlled by the transcription factor Egr-2 (Okamura *et al.*, 2009). The ectopic expression of Egr-2 in naïve CD4 T cells using retroviral transduction methods was sufficient to induce LAG-3 and IL-10 expression, and these cells were capable of suppressing antigen-specific responses *in vivo* (Okamura et al., 2009). It is interesting to note that Egr-2, a transcription factor required for the induction of T cell anergy (Zheng *et al.*, 2012), drives LAG-3 and IL-10 expression. In naïve CD4 T cells, the expression of Egr-2 is triggered by IL-27, and this transcription factor binds to the *Prdm1* promoter, inducing the expression of Blimp-1 (encoded by *Prdm1*), which is the indispensable transcription factor required for the transactivation of *Il10* gene (Iwasaki *et al.*, 2013). Additionally, $CD4^+$ $CD25^-$ $LAG\text{-}3^+$ T cells are known to suppress humoral responses in autoimmune diseases through the production of TGF-β3 in both mice and humans (Okamura *et al.*, 2015). These cells represent one of the few populations of T cells regulating B cell responses. In the context of transplantation immunology, the Tr1 cells expressing LAG-3 and producing IL-10 are considered to play a

beneficial role as they are responsible for calming down host immune responses, thereby maintaining immune homeostasis and preventing graft-rejection (Song *et al.*, 2021). However, the role of LAG-3 expressing T cells in infections are much more complicated as is their IL-10 production, and these topics discussed in the next section.

1.8.3.3 LAG-3-expressing T cells in infections

Upregulation of LAG-3 on T cells often correlates with either an increase in pathogen load and/or disease progression in many long-lasting conditions caused by viruses, bacteria and parasites such as HIV infection (Juno *et al.*, 2015; Tian *et al.*, 2015), Hepatitis B virus infections (Li *et al.*, 2013), SARS-CoV-2 infection (Herrmann *et al.*, 2020; Zheng *et al.*, 2020), *Mycobacterium tuberculosis* infection (Phillips *et al.*, 2015), and *Plasmodium falciparum* malaria (Butler *et al.*, 2011; Illingworth *et al.*, 2013). During these infections, in addition to the activated T cells and regulatory T cell subtypes, the other important category of T cells expressing LAG-3 are the exhausted T cells. Almost in all of these infections, LAG-3 expression is also associated with either T cell exhaustion or IL-10 production or both. The term “exhaustion” refers to the loss of proliferation and cytokine-production capacities by T cells due to excessive immune activation as a consequence of persistent pathogen-exposure (Blank *et al.*, 2019). LAG-3, when expressed along with other co-inhibitory receptors such as TIM-3, PD-1, TIGIT, CTLA-4 has been described as a characteristic marker for T cell exhaustion (Blank *et al.*, 2019). It is interesting to note that CD8 T cells are known to highly express LAG-3 compared to CD4 T cells (Huard *et al.*, 1994a). A recent research article suggested LAG-3 acts as a “rheostat” – or a mechanism of variable resistance – in activated T cells, as the strength of TCR signaling directly correlated to level of LAG-3 expression (Maeda *et al.*, 2019). In other words, the stronger the TCR signalling, as observed in the case of exhausted T cells in infections, the higher is the LAG-3 expression on these T cells. Similarly, weaker TCR signalling, as observed in the case of tumor-infiltrating T cells that turn out to be regulatory, results in lower levels of LAG-3 expression (Burnell *et al.*, 2021). Overall, higher level of LAG-3 expression associates more with exhausted T cell phenotype and lower level of LAG-3 expression associates more with regulatory T cell phenotypes.

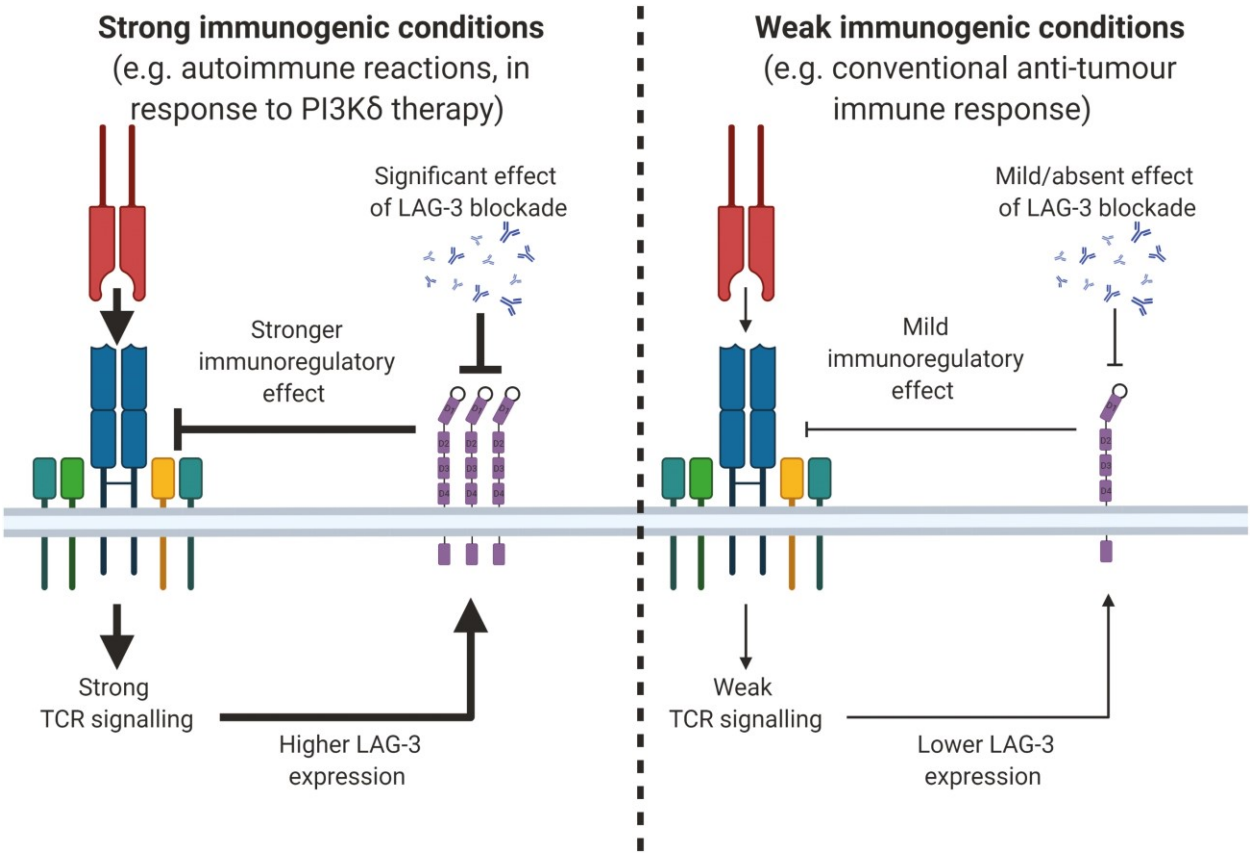

Figure 1.10 The relation between strength of immunogenic conditions and LAG-3 expression in T cells

Reproduced from (Burnell et al., 2021).

In CD8 T cells, the expression of LAG-3 alongside many other inhibitory co-receptors such as PD-1 and TIM-3 has been associated with T cell exhaustion in mouse models of lymphocytic choriomeningitis (LCMV) infection. One of the earliest studies revealed that although LAG-3 is upregulated by CD8 T cells during both acute and chronic LCMV infection in mice, inhibiting LAG-3 either *in vitro* or *in vivo* did not result in amelioration of cytokine production and proliferation abilities of antigen-specific CD8 T cells. Therefore, these authors suggested that LAG-3 expression *per se* does not contribute to CD8 T cell exhaustion in this infection model (Richter *et al.*, 2010). Similarly, another study performed on lymphocytes from the peripheral blood of HIV-infected individuals demonstrated that LAG-3 does not contribute to T cell exhaustion in HIV-infection, although it causes dysfunction of innate immune cells such as natural killer (NK) or invariant NKT (iNKT) cells (Juno *et al.*, 2015). Recently, it was also demonstrated that dual blockade of PD-1 and LAG-3 was most effective in reversing exhaustion of iNK-T cells during HIV infection (Juno *et al.*, 2015). The anti-PD-1 treatment alone was also effective, while the anti-LAG-

3 treatment alone did not have any effect, and it was the combination therapy with the blockade of both of these inhibitory receptors that proved to be the most effective (Balasko *et al.*, 2023). Therefore, inhibition of multiple immune checkpoint molecules rather than one at a time proves to be a promising strategy for the treatment of infectious diseases. Additionally, these can have a positive effect in improving not just T cell responses but can potentially impact other immune cells such as NK cells and B cells and these aspects are discussed in the review by Cox et al (Cox *et al.*, 2017).

2 RATIONALE AND OBJECTIVES

CD4 T cells play a vital role in pathogen clearance during infections. The maintenance of CD4 T cell responses is quintessential for protection of the host against chronic infections. One such potentially fatal chronic parasitic infection is VL. It is caused by the protozoan parasites *Leishmania donovani* and/or *L. infantum*. In VL, the parasites dwell within infected macrophages, and the cytokines IFNγ- and TNF produced by CD4 T helper type 1 (Th1 cells) and CD8 T cells trigger the leishmanicidal capacity in these macrophages (Murray & Delph-Etienne, 2000). Particularly, in the chronic phase of VL, CD4 T cells serve as a major cellular source of these cytokines as the CD8 T cells become dysfunctional and exhausted (Hammami *et al.*, 2015b; Joshi *et al.*, 2009a). The CD4 T cell responses are also prone to mechanisms of suppression as well. These cells undergo functional exhaustion (Esch *et al.*, 2013b) and cell death due to damage sensing (Fabié *et al.*, 2018). Their priming is inhibited by hypoxia (Hammami *et al.*, 2017), as well as by cytokines such as type-I IFNs (Kumar *et al.*, 2020) and IL-10 (Murphy *et al.*, 2001). Hence, there is functional and numerical decline of these protective immune cells, during VL, which demands for the existence of compensatory mechanisms to replenish the CD4 T cell compartment. Therefore, it is plausible that there are alternative coping mechanisms in place, to sustain the protective CD4 T cell responses during chronic infections.

LAG-3 is an inhibitory receptor upregulated following T cell activation (Bruniquel *et al.*, 1998) and is associated with different T cell types including Foxp3-dependent and independent regulatory T cells as well as exhausted T cells (Aggarwal *et al.*, 2023). As several subtypes of CD4 T cells express this marker, clearly, the LAG-3-expressing CD4 T cells constitutes a heterogeneous pool of CD4 T cells. However, the functional role of LAG-3 expressing CD4 T cells in VL and whether or not these cells influence disease outcome, still remains unknown. LAG-3 is expressed by CD4 T cells in VL patients, and it is among the top 50 upregulated genes in CD4 T cells obtained from VL patients compared to healthy controls (Kumar *et al.*, 2020). Our preliminary experiments showed that the frequency and number of LAG-3 expressing CD4 T cells increases in *L. donovani* infected mice, even during the chronic phase of the infection, when there is excessive CD4 T cell death due to damage-sensing. Therefore, we decided to examine the LAG-3^{+} CD4 T cell subsets in VL, in search of alternative mechanisms to replenish the effector CD4 T cell responses. The specific objectives of this study are

(i) the phenotypic and molecular characterization and

(ii) the functional characterization of LAG-3-expressing CD4 T cells in VL

In addition to VL, we also examine the role of LAG-3-expressing CD4 T cells in LCMV Cl13 and *H.polygyrus bakeri* infections. Overall, this work aims to know whether LAG-3-expressing CD4 T cell subsets are involved in sustaining CD4 T cell responses in chronic infections

3 ARTICLE I

LAG-3- and CXCR5-expressing CD4 T cells display progenitor-like properties during chronic visceral leishmaniasis

Titre: Les lymphocytes T CD4 exprimant LAG-3 et CXCR5 présentent des propriétés semblables aux cellules de type progénitrices lors de la leishmaniose viscérale chronique

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Figures: 7 primary, 4 supplemental

The authors declare no competing financial interests.

Author contributions

S.Sw., S.St., I.L.K. designed the study, S.Sw., L.T.M., A.P.M., L.C.P. conducted experiments. S.Sw., L.T.M. analyzed data, S.Sw., S.St., I.L.K. interpreted results, T.C., A.L. provided experimental material, S.Sw., S.St. wrote the manuscript with comments from all authors.

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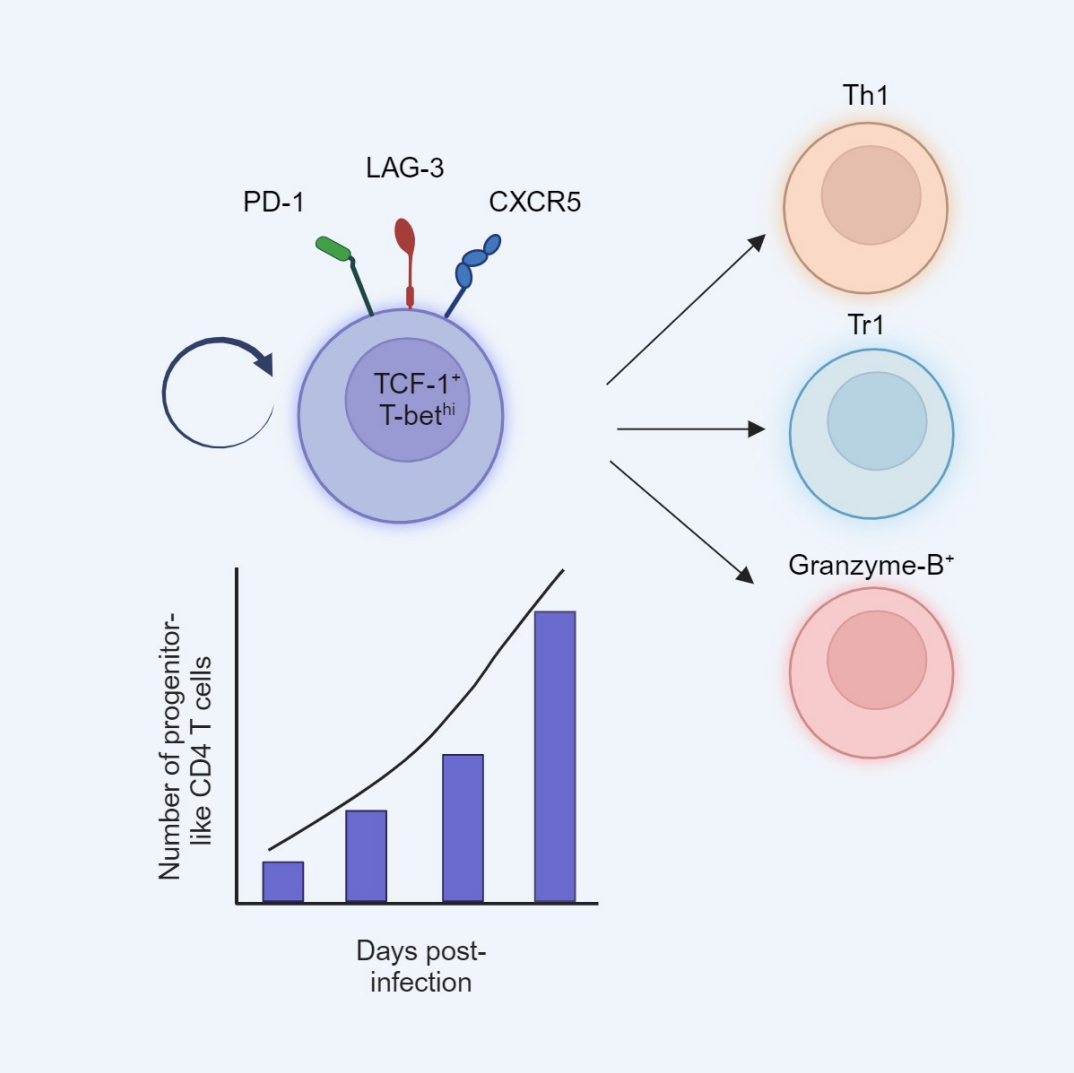
3.1 Graphical abstract



3.2 In brief

Swaminathan et al. identified a CD4 T cell subset that expresses LAG3^{+}CXCR5^{+} PD-1$^{lo/int}$ and expands during chronic visceral leishmaniasis. These cells display progenitor-like capacity, express genes associated with self-renewal, and differentiate into effector as well as regulatory CD4 T cell subsets, conferring protection upon adoptive transfer.

3.3 Summary

Maintenance of CD4 T cells during chronic infections is vital for limiting pathogen burden and disease recrudescence. Although inhibitory receptor expression by CD4 T cells is commonly associated with immune suppression and exhaustion, such cell-intrinsic mechanisms that control activation are also associated with cell survival. Using a mouse model of visceral leishmaniasis (VL), we discovered a subset of Lymphocyte activation gene 3 (LAG-3)-expressing CD4 T cells that co-express CXCR5. Although $LAG3^{+}CXCR5^{+}$ CD4 T cells are present in naïve mice, they expand during VL. These cells express gene signatures associated with self-renewal capacity, suggesting progenitor-like properties. When transferred into *Rag1*$^{-/-}$ mice, these $LAG3^{+}CXCR5^{+}$ CD4 T cells differentiated into multiple effector types upon *L. donovani* infection. The transcriptional repressor B cell lymphoma (BCL)-6 was partially required for their maintenance. Altogether, we propose that the $LAG3^{+}CXCR5^{+}$ CD4 T cell subset could play a role in maintaining CD4 T cell responses during persistent infections.

3.4 Introduction

In response to infection, CD4 T cells undergo activation and differentiation into several subtypes. These include effector cells (T helper type 1 or Th1, Th2, Th17) that are implicated in pathogen clearance as well as T follicular helper cells (Tfh) that assist in humoral immunity and regulatory T cells (such as conventional Foxp3-expressing Tregs and Foxp3-independent Tr1 cells) that suppress effector functions to prevent tissue damage and immunopathology [1]. Maintenance of CD4 T cells' number and function is crucial for an effective host response to chronic infection. The numerical and functional decline of CD4 T cells, due to immune activation by persistent pathogens, leads to altered immune responses unfavourable to the host, as observed in chronic viral [2], parasitic [3] and bacterial infections [4].

Visceral leihmaniasis (VL) is one such possibly fatal chronic infection, caused by *Leishmania donovani* and/or *Leishmania infantum/chagasi.* Symptoms of this disease include hepatosplenomegaly, hypergammaglobulinemia, anemia, cachexia, and immunosuppression [5]. Hepatosplenomegaly is also present in mice infected with *L. donovani*, which can control the infection in the liver but not in the spleen [5]. Amastigotes mainly reside in infected macrophages and production of reactive oxygen and nitrogen intermediates in response to activation triggers intracellular pathogen clearance in these cells [6]. Cytokines such as IFNγ and TNF produced by CD4 and CD8 T cells play a crucial role in the activation of these parasite-infected macrophages [7]. In VL, there is limited expansion of CD8 T cells as well as impairment of their functions [8,9] which results in the IFNγ-producing CD4 T cells being the major players in pathogen clearance during the chronic phase. However, there are several mechanisms suppressing the protective CD4 T cell responses in VL. These cells undergo functional exhaustion [10] and cell death due to damage sensing [11]. Th1 responses in VL are inhibited by hypoxia [12], type-I interferons [13] as well as IL-10 [14]. During VL, IL-10 is produced by many cells [15,16] but the major source of this cytokine are the IFNγ^{+}IL-10^{+} type 1 regulatory (Tr1) cells [17-19]. Tr1 cells not only suppress Th1 responses, but they are also essential for controlling pathological tissue damage in chronic VL [20] and in other parasitic infections [21,22]. Therefore, the mechanisms regulating the maintenance of protective CD4 T cell responses during chronic infections, particularly those involving regulation by Tr1 cells, are certainly complicated and require investigation.

Tr1 cells are also characterized by the co-expression of the cell surface markers Lymphocyte activation gene 3 (LAG-3) and CD49b [23]. LAG-3 is an inhibitory receptor expressed on the CD4 T cell membrane and binds to MHC-II with higher affinity than CD4 [24]. Although the exact mechanisms by which LAG-3 regulates T cell activation and clonal expansion is unknown, a

conserved KIEELE motif on the intracellular domain of this receptor is mainly responsible for its inhibitory functions [25,26]. LAG-3 is an immune checkpoint molecule expressed in response to TCR activation [27] and its upregulation is a cell-intrinsic mechanism employed by activated T cells for keeping proliferation in check. It is also co-expressed with other immune checkpoint molecules such as PD-1 and TIM-3 in exhausted T cells [25]. Additionally, LAG-3 expressed on Tregs can engage with MHC-II and is indispensable for Treg-mediated direct inhibition of effector CD4 T cells [28,29].

In the current study using experimental VL, we identified a subset of LAG-3$^+$ CD4 T cells expressing CXCR5 that expands during chronic infection and has progenitor-like properties. Indeed, these LAG-3$^+$CXCR5$^+$ T cells expressed a transcriptional signature associated with self-renewal and could differentiate into multiple T-effector lineages, particularly Tr1 cells. Furthermore, these cells were also present in other models of chronic infection, including LCMV Cl13 and *Heligmosomoides polygyrus bakeri* infection. We also demonstrated that these cells partially require the transcription factor BCL-6 for their maintenance. Owing to their progenitor-like properties, we propose that these cells could serve as a compensatory mechanism to sustain the declining CD4 T cell responses during chronic infections and could serve as targets for the development of therapeutic tools.

3.5 Results

3.5.1 Identification of a subset of LAG-3$^+$ CD4 T cells

In search of pathways that regulate CD4 T cell responses to infections, we tracked the kinetics of splenic LAG-3-expressing CD4 T cells in the experimental model of VL, as this marker is expressed by several subsets of regulatory cells [23,29,30]. LAG-3-expression is also an indication of T cell activation *in vivo* upon antigen exposure in chronic infections, eventually contributing to T cell exhaustion [31]. As shown in Figure 3.1a and 3.1b, the frequencies and numbers of LAG-3-expressing splenic CD4 T cells increased significantly starting from day 21 post-infection (p.i.) in mice infected with *L. donovani* and continued to increase as the infection progressed to the chronic stage. Several LAG-3-expressing subsets of CD4 T cells have been described. These include Foxp3-dependent regulatory T cells (Tregs) [29] and Foxp3-independent, IL-10 producing regulatory Tr1 cells [23], Tregs that regulate B cell responses [30] and exhausted T cells that co-express LAG-3 and other checkpoint inhibitors, such as TIM-3, PD-1 or TIGIT [32]. We noticed that at 21 days p.i., the proportion of regulatory cells (Foxp3$^+$CD25$^+$, Foxp3$^-$CD25$^+$, LAG-3$^+$CD49b$^+$) and exhausted cells (LAG-3$^+$ TIM-3$^+$), altogether accounted for less than ~50% of the total LAG-

3^+ CD4 T cells (Figure 3.1c). Therefore, we explored what other CD4 T cell subpopulations express LAG-3. Interestingly, we identified a subset that co-expressed LAG-3 and the follicle-homing chemokine receptor CXCR5, also highly expressed on T follicular helper (Tfh) cells. Notably, LAG-3^+ $CXCR5^+$ CD4 T cells were a distinct population, after excluding from the analysis the $CXCR5^+$ PD-1^{hi} germinal center Tfh cells (GC-Tfh)[33,34] that help in B cell maturation and antibody production as shown in Figure 3.1d. Interestingly, LAG-3^+CXCR5^+ CD4 T cells co-expressed several Tfh markers, such as PD-1, ICOS, and TCF-1 (Figure 3.1e). We also observed that the frequency of $CXCR5^+$ PD-1^{hi} GC-Tfh cells in infected mice spleens declines starting from 21 days post-infection (Figure 3.1f), which is in line with a previous study involving rhesus macaques infected with *Leishmania infantum* [35]. Although the proportion of Tfh cells diminishes, the frequency of LAG-3^+CXCR5^+ CD4 T cells peaks at day 21 p.i. (Figure 3.1g), indicating that these two cell populations have opposing dynamics. Moreover, the number of splenic LAG-3^+CXCR5^+ CD4 T cells continues to increase even during the chronic stage of VL (Figure 3.1h). Interestingly, these cells were all $CD44^+$, indicating that they have, most likely, been exposed to antigens (Figure 3.1i). Furthermore, we examined the antigen specificity of these cells using the tetramers specific for the peptide $PEPCK_{335-351}$, which is a derivative of a metabolic enzyme expressed by *Leishmania* - glycosomal phosphoenolpyruvate carboxykinase (PEPCK) [36]. We observed that over 10% of the LAG-3^+ $CXCR5^+$ PD-$1^{lo/int}$ cells were PEPCK-specific (Figure 3.1j) compared with ~2% PEPCK-specific cells among the total splenic CD4 T cell population. These findings suggest that the LAG-3^+ $CXCR5^+$ PD-$1^{lo/int}$ cells are most likely antigen-specific.

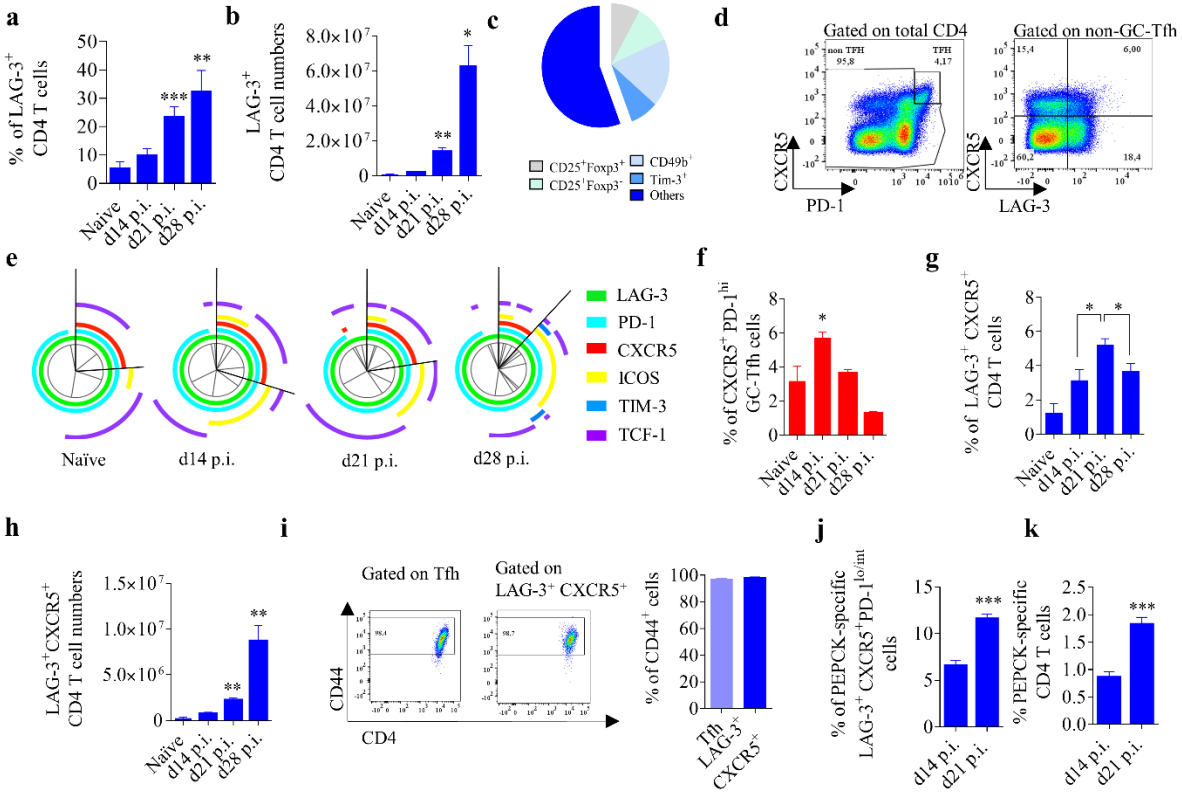


Figure 3.1 Identification of a subset of LAG-3⁺ CD4 T cells

Mice were infected with L. donovani amastigotes intravenously and euthanized at various time points after infection. Graphs show (a) the frequencies and (b) numbers of LAG-3-expressing CD4 T cells during infection; (c) the distribution of various CD4 T cell subsets within the LAG-3⁺ CD4 T cell population; (d) representative FACS plots showing gating strategy for identifying non-GC-Tfh CD4 T cells (left), and CXCR5 and LAG-3 expression by non-GC-Tfh CD4 T cells at day 21 p.i. (right). (e) SPICE analysis of flow cytometry data showing heterogeneity of the LAG-3⁺ CD4 T cells expressing the indicated markers over the course of experimental VL. Graphs show (f) frequencies of CXCR5⁺ PD-1^{hi} GC-Tfh cells, (g) frequencies and (h) numbers LAG-3⁺CXCR5⁺ CD4 T cells during VL. (i) Representative FACS plots and percentage of CD44⁺ Tfh and LAG-3⁺CXCR5⁺ CD4 T cells at 21 days p.i.; graphs show the frequencies (j) PEPCK-specific LAG-3⁺CXCR5⁺PD-1$^{lo/int}$ CD4 T cells and (k) splenic CD4 T cells during experimental VL. Data represent mean ± SEM of one of 3 independent experiments, with n = 4 per group for each experiment. *p < 0.05, **p < 0.01, ***p<0.001.

3.5.2 $T_{LCPlo/int}$ cells display an enrichment of memory-like genes

To characterize these cells further, we purified the LAG-3⁺CXCR5⁺ PD-1$^{lo/int}$ CD4 T cells (referred to as $T_{LCPlo/int}$ hereafter) and compared their transcriptomic profile to that of LAG-3⁻CXCR5⁺ PD-1^{hi} Tfh cells (GC-Tfh) by bulk RNA sequencing. Due to their similarity in surface marker expression to GC-Tfh and their opposite expansion kinetics, we chose to compare the $T_{LCPlo/int}$ cells to the GC-Tfh cells. Both cell populations were purified by cell sorting (according to strategy shown in Sup. Figure 3.8a), from the spleens of mice challenged with *L. donovani* at 21 days p.i. (Figure

3.2a). As the majority of the LAG-3^{+}CXCR5^{+} cells were PD-1$^{lo/int}$ (Sup. Figure 3.8b), we decided to sort only PD-1$^{lo/int}$ and not PD-1^{hi} cells, which constituted just a small percentage of LAG-3^{+}CXCR5^{+} CD4 T cells. Bulk-RNA sequencing revealed that there were 5561 differentially regulated genes (DEGs) between $T_{LCPlo/int}$ cells and Tfh cells (with Δlog2FC > 1.5, $p<0.05$) among which 3456 were upregulated and 2105 were down regulated, as shown in the volcano plot (Figure 3.2b). The principal component analysis (PCA) for the top 500 varying genes showed a clear lack of association between these two populations (Sup. Figure 3.8c). These findings confirmed that the $T_{LCPlo/int}$ cells were very distinct from Tfh cells. To test if LAG-3^{+}CXCR5^{+} CD4 T cells had similarities to any of the known T cell types, we performed gene set enrichment analysis (GSEA). We chose the gene sets for the enrichment analysis from the MiSGDB, based on previously published articles comparing effector and memory Th1 and Tfh cells as well as those comparing T effectors and Tregs [37,38]. These available gene sets on the MiSGDB, utilize the assembled gene lists that were overrepresented in each known CD4 T cell subset (such as Th1 effector) in comparison to another (such as Tfh effector). Our analysis based on these relative comparisons revealed that the $T_{LCPlo/int}$ cells were more closely related to Tfh cells rather than Th1 effectors (Figure 3.2c), and Tfh memory cells rather than Th1 memory cells (Figure 3.2d). In addition, these cells shared more similarities with T effectors compared to Treg cells (Figure 3.2e). Using BubbleGUM (GSEA Unlimited Map) software, we generated a bubble map based on the normalized enrichment scores and FDRs. As shown by the map in Figure 3.2f, out of all the described T cell subsets, Tfh memory-associated genes were the most enriched in the $T_{LCPlo/int}$ cells. Nevertheless, the normalized enrichment score is relatively low, suggesting that the $T_{LCPlo/int}$ cells are a distinct population and have few similarities with Tfh memory cells.

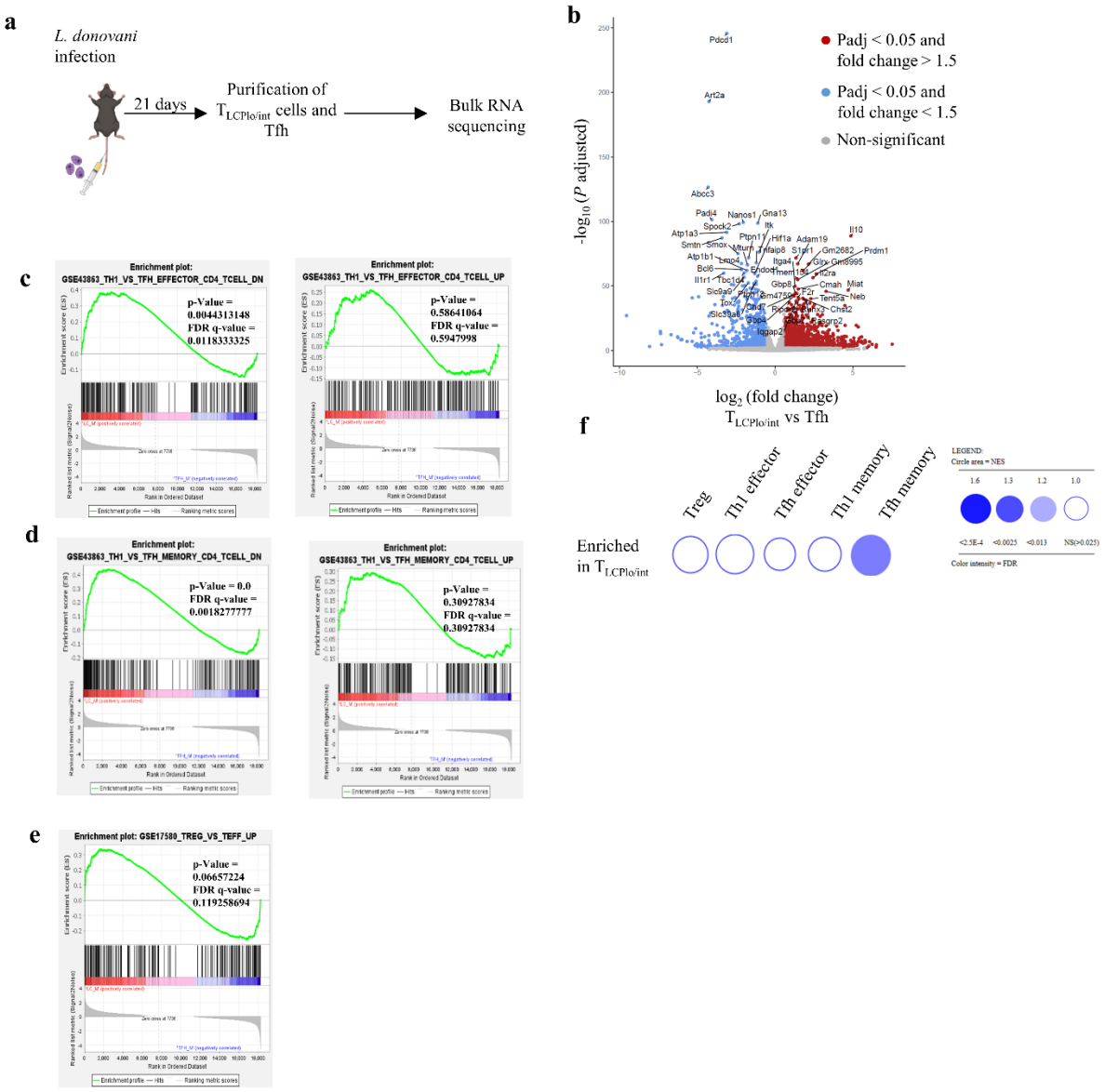


Figure 3.2 $T_{LCPlo/int}$ cells display an enrichment of memory-like genes

(a) Schematic showing experimental design for bulk RNA-sequencing of $T_{LCPlo/int}$ cells versus Tfh cells. (b) Volcano plots showing DEGs between $T_{LCPlo/int}$ cells versus Tfh cells. A threshold of Δlog2FC > 1.5 and FDR cut-off of 5% (P adjusted < 0.05) was used to determine significant DEGs. (c-e) Enrichment plots generated using the GSEA software to check for similarities between $T_{LCPlo/int}$ cells and different CD4 T cell subsets, (c) Th1 and Tfh effector cells, (d) Th1 and Tfh memory cells, and (e) Tregs. (f) Maps generated using the BubbleGUM software to visualize the GSEA performed. NES = normalized enrichment score. Data were generated from two independent infection experiments with n = 3 each. Also see Sup. Figure 3.8.

3.5.3 Molecular gene signatures of $T_{LCPlo/int}$ cells

Taking a closer look at the transcriptional profile of the $T_{LCPlo/int}$ cells, we observed that these cells expressed higher levels of co-inhibitory receptors such as *Ctla4* and *Cd244a* (encoding for SLAMF4, also known as 2B4) relatively to GC-Tfh cells (Figure 3.3a). Some of the co-stimulatory receptors such as *Cd40* and *Cd86* were highly expressed by these cells relatively to GC-Tfh cells as well, although other such as *Cd28* and *Cd80* were not. Despite having higher levels of co-inhibitory receptor expression compared with GC-Tfh cells, these cells also expressed higher levels of cytokines (notably interferon-γ, IL-10, TNF), chemokines and effector molecules (granzymes and perforins), as shown in Figure 3.3b, which suggested that they may be functionally active. These cells also expressed higher levels of several transcription factors characteristic of Th1 cells, such as *Prdm1*, *Tbx21*, *Eomes, Runx3* and *Id2* (Figure 3.3c, [39]), relatively to GC-Tfh cells. The $T_{LCPlo/int}$ cells expressed lower levels of canonical GC-Tfh-related transcription factors *Bcl6* and *Tcf7* compared to Tfh cells. However, it is important to note that these cells clearly expressed *Bcl6* and *Tcf7* as the TPM (Transcripts Per Kilobase Million) values for these transcripts were 19.67±1.296SE and 594.9±51.25SE respectively using STAR analysis [40]. Nevertheless, they were significantly less than the TPM values observed for the GC-Tfh cells (BCL-6 74.24±6.996SE and TCF-1 1160.782±107.7SE). Similarly, CXCR5 was expressed at lower levels in $T_{LCPlo/int}$ cells when compared to the Tfh cells (TPM value: 215.4±14.03SE and 552.9±45.87SE respectively). Importantly, many of the memory-associated molecules such as *Ccr7*, *Il2ra*, *Il7r*, *Ly6a* and *Slamf1* were also highly expressed by the $T_{LCPlo/int}$ cells (Figure 3.3d). Taken together these data suggested that the $T_{LCPlo/int}$ cells had similarities to the Th1 lineage, although these cells expressed several memory-like markers.

3.5.4 $T_{LCPlo/int}$ cells are HSC-like and express gene signatures associated with self-renewal capacity

Recently, a progenitor-like CD8 T cell population with self-renewal capacity has attracted much attention as it resembles exhausted cells, expresses CXCR5, and gives rise to effector CD8 T cells [41-45]. Since the $T_{LCPlo/int}$ cells also expressed markers such as CXCR5, BCL-6, TCF-1 and had high expression of co-inhibitory receptors (activated, exhausted-like), we tested whether these cells are the CD4 T cell analogs of the progenitor-like $CXCR5^+$ CD8 T cells. Therefore, we performed GSEA to compare the $T_{LCPlo/int}$ cells to hematopoietic stem cells (HSCs). Strikingly, the HSC-related genes were significantly enriched in the $T_{LCPlo/int}$ cells (Figure 3.3e). Furthermore, these cells highly expressed several important HSC-associated self-renewal genes, such as *Myb*, *Kit*, *Notch1*, *Fzd6*, and *Sox*4 (Figure 3.3f). To confirm that $T_{LCPlo/int}$ cells were proliferating, we

assessed Ki67 expression in $T_{LCPlo/int}$ cells present in the spleen at d28 after *L. donovani* infection and found that they indeed expressed significant levels of Ki67 (Figure 3.3g). Considering that these cells had a combination of an HSC-associated transcriptional signature and memory-like phenotype and that they express the proliferation marker Ki67, our data suggest that $T_{LCPlo/int}$ cells may represent a CD4 T cell precursor population.

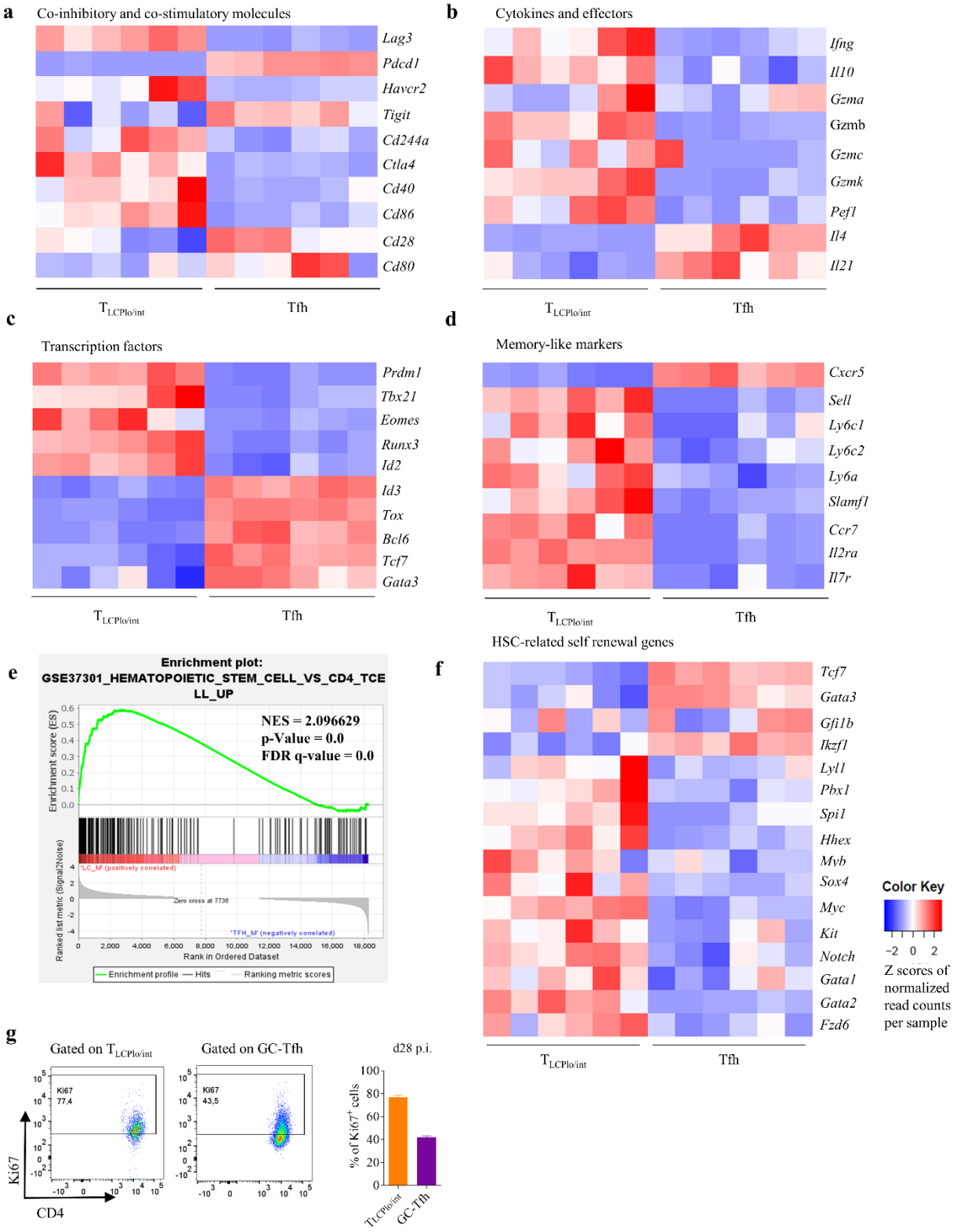


Figure 3.3 Molecular gene signatures of $T_{LCPlo/int}$ cells

Heat maps show relative up- or down-regulation of selected genes in $T_{LCPlo/int}$ cells versus Tfh cells from *L. donovani* infected mice at 21 days p.i.: (a) Co-inhibitory and co-stimulatory molecules, (b) cytokines and effector molecules, (c) transcription factors, and (d) memory-like markers. (e) Enrichment plots generated using GSEA software to check for similarities between $T_{LCPlo/int}$ cells and HSCs. (f) Heat maps showing relative up- or down-regulation of selected self-renewal genes (usually expressed by HSCs) in $T_{LCPlo/int}$ cells versus Tfh cells from *L.donovani* infected mice at 21 days p.i. (g) Mice were infected with *L. donovani*. Graph shows Ki67 expression (mean ± SEM) by $T_{LCPlo/int}$ cells at d28 p.i.. Data were generated from two independent infection experiments with n = 3 each.

3.5.5 $T_{LCPlo/int}$ cells can differentiate into several CD4 T effector lineages

To evaluate the expansion capacity and progenitor-like properties of the $T_{LCPlo/int}$ cells, we designed adoptive transfer experiments. Specifically, $T_{LCPlo/int}$ cells purified from *L. donovani*-infected C57BL/6 mice at d21p.i. or naïve CD4 T cells were adoptively transferred into naïve *Rag1*$^{-/-}$ mice and either analyzed 21 days post transfer or the *Rag1*$^{-/-}$ recipient mice were infected with *L. donovani* one day after transferring cells and then analyzed at different time points post-transfer (Figure 3.4a). Surprisingly, the $T_{LCPlo/int}$ cell displayed a limited expansion capacity in naïve *Rag1*$^{-/-}$ mice compared with the naïve CD4 T cells as shown in Figure 3.4b and 3.4c. However, the observations were very different when the *Rag1*$^{-/-}$ recipient mice were infected with *L. donovani* one day after transferring cells - the $T_{LCPlo/int}$ cells expanded in the infected *Rag1*$^{-/-}$ mice similarly to naïve CD4 T cells. Indeed, no significant differences in CD4 T cell frequencies were observed between the group that received naïve CD4 T cells and the $T_{LCPlo/int}$ group at any time point analyzed (Figure 3.4d). We also stained adoptively transferred cells for Ki67 to monitor their proliferation and could not find significant differences between the two groups in the frequencies of Ki67 expressing cells at any time point analyzed (Sup. Figure 3.9). Furthermore, the $T_{LCPlo/int}$ cells do not expand when transferred in naïve *Rag1*$^{-/-}$ recipients, indicating that this sorted population of cells is not contaminated with naïve CD4 T cells, as the results in 4b and 4c clearly show that the naïve CD4 T cells expand in naïve *Rag1*$^{-/-}$ recipients.

However, when we analyzed the number of adoptively transferred cells present in the spleen at various time points of infection, we found significant differences between the two groups at d21 and 28 p.i. (Figure 3.4e). These differences were due to the fact that mice that received $T_{LCPlo/int}$ cells had smaller spleens than those that received naïve CD4 T cells (Figure 3.4f). We next assessed the splenic parasite burden to determine whether the differences in spleen weight were resulting from differences in the number of parasites present in that organ. Interestingly, we found significantly lower parasite burdens at d21 and 28 p.i. in mice that received $T_{LCPlo/int}$ cells compared

with the control group (Figure 3.4g), suggesting that $T_{LCPlo/int}$ cells had a superior capacity to provide protective immunity.

Interestingly, the $T_{LCPlo/int}$ cells differentiated into many different functional CD4 T cell subsets (Figure 3.4h-3.4l). Most strikingly, these cells not only gave rise to significantly increased frequencies of IFNγ^{+}IL-10^{+} Tr1 cells (Figure 3.4h) but these Tr1 cells produced higher levels of IL-10 than the Tr1 cells derived from adoptively transferred naïve CD4 T cells (Figure 3.4i). Additionally, after re-stimulation with *L. donovani*-primed bone marrow-derived dendritic cells, $T_{LCPlo/int}$ cells differentiated into antigen-specific IFNγ-producing Th1 cells (Figure 3.4j, Sup. Figure 3.10a) at frequencies similar to those found for Th1 derived from naïve CD4 T cells, with exception of frequencies observed at d21 p.i. These $T_{LCPlo/int}$ cells also gave rise to TNF^{+} CD4 T cells (Figure 3.4k, Sup. Figure 3.10b) and Granzyme-B^{+} CD4 T cells (Figure 3.4l, Sup. Figure 3.10c). Altogether, these results confirmed the ability of $T_{LCPlo/int}$ cells to differentiate into multiple effector lineages in response to chronic infection, thereby validating their progenitor-like properties.

We also compared CD4 T cell responses in *Rag1*$^{-/-}$ mice adoptively transferred with $T_{LCPlo/int}$ or Lag3^{neg} CD4 T cells purified at d21 p.i. from the spleen of *L. donovani* infected mice. As for the adoptive transfer experiment described above, we only transferred 10^4 cells one day prior to infection with *L. donovani*. Remarkably, the $T_{LCPlo/int}$ cells displayed superior expansion capacity compared with Lag3^{neg} CD4 T cells at d14 p.i., in frequencies (Sup. Figure 3.10d) and numbers (Sup. Figure 3.10e) but gave rise to similar frequencies of IFN-y^{+} (Sup. Figure 3.10f) and IFN- y^{+} IL-10^{+} (Sup. Figure 3.10g) cells.

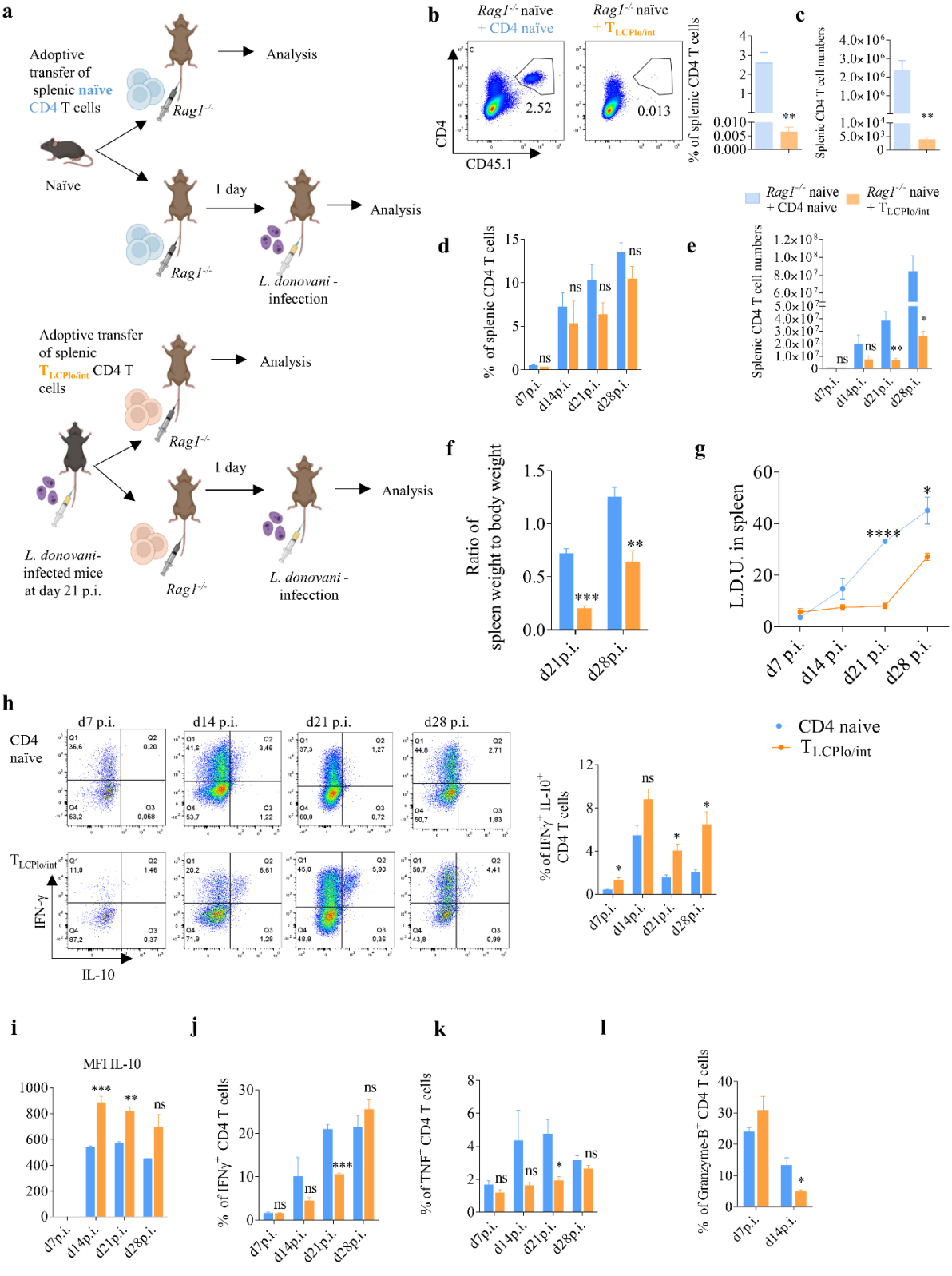


Figure 3.4 $T_{LCPlo/int}$ cells can differentiate into several T effector lineages

(a) Schematic showing design of adoptive cell transfer experiment. Graphs show (b) the percentage of total splenocytes that are $CD4^+$ and (c) the number of splenic $CD45.1^+$ $CD4^+$ T cells at day 21 p.i., in uninfected *Rag1*$^{-/-}$ mice; the frequencies (d) and the numbers (e) of splenic $CD45.1^+$ $CD4^+$ T cells in *Rag1*$^{-/-}$ mice infected with *L. donovani* that received either $T_{LCPlo/int}$ cells from *L. donovani*-infected mice or naïve CD4 T cells; graphs represent the percentage of spleen weight relative to body weight (f) and the parasite burden in the spleen (expressed as Leishman Donovan Units, LDU) (g) of *Rag1*$^{-/-}$ mice adoptively transferred with naïve CD4 T cells or $T_{LCPlo/int}$ cells a day prior to infection with *L. donovani*; (h) representative FACS plots showing expression of IFNɣ and IL-10 by adoptively transferred CD4 T cells (gated on $CD45.1^+$ $CD4^+$ cells) in *Rag1*$^{-/-}$ recipient mice infected with *L. donovani* and frequencies of splenic $IFN\gamma^+$ $IL\text{-}10^+$ Tr1 cells; (i) mean fluorescence intensity of IL-10 in Tr1 cells; (j) percentage of antigen specific IFNγ-producing, (k) TNF-producing and (l) Granzyme-B-producing splenic CD4 T cells from *Rag1*$^{-/-}$ recipient mice infected with *L. donovani*. Data represent mean ± SEM from one out of two or three independent infections with four mice per group per experiment. *p < 0.05, **p < 0.01, ***p<0.001, ns (not significant). Also see Sup. Fig. S2 and S3.

3.5.6 LAG-$3^+$$CXCR5^+$$PD1^+$ CD4 T cells also expand in other chronic infections

Subsequently, we tested if $T_{LCPlo/int}$ cells expand in other chronic infections. For this purpose, we used mouse models of LCMV clone-13 (Cl13) and *Heligomosomoides polygyrus bakeri* infection. We observed that the frequency of LAG-3-expressing splenic CD4 T cells increased significantly starting from day 8 post-infection (p.i.) in LCMV Cl13 infected mice (Figure 3.5a), similar to *L. donovani* infection. We also found that the frequencies of LAG-$3^+$$CXCR5^+$ CD4 T cells, which did not include GC-Tfh ($CXCR5^+PD\text{-}1^{hi}$) cells, increased significantly starting from d8 p.i. (Figure 3.5b and 3.5c). As observed in *L. donovani* infection, the LAG-$3^+$$CXCR5^+$ CD4 T cells in LCMV Cl13 infected mice also expressed Tfh markers such as PD-1, ICOS, and TCF-1 (Figure 3.5d). Furthermore, $T_{LCPlo/int}$ cells were also present in *H. polygyrus bakeri* infection (Figure 3.5e). Interestingly, in each of these chronic infections, these cells expressed different levels of PD-1 at the CD4 T cell peak expansion phase (Figure 3.5f). In *L. donovani* infection, most of these cells expressed intermediate levels of PD-1 (Figure 3.5f, top panel); in LCMV Cl13 infected mice, most of these cells were $PD\text{-}1^{hi}$ (Figure 3.5f, middle panel); while in *H. polygyrus bakeri* infection most of these were $PD\text{-}1^{lo}$ (Figure 3.5f, bottom panel). Taken together these data confirmed that the $T_{LCPlo/int}$ cells existed in several infections, although differing in their PD-1 expression, suggesting that the development of these cells could be context-dependent and might differ in varying types of infections.

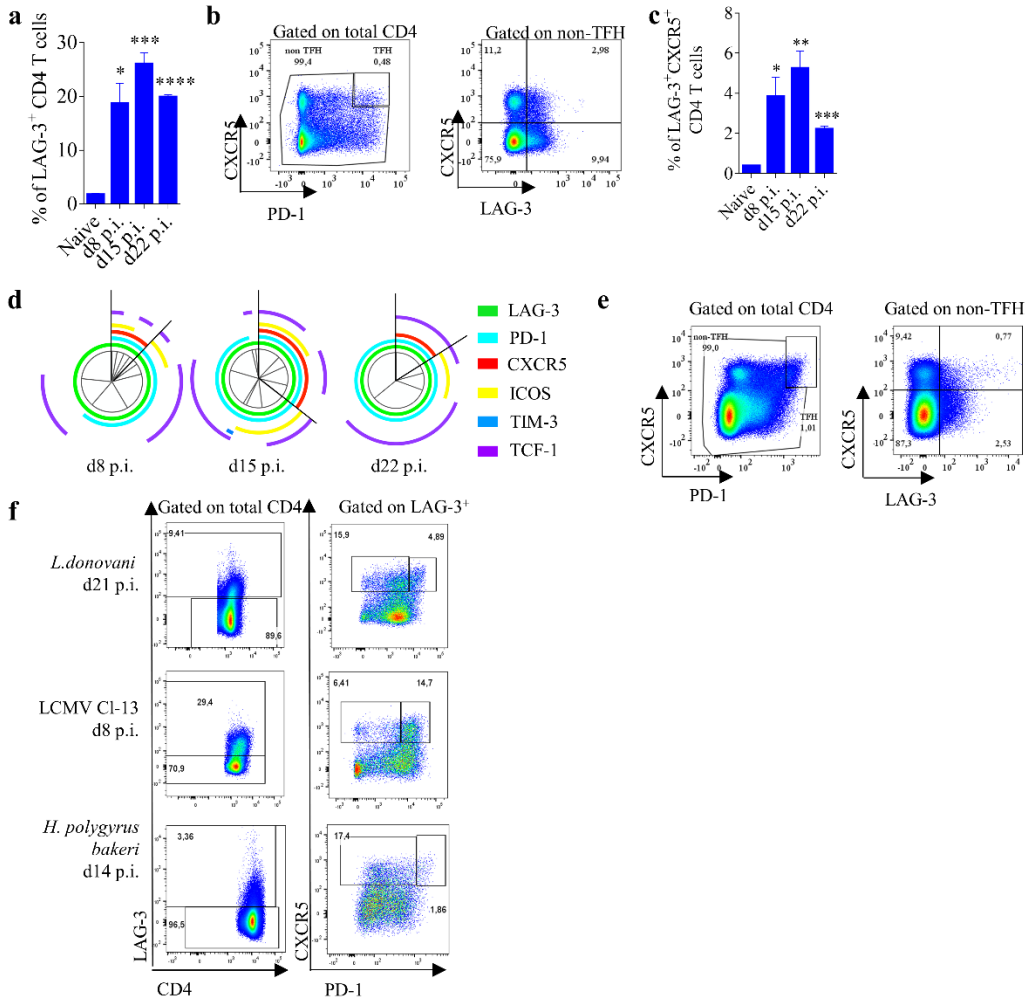


Figure 3.5 LAG-3⁺CXCR5⁺PD1⁺ CD4 T cells also expand in other chronic infections

Mice were infected with LCMV Cl-13 intravenously and euthanized at various time points after infection. (a) Graph shows the frequency of LAG-3-expressing CD4 T cells over the course of infection. (b) Representative plots showing gating strategy to identify non-Tfh CD4 T cells (left) and LAG-3 and CXCR5 expression by non-Tfh CD4 T cells (right) in LCMV Cl-13 infected mice at 8 days p.i. (c) Graph represents the frequencies of CD4 T cells expressing LAG-3 and CXCR5 during LCMV Cl-13 infection. (d) SPICE analysis of flow cytometry data showing heterogeneity of the LAG-3⁺ CD4 T cell pool expressing various markers in LCMV Cl-13 infection. Mice were infected with *H. polygyrus bakeri* by administering 200 L3 stage larvae by gavage and were euthanized 14 days p.i.. (e) Representative plots showing the gating strategy for identifying non-Tfh CD4 T cells (left) and the expression of LAG-3 and CXCR5 by non-Tfh CD4 T cells (right) in the mesenteric lymph nodes of *H. polygyrus bakeri* infected mice at day 14 p.i. (f) Representative plots to demonstrate the differences in PD-1 expression in the $T_{LCPIo/int}$ cells in the three different infection models used in this study. Left column: expression of LAG-3 in CD4 T cells; right column: expression of CXCR5 and PD-1 in LAG-3⁺ CD4 T cells. Time points were chosen based on the CD4 expansion phase in each infection model. Data represent mean ± SEM

from one of two independent infections with three to five mice per group per experiment. *p < 0.05, **p < 0.01, ***p<0.001, ****p<0.0001.

3.5.7 $T_{LCPlo/int}$ cells are distinct from T_{LCPhi} cells in experimental VL

We noticed that in addition to the $T_{LCPlo/int}$ cells, a subset that is LAG-3^{+}CXCR5^{+}PD-1^{hi} (henceforth referred to as T_{LCPhi} cells) also existed in experimental VL (Figure 3.6a). Flow cytometric analysis revealed the differential expression of several markers between these two populations. The $T_{LCPlo/int}$ cells expressed low levels of both the canonical Tfh transcription factors BCL-6 (Figure 3.6b and Sup. Figure 3.11a) and TCF-1 (Figure 3.6c and Sup. Figure 3.11b), while they expressed higher levels of T-bet (Figure 3.6d and Sup. Figure 3.11c), compared to the Tfh and the T_{LCPhi} cells. These cells expressed lower levels of CD69 and higher levels of the markers CD150 and CXCR6 (Figure 3.6e-g and Sup. Figure 3.11d-f), compared to the other two populations. A recent publication described a subset of CD4 T cell precursors that were BCL-6^{lo}TCF-1^{+} and whose development is associated with Tfh development [34]. The BCL-6^{lo}TCF-1^{+} progenitor cells described by Xia et al. were expressing lower levels of CD69, TIM-3, CXCR6, and CD150 and did not express the transcription factor Blimp-1. In contrast, the $T_{LCPlo/int}$ cells highly expressed *Prdm1* (BLIMP-1, Figure 3.3c). These cells also expressed lower levels of CD69 and higher levels of the markers, CD150 and CXCR6 (Figure 3.6e-g), suggesting that these $T_{LCPlo/int}$ cells were different from the progenitors described by Xia et al. However, the T_{LCPhi} cells looked very similar to the BCL-6^{lo}TCF-1^{+} progenitors (Figure 3.6e-g).

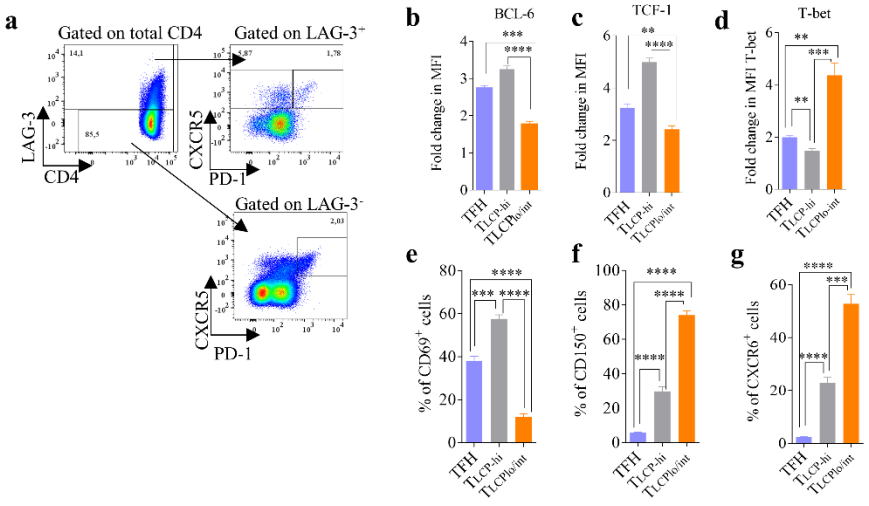


Figure 3.6 $T_{LCPlo/int}$ cells are distinct from T_{LCPhi} cells in experimental VL

Mice were infected with *L. donovani* amastigotes intravenously and euthanized at d21 p.i. (a) Representative plots showing gating strategy for T_{LCPhi} and Tfh cells. Graphs show the mean fluorescence intensity of (b) BCL-6, (c) TCF-1, and (d) T-bet expression in Tfh vs T_{LCPhi} vs $T_{LCPlo/int}$ cells, and percentages of (e) $CD69^+$, (f) $CD150^+$, and (g) $CXCR6^+$ Tfh vs T_{LCPhi} cells vs $T_{LCPlo/int}$ cells. Data represent mean ± SEM from one of two independent infections with four mice per group per experiment. **p < 0.01, ***p<0.001, ****p<0.0001. Also see Sup. Fig. S4.

3.5.8 Absence of BCL-6 partially affects the maintenance of $T_{LCPlo/int}$ cells and drives Tr1 cell differentiation

Since the progenitor cells described by Xia et al. were BCL-6-dependent [34], we wanted to know if this transcription factor is required by the $T_{LCPlo/int}$ cells and the T_{LCPhi} cells. Therefore, we challenged *Bcl6*$^{fl/fl}$*xCd4-Cre*$^-$ and *Bcl6*$^{fl/f}$*xCd4-Cre*$^+$ mice with *H. polygyrus bakeri* and analyzed the CD4 T cells from mesenteric lymph nodes at d14 p.i. Interestingly, despite the fact that we observed higher percentages of LAG-3^+ CD4 T cells in CD4 T cell-specific *Bcl6*$^{-/-}$ mice compared to *Bcl6* sufficient mice (Figure 3.7a), the frequency of $T_{LCPlo/int}$ cells decreased significantly and the T_{LCPhi} population disappeared in the absence of BCL-6 (Figure 3.7b), suggesting that this transcription factor could play a role in the maintenance of both populations. To know whether the absence of BCL-6 affects the induction of the various CD4 T cell subsets, we performed adoptive transfer experiments using the mouse model of visceral leishmaniasis. Total splenic CD4 T cells were purified from *Bcl6*$^{fl/fl}$*xCd4-Cre*$^-$ mice and *Bcl6*$^{fl/f}$*xCd4-Cre*$^+$ mice and transferred into *Rag1*$^{-/-}$ mice a day prior to *L. donovani* challenge; the adoptively transferred cells were analyzed in the spleen of mice at 21 days p.i. (Figure 3.7c). Strikingly, mice that received *Bcl6*$^{fl/f}$*xCd4-Cre*$^+$ CD4 T cells showed significantly increased frequency of IFNγ$^+$IL-10^+ Tr1 cells compared to those that received *Bcl6*$^{fl/fl}$*xCd4-Cre*$^-$ T cells (Figure 3.7d). This observation was substantiated by the fact that in the absence of BCL-6, higher percentages of LAG-3^+CD49b$^+$ CD4 T cells (Figure 3.7e) were observed. In addition, *Bcl6*$^{fl/f}$*xCd4-Cre*$^+$ CD4 T cells generated significantly fewer antigen-specific IFNγ-producing Th1 cells (Figure 3.7f), similar percentages of TNF^+ CD4 T cells (Figure 3.7g), and significantly higher frequency of Granzyme-B$^+$ CD4 T cells (Figure 3.7h) compared to *Bcl6*$^{fl/fl}$*xCd4-Cre*$^-$ CD4 T cells. Taken together, our results suggest that BCL-6 is partially required for the maintenance of $T_{LCPlo/int}$ cells but essential for the maintenance of T_{LCPhi} cells; moreover, the absence of BCL-6 favoured the differentiation into LAG-3^+ CD4 T cells, Granzyme-B$^+$ CD4 T cells and Tr1 cells.

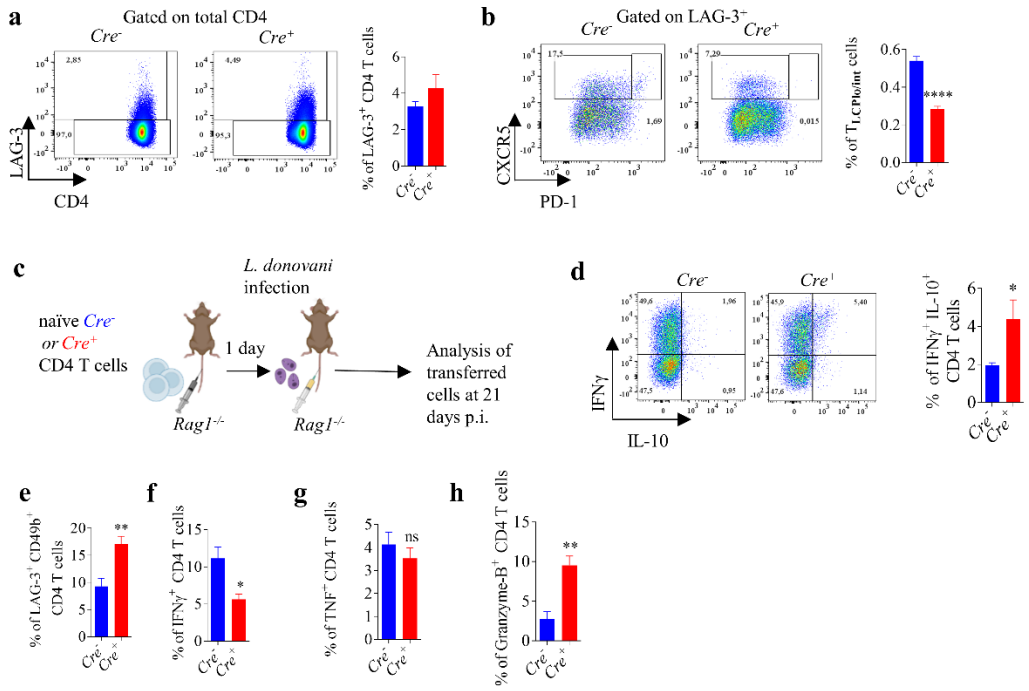


Figure 3.7 Absence of BCL-6 partially affects the maintenance of $T_{LCPlo/int}$ cells and drives Tr-1 cell differentiation.

Mice were infected with *H. polygyrus bakeri* by administering 200 L3 stage larvae by gavage and were euthanized 14 days p.i. Representative plots and graphs showing (a) frequencies of LAG-3$^+$ CD4 T cells and (b) expression and frequencies of PD-1$^+$ and CXCR5$^+$ among LAG-3$^+$ CD4 T cells in the mesenteric lymph nodes of *H. polygyrus bakeri* infected mice. (c) Schematic showing the design of adoptive transfer experiments to test the impact of BCL-6 ablation on the induction of the various CD4 T cell subsets during experimental VL. (d) Representative plots showing IFNγ and IL-10 expression in splenic CD4 T cells and percentages of Tr1 cells in *Rag1*$^{-/-}$ recipient mice infected with *L. donovani*. Graphs show the percentage of (e) LAG-3$^+$CD49b$^+$ cells, (f) antigen specific IFNγ$^+$, (g) TNF$^+$ and (h) Granzyme-B$^+$ splenic CD4 T cells from *Rag1*$^{-/-}$ recipient mice infected with *L. donovani*. Data represent mean ± SEM from one out of two or three independent infections with four mice per group per experiment. *p < 0.05, **p < 0.01, ***p<0.001. *Cre*$^-$ refers to *Bcl6*$^{fl/fl}$*xCd4-Cre*$^-$ mice and *Cre*$^+$ refers to *Bcl6*$^{fl/fl}$*xCd4-Cre*$^+$mice.

3.6 Discussion

In chronic infections, CD4 T cells either undergo apoptosis, or their functions are inhibited by several regulatory mechanisms. A sustained CD4 T cell response is crucial for continued host protection as these cells are central players in mediating pathogen clearance. We discovered a subset of CD4 T cells with progenitor-like self-renewal and differentiation capacities. These cells

can give rise to both regulatory and effector T cell subsets. We propose that these cells could be involved in the maintenance of CD4 T cell responses during chronic VL.

The subset of CD4 T cells expressing LAG-3, CXCR5, and PD-1 that we have characterized in this study and named $T_{LCPlo/int}$ cells strikingly resembles a population of CD8 T cells known as the T_{PEX} (T precursor exhausted). These T_{PEX} cells have progenitor-like potential and are characterized by the expression of TCF-1 and CXCR5.[41–45] $T_{LCPlo/int}$ cells also have detectable protein expression of TCF-1, a transcription factor that is associated with self-renewal in memory CD4 T cells[46] and regulates the switch between differentiation and self-renewal in HSCs.[47] In addition, $T_{LCPlo/int}$ cells resemble the progenitor-like CD8 T cells that respond to anti-PD-L1 therapy, in terms of PD-1 and T-bet expression as well, as it has been reported that PD-1^{int} T-bet^{hi} CD8 T cells behave as progenitors, while the PD-1^{hi}T-bet^{lo} cells are terminally exhausted cells.[48–50] Interestingly, both T_{PEX} cells and $T_{LCPlo/int}$ cells express LAG-3 and other co-inhibitory receptors, several cytokines, and various self-renewal genes, including the transcription factor Myb, which controls the long-term self-renewal potential of $CXCR5^{+}$ precursor CD8 T cells.[51] T_{PEX} can give rise to all CD8 T cell effector populations, and similarly, the $T_{LCPlo/int}$ cells differentiate into multiple CD4 T cell effector and regulatory lineages during chronic infections. Hence, our results suggest that $T_{LCPlo/int}$ cells could be the analog of the T_{PEX} CD8 T cells. An important characteristic of T_{PEX} cells is that they proliferate and replenish CD8 T cell responses following anti-PD-1 therapy.[44] It still remains unknown whether the $T_{LCPlo/int}$ cells are also capable of proliferating after immunotherapy, and this warrants further investigations.

Interestingly, the $T_{LCPlo/int}$ cells also expressed memory-like markers and displayed enrichment of gene sets associated with Tfh memory cells. These properties resemble a Th1 memory cell population expressing *Id3*, recently described by Shaw et al.[52] The $T_{LCPlo/int}$ cells, however, have lower expression levels of *Id3* and similar expression levels of *Zbtb7b* (THPOK, log2 fold change = 0.155457 and adjusted p value = 0.223661) compared to Tfh cells. These are two major CD4 T cell memory-associated transcription factors. ID3 is essential for defining both Th1 and Tfh memory CD4 T cells,[52] and the $T_{LCPlo/int}$ cells highly express *Id2*, the antagonist of *Id3*, which was previously described to strengthen Th1 lineage commitment.[39] Additionally, the $T_{LCPlo/int}$ cells do not express high levels of THPOK, which is required for memory T cell development.[53] Although $T_{LCPlo/int}$ cells are $CXCR5^{lo}SLAM^{hi}$, indicating some resemblance to memory Th1 cells, we speculate that they are not memory cells due to the fact that they also exist in naive mice and start expanding at day 14 p.i. in *L. donovani*-infected mice, a time point when CD4 T cell responses are just beginning to develop.[11] Moreover, recent studies demonstrated that multiple

CD4 T cell lineages are programmed to upregulate a memory-associated genes during chronic LCMV infection, possibly for controlling immunopathology.[54,55] Thus, it is possible that the expression of a memory-associated transcriptional program is induced by persistent infection and that the $T_{LCPlo/int}$ cells are not bona fide memory cells. Further investigations are warranted to determine whether these cells are also present during acute infection or result from an altered gene expression program promoted by chronic VL.

LAG-3^{+}CXCR5^{+} CD4 T cells expanded in different models of infections (viral and parasitic); however, their PD-1 expression varied depending on the pathogen. This difference in PD-1 expression is context dependent, i.e., it is plausible that these $T_{LCPlo/int}$ cells give rise to different types of effector lineages, depending on the predominant type of T cell response to a particular infection. The effector T cell response in LCMV Cl13 is skewed toward a robust Tfh response. The bona fide GC-Tfh cells are PD-1^{hi},[56] and PD-1hi cells constituted the majority of the LAG-3^{+} CXCR5^{+} CD4 T cells in this infection model. In *H. polygyrus bakeri* infection, the effector response is directed more toward the Th2 lineage,[57] and PD-1^{lo} cells were more abundant. In VL, the Tfh response crashes after day 21 p.i.,[35] and Th1, Th2, and Tr1 responses dominate during chronic infection. Most of the LAG-3^{+}CXCR5^{+} CD4 T cells in experimental VL were PD-1^{int}, and only a small percentage were PD-1^{lo} or PD-1^{hi}. Whether the PD-1^{lo} population retains stemness properties and can differentiate into various CD4 T cell subsets remains to be determined. The T_{LCPhi} population showed similarities in the expression of several characteristic markers, particularly BCL-6 and TCF-1, to a recently described CD4 progenitor population characterized as CXCR5^{+}BCL-6^{lo} TCF-1^{+}.[34] The $T_{LCPlo/int}$ cells express TCF-1 at lower levels compared to the T_{LCPhi} population and have a different expression profile for a set of markers that both T_{LCPhi} and CD4 T cell progenitors described by Xia et al. had in common. TCF-1 is crucial for the progenitor-like properties of CXCR5^{+} precursor CD8 T cells.[58] Therefore, owing to the high TCF-1 expression of the T_{LCPhi} population, these might potentially be the precursors for the $T_{LCPlo/int}$ cells, which will then be an intermediate precursor population. On the other hand, it is also possible that the T_{LCPhi} cells are the same cells described by Xia et al.[34] and maintain the Tfh response during chronic infection, as it is well established that TCF-1 is required for Tfh differentiation.[59,60] It is also possible that the T_{LCPhi} cells could serve as precursors for Tfh cells as well as $T_{LCPlo/int}$ cells. Further investigations are warranted to validate these possibilities.

$T_{LCPlo/int}$ cells also share some similarities with the recently described pre-Tfh cells.[54] Although we do not know whether $T_{LCPlo/int}$ cells can give rise to Tfh, pre-Tfh cells were not reported to express *Prdm1* or *Lag3*, suggesting that they are most likely two different CD4 T cell subsets.

Furthermore, BCL-6 is only partially required for the maintenance of the $T_{LCPlo/int}$ cells, as the frequency of these cells decreased by almost half in its absence. However, this transcription factor is definitely required by the T_{LCPhi} cells, as these cells are non-existent in the absence of BCL-6, highlighting another similarity to the cells described by Xia et al. In the absence of BCL-6, we also observed the increased frequency of $LAG3^+$ CD4 T cells and Tr1 cells. Indeed, this corroborates previous reports that BLIMP-1 and BCL-6 are mutual antagonists[61] and that BLIMP-1 is a transcription factor indispensable for Tr1 cell differentiation.[62] Hence, we could speculate that BCL-6 limits the differentiation into regulatory CD4 T cell lineages and that, in the absence of BCL-6, progenitor cells differentiate into Treg cells by default. It remains to be clarified why $T_{LCPlo/int}$ cells, but not T_{LCPhi} cells, are partially maintained in the absence of BCL-6 and whether T_{LCPhi} cells develop at all without BCL-6. This could help in understanding the ontogeny of these various subsets of precursor-like cells.

An interesting observation in this study is the discovery of a precursor population that gives rise to Tr1 cells, partially requiring BCL-6 for its maintenance and highly expressing *Prdm1*. The IL-10-producing Tr1 cells play a complicated dual role in chronic infections. Several studies show that IL-10 derived from Tr1 suppresses Th1 responses in VL,[17–19] leading to disease progression in humans.[63,64] However, there is also evidence showing Tr1-mediated suppression of TNF-producing pro-inflammatory cells, which is essential for controlling pathological tissue damage in VL.[20] Similarly, in other intracellular parasitic infections, such as those caused by Toxoplasma or Plasmodium infections, the Tr1 cells are shown to protect the host against severe immunopathology.[21,22] In VL, Tr1 cells produce IL-10 and IFNγ and are induced by IL-27.[6][5] In malaria, expression of BLIMP-1 was also suggested to be essential for the development of these cells[20] and, more recently, for the expression of inhibitory receptors.[62] However, little is known about their origin. Therefore, understanding the peripheral generation of Tr1 cells during chronic infections and the cues necessary to drive or block their differentiation is quintessential for designing therapeutic tools targeting IL-10, and this study has taken us further ahead in this aspect.

A major highlight of this study is the discovery of a progenitor-like population of CD4 T cells that only expands in the presence of antigen. There is functional and numerical decline of the protective CD4 T cells during chronic infections, which demands the existence of compensatory mechanisms to replenish the CD4 T cell compartment.[11–13] Therefore, it is plausible that this progenitor-like population of CD4 T cells expressing LAG-3 and CXCR5 constitutes an alternative coping mechanism, other than differentiation from naive CD4 T cells, to compensate for the loss

of CD4 T cells during chronic infections. We propose that $T_{LCPlo/int}$ cells could be bearing the weight of differentiation into CD4 T cell effectors during the chronic stages of VL.

In conclusion, we have provided a phenotypic and molecular description as well as demonstrated the differentiation potential and progenitor-like properties of a progenitor CD4 T cell population. We have confirmed that similar populations exist in viral and parasitic infections, although they differ in PD-1 expression. We propose that these progenitor-like cells could be involved in the maintenance of CD4 T cell responses during *L. donovani* infection and could be exploited for therapeutic purposes.

Limitations of the study

Although we showed that adoptive transfer of $T_{LCPlo/int}$ cells in *Rag1*$^{-/-}$ mice confers a higher degree of protection than naive WT CD4 T cells upon *L. donovani* infection, we could not firmly demonstrate a protective role for these cells during chronic VL, and we only provide insights on potential mechanisms supporting CD4 T cell maintenance.

Additionally, performing single-cell RNA sequencing with trajectory analysis and time series experiments of LAG-3-expressing CD4 T cell populations might provide more information on how this population changes over time and transitions or differentiates into different entities over time and whether it is linked to other T cell populations such as pre-Tfh.

3.7 Acknowledgements

We thank the Canadian Institutes of Health Research (PJT-190001 to S. Stäger and PJT-362757 to I.L.K.) for financial support. We thank Dr. Jude Uzonna, Univeristy of Manitoba, Winnipeg, Manitoba, Canada and the NIH Tetramer Core Facility, Emory University, Atlanta, Georgia, USA, for generously donating the I-A(b)-$PEPCK_{335-351}$ and the corresponding negative control tetramers. We thank our animal care technician Annie Salesse, who cared for the mice used in this study. S.Sw. was supported by a scholarship from the Fondation Armand-Frappier. I.L.K. holds a Canada Research Chair in Barrier Immunity. A.P.M. holds a Human Frontiers Postdoctoral Fellowship.

3.8 STAR Methods

Table 3.1 Key resource table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
BV421 anti-mouse CD3	Biolegend	Catalog: 100227; RRID: AB_10900227
Alexa Fluor 700 anti-mouse CD3	Biolegend	Catalog: 100216; RRID: AB_493696
FITC anti-mouse CD4	BD Biosciences	Catalog: 553729; RRID: AB_395013
PE anti-mouse CD4	BD Biosciences	Catalog: 557308; RRID: AB_396634
BV711 anti-mouse CD4	BD Biosciences	Catalog: 563050; RRID: AB_2737973
PE-Cyanine7 anti-mouse LAG-3	eBioscience	Catalog: 25-2231-82
APC anti-mouse CD185 (CXCR5)	Biolegend	Catalog: 145506; RRID: AB_2561969
Biotin anti-mouse CD279 (PD-1)	Biolegend	Catalog: 135212; RRID: AB_10640124
Brilliant Violet 605 anti-mouse CD366 (Tim-3)	Biolegend	Catalog: 119721; RRID: AB_2616907
PerCP/Cyanine5.5 anti-mouse CD278 (ICOS)	Biolegend	Catalog: 117424; RRID: AB_2832419
PE anti-mouse CD25	BD Biosciences	Catalog: 553866; RRID: AB_395101
FITC anti-mouse CD49b (Integrin alpha 2)	Biolegend	Catalog: 103503; RRID: AB_313026
Biotin anti-mouse CD44	BD Biosciences	Catalog: 553132; RRID: AB_394647
PerCP/Cyanine5.5 anti-mouse CD150	Biolegend	Catalog: 115922; RRID: AB_2303663

PE anti-mouse CD45.1	BD Biosciences	Catalog: 553776; RRID: AB_395044
FITC anti-mouse CD45.1	BD Biosciences	Catalog: 553775; RRID: AB_395043
BV711 anti-mouse CXCR6 (CD186)	Biolegend	Catalog: 151111; RRID: AB_2721558
PerCP-Cyanine5.5 anti-mouse CD69	eBioscience	Catalog: 45-0691-82
BV421 Streptavidin	BD Biosciences	Catalog: 563259; RRID: AB_2869475
FITC Streptavidin	BD Biosciences	Catalog: 554060;
PerCP-Cy5.5 Streptavidin	BD Biosciences	Catalog: 551419;
Alexa Fluor 647 anti-mouse Foxp3	Biolegend	Catalog: 126408; RRID: AB_1089115
PE anti-mouse TCF-1	Biolegend	Catalog: 655208; RRID: AB_2728491
BV421 anti-mouse BCL-6	BD Biosciences	Catalog: 563363; RRID: AB_2738159
PerCP-Cy5.5 anti-mouse Tbet	eBioscience	Catalog: 45-5825-82;
APC anti-mouse IFN-γ	BD Biosciences	Catalog: 554413; RRID: AB_398551
PE anti-mouse IL-10	BD Biosciences	Catalog: 554467; RRID: AB_395412
PE-Cy7 anti-mouse TNF	BD Biosciences	Catalog: 561041; RRID: AB_10562571
PE anti-mouse TNF	BD Biosciences	Catalog: 554419; RRID: AB_395380
Alexa Fluor 700 anti-human/mouse Granzyme-B	Biolegend	Catalog: 372222; RRID: AB_2728388
FITC anti-mouse Ki67	Thermo Fisher Scientific	Catalog: 11-5698-82
APC anti-mouse Ki67	Thermo Fisher Scientific	Catalog: 17-5698-82

Chemicals, peptides, and recombinant proteins		
Zombie Aqua Fixable Viability Kit	Biolegend	Catalog: 423102
I-A(b) L. major GPC 335-351 NDAFGVMPPVARLTPEQ	Dr. Jude Uzonna, Univeristy of Manitoba, Winnipeg, Manitoba, Canada; Mou et al. [36], NIH Tetramer Core Facility	GenBank: XP_003721987.1; Uniprot : E9ADF8
I-A(b) human CLIP 87-101 PVSKMRMATPLLMQA	Dr. Jude Uzonna, Univeristy of Manitoba, Winnipeg, Manitoba, Canada; Mou et al. [36], NIH Tetramer Core Facility	GenBank: NP_001020329.1; Uniprot: P04233
GolgiPlugTM Protein Transport Inhibitor	BD Biosciences	Catalog: 555029
Phorbol 12-myristate 13-acetate	Sigma Aldrich	Catalog: P8139
Ionomycin calcium salt from Streptomyces conglobatus	Sigma Aldrich	Catalog: I0634
Trizol	Invitrogen	Catalog: 15596026
Critical commercial assays		
CD4+ T Cell Isolation Kit, mouse	Miltenyi Biotec	Catalog: 130-104-454
RNeasy mini kit	Qiagen	Catalog: 74104
KAPA RNA HyperPrep	Roche	Catalog: 08098107702
Poly-A selection	Thermo Scientific	Catalog: 61005
Deposited data		
RNAseq $T_{LCPlo/int}$ versus Tfh	This paper	GEO: GSE255333
TREG_VS_TEFF	Layland et al [38]	GSE17580
HSC-related genes	Ramirez et al [67]	GSE37301

Expression profile of T cell subsets after infection	Hale et al [37]	GSE43863
Experimental models: Organisms/strains		
B6.129S7-*Rag1*^{tm1Mom}	The Jackson Laboratory	002216
B6-Ly5.1	The Jackson Laboratory	002014
B6-Ly5.2	The Jackson Laboratory	000664
B6-*Cd4-Cre*	The Jackson Laboratory	022071
B6-*Bcl6*$^{fl/fl}$	Dr. Toshitada Takemori, Riken Institute, Yokohama, Japan. Kaji et al [68]	NA
Software and algorithms		
Flowjo v10.7	Flowjo	https://www.flowjo.com/solutions/flowjo/downloads
SPICE	NIH	https://niaid.github.io/spice/
Graphpad Prism v7.0	Graphpad Inc.	https://www.graphpad.com/how-to-buy/
Trimmomatic version 0.35	Bolger et al [69]	http://www.usadellab.org/cms/index.php?page=trimmomatic
STAR version 2.7.1a	Dobin et al [70]	http://code.google.com/p/rna-star/
DESeq2 version 1.30.1	Love et al [71]	http://www.bioconductor.org/packages/release/bioc/html/DESeq2.html
GSEA v4.2.2	Subramanian et al [72]	https://www.gsea-msigdb.org/gsea/index.jsp

BubbleGUM (GSEA Unlimited Map) v1.3.19	Spinelli et al [73]	http://www.ciml.univ-mrs.fr/applications/BubbleGUM/index.html
Other		
Leishmania donovani	Internal stock	NA
LCMV clone 13	Internal stock	NA
Heligmosomoides polygyrus bakeri	Internal stock	NA

The above table provides an easy access to important resources and materials used in this work.

Resource availability

Lead contact

Any further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact Simona Stäger (Simona.Stager@inrs.ca).

Materials availability

No new materials were generated in this study.

Data availability

- RNAseq data have been deposited at GEO and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. This paper also analyzes existing, publicly available data. These accession numbers for the datasets are listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

3.8.1 Experimental model and study participant details

3.8.1.1 Mice

B6.129S7-*Rag1*^{tm1Mom} mice (henceforth referred to as *Rag1*$^{-/-}$ mice), congenic B6-Ly5.1 mice, and B6-Ly5.2 mice were purchased from The Jackson Laboratory. All mice were housed and bred at the INRS animal facility under specific pathogen-free conditions and were used at 6 -10 weeks of age. *Cd4-Cre* and *Bcl6*$^{fl/fl}$ mice on a C57BL/6 background were bred and kept under specific pathogen-free conditions at McGill University. *Cd4-Cre* mice were obtained from The Jackson Laboratory (Strain #:022071) and *Bcl6*$^{fl/fl}$ mice were originally provided by Dr. T. Takemori (Riken Institute, Yokohama, Japan) [68]. CD4 T cell -specific *Bcl6*$^{-/-}$ mice were obtained by breeding *Bcl6*$^{fl/fl}$ with *Cd4-Cre* mice; *Bcl6*$^{fl/fl}$-*Cre*$^{-}$ mice were used as controls. No differences in the course of *L. donovani* infection were observed between male and female mice, so male and female mice were used indiscriminately. Nevertheless, mice were always sex-matched and compared accordingly for various experimental groups. Splenic parasite burdens were determined by examining methanol-fixed, Giemsa-stained tissue impression smears. Data are presented as Leishman–Donovan Units (LDUs)[11].

3.8.2 Method details

3.8.2.1 Pathogens

Leishmania donovani (strain LV9) was maintained by serial passage in B6.129S7-*Rag1*^{tm1Mom} mice. Amastigotes were isolated from the spleen of infected animals [74]. Mice were infected by injecting 2×10^7 amastigotes intravenously (i.v.) via the lateral tail vein and were euthanized at 7, 14, 21 or 28 days p.i. 2×10^6 pfu of LCMV Cl13 was administered i.v. and the mice were euthanized 8, 15 or 21 days p.i. Infections with *Heligmosomoides polygyrus bakeri* were performed by administering 200 L3 stage larvae by gavage and the mice were euthanized 14 days p.i.

3.8.2.2 Study approval

Protocols #1910-01 and #2003-02 approved by the Comité Institutionnel de Protection des Animaux of the INRS-Institut Armand-Frappier were strictly followed for infection experiments involving mice (*L. donovani* and LCMV Cl13 infections). For the *H. polygyrus bakeri* infections, experiments were done in accordance with the McGill University Health Centre Research Institute

Animal Resource Division with approved animal use protocol #7977. These protocols respect good animal practice provided by the Canadian Council on Animal Care.

3.8.2.3 Cell purification

Splenic LAG-3^{+} CXCR5^{+} PD-1$^{lo/int}$ cells and Tfh cells from day 21 infected mice were purified by flow cytometry for RNA sequencing and adoptive transfer experiments. Spleens were harvested from infected mice and CD4 T cells were isolated by magnetic activated cell sorting (MACS) (Miltenyi Biotech) [11]. Isolated CD4 T cells were then stained and sorted using BD FACSAria IIu cell sorter. Gating strategies are shown in Sup. Fig S1b. Naïve CD4 T cells from wild type B6 mice or *Bcl6$^{fl/fl}$xCd4-Cre^{-}* or *Bcl6$^{fl/fl}$xCd4-Cre^{+}* mice were purified by MACS for adoptive transfer experiments with purity > 95%.

3.8.2.4 Adoptive transfer

Naïve or *L. donovani*-infected C57BL/6-Ly5.1 mice at 21 days p.i. were used as donors of CD4 T cells or LAG-3^{+} CXCR5^{+} PD-1$^{lo/int}$ cells. 1x 10^{4} cells were adoptively transferred i.v. into *Rag1$^{-/-}$* mice a day prior to *L. donovani* infection (i.v.). Naïve *Bcl6$^{fl/fl}$* mice and naive *Bcl6 $^{fl/fl}$ xCd4-Cre* mice were also used as CD4 T cell donors for similar experiments. In this case, 0.5 x 10^{6} cells were transferred.

3.8.2.5 T cell stimulation

Antigen-specific re-stimulation was performed as follows – bone-marrow-derived dendritic cells (BMDCs) were differentiated *in vitro* in the presence of GM-CSF (X23 supernatant) and were plated with fixed *L. donovani* amastigotes overnight at 37°C; splenocytes from infected mice were co-cultured with fixed-parasite-treated BMDCs for 6 hours at 37°C. Brefeldin A (BD Biosciences) was added after 2 hours of culture. To observe the IL-10^{+} IFNγ^{+} Tr1 cells, splenocytes were treated with 5 ng/mL (PMA) and 500 ng/mL Ionomycin (Sigma) in the presence of Brefeldin A for 4 hours at 37°C. Following culture, re-stimulated cells were used for flow cytometry.

3.8.2.6 Flow cytometry

Cells were stained for cell-surface and intracellular markers [9]. The following anti-mouse antibodies were used for extracellular staining - anti-CD3 BV421 (17A2, Biolegend), anti-CD3 Alexa fluor 700 (Biolegend) anti-CD4 FITC (GK1.5, BD Biosciences), anti-CD4 PE (GK1.5, BD Biosciences),

anti-CD4 BV711 (GK1.5, BD Biosciences), anti-LAG-3 PE-Cy7 (C9B7W, eBioscience), anti-CXCR5 APC (L138D7, Biolegend), anti-PD-1 biotin (29F.1A12, Biolegend), anti-TIM-3 BV605 (RMT3-23, Biolegend), anti-ICOS PerCP-Cy5.5 (7E.17G9, Biolegend), anti-CD25 PE (PC61. BD Biosciences), anti-CD49b FITC (MI/59, eBioscience), anti-CD44biotin (IM7, BD Biosciences), anti-CD150 PerCPCy5.5 (TC15-12F12.2, Biolegend), anti-CD45.1 FITC, anti-CD45.1 PE (A20, BD Biosciences), anti-CXCR6 BV711 (BD Biosciences), anti-CD69 PerCP-Cy5.5 (H1.2F3, eBioscience), Streptavidin BV421 (BD Biosciences), Streptavidin FITC (BD-Biosciences), Streptavidin PerCP-Cy5.5 (BD Biosciences). Zombie Aqua Fixable Viability Kit (Biolegend) was used to according to manufacturer's recommendations to assess live versus dead cells. After extracellular staining, cells were fixed using 4% paraformaldehyde and permeabilized using saponin. The following antibodies were used for intracellular staining - anti-Foxp3 APC (MF-14, Biolegend), anti-TCF-1 PE (7F11A10, Biolegend), anti-BCL-6 BV421 (K112-91, Biolegend), anti-Tbet PerCP-Cy5.5 (4B10, eBioscience), anti-IFNγ APC (XMG1.2, BD Biosciences), anti-IL-10 PE (JES5-16E3, BD Biosciences), anti-TNF PE-Cy7 (MP6-XT22, BD Biosciences), anti-TNF PE (MP6-XT22, BD Biosciences), anti-Granzyme-B alexa-fluor700 (AQ16A02, Biolegend), anti-Ki67 APC, anti-Ki67 FITC (SolA15, eBioscience). Samples were acquired on BD LSRFortessa II cell analyzer (Becton Dickinson). Data was analyzed with Flowjo v10.6 or above. SPICE (simplified presentation of incredibly complex evaluations) v6.1001 was used to present data analyzed on Flowjo as pie charts.

3.8.2.7 PEPCK-tetramer staining

The following reagents were obtained through the NIH Tetramer Core Facility: I-A(b) *L. major* $GPC_{335-351}$ NDAFGVMPPVARLTPEQ and the corresponding negative control tetramer I-Ab–$CLIP_{87–101}$ PVSKMRMATPLLMQA. I-A(b) *L. major* $GPC_{335-351}$ NDAFGVMPPVARLTPEQ (PE-Labeled Tetramer), also known as I-A(b) $PEPCK_{335-351}$ [36], and the corresponding negative control tetramer I-Ab–$CLIP_{87–101}$ PVSKMRMATPLLMQA (PE-Labeled Tetramer) were used in experiments aimed at identifying antigen specific CD4 T cells. Splenocytes were stained with either the tetramer I-Ab–$PEPCK_{335–351}$ (1µg) or control I-Ab–CLIP87–101 (1µg) for 30 min at 37°C, washed and then stained with extracellular markers, fixed and stained for intracellular markers as already described.

3.8.2.8 RNA extraction and sequencing

Total RNA was isolated using TRIzol (Sigma Aldrich), followed by RNeasy mini kit (Qiagen) according to the manufacturer's instructions. RNA was quantified using Qubit (Thermo Scientific) and quality was assessed with the 2100 Bioanalyzer (Agilent Technologies). Transcriptome libraries were generated using the KAPA RNA HyperPrep (Roche) using a poly-A selection (Thermo Scientific). Sequencing was performed on the Illumina NextSeq500, obtaining around 20M single-end 75bp reads per sample. Six biological replicates were used for both LAG-3^{+} CXCR5^{+} PD-1$^{lo/int}$ cells and Tfh cells from infected mice. RNA extraction, library preparation and sequencing were all performed at the Institute for Research in Immunology and Cancer's Genomics Platform (University of Montreal).

3.8.2.9 RNA sequencing analysis

Adapter sequences and low-quality bases in the resulting FASTQ files were trimmed using Trimmomatic version 0.35 [69] and genome alignments were conducted using STAR version 2.7.1a [70]. The sequences were aligned to the mouse genome version GRCm38, with gene annotations from Gencode version M25 based on Ensembl 100. As part of quality control, the sequences were aligned to several different genomes to verify that there was no sample contamination. Expression levels were obtained as gene readcounts from STAR as well as estimated using RSEM [40], in order to obtain gene and transcript level expression in reads per transcripts per million (TPM) for these stranded RNA libraries. DESeq2 version 1.30.1 [71] was then used to normalize gene readcounts and compute differential expression between the various experimental conditions. Significant differentially regulated genes (DEGs) were chosen according to a false discovery rate (FDR) cut-off of 5% (padj < 0.05). Computing both a hierarchical sample clustering based on pearson correlation of normalized log readcounts and a principal component analysis showed that samples behave as expected, with no obvious outlier, as well as good consistency among replicates. All the above analyses were done by the genomics center at the Institute for Research in Immunology and Cancer's Genomics Platform (University of Montreal). Heat maps and volcano plots were generated using the public server at usegalaxy.org.

3.8.2.10 Gene set enrichment analysis (GSEA)

Readcounts normalized by DESeq2 were also used to perform functional analysis using GSEA v4.2.2 [72]. The following gene sets were chosen for the analysis based on previously published

articles – GSE17580 [38], GSE37301 [67], GSE43863 [37]. BubbleGUM (GSEA Unlimited Map) [73] v1.3.19 was used to visualize the analysis with FDR < 0.025.

3.8.3 Quantification and statistical analysis

Statistical analyses were performed using unpaired Student's t test and were conducted using Graphpad Prism (Graph-Pad). Differences were considered to be statistically significant when $p < 0.05$. All experiments were conducted independently at least two or more times with 3 or more mice per group per repeat.

3.8.4 Declaration of interest

The authors declare no competing financial interests.

3.9 Supplemental information

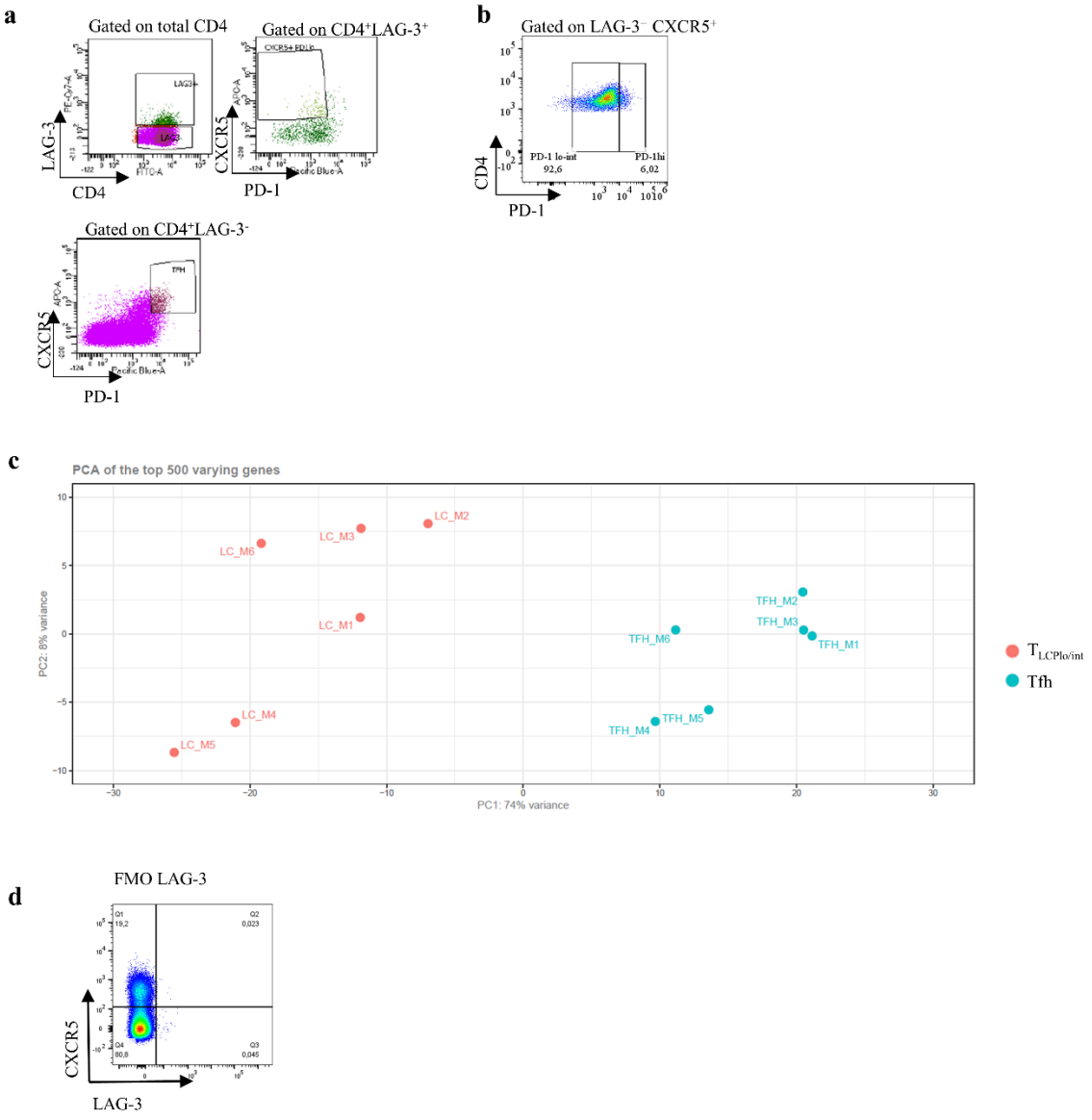


Figure 3.8 Additional information on gating strategies and RNA-seq analysis

(a) Gating strategy for purification of $T_{LCPlo-int}$ cells and Tfh cells for bulk-RNA-seq from the spleen of *L. donovani* infected mice at 21 days p.i. (b) Representative plot showing that at day 21 p.i. in experimental VL, the majority of the LAG-3$^+$CXCR5$^+$ non-Tfh CD4 T cells are PD-1$^{lo/int}$. (c) Principal component analysis (PCA) plots of the top 500 varying genes between the two cell populations GC-Tfh and $T_{LCPlo/int}$ (d) FMO control for LAG-3 antibody, used to establish the gates shown in Figure 3.1d and sup. Figure 3.8.1b.

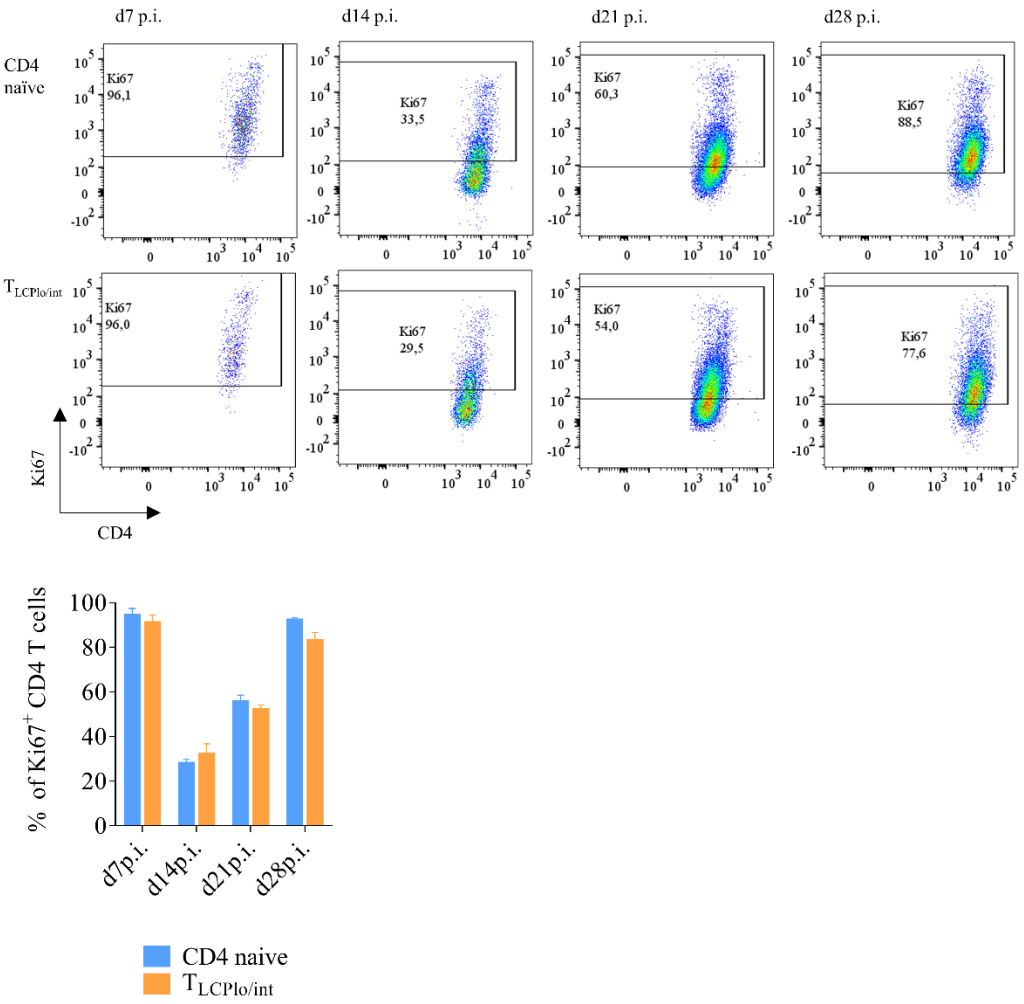


Figure 3.9 Ki67-expression by $T_{LCPlo/int}$ cells after adoptive transfer

Mice were infected with *L. donovani* and euthanized at various time points after infection. Graph shows mean ± SEM of the percentage of Ki67$^+$ $T_{LCPlo/int}$ cells. Expreiments Experiments were conducted independently at least two or more times with 3 or more mice per group per repeat.

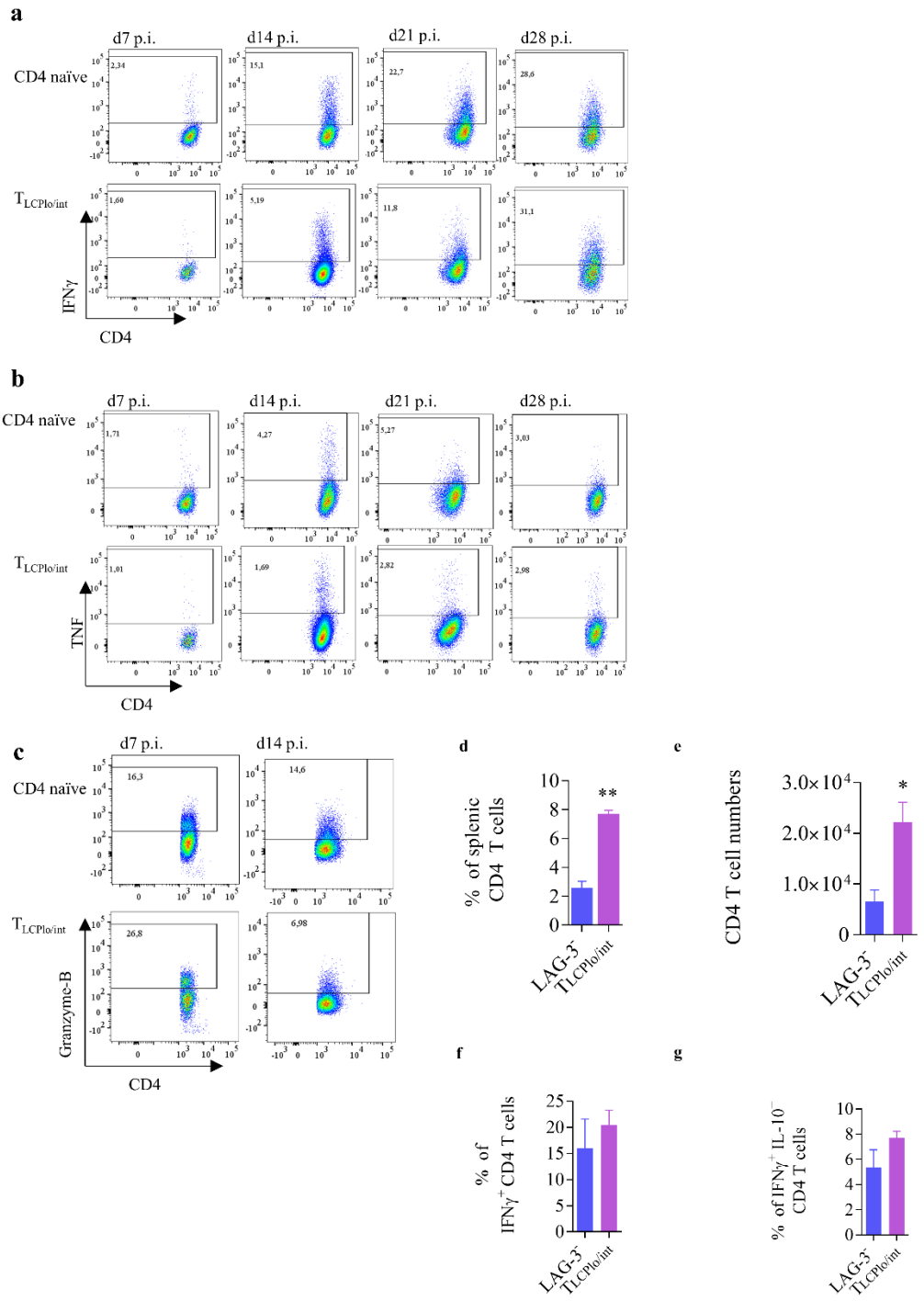


Figure 3.10 Additional information on adoptive transfer experiments in section 3.3.5

Representative plots showing, over the course of infection, frequencies of splenic (a) IFNγ$^{+,}$ (b) TNF^{+} and (c) Granzyme-B^{+} CD4 T cells in *Rag1*$^{-/-}$ recipient mice infected with *L. donovani* that received either $T_{LCPlo/int}$ cells from day 21-infected mice or CD4 T cells from naïve mice. (d-g) *Rag1*$^{-/-}$ mice were adoptively transferred with LAG3^{-} or $T_{LCPlo/int}$ cells purified at d21 p.i. from mice infected with *L. donovani*. Graphs show mean ± SEM of the percentage (d) and the number (c) of CD45.1^{+}CD4^{+} T cells present in the spleen at d14 p.i., and the

percentage of IFN-ɣ (f) and IFNγ^{+} IL-10^{+} (g) CD45.1^{+}CD4^{+} T cells at d14 p.i. Experiments were conducted independently at least two or more times with 3 or more mice per group per repeat.

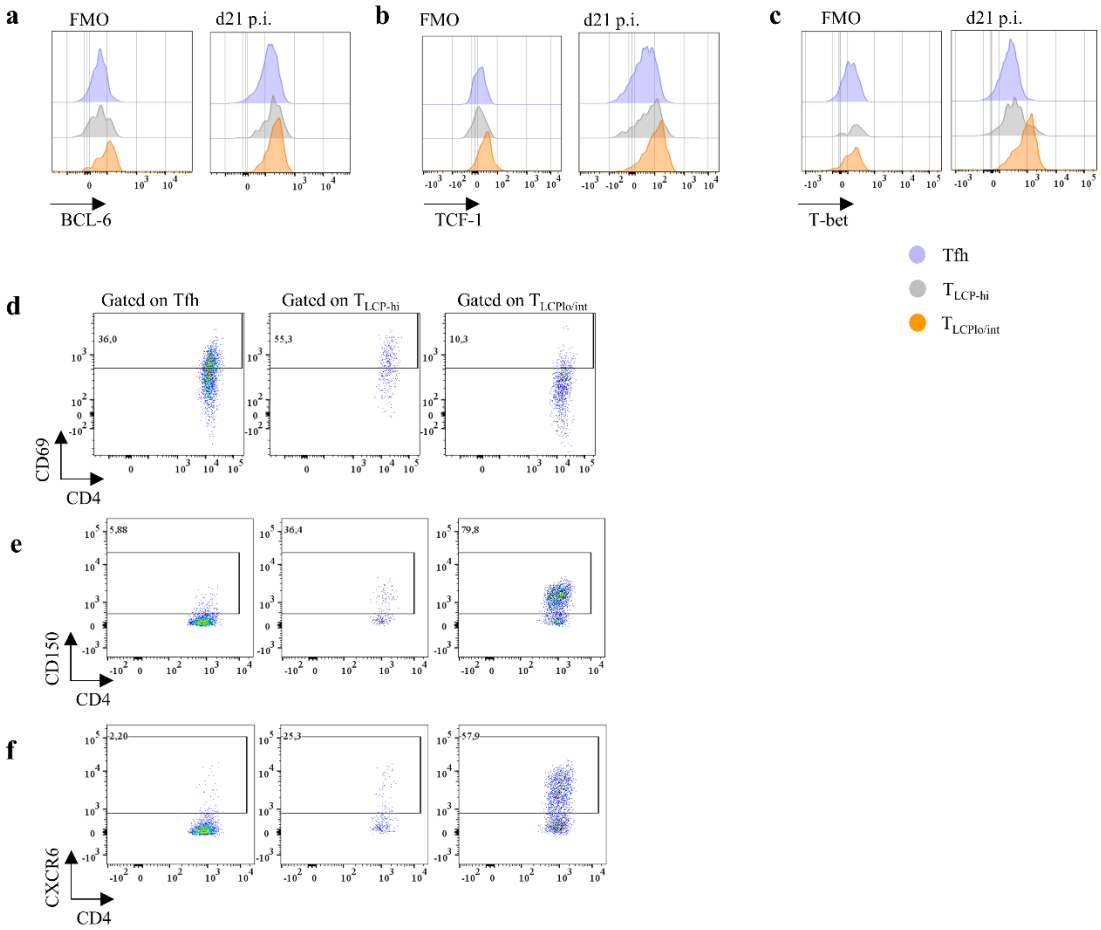


Figure 3.11 FACS plots related to section 3.3.7

Representative plots showing (a) BCL-6 (b) TCF-1 and (c) T-bet (d) CD69 (e) CD150 and (f) CXCR6 expression on Tfh vs T_{LCPhi} vs $T_{LCPlo/int}$ cells from mice infected with *L. donovani* at 21 days p.i.

3.10 References

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4 GENERAL DISCUSSION

The immune responses to infectious agents are diverse and multifaceted. Effective immune responses to infections are those that favour the host and not the pathogen. One of the vital cellular components that contributes towards pathogen elimination is the CD4 T cell response. However, in many chronic viral, parasitic and bacterial infections, there is a general decline in the CD4 T cell response either due to apoptosis or due to other suppressive mechanisms. Herein we describe a subset of CD4 T cells, characterized by the expression of the markers LAG-3, CXCR5 and PD-1. These cells have the potential to self-renew, can proliferate and differentiate to give rise to both effector and regulatory subsets of CD4 T cells during chronic visceral leishmaniasis. Therefore, we propose that this population of CD4 T cells could contribute towards maintaining the CD4 T cell response during chronic infections.

The subset of CD4 T cells characterized in this work, are proposed as the functional analogs of Tpex CD8 T cells. These Tpex CD8 T cells expand rapidly following anti-PD-1 therapy, and their primary function is to give rise to effector CD8 T cells and thereby sustain the CD8 T cell response to chronic infections as demonstrated in both mice and humans (Im *et al.*, 2016b; Miller *et al.*, 2019; Sade-Feldman *et al.*, 2018). In the last year, attempts have been made to search for and to characterize a CD4 T cell population that serves a similar function (Shaw *et al.*, 2022b; Xia *et al.*, 2022; Zander *et al.*, 2022b). A short summary of the CD4 T cell progenitor-like populations described by other authors is provided in the Table 4.1 below.

Table 4.1 List of publications describing progenitor-like CD4 T cell populations

Reference	**Description of similar populations of CD4 T cells**	**Characteristic markers**
Wei et al., 2019	Non-canonical or atypical phenotypes of CD4 T cells observed after anti-CTLA-4 treatment	Co-expressing ICOS, T-bet GATA-3, and RORγt
Shaw et al., 2022b	Memory Th1 cells, with “multipotent recall potential; or the ability to generate Th1 and Tfh effectors”	Id3-GFP^{hi} SLAM^{hi}CXCR5^{lo}

Xia et al., 2022	Progenitor $CD4^+$ T cells that give rise to T effectors and Tfh cells; these are critical for sustained CD4+ T cell responses to chronic antigen	BCL-6^{lo}TCF-1^+ T-bet^{lo} $CD69^{hi}CD150^{lo}CXCR6^{lo}$
Zander et al., 2022b	Several subsets of antigen-specific CD4 T cells in acute versus chronic LCMV infection	$Slamf6^+$TCF-1^{hi} memory-like CD4 T cells

The profile of CD4 T cell exhaustion during viral infections is different from CD8 T cells in many aspects. Studies involving anti-tumor immune responses have already demonstrated that it is mostly the CD8 T cells that respond to anti-PD-1 therapy while the CD4 T cells respond more to anti-CTLA-4 therapy (Wei *et al.*, 2017). One of the differences between CD4 versus CD8 T cell responses is the extent of inhibitory receptor expression, which appears to be less pronounced in CD4 T cells (Crawford *et al.*, 2014). Snell et al. proved that in CD4 T cells, PD-1 acts mainly to inhibit proliferation of Th1 cells and the blockade of PD-1-signaling amplified Treg responses simultaneously, thereby not contributing towards improved Th1 response. Additionally, the TCF-1+ CD4 T cell population could not be targeted by blockade of PD-1-PD-L1 signaling (Snell *et al.*, 2021). Therefore, the CD4 T cell response to immune checkpoint inhibition therapy appears to be complicated as more than one CD4 T cell subset can express these immune checkpoint molecules which altogether reflect on the disease outcome. Furthermore, inhibitory receptors can have other functions beyond serving as a marker for T cell exhaustion. In fact, the immune checkpoint molecules LAG-3 and PD-1 are known to be responsible for “fine-tuning” the immune response to retroviral infections (Donnarumma *et al.*, 2016). Therefore, these molecules serve as regulatory mechanisms that are part of a second layer of control, in addition to the classic expression of lineage determining transcription factors during infections. Understanding how these immune checkpoint molecules might participate in or influence CD4 T cell differentiation during infection is a pre-requisite for designing effective therapeutic tools. In these lines, the $T_{LCPlo/int}$ cells characterized in this study demonstrate that LAG-3, PD-1 and CXCR5 are markers indicative of cells with proliferation capacity and ability to give rise to effector and regulatory CD4 T cell types. Based on these results, perhaps inhibiting LAG-3 or PD-1 pathways or both during VL might not prove to be the most effective treatment strategy for host protection, as this might possibly interfere with the “progenitor-like” $T_{LCPlo/int}$ cells. However, these inferences are proposed

based on results obtained from a mouse model of VL, and require further demonstration in VL patients. Nevertheless, these findings obtained provide useful insights into the mechanisms underlying maintenance of CD4 T cells. It also underpins the idea that inhibitory receptor expression could also be related to cells with proliferative and differentiation capacity, and operate to regulate CD4 T cell differentiation during chronic infections rather than merely functioning as indicators of T cell exhaustion during chronic infections.

The concept that inhibitory receptors serve as important tools in differentiation has been the theme of very few studies in recent years involving anti-tumor immunity. Wei et al showed recently that one of the main functions of the negative costimulatory molecule CTLA-4 is to restrain the peripheral CD4 T cells to specific phenotypic boundaries. These authors observed the presence of atypical phenotypes of CD4 T cells such as a subset co-expressing ICOS, T-bet GATA-3, and RORγt, that existed and expanded only in mice lacking CTLA-4 (Wei *et al.*, 2019). Previously in 2017, the same group also showed that similar subsets of CD4 T cells expressing ICOS, T-bet and behaving like Th1 cells were expanding in cancer patients treated with anti-CTLA-4 therapy (Wei *et al.*, 2017). Taken together these scenarios illustrate that the blockade of inhibitory receptors leads to the transgression of conventional CD4 T cell differentiation pathways, resulting in the emergence of non-canonical phenotypes. However, significance of these subsets and their contribution towards anti-tumor immune responses were not discussed in these studies, and remain to be explored. The $T_{LCPlo/int}$ cells also expresses the co-stimulatory molecule ICOS and CTLA-4. Therefore, it will be interesting to investigate whether these cells respond to anti-CTLA-4 therapy and if they are similar to or different from the non-canonical subsets described by Wei et al. In the same lines, it would be interesting to evaluate whether or not these $T_{LCPlo/int}$ cells expand in response to anti-CTLA-4 therapy, either in the context of tumors or in the case of infectious diseases. Additionally, ICOS has been described to function as an important molecule dictating differentiation of T helper subsets during infection. It is required for the maintenance of Tfh responses during the chronic stage of *Plasmodium* infection. A study carried out using mouse model of malaria reported that although the *Icos*$^{-/-}$ mice had a normal Tfh response during the initial stages of the infection, these mice were unable to maintain Tfh cell numbers during the chronic phase of the infection and this in turn impacted the quality of humoral responses (Wikenheiser *et al.*, 2016). Briefly, all these studies point towards inhibitory receptors gaining more relevance in sustaining the CD4 T cell responses during chronic infections.

Understanding the transcription factors expression in CD4 T cells that expand following immune checkpoint therapy has gathered much interest as this is key for designing effective therapies. One such transcription factor that is upregulated in expanding CD4 T cells after immune checkpoint inhibition is the Bhlhe40 (Salmon *et al.*, 2022). This protein is also associated with many aspects of CD4 T cell differentiation. In many infection models such as *Plasmodium yoelii*, *Mycobacterium tuberculosis,* and *Toxoplasma gondii*, Bhlhe40 repress IL-10 and promotes IFNγ production by CD4 T cells, helping in pathogen clearance (Huynh *et al.*, 2018; O'Neal *et al.*, 2023; Yu *et al.*, 2018). Recently, it was also shown that Bhlhe40 is an important transcription factor controlling the germinal center reaction as it is expressed by and influences both the B cells and Tfh cells. It serves as a proliferation break in activated CD4 T cells, and restrains the production of Tfh cells, as CD4-T-cell-specific *Bhlhe40*$^{-/-}$ mice displayed increased percentage and number of Tfh cells (Rauschmeier *et al.*, 2021). The $T_{LCPlo/int}$ cells, which express some phenotypic markers of Tfh cells, but give rise to both Th1 cells and Tr1 cells, also express transcripts of *Bhlhe40* as revealed by the RNAseq analysis. Therefore, it is possible that Bhlhe40 is involved in the regulation of pathways that direct the differentiation of $T_{LCPlo/int}$ cells. Our group previously showed that the IFNγ-producing CD4 T cell in VL start to undergo apoptotic cell death starting from day 28 post-infection, which is mediated by the transcription factor IRF-5, in response to inflammation-induced damage (Fabié *et al.*, 2018). Interestingly, at this time point, the number of $T_{LCPlo/int}$ cells continues to increase. As we propose that $T_{LCPlo/int}$ cells exist to maintain the CD4 T cell responses, the fact that these cells continue to expand at day 28 aligns with the effector cells undergoing apoptotic cell death. However, we noticed that the relative mRNA expression of IRF-5 in $T_{LCPlo/int}$ cells (TPM 23.17±3.35SE) is higher compared to the Tfh cells (TPM 7.90±0.96SE). Therefore, it would be interesting to investigate the role of IRF-5 in the $T_{LCPlo/int}$ cells as it might possess a completely different role (not triggering cell death) in the $T_{LCPlo/int}$ cells.

Additionally, our team recently showed that IRF-5 is upregulated in memory CD4 T cells in people-living with HIV who are under antiretroviral therapy (ART). These IRF-5-expressing memory CD4 T cells were more prone to Fas-mediated apoptosis. This suggests a role for IRF-5 to serve as an “imprint” which renders memory CD4 T cells more susceptible to cell death (Carmona-Pérez *et al.*, 2023). Considering that memory CD4 T cells in HIV are more likely to undergo cell death due to residual inflammation, opens up the possibility of compensatory mechanisms to cope for the excessive CD4 T cell loss. Therefore, it would be interesting to know whether or not the $T_{LCPlo/int}$ cells exist in people-living with HIV who are under antiretroviral therapy (ART) and if they exist, it would be interesting to explore if these cells express IRF-5, and the potential role of this

transcription factor in these cells. However, in HIV infection, the expression of coinhibitory receptors such as LAG-3 and PD-1 are associated with viral persistence (Fromentin *et al.*, 2016). Particularly, the PD-1-expressing CD4 T cells, mostly constituting the Tfh cell population are one of the major cellular reservoirs of HIV (Banga *et al.*, 2016). The $T_{LCPlo/int}$ cells express low/intermediate levels of PD-1 and LAG-3 and express other markers such as TCF-1 and ICOS similar to Tfh cells and these cells have the capacity to self-renew. Based on these findings, it is possible that if the $T_{LCPlo/int}$ cells exist in people living with HIV, these cells might be potential viral reservoirs and might be acting alongside the virus, establishing persistence.

In vitro experiments show that IRF-5 is upregulated in T cells following TCR stimulation – in early times following TCR stimulation, it is involved in the efficient assembly of TCR-triggered signalling complexes and at later times, it promotes the production of Th1 and Th17 associated cytokines (Yan *et al.*, 2020). Owing to its potential role in T cell differentiation, studying IRF-5 expression in $T_{LCPlo/int}$ cells could be very relevant.

A few more transcription factors expressed by the $T_{LCPlo/int}$ cells are linked to regulation of CD4 T cell differentiation during infection. Firstly, the transcriptional repressor BCL-6 and its antagonist Blimp-1 are well known for their opposing roles in CD4 T cells (Johnston *et al.*, 2009). We observed that the $T_{LCPlo/int}$ cells express BCL-6 at low levels, and the frequency of $T_{LCPlo/int}$ cells diminished by almost half in the absence of BCL-6, indicating that this protein is required for the maintenance of these cells. Additionally, the $T_{LCPlo/int}$ cells highly expressed *Prdm1* which encodes for Blimp-1, and this protein dictates IL-10 production in Th subsets (Neumann *et al.*, 2014). Studying the role of BCL-6 and Blimp-1 in detail in the $T_{LCPlo/int}$ cells will provide us with important information on the directional cues governing the differentiation of these progenitor cells into various CD4 T cell subsets.

The “pre-Tfh” cells are a population of CD4 T cells that serve as progenitors for Tfh cells. Some authors describe the pre-Tfh cells as expressing both Th1 and Tfh-like markers (Song & Craft, 2019; Zander *et al.*, 2022b). Although the $T_{LCPlo/int}$ cells appear to resemble the pre-Tfh cells in this aspect, the latter do not express *Prdm1* and *Lag3*, which are clearly upregulated by the $T_{LCPlo/int}$ cells. Therefore, these are two distinct cell populations. However, it would be interesting to find out if the $T_{LCPlo/int}$ cells give rise to or derive from the pre-Tfh. Another interesting study has recently shown that Tfh-cells are “plastic”, and can give rise to Tr1 cells *in vivo* during chronic antigen-stimulation and this reprogramming process is tightly regulated by the transcription factor Blimp-1 (Solé *et al.*, 2023). Based on this study, it is intriguing to speculate that the $T_{LCPlo/int}$ cells are

indeed plastic Tfh cells, in intermediary stages of transforming into Tr1 cells, as a consequence of chronic antigen stimulation.

The strength of TCR stimulation is an important parameter in the differentiation of T-helper subtypes during chronic infection. A strong pMHC-TCR interaction leads to Th1 differentiation while Tfh and Th2 subsets require only a relatively lower pMHC-TCR interaction (Bouchery *et al.*, 2014). There is also a link between thymic positive selection of T cells and their high TCR-avidity in response to foreign antigens – T cells which have TCRs with high avidity during positive selection (yet not surpassing the limit for negative selection), appear to be dominating the response to infections (Mandl *et al.*, 2013). Additionally, there is increasing evidence that supports the idea that naïve CD4 T cell population is not homogenous even prior to antigen-exposure and there are pre-existing biases, based on TCR-self antigen recognition that affect the functionality of T cells and their interaction with foreign antigens (Mandl *et al.*, 2013; Persaud *et al.*, 2014; Sood *et al.*, 2019). It is therefore possible that a "progenitor-like" cell population such as the $T_{LCPlo/int}$ cells with the potential to give rise to many different effector as well as regulatory Th subsets might comprise a heterogeneous pool of cells differing in their TCR avidity. Performing simultaneous single-cell transcriptome and TCR-repertoire sequencing of $T_{LCPlo/int}$ cells could provide insights on how TCR avidity affects the differentiation of $T_{LCPlo/int}$ cells. Furthermore, the $T_{LCPlo/int}$ cells exist in naïve mice, although in very small numbers, and it is known that thymic selection mechanisms predispose and influence CD4 T cell differentiation. Altogether this raises the interesting possibility of origin of the $T_{LCPlo/int}$ cells – whether these constitute a naïve T cell population arising from the thymus with the predisposition (in terms of TCR strength) to act as a "progenitor-like" cell population during chronic VL. Studies conducted in naturally infected dogs and in malnourished mice infected with *L. infantum* show the presence of parasites in the thymus, causing changes in the tissue microarchitecture (da Silva *et al.*, 2020). These studies also raise the possibility of $T_{LCPlo/int}$ cells arising from heterogeneous naïve T cells that have already encountered parasite-antigens in the thymus.

We demonstrated that these $T_{LCPlo/int}$ cells are present in three different models of infection, although we only characterized the functional properties of these cells during *L. donovani* infection. Interestingly, these cells also conferred enhanced protection from infection in *Rag1*$^{-/-}$ mice that received these cells by adoptive transfer. We speculate that this resistance is due to the increased levels of IL-10 in these *Rag1*$^{-/-}$ recipients, which had higher frequencies of Tr1 cells producing higher levels (MFI) of IL-10, which resulted in significantly lowered IFNγ. Therefore,

these $T_{LCPlo/int}$ cells appear to be essential for limiting the IFNγ-mediated inflammation, and thereby preventing excessive tissue damage as well as enhancing host protection. This in turn results in a smaller spleen, which impacts the parasite burden. However, there are other important aspects which might reflect on the parasite burden that are to be considered. In parasitic infections, the $Ly6C^{hi}$ monocytes from the bone marrow are recruited to the secondary lymphoid organs and other inflamed sites, to differentiate into either dendritic cells or macrophages. During VL, in the bone marrow, the $Ly6C^{hi}$ monocytes are primed by IFNγ-producing CD4 T cells in the presence of the parasite (Romano *et al.*, 2021). However, these $Ly6C^{hi}$ monocytes further contribute to parasite persistence when they migrate to the spleen, as they possess a regulatory phenotype expressing Sca-1 (Abidin *et al.*, 2017). Although the Th1 cells activate monocytes in the bone marrow, they might also induce inflammation mediated pathology (Pinto *et al.*, 2017b; Preham *et al.*, 2018). Taking all of this into account, it will be interesting to explore the impact of $T_{LCPlo/int}$ cells, which give rise to IL-10-producing Tr1 cells as well as Th1 cells, in regulating the bone marrow niche. This will also play a crucial role in designing effective therapeutic tools for VL.

In conclusion, we propose that the LAG-3- and CXCR5-expressing progenitor-like CD4 T cells described in this work could be the central players responsible for the continuation of CD4 T cell responses during chronic infection. These cells might respond to immune checkpoint inhibition therapy. Therefore, these cells might potentially constitute a part of effective treatment strategies in the future, for many other disease such as cancer and autoimmune conditions, in addition to chronic infections.

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6 APPENDIX I

List of publications from collaborations

1. Mai LT, **Swaminathan S**, Nguyen TH, Charpentier T, Loucif H, Carmona-Pérez L, Lamarre A, Heinonen KM, Fritz JH, Stäger S (2024) The transcription factor IRF-5 is essential for the metabolic rewiring of CD8 T cells during chronic infection. bioRxiv 10.1101/2024.01.29.577789:2024.2001.2029.577789.
2. Meli AP*, Russell GA*, **Swaminathan S***, Weichselbaum L, MacMahon CA, Pernet E, Karo-Atar D, Rogers D, Rochette A, Fontes G, Mandl JN, Divangahi M, Klein OD, Gregorieff A, Stäger S, King IL (2023) Bcl-6 expression by CD4(+) T cells determines concomitant immunity and host resistance across distinct parasitic infections. *Mucosal Immunol* 10.1016/j.mucimm.2023.08.004. *equal contribution
3. Carmona-Pérez L, Dagenais-Lussier X, Mai LT, Stögerer T, **Swaminathan S**, Isnard S, Rice MR, Barnes BJ, Routy J-P, van Grevenynghe J, Stäger S (2023) The TLR7/IRF-5 axis sensitizes memory CD4+ T cells to Fas-mediated apoptosis during HIV-1 infection. JCI Insight 8(13).
4. Stögerer T, Silva-Barrios S, Carmona-Pérez L, **Swaminathan S**, Mai LT, Leroux LP, Jaramillo M, Descoteaux A, Stäger S (2023) Leishmania donovani Exploits Tunneling Nanotubes for Dissemination and Propagation of B Cell Activation. *Microbiol Spectr* 11(4):e0509622.