

Cellular dynamics of immune evasion during
***Leishmania major* infection**

by

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Abstract

Despite the generation of a strong T cell response, clearance of *Leishmania major* is incomplete and leaves a pool of chronically infected cells. Understanding of the persistence mechanisms is lacking, but *Leishmania major* driven induction of the immunosuppressive microenvironment through recruitment of regulatory T cells at the site of infection has been proposed to prevent parasite clearance *in vivo*. In the presented thesis, I used a novel TCR transgenic mouse model, where CD4⁺ T cells recognize an immunodominant peptide derived from *Leishmania*- glycosomal phosphoenolpyruvate carboxykinase (PEPCK), as a sensitive tool to characterize the dynamics of anti-*L. major* CD4⁺ T cell responses and to characterize mechanisms which restrain their effector function. Intravital microscopy studies characterizing *L. major*-specific CD4⁺ T cell migration dynamics within skin lesions directly in live mice show a significant recruitment of adoptively transferred effector T cells to the lesion site *in vivo*, displaying cellular behaviors consistent with antigen recognition at early and late stages of infection. However, cellular dynamics are augmented at the healed stage, indicating a fundamentally altered environment. I show that *Leishmania*-specific Tregs display higher suppressive activity compared to polyclonal control Tregs, and that this suppression is mediated through IL-10 and not through disrupting cell-cell contacts or antigen presentation. Challenge of healed mice with *L. major* antigen results in expansion of endogenous *Leishmania*-specific Tregs that lead to loss of lesion control in an IL-10 dependent manner. Lack of PEPCK antigen during challenge does not suppress effector Th1 response and parasite control. My data proposes a stochastic model of parasite survival, where inflammatory factors that control parasite numbers are counterbalanced by *Leishmania*-specific immunosuppressive factors that facilitate parasite persistence.

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Dedication

This thesis is dedicated to my husband Alex Barnes, my parents, Roman and Galyna Zayats, my sister Maryana Gange, and a special little boy who was in and under my heart throughout the writing of this work.

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Abbreviations

Acronym	Definition
2P-IVM	Two-Photon Intravital microscopy
AdV	Adenovirus vector
Aire	Autoimmune regulator
APC	Antigen-presenting cell
BCG	Bacillus Calmette-Guerin
BMDC	Bone marrow-derived dendritic cell
BMM	Bone marrow-derived macrophage
BSA	Bovine Serum Albumin
CACS	Central Animal Care Services
CCR	Chemokine receptor seven
CD	Cluster of differentiation molecules
CL	Cutaneous Leishmaniasis
CpG	Cytidine-phosphateguanosine
CR	Complement Receptor
cTECs	Cortical Thymic epithelial cells
CTL	Cytotoxic T cell
CTLA-4	Cytotoxic T-cell lymphocyte antigen-4
CXCR	Chemokine, CXC Motif, Receptor
DC	Dendritic cell
DCL	Diffuse Cutaneous Leishmaniasis

dLNs	Draining Lymph nodes
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic Acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
FACS	Flourescence Activated Cell Sorting
FBS	Fetal Bovine Serum
Fgl2	Fibrinogen-like protein 2
FoxP3	Forkhead box p3
GITR	Glucocorticoid-induced tumor necrosis factor-related receptor
GM-CSF	Granulocyte-macrophage colony stimulating factor
HIV	Human Immunodeficiency Virus
IFN γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
KO	Knock Out
LACK	Leishmania homologue of receptors for Activated C Kinase
LAG-3	Lymphocyte activation gene-3
LPG	Lipophospoglycan
LST	Leishmanin Skin Test

M-CSF	Macrophage-colony stimulating factor
M199	Medium 199
MA	Meglumine Antimoniate
MHC I	Major Histocompatibility Complex I
MHC II	Major Histocompatibility Complex II
mTECs	Medullary Thymic epithelial cells
NK	Natural Killer cell
NO	Nitric Oxide
OX40	Tumor necrosis factor receptor superfamily, member 4
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEPCK	Phosphoenolpyruvate carboxykinase
PIS	Press-Imprint-Smear
PKDL	Post-kala-azar Dermal Leishmaniasis
PPP	Pentose phosphate pathway
PRR	Pattern recognition receptor
pTregs	Peripherally derived Tregs
PVR	Poliovirus receptor
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute Medium
RPT	Rapid Diagnostic Test

SCFAs	Short-chain fatty acids
SLO	Secondary Lymphoid organs
SSG	Sodium Stibogluconate
STAT	Signal transducer of activator of transcription
T-bet	T box transcription factor
Tcm	Central memory T cells
TCR	T Cell Receptor
Tem	Effector memory T cells
Tfh	T follicular helper cells
TGF- β	Transforming Growth Factor Beta
Th	T helper
TIGIT	T-cell mediated immunoreceptor with Ig and ITIM domains
TLR	Toll-like receptor
TME	Tumor microenvironment
TNF	Tumor necrosis factor
Tregs	Regulatory T Cells
Trm	Tissue-resident memory T cells
tTregs	Thymic-derived Tregs
VAT	Visceral adipose tissue
VL	Visceral Leishmaniasis
WHO	World Health Organization
WT	Wild Type

Chapter 1. Introduction

1.1. Parasite-host coevolution

Parasite-host coevolution occurs when a parasite and a host continually adapt to each other. The host adapts to avoid or survive the infection through immune defences such as resistance or tolerance. In turn, parasites evolve a plethora of characteristics to evade host immunity, such as numerous life cycles, infective states, and transmission mechanisms. Given the ubiquity of parasites and the impactful role they play in the natural world, this biological phenomenon is likely only explained by selective pressure to survive by constantly adapting to their hosts. The evolution of innate and adaptive immune responses themselves are suggested to develop from coevolution, as the key determinants of optimal immune strategies are found to be the statistical features of pathogen occurrence¹. An environment with a broad spectrum of pathogens will support an immune system with a variety of responses, consisting of innate and adaptive immune responses. Many immune response features have evolved independently but share the same general functions between jawed and non-jawed vertebrates¹.

Hosts and parasites evolve through continual reciprocal selective pressure. The fluctuating selection hypothesis suggests that these interacting genotypes undergo cyclic changes through negative frequency-dependent selection². For millions of years, malaria parasites have co-existed with their hosts. As hosts activate numerous immune mechanisms to clear the parasites, the parasites diversify their genome and switch the expression of immune system targets to evade host immunity³. Similarly, first records of leishmaniasis date back 1500-2500 BCE and leishmanial mitochondrial DNA was found in mummies from Middle Kingdom tomb in West Thebes^{4,5}. The evolution of leishmaniasis is intricately tied with humans, from early migration and the

domestication of dogs to emergence of new forms of leishmaniasis in more recent history. The Ebers Papyrus, a collection of ancient Egyptian medical documents, reports a skin condition called “Nile pimple” in 1500 BCE⁵ and *Leishmania*-infected macrophages were detected in a Peruvian mummy of a 6-year-old girl from 800 BCE⁶. Molecular evidence of leishmaniasis was detected in four skulls dating back to the 11th century in high-altitude northern Chile, not endemic to the disease⁷, implicating human migration in spreading of the illness. In 1756 Alexander Russel published a clinical account on the “Oriental sore” while practicing in Aleppo⁵. He noted that no treatment was beneficial and that the lesions generally heal in 8 to 12 months. In 1903 William Boog Leishman, a Scottish pathologist serving with the British Army in India, published an observation of ovoid bodies in a post-mortem spleen sample of a soldier⁸. A few weeks later another publication reporting similar ovoid bodies taken during life and at autopsy from native Indian subjects was published by Charles Donovan, an Irish doctor working as a professor at Madras Medical College⁹. In Brazil, first New World leishmanial parasites were described in 1909⁵. Initially thought to be identical to already known *L. tropica*, morphological differences led to the conclusion that it was a new species. New species arise in modern times as well. *L. martiniquensis* was first isolated in 1995 and named in 2014¹⁰ and has been associated with horses and cattle in Europe and USA¹¹.

The evolution of leishmaniasis is fundamentally tied to human history and activity. Cultural, environmental, and socio-economic factors play an important role in fluctuation of case numbers. Evolution under constant selective pressure endowed *Leishmania* with remarkable immune evasion strategies, and considering the mathematical model of adaptation of parasite to host, the recent increase in incidence might be sufficient to indicate that another adaptation is possible¹². Acceleration of recent ecological changes, such as deforestation, global warming, and

armed conflicts, is changing our relationship with the parasite, highlighting the importance of understanding the parasite survival strategy to eradicate this disease.

1.2. *Leishmaniasis*

1.2.1. *Epidemiology*

Leishmaniasis is a complex parasitic disease caused by more than 20 protozoan species of the genus *Leishmania*. The infection is endemic on three territories, five continents and in more than 98 countries¹³. It is estimated that globally, the annual number of new cases is between 0.7 and 1.2 million a year. With conservative assumptions, between 70 to 75% of leishmaniasis cases are reported in Afghanistan, Algeria, Brazil, Colombia, Costa Rica, Ethiopia, Iran, North Sudan, Peru, and Syria¹⁴. Twenty-seven members of the European Union are also classified as endemic states for leishmaniasis¹⁵. In the predominantly urban areas, humans are the major reservoir for the parasite, leading to severe outbreaks in the densely populated cities^{13,16}. Major outbreaks occur in refugee camps, war zones, and large-migration areas. Leishmaniasis is the ninth largest disease burden amongst the individual infectious illnesses, yet, despite this, it remains a neglected tropical disease¹⁷. With regard to the mortality rate due to a parasitic infection, leishmaniasis is second only to malaria¹⁸. Approximately 20,000 to 30,000 deaths occur each year¹³.

Leishmaniasis is extremely widespread, and, along with other tropical infections, is generally regarded as a neglected tropical disease due to the lack of affordable, accessible, and effective treatments. Most of the affected patients live in developing countries, are in the poorest segments of the population, and have difficulty accessing diagnosis and treatment. There is little investment in developing treatments and because most of the patients are poor and rural, treatment

and control of the disease are not generally regarded as high priority¹⁹. Leishmaniasis is epidemiologically unstable and result in unpredictable fluctuations in the number of cases, often based on human activity, and thus major epidemics are frequent. Leishmaniasis can be asymptomatic, and if symptoms do present, they vary depending on the type of immune response mounted by the patient and the species of *Leishmania* parasites. Leishmaniasis presents in three different manifestations, known as cutaneous (CL), mucocutaneous, and visceral (VL), commonly known as kala-azar, depending on the parasite species, affected organs, and symptoms. In the Americas there are various *Leishmania* species occupying the same endemic areas, which leads to a complicated epidemiology, as vectors, transmission cycles, and great variance in clinical manifestations¹³. With the recent spike in the numbers of Human Immunodeficiency Virus (HIV) and leishmaniasis co-infected patients²⁰⁻²², the disease symptoms are becoming increasingly atypical, creating a greater need for understating the disease pathology^{21,23-25}.

1.2.2. Visceral Leishmaniasis

Visceral Leishmaniasis (VL) is fatal if left untreated, and presents with fever, anorexia, and anemia²⁶. VL is caused by *L. donovani*, *L. infantum*, and *L. chagasi*, which can disseminate into the spleen, liver, and bone marrow, causing substantial damage. Granuloma development, which is the distinguishing feature of the liver's response to leishmaniasis, consists of mononuclear cells that surround the infection site and prevent the spread of parasites. 2-8 months after infection and initial skin lesions, inflammatory reactions within the viscera begin developing²⁶ and if left untreated, the mortality rate is 75-95% within 2 years^{13,27}.

Post-kala-azar dermal leishmaniasis (PKDL) is a condition in a subset of patients, who have been successfully treated for VL caused by *L. donovani*²⁸, where dermal lesions become heavily parasitized. This condition remains asymptomatic for months to years, then parasites begin

to proliferate within the skin and form diffuse macular, nodular, and maculopapular lesions anywhere on the body, but most often on the face²⁷.

1.2.3. Mucocutaneous Leishmaniasis

Mucocutaneous form of Leishmaniasis is relatively rare and is caused by *L. panamensis*, *L. braziliensis*, and *L. guyanensis*²⁹. It can lead to partial or complete destruction of the oral, nasal, and tracheal mucosa. Mucosal diseases are observed in patients who have been affected by cutaneous leishmaniasis within the previous two years and rarely in patients without a skin condition³⁰. Disease severity is variable and generally depends on the duration of infection. While it was more common to observe patients with extremely destructive lesions in the past, nowadays, access to health services is improving and mucosal damage is diminishing³¹.

1.2.4. Cutaneous Leishmaniasis

CL is the most widespread form and the infection commonly manifests with multiple severely inflamed ulcers, which may coalesce, heal slowly, and leave disfiguring scars¹⁸. It occurs after an infection with different *Leishmania* species, but most commonly by *L. major*, *L. mexicana*, and *L. tropica*. Initial symptoms of CL include small red swelling, termed “erythema”, which develop at the site of fly bite, eventually transforming into nodule. This nodule becomes ulcerated at 2 weeks to 6 months and later transforms into hallmark *Leishmania* lesion. However, unusual clinical cases do present, wherein *L. major* can mimic other CL forms caused by different *Leishmania* species, or present in a form resembling other inflammatory skin disorders or infections³². Current CL treatments are expensive, toxic, and often fail³³. Healed patients do develop resistance towards future infections, yet it requires a small persistent parasite population, which remains in the lesion indefinitely^{18,33,34}. Aside from being a clear hurdle for vaccine

development, as live parasites are required to maintain immunity, a persistent *L. major* population presents high risks of reactivation, especially in immunocompromised patients.

1.3. Overview of *Leishmania major* life cycle

Leishmania major is a protozoan parasite that undergoes a digenetic life-cycle, as it alternates between inhabiting a phlebotomine sand fly vector and a mammalian host³⁵. During a blood meal acquisition, an infected sand fly breaks the skin (Figure 1), induces bleeding, and regurgitates the infective metacyclic promastigote form of the parasite into the skin of the mammalian hosts, such as rodents, dogs, and humans^{33,35}. The flagellated promastigotes are then consequently engulfed by the phagocytic cells of the immune system. Neutrophils are the initial cells to localize to the infection, yet macrophages become the main parasite hosts³³. Following phagocytosis, the parasites transform into the non-flagellated amastigote form³³ within the phagolysosome. The life cycle of the parasite completes when the sand flies ingest infected macrophages from another blood meal.

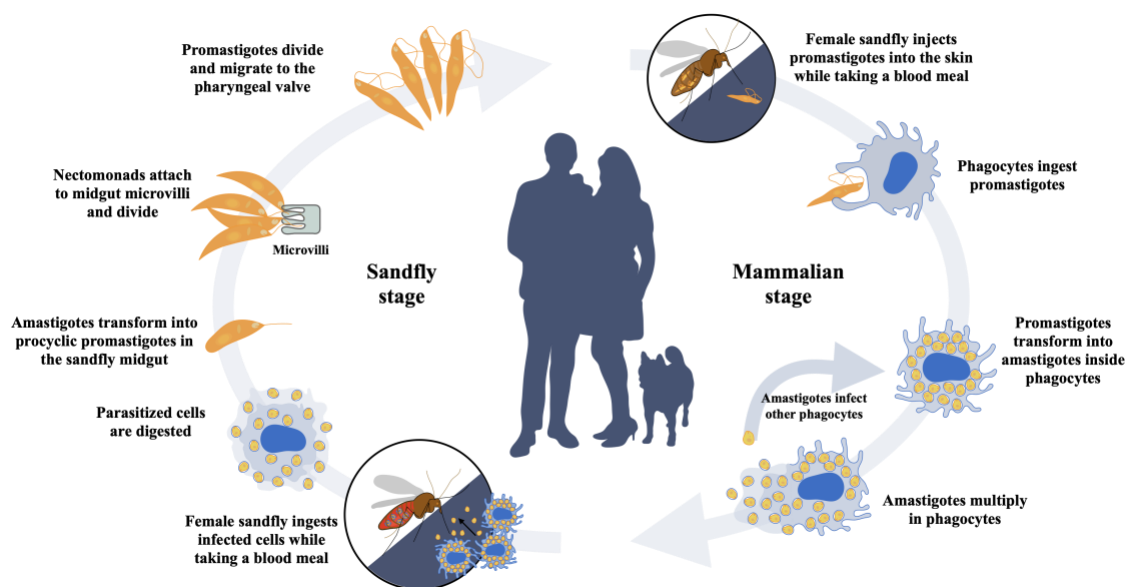


Figure 1. *Leishmania* life cycle

Leishmaniasis can be transmitted by the female vector from infected animals. Canids and rodents act as the main reservoirs, but leishmaniasis can also be transmitted from infected humans. As it is an example of anthroponosis, leishmaniasis has significant implications on both human and animal health. Vectors for a given *Leishmania spp.* vary between the sandfly species and prevalence of disease varies between regions^{36,37}. Phlebotomine sandflies belong to the family Psychodidae and have the average body length of 2 to 3 mm¹⁹. Both males and females feed on sugary secretions from plants, but females require at least one bloodmeal to complete the development of viable eggs. Unlike mosquitos, which are 6 to 12 mm in length, sandflies are also silent, and thus an attack is harder to detect. Among the 800 phlebotomine sandfly species, 98 species of *Phlebotomus* and *Lutzomyia* genera can spread leishmaniasis¹⁹.

1.3.1. Growth inside the sandfly

Inside the sandfly the continuation of the *Leishmania* lifecycle is mainly restricted to the midgut. Development is initiated when macrophages harboring amastigotes are ingested with the blood. The blood meal becomes incased in the peritrophic matrix and separated from the midgut epithelium³⁵. The decrease in temperature and an increase in pH of the environment triggers the transformation of amastigotes into the procyclic promastigote form. Procyclic promastigotes are the first replicative form of the parasite. They possess a short flagellum and are weakly motile. Around 48-72 hours replication slows down and parasite transition into the elongated and motile nectomonad form (Figure 1)³⁸. This form is able to escape the matrix-encased blood and transition into the lumen where they attach to the microvilli of the midgut through the binding of *Leishmania* lipophosphoglycan (LPG) and replicate³⁹. Nectomonads travel towards the anterior midgut and develop into the short leptomonad promastigote form, entering another proliferative state^{38,40-42}. As the parasites detach and then colonize the

stomodaeal valve of the sandfly, they ultimately transition into the infective metacyclic stage, waiting to be delivered to the skin of vertebrate host^{35,43}.

1.3.2. Growth inside the mammalian host

At the late stage of development inside the sandfly vector, numerous nectomonad forms of the parasite start secreting filamentous proteophosphoglycan, This begins to obstruct the thoracic midgut and facilitates reflux of parasites when the fly takes a subsequent bloodmeal³⁵. While the number of parasites injected into the host can vary from 100 to 100 000 per bite, it has been reported that a large proportion of bites transmit 600 parasites and the rest transmit much higher doses⁴⁴. Bimodality of infection can be reflected in the disease outcome, where high dose of infection causes stronger inflammatory responses, while low dose infections lead to a larger population of parasites persisting in the chronic stages of infection⁴⁵.

Host phagocytic cells, such as neutrophils, monocytes, macrophages, and dendritic cells engulf *Leishmania major* parasites. Leishmania parasites thrive and multiply in macrophages, which as a barrier between them and the host's immunological response. Promastigotes differentiate into non-motile amastigote forms inside the macrophage parasitophorous vacuole. Upon exiting macrophages, amastigotes can infect nearby cells.

The severity of human disease progression is dictated by a number of factors, from the composition of sand fly saliva⁴⁶, degree of tissue damage⁴⁷, to host skin microbiome and sand fly gut microbiota⁴⁸. One of the most important aspects, however, is the type of immune response developed by the patient. *Leishmania* parasites are not neutralized by antibodies, therefore, patients with a predominantly humoral response cannot control the parasite load and exhibit a severe form of the disease called diffuse cutaneous leishmaniasis^{33,49}. Individuals that

develop a strong T cell response, which is characterized by high levels of IFN- γ and strong delayed-type hypersensitivity, are able to control the disease, with the parasites killed by IFN- γ -activated macrophages⁴⁹⁻⁵¹. Nevertheless, an exaggerated T cell response can lead to immunopathology which can lead to mucocutaneous leishmaniasis in severe cases⁵⁰. Patients in between the high ends of the spectrum may develop chronic or self-healing lesions³³.

1.4. Laboratory diagnosis

Diagnosis of CL is based on clinical features and laboratory testing. There are numerous methods which vary in their accuracy, including direct parasitological examination through microscopy, histopathology, or parasite culture, and indirect testing through serology and molecular diagnostics⁵². Method of detection is generally determined by recourse and infrastructure availability.

1.4.1. Direct testing

Direct parasitological diagnosis is considered the optimal form of detection because it is highly specific, although it may not be sufficiently sensitive⁵². This method relies on the expertise of the health staff in order to collect the specimen and perform the tests. Direct diagnosis can be time consuming and requires an established laboratory structure⁵³. To achieve the highest yield of amastigotes, fine needle aspiration cytology can be done, in addition to scraping smears⁵⁴, both of which are invasive and can be painful. Another collection method is a Press-Imprint-Smear (PIS), which is also considered to be rapid and relatively sensitive for the diagnosis of CL⁵⁵. With light microscopy, amastigotes can be visually identified in lesional smears of biopsies by Geimsa's method. An easier detection can be done with immunohistochemistry⁵³. Parasite cultures in growth mediums from suspected lesions are more difficult, require advanced scientific technique,

are prone to contamination, and are time consuming⁵⁶. Sensitivity of this detection method can also be highly variable.

1.4.2. Indirect serological diagnostic methods

The pillar formats for serological CL testing are indirect fluorescent antibody (IFA), enzyme-linked immunosorbent assay (ELISA), western blot, and lateral flow assay. However, as humoral response to the parasite is poor, the use of serology for the diagnosis of CL is not recommended by the recent guidelines due to the low sensitivity of these tests⁵⁷. Still, there have been successful recent attempts to improve serological testing. The use of crude antigens derived from amastigotes of the local *Leishmania* strains has been relatively successful⁵⁸. More than 70 antigens have been evaluated as potential diagnostic markers for ELISA detection⁵⁹. Rapid diagnostic tests (RPTs) have also been gaining traction due to a good diagnostic performance. Several RDTs have been developed based on 39 amino acid sequence repeats of kinesin-related protein of *L. donovani*⁵⁷, although only a few have been developed for CL. A new promising RTD based on the membrane-based qualitative immunoassay using polyclonal antibodies against amastigote peroxidoxin has been evaluated in 5 countries⁵⁷. Termed CL Detect™ Rapid Test, it mainly detects *L. major*⁶⁰.

1.4.3. Leishmania Skin Test

Leishmania skin test detects cellular immune response against *Leishmania* and is positive in more than 90% CL cases⁶¹. The test is comparable to Mantoux tuberculosis test, where *Leishmania* extracts are injected intradermally to detect a cutaneous reaction, identifying a delay-type hypersensitivity response due to prior infection. If the skin reaction is bigger or equal to 5 mm, the test is considered positive. Patients already diagnosed with CL with a negative LST have

a higher chance of relapse or treatment failure, due to inadequate T-cell mediated response⁶¹. The use of LST has declined over the last decade, despite it being available for over a century, due to lack of standardised antigen.

1.4.4. Nucleic acid amplification testing

In comparison to previous methods, molecular approaches, in particular polymerase chain reaction (PCR), possess far superior sensitivity and specificity. The main targets for PCR have been ribosomal or kinetoplast DNA. Importantly, smear samples are sufficient for a reliable PCR result without requiring invasive sampling methods⁶². Moreover, quantified PCR can be used to assess CL patient treatment response and predict treatment failures⁶³. PCR diagnostics require for advanced laboratory infrastructure and personnel, making them less suitable for disease endemic countries.

1.5. Treatment

While CL is generally self-healing, irreversible disfiguring scarring occurs often, and complications can lead to lasting disability and permanent destruction of tissue. Secondary bacterial infections are common, hinder recovery, and can lead to functional impairment. CL has been largely neglected for drug development and therapy options generally rely on local expertise. Most current treatments are decades old and present with high toxicity and side effects⁶⁴. Moreover, drug resistance is an emerging problem in controlling CL⁶⁵. There are several treatment options for CL, with the most common treatment in the majority of endemic countries being pentavalent antimonials. Alternative treatments include antifungal agents, miltefosine, and heat or cryotherapy⁶⁵.

1.5.1. Physical treatments

Cryotherapy leads to destruction of infected tissue by exposing the lesions to extreme temperatures using liquid N₂ or CO₂. While painful in the process, it does not cause adverse side effects compared to other drugs. It is simple and inexpensive and presents with low rates of relapse⁶⁶. However, some patients report burning and secondary infections after treatment. Thermotherapy is a cost-effective treatment with minimal side effects and useful in areas with limited resources^{67,68}. Complications may include burns and the need for local anaesthesia.

1.5.2. Antimony

Chemical element antimony has the atomic number 51 and symbol Sb. Sodium stibogluconate (SSG) and meglumine antimoniate (MA) are the two major antimonial formulations used in CL treatment. In addition to other mechanisms, antimonials activate macrophages to kill the parasites and lead to targeting of trypanothione reductase which leads to parasite killing^{69,70}. Different *Leishmania* species show varied responses and antimony-resistant phenotypes are developing⁷¹, with Indian subcontinent reaching 60% unresponsiveness. Patients receiving antimonial experience pain during treatment and severe side effects, including cardiotoxicity, pancreatitis, hepatotoxicity, and nephrotoxicity⁷².

1.5.3. Miltefosine

Miltefosine, an alkyl phosphocholine derivative was first used as an anticancer agent before antileishmanial properties were detected⁷³. Miltefosine negatively affects sterol and phospholipid biosynthesis and cell signal transduction in parasites⁷⁴. While effective, is also results in high probability of relapse, in particular with a new infection⁷⁵. Miltefosine has a long half-life and is a teratogen⁷².

1.5.4. Amphotericin B

Amphotericin B is commonly used as an alternative drug with a broad spectrum antiparasitic and antifungal effects⁷⁶, used intralesionally or intravenously. It binds a major sterol of the protozoal cell membrane, ergosterol, and promotes ion leakage, pore formation, membrane permeability and cell death⁷⁷. However, similar to antimonials, it presents with high toxicity⁷².

1.5.5. Pentamidine

Pentamidine inhibits mitochondrial topoisomerase II, calcium transport, lysin-arginine transport, and impedes mitochondrial membrane potential, leading to parasite death⁷⁸. It also causes cardiotoxicity, reduction in blood pressure, and irreversible insulin-dependent diabetes⁷⁹.

1.5.6. Paromomycin

Paromomycin is an aminoglycoside antibiotic which interferes with 16S ribosomal RNA of the target organism⁸⁰. It does not prevent with severe toxicity, yet high resistance emerges when it is used in monotherapy⁷².

Current chemotherapy for leishmaniasis presents with severe limitations including cost, toxicity, drug resistance, routes of administration, and clinical failure. Drug resistance has increased alarmingly, especially in the endemic areas. It has also been observed that while the drugs are capable of resulting in a clinical cure, infected individuals are not cured parasitologically and remnants of disease remain in the population. Relapses have been reported in cutaneous and visceral Leishmaniasis, particularly in immunocompromised patients. The lack of complete clearance and inability to induce a long-term memory are the reasons for failure to eradicate the disease.

1.6. Vaccination effort

Conventional vaccination strategy involves the generation of long-lasting, antigen specific memory responses after challenge with a killed or attenuated pathogen. Generally speaking, vaccines that illicit strong memory B cell responses provide long-lasting protection against a number of viral infections, including polio, smallpox, and measles⁸¹. However, antibody responses are not sufficient to protect against intracellular parasitic infections, such as leishmania for which a viable vaccine is still not available⁸².

Recovery from natural or experimental *Leishmania* infection leads to the development of memory responses to secondary infection, and is the basic principle behind leishmanization, where individuals are deliberately injected with live parasites. This practice attempts to induce a protective response against potentially more serious ulcers acquired from natural infection later in life⁸³. Mice previously infected with *L. major* are protected against detrimental visceral leishmaniasis-inducing *Leishmania infantum*⁸⁴. This phenomenon is now defined as concomitant immunity, where the immune response cannot clear the initial infection but is able to provide powerful immunity to secondary infection. Existence of concomitant immunity does indicate that immunological mechanisms play a role in shaping the disease, and the scientific community hopes to use the mechanisms as a roadmap for the development of an effective vaccine strategy. However, there are numerous barriers for leishmaniasis vaccine development and the fact that this disease mainly affects low- and lower-middle-income populations does not invite commercial developers to invest in vaccine development⁸⁵. An ideal vaccine must be safe, stable, reproducible, easily stored and administered, and induce long-term immunologic memory⁸⁵. No leishmaniasis vaccine meets these standards yet⁸⁶.

First-generation vaccines have shown some therapeutic promise but have not been

developed further. They consist of whole killed *Leishmania* promastigotes, adjuvanted with BCG and/or Alum. While there is evidence of immunogenicity, it does not translate to protection⁸⁷. Second-generation subunit vaccines include peptides and proteins with differing adjuvant and delivery systems⁸⁸. Unfortunately, complete protection has rarely been demonstrated. Third-generation DNA-based vaccines have been shown to be effective in rodent and simian models⁸⁹, and there is an ongoing trial for patients with persistent PKLD⁹⁰. Lastly, mRNA vaccines have risen in popularity and are yet to be investigated in the context of leishmaniasis.

1.6.1. Leishmanization

Leishmanization is an ancient practice and is defined by administering a low dose of live virulent *L. major* parasites leading to a single lesion⁹¹. Exudate from an active cutaneous leishmaniasis lesion was collected with a sharp knife and inoculated into a naïve individual, usually a child, into a part of the body that is usually covered⁹¹. This vaccination practice provides greater than 90% protection against reinfection, yet comes along with numerous safety concerns, including uncontrollable persistent skin lesions, the spread of HIV, use of immunosuppressive drugs, and persistence of live parasites⁹². This vaccination practice is the least safe option, cannot be administered to immunosuppressed individuals, and will lead to medical issues if the patient does become immunocompromised. In 1989, at the second meeting of the expert committee on the control of leishmaniasis, it was recommended that leishmanization is only used as a last resort, when all other control measures have failed⁹¹. While there are several studies devoted to finding a good vaccine alternative, current knowledge is mainly based on animal models which cannot be easily extrapolated to humans⁹³. Cost, antigenic complexity, genetic variability and differing types of immune response severely limit progress⁹⁴. At the same time, because dead parasites,

recombinant DNA, or protein vaccines fail to elicit a memory response⁹⁵, for which parasite persistence is a necessity, researchers are beginning to revisit leishmanization. Leishmanization has been used in the Middle East and former Soviet Union. At the same time, modern proposal is to utilize live, genetically modified *Leishmania* parasites⁹⁶.

1.6.2. First generation vaccines

First-generation vaccines are composed of whole-killed parasites or fractionated *Leishmania* antigens. These vaccines are cheap and easy to develop, do not require advanced technology, and cause no risk of lesion development or virulence. However, results provide inconsistent effect and adjuvants such as BCG have been used in many studies. Still, first-generation vaccines are the only *Leishmania* vaccine candidates to reach Phase III clinical trials. Killed parasite vaccines provide a large assembly of parasite antigens, however they induce a weaker Th1 response than live parasites and fail to protect against natural exposure⁹⁷. There are four fractionated *Leishmania* antigen vaccines that have been licensed for prevention of canine leishmaniasis and commercialized⁸². In humans, failure to protect against reinfection renders killed *Leishmania* parasites an inadequate vaccine candidate.

1.6.2.1. Live attenuated vaccines

Live attenuated *Leishmania* vaccines include parasites with genes responsible for their survival and/or virulence being modified or deleted. Infection with live attenuated *Leishmania* is similar to infection with virulent parasites, but with an advantage of preventing disease. There have been several promising studies that demonstrated live-attenuated *Leishmania* as a good strategy for promoting long-lasting immunity⁹⁸⁻¹⁰⁴. Since they mimic natural infection more closely, they induce better protection¹⁰⁵. The emergence of CRISPR/Cas gene editing technology has allowed for a precise selection of safer attenuated parasites^{86,106}. Since 1995 there have been 54 selected

gene targets for live-attenuated *Leishmania* parasites. Still, 70% of these studies were carried out using *L. major* Friedlin and *L. donovani* BPK282A1 strains, and only 10% of live-attenuated *Leishmania* knockout mutants have proven to induce both a protective immune response and protection upon reinfection⁹⁶. Very few studies have moved past the use of non-rodent models, and most of these studies concentrate on the proof of concept without addressing induction of protective immune response.

1.6.3. Second generation vaccines

Second generation vaccines include recombinant proteins that are produced in genetically modified cells such as viruses or bacteria, purified native protein fractions of parasite antigens, synthetic peptides, and genetically altered parasites¹⁰⁷. These vaccines are accessible to large-scale and cost-effective production. Many subunit vaccine candidates pose no risk of infection and are safe for immunocompromised individuals. There have been numerous subunit vaccine candidates using notable *Leishmania* antigens, including gp63, p36/LACK and others¹⁰⁸. 3 recombinant antigen vaccines have reached phase II trials⁸². The caveat, however, is that pure proteins induce a weak cell-mediated response. Therefore, these vaccines often require adjuvants and multiple doses, which is a disadvantage.

1.6.3.4. Saliva vaccine

Sandfly saliva has been shown to enhance the infection caused by *Leishmania* spp through preventing hemostasis. Interestingly, injection of sand fly salivary gland extract into mice results in increase in IFN- γ and IL-12 production at the site of inflammation, and pre-exposure to saliva was shown to protect mice against infection¹⁰⁹. Other studies showed that immunization with a vector expression salivary protein PpSP15 showed positive protection results against CL and VL^{109,110} in rodents and non-human primates. However, sera and PBMCs of individuals exposed

to sandfly bites could detect PpSP15 through antibodies, there was no IFN- γ response detected in PBMCs after stimulation with the protein¹¹¹.

1.6.4. Third generation vaccines

Third generation vaccines employ direct injection of nucleic acids, as naked or encapsulated DNA, or mRNA. A large variety of *Leishmania* antigens have been investigated and the most prevalent are DNA vaccines encoded with previously tested recombinant vaccine candidates^{108,112}. Unfortunately, these vaccines remain in pre-clinical trials. The first DNA vaccine developed for leishmaniasis encoded gp63 and has been shown to induce a Th1 response in mice¹¹³. A DNA vaccine containing LACK has delivered various protection in cutaneous and visceral infections in mice and canines^{112,114}. Overall, while successful in animal models, DNA vaccines have not translated well to humans because they fail to generate significant clinical benefits.

1.6.4.1. Adenovirus-vectored vaccines

Viral vectors, especially adenovirus (AdV) vectors, are highly immunogenic and able to drive a CD8⁺ T cell response. Two AdV-vectored leishmaniasis vaccines have been developed in the past ten years^{90,115,116}. HAdV-5 vector expressing *L. donovani* amastigote specific antigen A2 was tested in non-human primates¹¹⁵ and recombinant A2 protein is the basis for one of currently approved vaccines for canines, Leish-Tec¹¹⁷. In primates, at 6 weeks 100% of vaccinated macaques were asymptomatic but had granulomas, yet at 24 weeks all developed some form of disease. While protective to a degree, this vaccine did not induce sterile immunity. The second AdV-vector vaccine consists of a ChAd63 vector and a fusion protein made of synthetic *L. donovani* antigens. This vaccine was designed as a therapeutic for patients with persistent PKDL⁹⁰. After vaccination follow up, 47.8% has up to 25% clinical improvement⁹⁰. A phase IIb randomized placebo-controlled trial began in 2019 and was concluded in February 2023, results pending.

1.7. Immune response to Leishmania major and immunomodulation by the parasite

The interplay between *Leishmania* and host immune response is complex and variable. Numerous experimental models and techniques have shed some light on the cellular dynamics between the parasite and host immunity, from initial infection to generation of the adaptive immune response. Given the long history of Leishmaniasis, parasites have evolved sophisticated immune-evasion strategies, utilizing the functions of immune system to its benefit. In this section, the interplay between the host immune response and the parasite will be presented.

1.7.1. Innate immune response

As the sandfly injects the proboscis into the tissue, it damages the microvasculature of the dermis and the epidermis, which leads to a local inflammatory response. Injection directly into the tissue circumvents the skin barrier and exposes the ejected parasite to the second immune line of defence, the complement system. The complement system is a complex of biochemical processes with the aim of efficient detection and elimination of pathogens. Activation of the complement cascade results in inflammation, opsonization and lysis of the invading pathogen. *Leishmania*, however, has evolved to resist the lysis branch of the cascade and utilize opsonization as a factor for receptor-mediated entry into its target cells. LPG of the metacyclic promastigotes acts as a direct barrier to prevent insertion of the C5b-C9 membrane attack complex in the parasites and saves them from lysis^{118,119}. At the same time as C3b is cleaved from C3, it binds the pathogens and the complement receptors CR1 and CR3 on phagocytes, such as neutrophils and macrophages. While the aim of opsonization is to promote phagocytosis and lysis of pathogens, in the case of *Leishmania* infection it aids the parasite in infecting its target cells. *Leishmania* surface metalloprotease GP63 enhances the cleavage of C3b to iC3b, which loses its proteolytic activity

and is only able to aid in opsonization but cannot activate complement-mediated cytotoxicity¹²⁰. Ligation of CR3 on macrophages also downregulates production of IL-12, which is a key cytokine in inducing Th1 response¹²¹. Additionally, *L. major* can become bound by cross-reactive anti-phospholipid IgG antibodies, which also enhance opsonization¹²². *L. major* also first encounters resident cells, such as dermal macrophages and Langerhans cells, which are subsequently infected^{123,124}.

1.7.1.1. Natural Killer cells

Together with other phagocytes, Natural Killer cells (NK) form the initial line of defence against infections. NK cells both induce cytolytic destruction of infected cells and secrete pro-inflammatory cytokines IFN- γ , TNF α . In the resistant C57BL/6 mouse model, NK cells were shown to be dispensable in the generation of Th1 response, yet their presence in the susceptible Balb/c model delays the onset of disease and diminished parasite burden¹²⁵. In vivo intravital microscopy showed that during *L. major* infection, NK cells are recruited to the paracortex of the draining lymph nodes, where they can interact with DC, promoting DC maturation, and regulate co-localized CD4 T cell responses^{126,127}. In human subjects, higher NK cell numbers and activity are associated with protection^{128,129}.

1.7.1.2. Neutrophils

Neutrophils are polymorphic granulocytes which are the earliest innate immune cells recruited to the site of infection. Neutrophil chemotaxis is induced by complement C3¹³⁰, chemokine secretion by tissue resident cells¹³¹, and sandfly- and *Leishmania*-derived factors^{47,132}. Rapid recruitment and swarming behaviors of neutrophils, which are aimed at eliminating the parasite, also contribute to further parasitic dissemination⁴⁷. Neutrophils may be responsive to factors produced by the parasite themselves or to the sand fly bite, as skin piercing

alone can cause neutrophil recruitment in mouse skin¹³³. It is known that phagocytosed parasites are able to survive within neutrophils and can modulate function, for example the ability of neutrophils to produce reactive oxygen species (ROS) necessary for parasite killing¹³⁴. Additionally, *L. major* prolongs the naturally short lifespan of neutrophils, inducing them to secrete chemoattractants such as MIP-1 β for a longer duration of time and attracting more macrophages to the site of infection¹³⁵. Thus, infected dying neutrophils act as “Trojan horses” for the incoming phagocytes to facilitate spread and perpetuate the infection.

1.7.1.3. The role of Monocytes

Bone-marrow derived monocytes constitute the majority of mononuclear phagocytes in the blood, and they emigrate and differentiate into peripheral cell subsets upon maturation¹³⁶⁻¹³⁹. Following recruitment into a site of infection, the fate of monocytes depends on the local environmental conditions. Recognition of pathogen-associated molecular patterns (PAMPs)¹⁴⁰ through pattern recognition receptors (PRRs) on their surface and the cytokines present in the environment can induce effector, suppressor, or reparative functions in monocytes¹⁴¹. At peripheral sites of infection, monocytes can acquire a range of properties and phenotypes, and they can displace pre-existing phagocyte populations and become the main phagocytic cells¹⁴². In the skin, IFN γ drives monocyte recruitment and activation, and induces the production of NO and ROS, which are important for *L. major* elimination¹³⁸. CCR2⁺Ly6C⁺CX3CR1⁺ inflammatory monocytes have recently been shown to represent a dual role in primary vs secondary *L. major* infection¹⁴³. When healed mice are challenged with a secondary infection at the distal site, inflammatory monocytes represent 80% of iNOS⁺ cells and, therefore, play a major role in parasite clearance. Exposure of monocytes to IFN γ prior to establishment of the pathogen niche endows them with ability to execute leishmanicidal activity¹⁴⁴. However, the

same cells display low MHC II expression and act as permissive hosts for parasite at the original, healed lesion^{143,145,146}, potentially due to decreased IFN γ and increase IL-10 exposure.

1.7.1.4. Leishmania-macrophage interactions

The most central aspect of *Leishmania* intracellular lifecycle is the capacity of parasites to access host phagocytic cells to establish a chronic infection. Upon infection with *L. major*, neutrophils secrete high levels of CCL3 and CCL4^{147,148}, which attract monocytes, macrophages, and even DCs to the site of infection. *Leishmania* induces the expression of apoptotic markers on the neutrophil cell surface, and upon apoptosis in the skin, parasites are engulfed.

At the site of infection, macrophages are the primary hosts for *Leishmania major*. Parasites survive within phagolysosomal vesicles and macrophages require strong Th1 responses to help clear the infection^{33,149,150}. In fact, alternative activation of macrophages by IL-4 and IL-13, produced by Th2 cells or eosinophils, can induce a more favorable environment for parasites to proliferate^{49,135,151}. As a survival mechanism, the transition of parasites from the promastigote to the amastigote stage, a process which remains incompletely understood, has been shown to significantly upregulate Th2-associated cytokine production within macrophages¹⁵². Additionally, parasites survive within macrophages with the aid of pathogenicity factors, such as lipophosphoglycan (LPG), which modify the phagosome into a parasitophorous vacuole¹⁵², and GP63, which interferes with phagosome proteolytic activity and *Leishmania* antigen presentation on MHCI¹⁵³. These modifications weaken vacuole acidification and induce actin accumulation, which creates a physical protective barrier around the vacuoles^{152,154}. Moreover, GP63 cleaves various signaling proteins within the phagocyte, causing a decrease in secretion of TNF, IL-12, and NO¹⁵², and rendering the macrophage

response inadequate to kill the parasites. Recently, GP63 has been shown to degrade CXCL9, 10, and 11 and diminish recruitment of Th1 cells¹⁵⁵.

Following recruitment to the site of infection, monocytes acquire various functions depending on the local environmental cues and develop into the pre-existing phagocyte populations¹⁴². In case of *Leishmania major* infection, monocytes can be recruited through the production of NO, ROS, or IFN- γ -facilitated production of CXCL9 and CXCL10¹³⁸. Interestingly, at the site of primary infection monocytes have a lower expression of MHC II, compared to the same cells at the site of secondary challenge, making them permissible hosts for the parasites^{143,145,146}.

1.7.1.5. Dendritic cells

Monocyte-derived DCs play a pivotal role during *Leishmania* infection as they help induce antigen-specific Th1 responses¹⁵⁶⁻¹⁵⁸. In order to mount an appropriate CD4⁺ T cell response against intracellular pathogens, DCs must engulf parasites at the site of infection, migrate to the draining lymph node and physically engage naïve CD4⁺ T cells in an antigen-specific manner¹⁵⁹. Activation of DCs through *L. major* interaction with TLR9 is associated with increased LN heterotrophy and a substantial naïve T cell recruitment¹⁶⁰. Optimal T cell activation requires two signals: Signal 1 is initiated downstream of the T cell receptor (TCR) after binding to peptide-bound major histocompatibility complex II (MHC-II) on DCs. Signal 2 is induced downstream of the CD28 molecule after ligation with co-stimulatory molecules CD80 and CD86 expressed on DCs that are upregulated upon pathogen recognition^{159,161}. Prolonged DC:T cell contacts induce rapid proliferation and differentiation into effector Th1 cells through the production of IL-12 by activated DCs^{161,162}. Activated Th1 cells then become endowed with chemokine receptors that allow them to migrate to the site of infection.

1.7.2. Adaptive Immune responses

1.7.2.1. CD8 T cells

CD8⁺ T cells provide protection against cytosolic intracellular infections, where proteins can be presented on MHC I and cannot kill pathogens which target the host phagosome and sequester most of the antigen¹⁶³⁻¹⁶⁵. While CD4⁺ Th1 cells are required for *L. major* control, CD8⁺ T cells play a multifaceted role in disease progression: CD8⁺ T cells can promote Th1 cell differentiation but also increase pathology at the site of infection. Intralesional CD8⁺ T cells fail to make IFN- γ due to an IL-12 deficit, but even with IL-12, they fail to control *Leishmania* infection in the absence of CD4⁺ T cells¹⁶⁶. While CD8⁺ T cells alone are unable to play a protective role¹⁶⁶⁻¹⁶⁸, IFN- γ production by the cytotoxic T cells contribute to the Th1 differentiation post-infection. Low dose parasite inoculation into the ear dermis of CD8⁺ T cell deficient C57BL/6 mice has been shown to induce a Th2 response and thus lead to an uncontrolled infection, which can be downregulated by IFN- γ -producing CD8⁺ T cells¹⁶⁹. However, frequency of CD8⁺ T cells is higher in ulcerated lesion and production of granzyme B is correlated with higher intensity of inflammation and immunopathology¹⁷⁰⁻¹⁷³.

1.7.2.2. CD4 T cells

1.7.2.2.1. Th cell activation

After selection in the thymus, naïve T cells enter the blood and travel to the secondary lymphoid organs (SLOs) in search of their cognate antigen presented on DCs. Expression of CCR7 and CD62L helps the cells localize to the SLOs¹⁷⁴ and naïve CD4⁺ T cells have 2-3 months to travel between the SLOs and become activated^{175,176}. CD44 is an important marker for T cell activation, which becomes upregulated after activation. It is a type I transmembrane glycoprotein which can mediate lymphocyte rolling under the physiologic flow to the site of

infection^{177,178}. There is a variety of Th cell subsets which naïve CD4⁺ T cells can differentiate into and the cell fate is determined by the infection-induced innate immune response and the cytokine milieu present in the environment. Activated T cells can differentiate into T follicular helper cells (Tfh), T helper 1 (Th1), T helper 2 (Th2), T helper 9 (Th9), T helper 17 (Th17), and T helper 22 (Th22) subsets. Cytokine stimulation leads to the activation of signaling transducer and activator of transcription (STAT) proteins, which drive Th cell differentiation. STAT4 is critical for Th1 cell differentiation and is activated downstream of IL-12^{179,180}. STAT6 drives the differentiation to Th2 phenotype and becomes induced downstream of IL-4^{181,182}. Naïve CD4 T cells undergo lineage polarization into effector subsets and this ability is mediated by master transcription factors, which induce expression of subset-specific genes while suppressing the expression of genes associated with alternate lineages. Importantly, T-bet is a master regulator for Th1 cell differentiation, and its expression can be used to identify Th1 cells¹⁸³. However, differentiated T helper cells retain the ability to adapt to the changing microenvironments and surrounding cues in order to provide proper immunity¹⁸⁴. In T helper cells, bivalent histone modifications are independent of the differentiation status and are maintained near master regulator genes¹⁸⁵. T helper cells retain plasticity to adopt alternative subsets upon receiving appropriate cues. Th1 cells can be induced to produce IL-4 under Th2 conditions¹⁸⁶ and Th2 cells can express IFN- γ when cultured with IL-12, type I IFNs and IFN- γ ¹⁸⁷.

1.7.2.2.2. Th1 cells

As the main sources of the cytokine IFN- γ aside from natural killer and CD8⁺ T cells¹³⁵, Th1 cells induce the classical activation of macrophages. Th1 cells predominantly drive protection against pathogens which reside in the phagosomes, such as *L. major*¹⁸⁸, *M. tuberculosis*¹⁸⁹, and *S. enterica*¹⁹⁰. Th1 cells aid parasitized macrophages in clearing *L. major* by releasing both IFN- γ and TNF α , which promote an increase in expression of ROS and nitric oxide (NO) that kill intracellular parasites¹⁹¹. Additionally, T cells upregulate costimulatory molecules CD40L, that bind CD40 on macrophages and act as a secondary activation signal¹⁹². To counter this, in the first 24 hours GP63 has been shown to cleave CD4 on T cells, as well as inhibiting MHC-I presentation, physically disabling activation of both CD4 and CD8 T cells¹⁵³. However, intravital microscopy studies have shown that effector Th1 responses do not necessarily require interactions with all infected cells, as paracrine expression of IFN- γ can have long-ranging effects, activating infected macrophages up to 80 microns away¹⁹³. Thus, effector Th1 responses are critical for the clearance of cutaneous *Leishmania* infections, evidenced by the rapid disease progression observed when Th2, rather than Th1 responses, are dominant⁴⁹.

Intravital microscopy studies have demonstrated the ability of effector T cells to enter the inflamed tissue irrespective of their antigen specificity. However, *Leishmania*-specific CD4⁺ T cells do exhibit a reduction in cell migration and accumulate at the site of infection¹⁹⁴, as described by their increased confinement. Previous microscopy studies using Balb/c mice, which exhibit a predominantly Th2 response and are highly susceptible to *Leishmania major*¹⁹⁵, showed large heterogeneity in T cell:infected phagocyte interactions, and only a small population of T cells form prolonged contacts with infected cells¹⁹⁴, indicating suboptimal immune synapse formation and responses. Interestingly, they observed a similarly

heterogeneous Th1 response using T cells derived from C57BL/6 mice, a result which suggests mechanisms in addition to the Th1/Th2 imbalance that influence anti-*Leishmania* T cell responses and possibly contribute to incomplete clearance of the parasites *in vivo*¹⁹⁶.

1.7.2.2.3. Memory CD4⁺ T cells

Patients exhibit a long-lasting, CD4⁺ T cell-dependent immunity to secondary challenge upon resolution of the primary *Leishmania major* infection, and a low number of parasites (10²-10⁴) has been shown to remain at the site of primary infection and in the draining lymph node following lesion resolution^{34,84,145,197}. These parasites seem vital in maintaining a population of effector CD4⁺ T cells which rapidly respond to secondary *Leishmania* challenge. Contrasting to viral immunity, which is mediated by the long-lived CD8⁺ T cells, the survival of CD4⁺ T cell-mediated memory against intracellular parasites remains poorly understood. Various subsets of these T cells have been identified, one of which is CD4⁺Ly6C⁺Tbet^{hi} T cells¹⁹⁷. These effector T cells are short lived in the absence of infection and migrate to the site of secondary challenge in order to aid parasite killing¹⁹⁷. Effector memory T cells (T_{EM}) have also been identified, but it not yet fully understood whether these cells also require a small pool of parasites for their long-term maintainance¹⁹⁸. In addition, central memory T cells (T_{CM}) have been shown to migrate to the draining lymph node through CD62L and CCR7, where they proliferate and differentiate into effector T cells and migrate back to the lesion site¹⁹⁹. T_{CM} serve as a source of *Leishmania*-specific T cells upon re-infection, though their maintenance seems to be parasite-independent¹⁹⁹. Adoptive transfer studies have shown that transferring T_{EM} and T_{CM} together or alone into a naïve mouse is insufficient to fight off infection, which suggests an additional role of tissue-resident memory T cells (T_{RM})^{197,199}.

1.7.3. The role of cytokines

Numerous cytokines are involved in various roles during immune response to *Leishmania major*. The interplay and balance between the opposing cytokines have an imperative impact on the outcome of the disease.

1.7.3.1. IFN- γ

Interferon gamma plays an imperative role in the elimination of *Leishmania major*, as it activates macrophages and induces the production of leishmanicidal molecules. The negative feedback in suppressing the detrimental Th2 response is another important role of IFN- γ . Administration of IFN- γ into mice at the time of *L. major* infection dramatically reduces lesion size and parasite burden²⁰⁰, while injecting anti-IFN- γ prior to infection leads to enhanced Th2 response and susceptibility in resistant mice²⁰⁰. Resistant C57BL/6 mice deficient in IFN- γ or IFN- γ receptor cannot control *L. major* proliferation²⁰¹. In human subjects, IFN- γ producing CD4 T cells can be detected in PBMCs of individuals with healed lesions²⁰². IFN- γ can be produced by various cell types, such as NK, NKT, and CD8⁺ T cells, but CD4⁺ T cells are the major source of IFN- γ in leishmaniasis.

1.7.3.2. TNF

Tumour necrosis factor (TNF) also plays a protective role in cutaneous leishmaniasis. TNF can be produced by T and B cells, and activated macrophages, DCs, and monocytes in response to various stimuli²⁰³. TNF synergises with IFN- γ in mediating leishmanicidal activity of macrophages by inducing the production of nitric oxide²⁰⁴. Administration of TNF into susceptible BALB/c mice challenged with the parasites lowers parasite burden, but at the same time neutralizing TNF plays no effect²⁰⁵. While TNF does play a protective role, it is not as essential as IFN- γ . TNF receptors are not necessary for the generation of Th1 response and subsequent NO production²⁰⁶.

1.7.3.3. IL-12

Interleukin 12 (IL-12) is heterodimeric cytokine, consisting of a bundle of four alpha helices in subunit IL12A and three beta sheet domains in IL12B. The physiological relevance of IL-12 came to light in 1995, when it was demonstrated that infected mice which were resistant to *L. major* produced high levels of IL-12, IFN- γ , and had activated natural killer cells²⁰⁷. Blockade or deletion of IL-12 in these mice resulted in susceptibility to *Leishmania*²⁰⁸, while injection of IL-12 into susceptible Balb/c mice led to a robust Th1 immune response and healing²⁰⁹. IL-12 is predominantly produced by DCs and macrophages, which are vital in activating CD4⁺ T cells. While the ability of macrophages to produce IL-12 has been shown to be decreased following cutaneous infection, DCs retain the capacity to secrete IL-12 that leads to differentiation and proliferation of Th1 cells and subsequent IFN- γ production that control parasite loads.

1.7.3.4. IL-4/IL-13

Interleukin-4 (IL-4) is predominantly produced by CD4⁺ Th2 cells and induces the differentiation of Th2 cells during activation, while suppressing Th1 induction²¹⁰. Susceptibility of BALB/c mice to cutaneous leishmaniasis is generally attributed to an increased number of IL-4 producing CD4⁺ T cells. Administration of anti-IL-4 neutralizing antibody renders susceptible BALB/c mice resistant to *L. major*^{211,212}. Interestingly, deletion of IL-4 gene in BALB/c mice does not lead to *L. major* resistance²¹³, while IL-4 receptor α deficient BALB/c mice are able to control the infection²¹⁴. These findings implicate signaling through the IL-4 receptor as pivotal in BALB/c susceptibility to *L. major*, rather than the cytokine itself. Interleukin-13 (IL-13) is another major Th2 cytokine which shares signaling through IL-4R α subunit²¹⁵. IL-13 promotes *L. major* pathogenesis by downregulating macrophage production of IL-12, iNOS, and TNF²¹⁶⁻²¹⁹. While IL-13 does exacerbate disease severity during the early stages of infection, it also enhanced

protection at the later stages²²⁰. Overall, the definitive roles of IL-4 and IL-13 in resistance and susceptibility to leishmaniasis is not fully understood, as robust Th2 responses can be induced in the absence of IL-4 and/or IL-13 and IL-4 and IL-13 mediate their action independent of other in both promoting susceptibility and resistance²²¹.

1.7.3.5. IL-10

Interleukin-10 (IL-10) mediates its effect through inhibition of Th1 cell development, suppression of Th1 production of IFN- γ , and blockade of macrophage activation by IFN- γ ²²²⁻²²⁴. IL-10 is a potent suppressor of Th1 and NK cells, both crucial cell types against intracellular pathogens²²⁵. However, the main role of IL-10 is to ameliorate the excessive activities of Th1 and CD8⁺ T cells by reducing immunopathology induced by proinflammatory responses. IL-10 can be produced by Tregs, monocytes, Th2 lymphocytes, mast cells, B cells, macrophages, NK cells, eosinophils, and under certain circumstances, activated T cells²²⁶. Th1 cells control themselves by producing IL-10 to diminish tissue damage, yet this self-control also limits the efficacy of immune responses and lead to a failure of parasite eradication. IL-10 deficient BALB/c mice are not susceptible to *L. major*, which implicates IL-10 in negatively regulating pathogenesis²²⁷. In C57BL/6 mice healing of the primary *L. major* infection is accompanied by parasite persistence²²⁸, which is controlled by regulatory T cells²²⁹. Increased numbers of Tregs and elevated levels of IL-10 lead to loss of immunity in healed resistant C57BL/6 mice²³⁰. T-cell specific IL-10 deficient C57BL/6 mice develop enhanced inflammation, while the same deficiency in BALB/c mice leads to a healing phenotype²³¹. Deletion of IL-10 production in macrophages and neutrophils had no effect. Taken together, IL-10 plays a central role in susceptibility, immunopathology, and *Leishmania major* parasite persistence. Additionally, IL-10 plays an important role in reducing

tissue fibrosis and accelerating wound healing²³². IL-10 inhibits excessive collagen deposition and prevents the formation of scars²³³.

1.7.3.6. TGF β

There are three isoforms of TGF β : TGF- β 1, - β 2, and - β 3, all encoded by different genes^{234,235}. TGF β is an immunosuppressive cytokine involved in the progression of *Leishmania* infection²³⁶. TGF β also inhibits IFN γ production and inactivates macrophages. High levels of IL-10 and TGF β can be detected in late human lesions of *L. major*²³⁷, though most studies have focused on the role of TGF β in *L. amazonensis* infections. Additionally, TGF β plays a role in wound healing by regulating a diverse set of profibrotic and anti-fibrotic cytokines. TGF β stimulates fibroblast synthesis and extracellular matrix contraction²³⁸.

Tissue healing and extracellular matrix remodeling

The skin is the largest organ in the body and forms a physical protective barrier between the organism and the outside world. It protects the body from microbial, chemical, and mechanical injury and consists of multiple layers: the epidermis, dermis, hypodermis and subcutis²³⁹. Epidermis is the top layer which creates the barrier from the outside world and consists of mainly keratinocytes, melanocytes, some Langerhans cells, and no blood vessels. The dermis is a fibrous layer and includes blood and lymphatic vessels, numerous glands, nerves, and hair follicles. The dermis is composed of collagen and elastic fibers, fibroblasts, and numerous immune cells, whereas the hypodermis and subcutis anchor the skin to the underlying muscles and contains nerves and blood vessels, fat cells, fibroblasts and macrophages.

Skin tissue regeneration and repair processes are complex sequences of cellular and molecular events which occur after the onset of tissue lesion in order to restore the damaged tissue.

Inflammatory, proliferative, and remodeling phases are sequential events in tissue repair. Extracellular matrix (ECM) proteins are essential in regulating tissue responses toward external and internal stimuli, and pathological conditions can become exacerbated if the ECM remodeling is out of equilibrium. The major regulators of ECM synthesis, deposition, degradation, and remodeling are immune cells and produce proteases, cytokines, growth factors, and ECM components during homeostasis and inflammation²⁴⁰. The major components of ECM in the skin is Collagen I, synthesised by fibroblasts²⁴¹. Other important component is the basal membrane, composed of collagen IV and laminin²⁴². When a sandfly injects its proboscis to acquire a blood meal, damage to the skin structure occurs, which leads to local rupture of dermis and the capillaries. This creates a blood pool containing ECM components from tissue and blood and surrounding cells^{243,244}. ECM components can induce inflammation and act as powerful chemoattractants: laminin fragments can act as danger associated molecular patterns (DAMPs), collagen fragments increase IL-1 β secretion in monocytes, elastin peptides chemoattract immune cells, and fibronectin can activate TLR signalling²⁴⁵. Thus, monocytes and neutrophils are the first cells to localise to the damage site and the rest of immune responses highlighted earlier follow. Acute phase may last between a few weeks to months, and no pathological change in epidermis is visible. The parasites then enter a proliferative stage where they multiply in the host cells²⁴⁶. Proliferative parasites evoke inflammatory reactions and recruitment of T cells, monocytes, neutrophils, DCs and Langerhans cells. This leads to inflammation-induced pathology and necrotic areas can be observed in the dermis¹⁴⁸. Most of our knowledge about immune response to leishmaniasis comes from the inflammatory state of infection, with very few studies focusing on the proliferative or tissue remodeling stages.

The proliferative stage seeks to reduce the area of tissue injury by recruiting fibroblasts and angiogenesis and reepithelialisation processes can be observed. Following the vigorous immune response to *Leishmania*, bystander damage leads to the development of dermal necrosis and an ulcerative lesion, which becomes covered by dead epidermal keratinocytes and other cells killed by apoptosis and necrosis, accompanied by dried exudate and both live and dead amastigotes²⁴⁷. T cells continue to kill off the parasites and lesion becomes defined as a dermal granuloma²⁴⁸. As lesion begins to heal, the tissue becomes remodeled with new epithelium and dermis becomes replaced by fibrotic tissue²⁴⁹.

The last stage involves tissue remodeling, but it is important to remember that all wound healing stages are not mutually exclusive, but rather may overlap over time. The remodeling stage is mediated by anti-inflammatory cytokines IL-10 or TGF- β 1²⁵⁰. This stage is marked by deep changes in the ECM, the resolution of inflammation, and the removal of foreign pathogens. Angiogenesis is the process of regrowing new blood vessels, which have been damaged during the injury or by the inflammatory stage. Granulation tissue begins to form through an increase in fibroblastic proliferation, collagenous and elastic biosynthesis, which creates a network of connective tissue, and the production of chemotactic factors and IFN-beta by fibroblasts²⁵⁰. As collagen composes a large portion of the connective tissue, fibroblasts are recruited to synthesize new collagen and during the repair stage, type III collagen is predominant, and becomes slowly replaced by type I collagen. At the same time, the collagen fibers that are replaced become thicker and placed in parallel, resulting in enhanced tensile strength. Collagen I has been shown to induce angiogenesis and from the pathophysiologic standpoint, mature type I collagen is primarily responsible for mechanical stability, while type III collagen is mostly considered juvenile collagen of the early wound healing phase²⁵¹.

Studies on *Leishmania amazonensis* infection have shown that collagen I is the predominant ECM component in the early-stage lesions²⁵² and in vitro studies have proven that promastigotes can attach to and move through collagen I scaffolds²⁵³. Importantly, the presence of parasites led to about 20% of collagen I degradation in 3D scaffolds, likely mediated by proteinases. It is possible that parasites secrete proteases to breakdown rigid collagen structures in the skin to facilitate migration and induce chemotaxis of target cells. What is particularly interesting is that as the presence of collagen I gradually decreased, type III collagen became the major component at the infection site at 90 days post-infection with *L. amazonensis*. These changes were more evident in susceptible BALB/c mice and type III collagen was deposited around the parasitized macrophages²⁵². The presence of collagen III was also reported in hepatic *Leishmania donovani* granulomas, which may contribute to restricting access by T cells. Under homeostatic conditions, collagen type III composes 5-20% of collagen content in the body²⁵⁴. A higher content of collagen III during leishmaniasis can induce the presence of a softer skin matrix, creating an easier path for parasite migration, although there have been no studies the effect of collagen III on parasite and lymphocyte trafficking. Growth factor stimulation coming from macrophages may have the most impact on collagen matrix remodeling by fibroblasts. While parasites can migrate on collagen I scaffolds, they migrate faster when macrophages are present in the system, possibly responding to secreted cytokines through chemotaxis²⁵³. Resistance of mouse strains to *L. major* is closely linked to the ability to heal wounds, with animals mounting a vigorous wound-healing response also showing resistance to lesion development²⁵⁵.

1.8. Immunomodulation by Regulatory T cells

1.9.1. Treg development

In 1969, Nishizuka and Sakakura *et al.*²⁵⁶ discovered that thymectomizing 3-day-old female mice lead to sterility but not when the procedure was performed on mice aged 7 days or older. The underlying cause for this phenomenon was later explained to be autoimmune oophoritis and that a suppressor cell population originated from the thymus during the first week after birth to prevent sterility²⁵⁷. T cells responsible for inducing immune tolerance were identified in 1995 as a subset of CD4⁺ T cells that constantly express IL-2 receptor CD25²⁵⁸. These cells were later further characterized by the expression of the transcription factor FoxP3^{259,260} and their impact in immune dysregulation diseases²⁶¹.

Prior to the discovery of Tregs, the widely accepted dogma was that clonal deletion of autoreactive T cells is the main driver of T-cell-mediated autoimmunity prevention, termed central tolerance. However, peripheral tolerance and the importance of Treg function has since been demonstrated in numerous diseases. Theoretically, T cells have the capacity to form $>10^{15}$ unique $\alpha\beta$ T-cell receptors²⁶², however humans contain only 10^{12} T cells²⁶³, which indicates that only a small part of possible TCR specificities is utilised at a given time. There are a plethora of unpredictable infections, therefore protective immunity relies on a high level of TCR cross-reactivity²⁶⁴. Consequently, a notion that T cells are purged of self-reactive specificities during thymic development is unsustainable, because removing cells that could cross-react with self-antigens would leave a significantly diminished protective repertoire.

Tregs can develop from two overarching mechanisms: either through development and differentiation in the thymus, or by differentiation of circulating CD4⁺ T cells. Thymic Tregs

(tTregs) develop in the thymus after thymocytes undergo both positive and negative selection during TCR rearrangement (Figure 2). Thymic epithelial cells are divided into cortical TECs (cTECs) and medullary TECs (mTECs), which are evolved in positive and negative selection of T cells, respectively. mTECs are mainly responsible for the regulation of Treg differentiation²⁶⁵. Hematopoietic progenitor cells enter the thymus at the cortical-medullary junction, migrate to cortex, return to the medulla and the thymus again through the junction. During negative selection self-reactive thymocytes are deleted or differentiate into Tregs. mTECs and tissue-restricted antigens presented by DCs mediate the late development of Tregs^{266,267}.

During selection, progenitor Treg populations are CD4 single-positive and CD25^{hi} and induce FoxP3 expression via stimulation by IL-2 and IL-15²⁶⁸ after TCR engagement. This finding underscores the importance of TCR activation as the main driver for determining Treg fate, rather than subsequent FoxP3 expression. Tregs that are specific for self-antigens must survive selection, but how this occurred was demonstrated using Nur77-GFP mice, where the GFP gene is placed within the immediate early gene Nr4a1 locus which is upregulated by TCR stimulation in thymocytes and T cells²⁶⁹. Thymocytes undergoing positive selection showed a twofold increase in Nr4a1 expression, while negative selection led to a 10-fold increase. Nr4a1 expression also showed that Tregs receive a stronger TCR signal than conventional T cells during negative selection, although not high enough for clonal deletion, and that stronger TCR signalling was associated with induction of Treg self-specific epigenetic changes and gene expression patterns²⁷⁰. This was later expanded on by demonstrating that T cells reactive to ubiquitously expressed antigens are likely to be deleted but clones reactive with tissue-restricted peptides developed into Tregs²⁷¹.

Tissue-specific antigens are induced by the transcription factor Aire (Autoimmune regulator) mainly expressed by mTECs and presented at low frequency in the thymus. Interestingly, while Aire is mainly expressed in the thymus, low levels of Aire have also been detected in spleen and lymph node tissue, embryonic liver, testis, and ovarian tissues²⁷². AIRE induces transcription of a wide selection of genes expressed in the peripheral tissues, leading to mTECs expressing antigen from other tissues in the organism. In the absence of Aire, clones that would normally differentiate into Tregs become conventional, autoimmune T cells²⁷³. Of note, the presentation of antigens induced by Aire occurs by both DCs and mTECs in a non-redundant manner, suggesting the importance of differential antigen presentation in Treg induction²⁷⁴.

The expression of tissue-specific genes due to Aire is unique to each mTEC in mice and peptide processing and presentation by mTECs can also be different between perinatal and adult mice, which generates age-specific Treg repertoire²⁷⁵. Thus, disparity in Treg TCR repertoires might explain differing susceptibilities to autoimmune diseases between individuals, suggesting that immune tolerance mechanisms may shift with age. However, not all Tregs rely on Aire for differentiation. Certain TCR clones of thymic Tregs can be induced by bone marrow-derived antigen presenting cells in the thymic medulla. These APCs, which are generally B cells and dendritic cells, migrate to the thymus after taking up blood-borne and peripheral tissue antigens²⁷⁶⁻²⁷⁸. While it is not clear whether these APCs display non-self-antigens derived from the microbiota or environmental antigens in the thymus, APCs have the capacity to carry foreign proteins to the thymus, as fluorophores painted on the skin of mice were found in thymic DCs²⁷⁹. This is consistent with the fact that a large proportion of Treg TCRs react with non-self-antigens²⁸⁰ in the thymus, arguing against the development of Tregs that strictly recognize self-antigens. Indeed, Tregs accumulate in neonatal skin and induce tolerance against *Staphylococcus epidermidis*²⁸¹.

Additional signals required for Treg development involve signaling through CD28 binding with CD80/86²⁸². *Cd28*^{-/-} or *Cd80*^{-/-}*86*^{-/-} mice on diabetes susceptible NOD background presented with exacerbated diabetes, and treatment of wild-type NOD mice with anti-CTLA-4 antibody, which blocked CD28 interaction, also led to a substantial loss of Tregs and accelerated diabetes²⁸². CD28 signalling, however, does not alter TCR specificity of Tregs, rather it insures the optimal differentiation and survival of these cells²⁸³. Lastly, substantial evidence suggests that thymic Treg differentiation requires IL-2, IL-15, and IL-7 signaling²⁸⁴. IL-2 plays an integral role in Treg cell development. Anti-IL-2 antibodies induce reduction of Treg numbers and development of organ-specific autoimmunity^{285,286}. Tregs development in mice lacking IL-2, IL-7, and IL-15 is completely abolished^{287,288}.

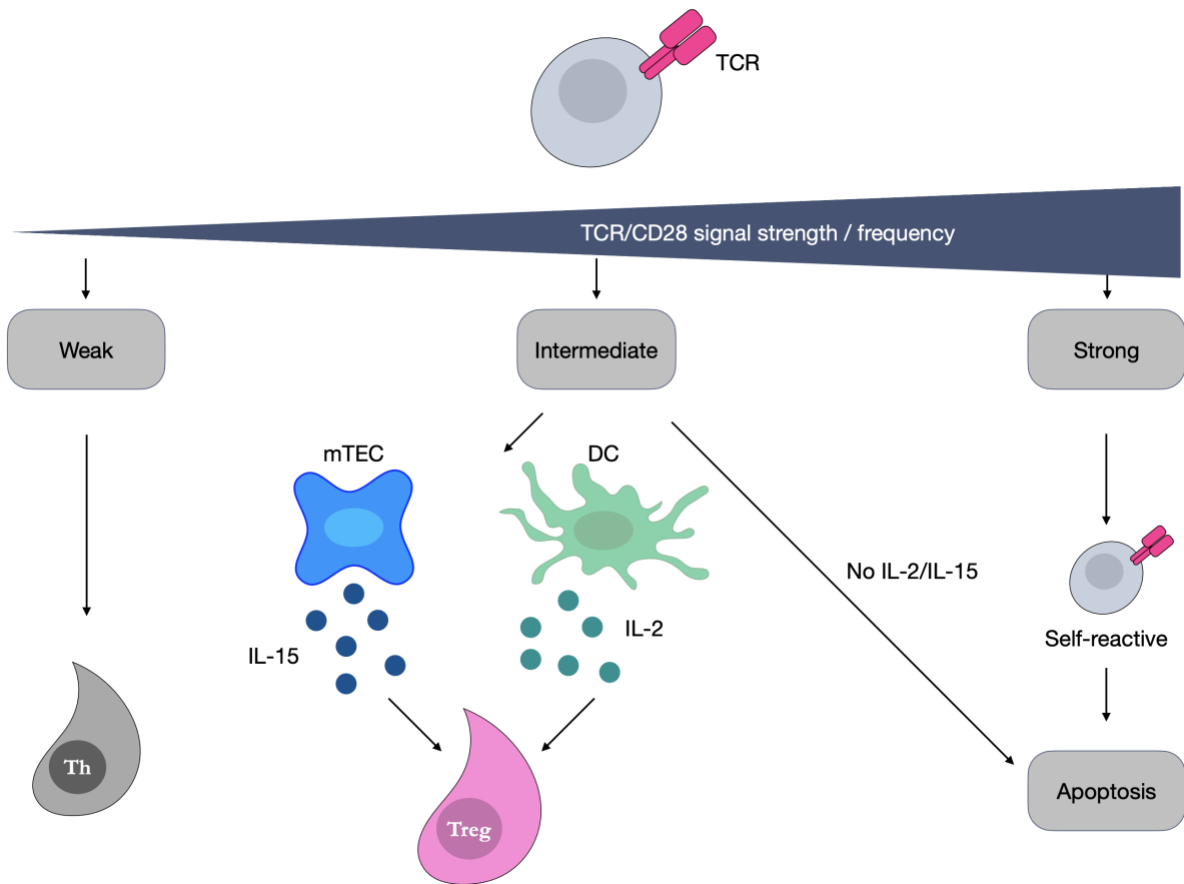


Figure 2. Treg development in the thymus.

During thymic development, weak TCR signals result in the generation of conventional T cells while strong signaling leads to clonal deletion of highly self-reactive thymocytes. Thymocytes are diverted into the lineage of Treg cells as a result of intermediate TCR signals. Further survival of these cells requires cytokine signaling. Treg precursors are saved from cell death and grow into mature $CD25^+Foxp3^+$ Tregs due to the production of IL-2 and IL-15 by DC and mTEC, respectively.

Treg cells can be thymus-derived (tTregs) or peripherally derived (pTregs) from FoxP3⁺CD4⁺ cells. Tregs expressing little to no Helios are thought to be peripherally derived, although some studies disagree whether it a true marker for tTregs²⁸⁹. Most tissue-specific Tregs are likely thymus derived^{290,291}, whereas placental and a large proportion of colonic lamina propria Tregs are peripherally derived coexisting with tTregs²⁹² and play an important role in immune tolerance at this mucosal site. Peripherally induced Tregs differentiate after conventional CD4⁺ T cell is exposed to low-dose foreign antigens and TGF- β signaling²⁹³, as can be seen in the Gut-Associated Lymphoid Tissue where pTregs are specific to gut microbiota²⁹⁴. FoxP3 expression is not sustained in pTregs if TGF- β is removed from the system²⁹³. The generation of peripherally-derived Tregs is not clear and direct TCR sequencing experiments yielded contradicting results. Some studies of intestinal Tregs show that colonic commensal bacteria influence the induction of peripherally-derived Tregs²⁹⁵, while others support the contrary²⁹⁶. It has been shown that CD4⁺ TCR T cells reactive to exogenous peptides could be induced to become pTregs using other various approaches: DCs from lamina propria can induce de novo Tregs via retinoic acid²⁹⁷ and short-chain fatty acids (SCFAs) can directly induce Tregs from naïve T cells^{298,299}. In vitro, Tregs can be developed from naïve CD4⁺ FoxP3⁺ T cells with TGF- β ³⁰⁰. In vivo, Tregs can be induced with chronic exposure of antigen in small doses, and these Tregs are indistinguishable from tTregs³⁰¹. Removal of pTregs leads to dysregulation of immune responses in the gastrointestinal tract and airway³⁰², and thus pTregs are thought to be responsible for immunologic tolerance towards non-pathogenic substances and commensal microbiota.

1.9.2. Skin Tregs

Regulatory T cells have been identified approximately 20 years ago^{259,260}, and our understanding of the various mechanisms by which Tregs exert their suppressive and homeostatic

functions continues to expand. Tregs are found in various lymphoid and non-lymphoid tissues and possess a breadth of phenotypic and functional characteristics as important regulators of tissue homeostasis. The first tissue specific Treg population was detected in adipose tissue, where they reside in spaces where adipocytes intersect with macrophages, dendritic cells, and other leukocytes^{303,304}. In obese mice, visceral adipose tissue (VAT) Treg numbers are vastly reduced and more concentrated in multiple adipocyte junctures³⁰³, which suggests that they are important regulators of local and systemic metabolism. The VAT Treg transcriptome differs from that of their lymphoid counterparts, with enrichment in chemokines and their receptors (*Cxcl2*, *Cxcr6*, *Ccr1* and *Ccr2*), cytokines and their receptors (*Il10*, *Il5*, and *Il9r*), and molecules associated with lipid metabolism (*Dgat1*, *Dgat2* and *Cd36*)³⁰⁵. VAT Tregs display a constrained TCR repertoire compared to lymphoid Tregs and some Tregs show clonal expansion, indicating response to local antigens²⁹¹. Skeletal muscles are vulnerable to mechanical stress and become injured frequently. Tregs play a major role in regulating muscle repair³⁰⁶. Muscles of healthy young mice harbour low Treg numbers. After injury, muscle Tregs rapidly expand, with highest numbers at day 3 or 4. Until day 14 they then proceed to rapidly decline, where they remain detectably elevated over baseline even a month after injury³⁰⁷. Muscle Tregs have a clonally expanded TCR repertoire, indicating recognition of local antigen drives specific Treg accumulation²⁹⁰.

Treg functions in the skin are remarkably diverse³⁰⁸ and their importance in skin was first demonstrated during *Leishmania major* persistence²²⁹. In perinatal mice, up to 90% of all skin CD4⁺ T cells are Tregs, where they promote skin tolerance to the resident microbiota^{281,309}. Tregs maintain skin homeostasis by inducing tolerance to self-antigens³¹⁰, restraining anti-pathogen responses to minimize collateral damage^{229,311,312}, and controlling innate and adaptive allergic reactions³¹³. Maintenance of self-tolerance is one of the most important functions of skin Tregs, as

evidenced by the fact that their absence or impaired function leads to severe autoinflammatory disease^{310,314-316}. Another important function of Tregs is to aid in wound healing. In the skin of healthy adult mice, Treg numbers range from 20% to 60% of CD4⁺ T cells depending on the stage of the hair growth cycle, as they concentrate around hair follicles and facilitate epithelial hair follicle stem cell differentiation³¹⁷. After injury, hair-follicle stem cells which are normally poised to contribute to cyclic rounds of hair generation, are recruited to support regeneration of the epithelium. Tregs also accumulate in the skin early after wounding and induce the surrounding expression of epithelial growth factor receptor, whereas Treg ablation results in delayed re-epithelialization and wound closure and increased pro-inflammatory macrophage accumulation³¹⁸. Wound healing is a highly inflammatory process, and Tregs have been shown to fine-tune this response. Th17 cells are prone to induce IL-17A during inflammation, which in turn increases CXCL5 expression in epithelial cells and recruits neutrophils. Neutrophil recruitment prevents the migration of hair-follicle stem cells to the damaged tissue and slows healing. Tregs prevent this delay by suppressing the Th17 inflammatory response from the start³¹⁹.

Skin Tregs upregulate transcripts encoding activation or memory markers. These Tregs preferentially express transcripts encoding skin-specific chemokine receptors, such as CCR2, CCR6, CCR8, CCR10, CXCR4 and CXCR6. Additionally, transcripts of IL-10, granzyme B, and amphiregulin, a ligand of epidermal growth factor receptor, are upregulated as well³²⁰. At the same time, bulk and single-cell RNA sequencing analysis of T cells in murine skin showed that not only did skin Tregs preferentially express GATA3 and other transcription factors usually associated with Th2 differentiation. It has been suggested that skin resident Tregs are poised to regulate Th2 immunity, and a subset of these cells is more differentiated toward reparative capacity³²¹. Curiously, skin Tregs also produce less pro-fibrotic TGF β compared to other tissue Tregs, while

also expressing higher levels of TGF β receptors³²¹. These Tregs could potentially be sequestering TGF β from activating fibroblasts and in turn utilizing it for self-maintenance and enhancement of regulatory capacity. Skin Tregs exhibit clonal expansion greater than that of corresponding CD25⁻ CD4⁺ T cell populations³²².

Tregs are seeded in the skin perinatally and localize around the hair follicle stem cells at steady state³¹⁷. Tregs are localized to the follicle by the production of CCL20, which can be further induced by the skin microbiota³⁰⁹. At this site, Tregs interact directly with stem cells via their expression of Jagged-1 binding to stem cell Notch. The effect of hair follicle stem cells on Tregs has not yet been described. Interestingly, maintenance of skin Tregs at steady state does not seem to rely on IL-2, but rather IL-7³²³, fitting with the high level of IL-7 production by keratinocytes. However, IL-2 could still play a role in skin Tregs, as it has a powerful capacity to expand the skin Treg compartment³⁰⁵. In humans, healthy adult skin has a substantial population of Tregs, about 5 times the frequency in the blood^{324,325}. As in mice, they localize near hair follicles and are highly activated and non-migratory³²⁵. Developing human skin has lower levels of Tregs, suggesting that skin Tregs may accumulate over time³²⁶. Alopecia, an autoimmune disease resulting T cell-driven hair follicle damage, is associated with FOXP3 promoter polymorphism³²⁷ and can be alleviated via low-dose IL-2 treatment³²⁷. Collectively, skin Tregs play a major role in regulating tissue-specific functions, such as tolerance to skin microbiota, hair follicle cycling, and epidermal repair.

1.9.3. Treg-immunomodulation through modifying dendritic cell function

Tregs have been described to mediate their suppressive activity by destabilizing DC:T cell contacts and thus preventing conventional T cell activation. Intravital microscopy in the lymph node has identified two non-overlapping populations of Tregs which continuously explore either the B follicles or the T cell zone³²⁸. Though much less abundant than conventional T cells, Tregs

are highly motile and frequently contact both DCs and conventional T cells in the lymph node. When activated DCs enter the lymph node, prolonged DC:Treg interactions are observed near the LN capsule, suggesting Treg activation³²⁸. During effector T cell priming, Tregs can engage in dynamic clusters with both T cells and DCs without fully stopping and destabilizing weak DC:T cell contacts in order to prevent suboptimal T cell activation. One established mechanism of Treg-mediated DC:T cell contact disruption is through the expression of CTLA-4, which binds CD80 and CD86 on APCs³²⁹, rendering them unable to stabilize and activate T cells. Interestingly, Tregs are able to trogocytose CD80 and CD86, transferring them from the APC cell surface to their own plasma membranes³³⁰, a phenomenon that has been demonstrated to occur in an antigen-dependent manner³³¹. The ability of Tregs to destabilize DC:T cell conjugates was demonstrated by the addition of anti-CTLA-4 antibody³²⁸. Upon activation, conventional T cells also upregulate CD80 and CD86, which renders them direct targets for Treg suppression. Treg activation, as mentioned above, has been shown to be antigen-dependent³³², but there is a gap of knowledge as to whether continual antigenic stimulation regulate optimal suppressive functions, especially during *L. major* infection.

1.9.4. Treg-immunomodulation through modifying macrophage function

Outside of the lymph node environment, Tregs can suppress immune responses through targeting multiple cell types³³³, utilizing both contact-dependent and independent mechanisms. Secretion of suppressive cytokines, such as IL-10, TGF β , and IL-35, and IL-2 consumption are the main contact-independent suppressive mechanisms employed by Tregs (Figure 3)³³³. In the context of *Leishmania major* infection, IL-10 and TGF β inhibit TNF and IFN γ production and function³³⁴, induce downregulation of co-stimulatory molecules CD40, CD80, and CD86 on DCs^{335,336}, inhibit NO synthesis³³⁷, and induce macrophages to secrete anti-inflammatory

cytokines. IL-10 produced by naturally occurring Tregs has proven essential in aiding *L. major* survival, although Tregs can also promote the early stage parasite survival in an IL-10 independent manner²²⁹.

Restoration of tissue homeostasis is an important phase of inflammation resolution. Tregs have been shown to secrete IL-13, which stimulates the production of IL-10 in macrophages and promotes macrophage phagocytosis of apoptotic cells, also known as efferocytosis³³⁸. IL-10 can also re-program macrophage metabolism in a way that promotes anti-inflammatory and resolution functions³³⁹. Specifically, IL-10 can inhibit LPS-induced glucose uptake and glycolysis, in turn promoting oxidative phosphorylation. A highly efficient oxidative phosphorylation in M2 macrophages leads to increased glucose levels, which promotes intracellular amastigote growth. IFN γ -activated M1 macrophages have a suboptimal glycolytic pathway and require an increased glucose uptake, whereby internalized glucose generates NADPH and ATP via the pentose phosphate pathway (PPP) which is critical for ROS metabolites and parasite killing^{340,341}. Suppressing glycolysis causes macrophages to switch to oxidative phosphorylation and subsequently decreases leishmanicidal activity.

Tregs have an impact on monocytes also, as monocytes cultured with Tregs for 6 days downregulate the expression of co-stimulatory and MHC II molecules and increase the expression of CD206 and secretion of IL-10³⁴², a hallmark of alternatively-activated monocytes and macrophages. Tregs have also been shown to exhibit suppressive activities in a cytotoxic fashion. Human Tregs have been demonstrated to induce cytolysis of target T cells in a perforin-dependent, Fas-FasL-independent manner in vitro³⁴³. In contrast, murine Tregs were able to kill responder cells in the granzyme B-dependent mechanism³⁴⁴. The most well-described mechanism is prevention of T cell-activation via CTLA-4 expression, which inhibits activation of co-stimulation

through CD28 by competitively binding of CD80/86. Together, Treg cells are master regulators of the immune response that are frequently implicated in diseases that associate with high (e.g. solid tumors) or low (e.g. multi-organ autoimmunity) Treg functions.

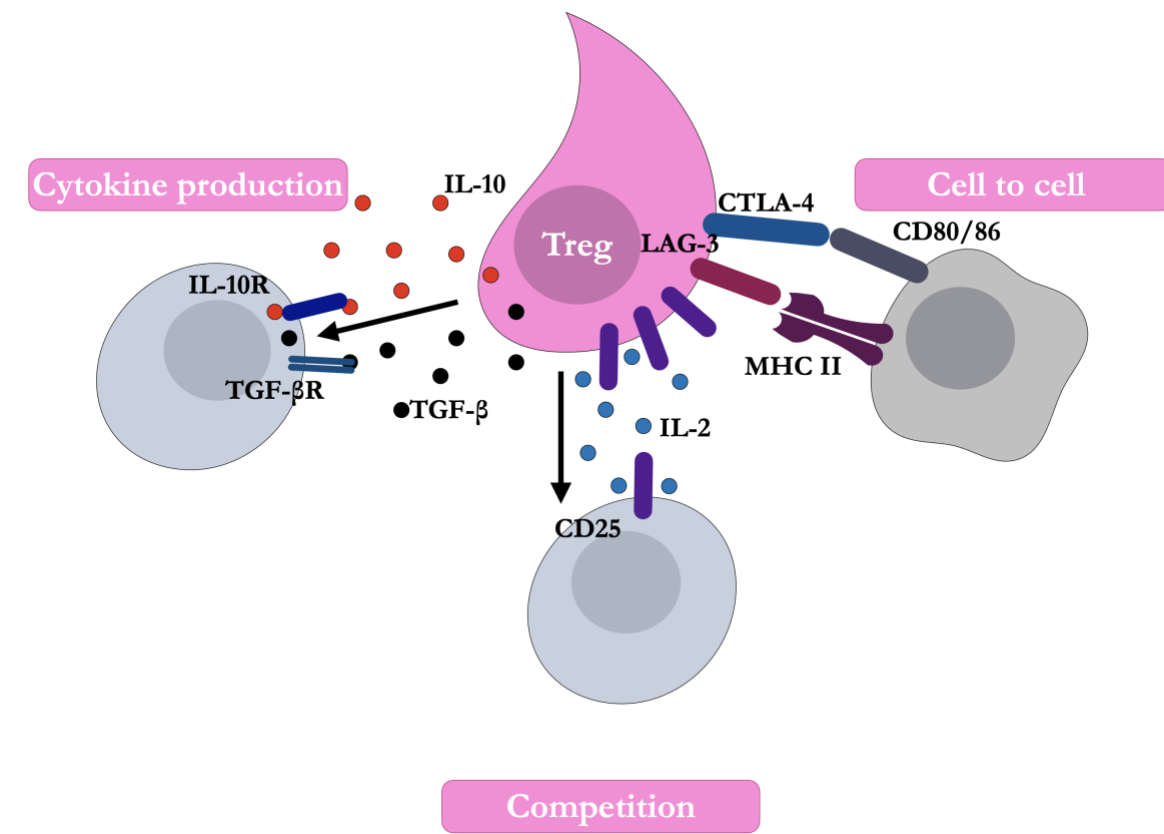


Figure 3. Simplified mechanisms of Treg suppression.

Cytokine production mechanisms of suppression include secretion of IL-10 and TGF β . Secretion of IL-10 results in suppression of IFN γ dependent activation of APCs. TGF β induces downregulation of IL-2 required for lymphocyte survival and upregulation of cell cycle inhibitors. High Treg expression of IL-2 receptor CD25 causes a more efficient consumption of the cytokine and leads to impaired T effector differentiation and survival signaling. Cell-contact dependent methods such as interaction of CTLA-4 with CD80/86 can lead to a blockage of these molecules on APCs, preventing T cell activation. LAG3:MHC II interaction can lead to impaired maturation of DCs, and anergy and arrest of effector T cell population.

1.9.5. Lessons learned from cancer research

Tumor-infiltrating Tregs are generally associated with the immunosuppressive state of tumour microenvironments (TME) and decreased survival rate of patients³⁴⁵. Tissue resident Tregs have a greater capacity for inducing tissue regeneration, which in turn allows the tumor to escape immune clearance³⁴⁶. Thus, the largest focus of cancer therapy has been on immune checkpoint inhibitors.

There are numerous mechanisms by which tumor infiltrating Tregs exert their regulatory function. High CD25 expression can deprive local environment of IL-2 and negatively impact expansion and function of effector T cells³⁴⁷ and NK cells³⁴⁸. Treg depletion leads to a pronounced expansion and activation of DCs, indicating Treg impact on antigen-presenting cells³¹⁶. This suppression may be caused by trans-endocytosis, through CTLA-4-dependent downregulation of CD80 and CD86³⁴⁹. Additionally, in human Tregs, CD3 and CD46 stimulation leads to the induction of granzyme A, which causes in the induction of apoptosis in target cells³⁴³. CD39 and CD73 are also highly expressed on Tregs, which facilitate the conversion of ATP and amplification of adenosine and cAMP and can directly affect the TME³⁵⁰. Adenosine signaling directly inhibits the proliferation of effector T cells and negatively regulates DCs. Lastly, Treg expression of an inhibitory cell surface receptor LAG-3 directly binds MHCII on DCs and suppresses effector T cell activation³⁵¹.

Tumour infiltrating Tregs display a highly activated phenotype (high CD45RO, low CD45RA) and a strong expression of costimulatory (OX40, ICOS, GITR) and inhibitory (CTLA-4, PD-1, LAG3) molecules³⁵²⁻³⁵⁴. Tregs are also recruited in large frequencies to tumor microenvironment through tissue-specific chemokine expression. For example, CCR5 leads to the recruitment of Tregs to squamous cell carcinomas and colorectal tumours, while hypoxia induces secretion of CCL28, recruiting Tregs into liver tumors, and initiates angiogenesis through VEGF

production³⁵⁵. CCR4 expression on Tregs has also been involved in trafficking to several nonlymphoid organs and tumors³⁵⁶. Importantly, there are similarities between tumor and tissue resident Tregs. These data suggest that intratumoral Tregs are derived from the surrounding tissue rather than from circulation³⁵⁴ and that environmental oxygen and metabolite availability may further contribute to differential Treg activation. It is important to note that there is limited overlap between the TCR repertoire of conventional T cells and Tregs in breast cancer tissue³⁵⁴ and human melanoma samples³⁵⁷, arguing that Treg have not been induced from the local Th1 cell repertoire, but recruited and retained in antigen-specific manner.

Tregs accumulate in tumor tissues through chemokines and upregulate FoxP3-regulated transcription factors to adapt to the TME. Metabolic signals significantly impact T cell differentiation and function³⁵⁸. The relative degree to which glycolysis and fatty acid oxidation is utilized in a naïve T cell after TCR engagement strongly impacts Treg differentiation^{359,360}. Cancer cells possess an enhanced glycolytic activity and create a microenvironment of glucose deficiency and lactic and fatty acid enrichment. Nutrient-poor, lactate-rich, hypoxic, and acidic environments significantly restrain effector T cells, which rely on glycolysis³⁶¹. FoxP3 in turn can inhibit glycolysis and reprogram T cell metabolism to enhance oxidative phosphorylation and fatty acid oxidation³⁶²⁻³⁶⁴. Additionally, Tregs have a higher resistance to reactive oxygen species in the TME compared to effector T cells. Aside from adapting to the unique demands of the TME, Tregs also actively shape it. Conversion of immunostimulatory ATP to suppressive adenosine by CD39 and CD73 directly inhibits T cell proliferation^{365,366}. Immunotherapy synergized with targeting this pathway can promote antitumor immune response³⁶⁷. Additionally, Treg activation can lead to increased catabolism by IDO, which contribute to attenuated antitumor immunity³⁶⁸. IDO activity reciprocally helps preserve Treg stability³⁶⁹.

Preclinical studies on mouse models demonstrate that Treg depletion leads to effective immune responses^{370,371}. However, Treg deletion strategy has serious drawbacks such as autoimmune toxicities and cannot be safely translated to humans. Similarly, blocking CD25 or IL-2 also leads to autoimmunity. Co-signaling molecules, however, have drawn a lot of attention in therapy (Figure 4). Conventional T cells require antigen exposure and activation to upregulate co-signaling molecules. However, Tregs express many of these markers constitutively and at higher levels, likely due to heightened TCR signaling. High surface expression of molecules such as PD-1, ICOS, TIGIT, and LAG3 is a rationale for antibody-driven Treg targeting^{372,373}.

1.9.6. Treg cell surface molecules

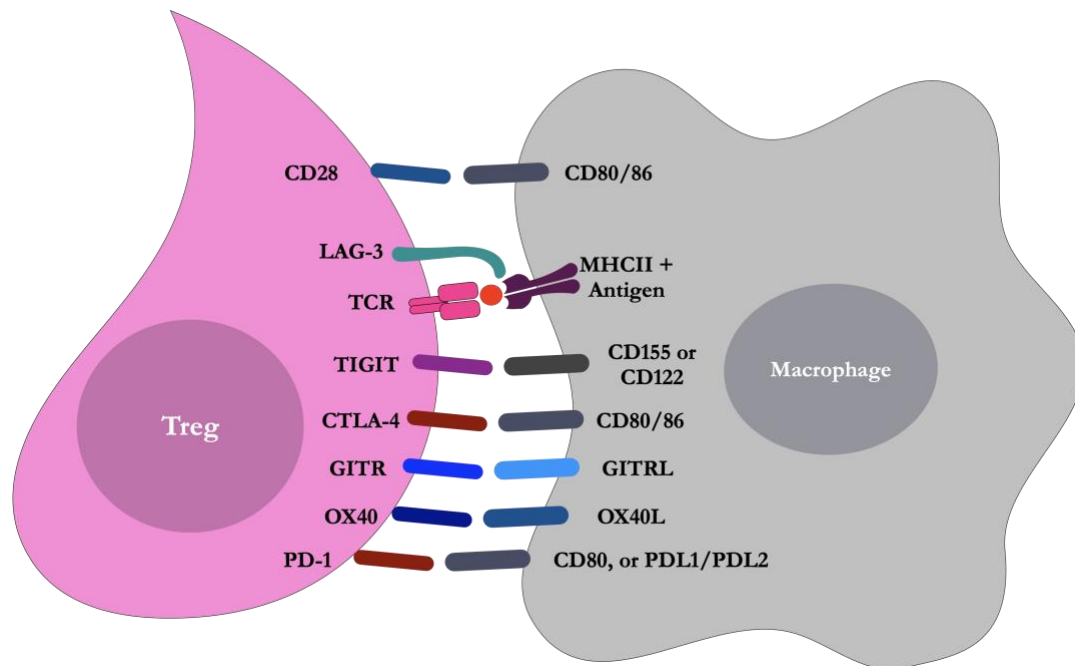


Figure 4. Treg cell surface molecules and their ligands

1.9.6.1. CD28

CD28 is a member of the Ig superfamily and is a cell surface homodimer. It plays a critical role in the initial activation of T cells, and therefore is constantly expressed on cell surface³⁷⁴. CD28 binds CD80 and CD86 on APC cell surface and essential for T cell development in the thymus³⁷⁵. CD28 signaling is required for the production of IL-2 by conventional T cells, which in turn is essential for the Treg maturation³⁷⁶. For Tregs, CD28 signaling is essential for upregulation of FoxP3 early in the differentiation process³⁷⁶ and for their full activation and proliferation in the periphery^{282,375}.

1.9.6.2. CTLA-4

Cytotoxic T-cell lymphocyte antigen-4 (CTLA-4) is closely related to CD28 and shares the same ligands. CTLA-4 is a suppressive molecule that is constitutively expressed on Tregs and knockout of CTLA-4 results in fatal autoimmunity and lymphoproliferation similar to what can be seen in FoxP3-deficient scurfy mice³⁷⁷. CTLA-4 competitively binds to CD80/86 and can deplete cell surface expression of these molecules^{335,378,379} and prevent subsequent APC:conventional T cell interaction. CTLA-4 may also play a role in arrest and enhancement of T cell and Treg mobility³⁸⁰. Treatment of tumour-bearing mice with anti-CTLA-4 antibody leads to tumor regression and CTLA-4 blockade has shown efficacy in human trials for the treatment of advanced melanoma^{378,381}. According to growing clinical evidence, checkpoint blocking antibodies may have a promising function in a variety of solid tumor malignancies, such as lung cancer, renal carcinoma, ovarian cancer, bladder cancer, head and neck cancer, and gastric cancer³⁸².

1.9.6.3. GITR

Glucocorticoid-induced tumor necrosis factor-related receptor (GITR) is expressed at low levels on naive T and NK cells and is upregulated upon activation³⁸². After activation, GITR is

constitutively expressed at a high level on Tregs and marks an activated Treg phenotype^{383,384}. GITR binds nectin and nectin-like adhesion molecules and mediates cell adhesion and signaling³⁸⁵. Anti-GITR antibodies can induce autoimmunity³⁸⁶ and overcome Treg cell-mediated suppression of the antitumor response³⁸⁷. GITR stimulation induces proliferation of both conventional T cells and Tregs, but overstimulation may lead to activation-induced cell death³⁸⁸.

1.9.6.4. OX40

OX40 is a costimulatory molecule expressed on activation by conventional T cells and constitutively on Tregs. OX40L is expressed on APCs, NK cells, and activated B, T, and NK T cells³⁸⁹. In conventional T cells, OX40 signaling leads to an increase in proliferation and cytokine production, but in Tregs the role is more complex³⁹⁰. While playing a mild role in proliferation of Tregs, OX40 also appears to interfere with FoxP3 expression³⁸⁰.

1.9.6.5. PD-1

Programmed cell death-1 (PD-1) is a coinhibitory cell surface receptor in the CD28 superfamily. PD-1 is expressed on activated T cells, B cells, NK cells, NKT cells, DCs, and monocytes. For effector T cells, level of PD-1 expression increases with antigen exposure^{391,392}, as PD-1 suppress co-stimulatory and TCR signals^{393,394}. High PD-1 expression can be associated with an exhausted T cell phenotype³⁹⁵. PD-1 is not essential for Treg development, but PD-1 has been both shown to promote³⁹⁶ and limit³⁹⁷ the differentiation of pTregs. PD-1 binds PD-L1 and PD-L2 expressed on T cells, B cells, DCs, and macrophages³⁸⁰ and the binding of PD-1 to its target is thought to suppress immune activity^{398,399}. However, checkpoint blockade studies in cancer have reported that PD-L1 blockage has actually enhanced Treg activity and led to hyper progression and metastasis⁴⁰⁰. There is growing evidence that PD-1 signaling can inhibit Treg cells^{401,402}. A recent study demonstrated that during inflammation, IFN- drives the upregulation of PD-L1 levels

on the surrounding APCs, which in turn diminish Treg function and allow for a more balanced parasite clearance⁴⁰³. It is likely that during systemic infection, PD-1 signaling creates negative feedback to balance the Treg:Th1 responses.

1.9.6.6. LAG-3

Lymphocyte activation gene-3 (LAG-3) is a transmembrane protein with a homology similar to CD4. LAG-3 binds MHC-II with a greater affinity than CD4⁴⁰⁴. LAG-3 regulates the expansion of activated T cells and the development of memory T cells⁴⁰⁵. Within activated T cells, LAG-3 is preferentially expressed and maintained by Tregs. In a model of lethal pneumonitis, it was shown that LAG-3 expression was crucial in allowing Tregs to suppress conventional T cells and protect mice from death⁴⁰⁶.

1.9.6.7. TIGIT

The co-inhibitory T-cell mediated immunoreceptor with Ig and ITIM domains (TIGIT) acts as an antagonist to a costimulatory receptor CD226 and regulates T cell responses utilizing a mechanism similar to CD28/CTLA-4⁴⁰⁷. TIGIT and CD226 both bind CD155 and CD112 and TIGIT binding stimulates IL-10 production and blocks IL-12 production by DCs, repressing Th1 immunity³⁸³. It is speculated that TIGIT is a Treg activation marker and TIGIT upregulation by Treg activation can be hindered by IL-12³⁸⁴. TIGIT⁺ Tregs possess a highly immunosuppressive phenotype, evidenced by the high expression of ICOS, PD-1, CTLA-4, and Lag3³⁸³. TIGIT⁺ Tregs also display a preferential suppression of Th1 and Th17 responses, but not Th2³⁸³. The main ligand for TIGIT is CD155, also known as Poliovirus receptor (PVR)⁴⁰⁸. It is highly expressed on DCs, macrophages, B cells, epithelial cells, and tumor cells³⁸⁵. CD155-TIGIT interaction suppresses immunological responses by increasing the production of IL-10 and decreasing release of IL-12³⁸², which induces a tolerogenic phenotype in DCs. On activated Tregs, TIGIT-CD155 interaction

leads to induction of effector molecule fibrinogen-like protein 2 (Fgl2), which promotes Treg-cell-mediated suppression of Th1 and Th17, but not Th2 cells^{383,409}. In vivo, *Rag*^{-/-} mice that received *Tigit*^{-/-} Tregs together with wild-type CD4⁺ and CD8⁺ T cells showed suppressed tumor growth⁴¹⁰. In human cancer, it is speculated that when the tumor already presents with immune infiltration, TIGIT becomes upregulated on T cells and promotes immunosuppressive environment, leading to metastasis³⁸⁵.

1.9.7. Mechanisms of Treg suppression: context matters

FoxP3 expression plays an imperative role in maintaining Treg functionality and phenotype. Loss of X chromosome encoded FoxP3 leads to a severe multi-organ autoimmune disease IPEX and a similar disorder in *scurfy* mice⁴¹¹. Later studies proved that *in vivo* FoxP3 is essential for differentiation of Tregs⁴¹². A member of the forkhead transcription factor family, FoxP3 suppresses NFAT and NFkB, which leads to suppression of expression of proinflammatory genes encoding effector cytokines. FoxP3 also activates transcription of genes encoding CD25, CTLA-4, GITR, and others⁴¹³.

An array of suppressive molecules and mechanisms have been identified, from the expression of immunomodulatory cytokines IL-10^{414,415} and TGF- β ⁴¹⁶ to expression of cell surface ectoenzymes CD39 and CD73 which convert extracellular ATP into its anti-inflammatory counterpart adenosine³⁵⁰. Single deficiency in genes encoding individual effector functions in Tregs does not lead to systemic autoimmunity at similar levels compared to total Treg depletion, indicating that there is redundancy in enforcing immune tolerance^{417,418}. Additionally, the majority of suppressive mechanisms are shared by multiple cell types and are rarely solely confined within the Treg population^{235,419,420}. Nevertheless, loss of suppressive functions by Treg cells cannot be fully compensated by other cells. Rather, we now have a deeper understanding that the action of a

single Treg mechanism of suppression exerts a nuanced, context-specific effect in controlling a particular aspect of immune response in a specific microenvironment it is in. For example, Treg cells deficient in IL-10 production fail to control skin inflammation or allergic airway despite the presence of IL-10 producing macrophages, B or T cells in those tissues⁴¹⁷. Treg-restricted deletion of *Ebi3* gene, which is expressed by various immune cells, leads to mice exhibiting decreased control of anti-tumor responses⁴²¹. An exacerbation to this trend seems to be the necessity of CD25 and CTLA-4; deletion of these molecules results in severe autoimmunity^{422,423}. This is likely attributed to the fact that CD25 and CTLA-4 are essential for Treg TCR selection, development, and survival^{378,422,423}. The primary role of CD25 is IL-2 depletion, which results in decrease in function of all cells which are highly sensitive to IL-2, such as CD4 and CD8 T cells and natural killer cells, and the role of CTLA-4 is thought to be interfering with DC-mediated T cell priming⁴²³⁻⁴²⁵. While Tregs do arguably express the highest levels of CD25, activated effector T cells, innate lymphoid cells, DCs, and even fibroblasts can also sequester IL-2 through CD25^{426,427}.

Tregs also contribute to tissue maintenance and repair. For example, Tregs help repair injured muscle and other tissues, and maintain hair follicle stem cells in the skin. No single molecule can be attributed as the most important hallmark of Treg-mediated suppression or maintenance of homeostasis. Rather, Tregs have the ability to deploy distinct mechanisms based on time and space, i.e. the mode of suppression is context dependent. When a model organism that has healed a prior *L. major* infection is challenged with *Leishmania* antigen, rapid expansion and recruitment of Tregs to the site of the original, healed infection can be observed²³⁰. This can be explained by the fact that Tregs that are activated under inflammatory conditions, such as during the primary Th1 response to *L. major*, are generally more potent suppressors, and are important in returning immune responses to baseline at the conclusion of inflammation^{226,428,429}. The outcome is increased

expression of genes promoting homing to non-lymphoid tissues in Tregs²²⁶, which creates a link between the ability of Tregs to maintain immune tolerance and heightened capacity for localization to peripheral tissues. Tregs activated in response to the highly inflammatory environment of *L. major* infection area have stronger suppressive capability and a better ability to rapidly localize to the site of infection.

1.9.8. Role of Antigen-specificity in Treg function

The Treg TCR repertoire is distinct from that of conventional CD4⁺ T cells^{275,430,431}. As discussed above, Tregs recognize a plethora of self-antigens due to the activity of AIRE in the thymus whereas the naïve T cell TCR repertoire is mainly focused on non-self-antigens. Indeed, it has been demonstrated that inducible ablation of the TCR results in Treg cell dysfunction, as TCR signalling was required for both Treg activation and to maintain expression of a limited set of genes expressed almost exclusively in activated Tregs, driving their homeostatic and suppressive functions. Given that antigen-activated conventional CD4⁺ T cells are thought to produce IL-2 in a spatially restricted manner, it is possible that Tregs use their TCR to correctly position themselves to gain preferential access to IL-2. Indeed, local IL-2 induction of STAT5 phosphorylation by Tregs is a part of a feedback circuit that limit effector T cell responses at various clusters throughout the lymph node⁴³². TCR-ablated Tregs remained naïve-like and did not populate non-lymphoid tissues, which suggests that effector differentiation and expansion are dependent on antigen activation. TCR activation is, therefore, crucial for optimal Treg suppressive functions.

All activated Tregs have immunosuppressive capability. For example, immunomodulation of Tregs in the colon does not depend on antigen⁴³³. However, antigen specificity of Tregs aids in orchestrating precise targeting for the delivery of enhanced suppressive functions. Ablation of PD-1, which is known to downregulate TCR and CD28 signaling, has been shown to enhance

immunosuppressive activity of Tregs^{403,434}, thus indicating that TCR stimulation further induces suppressive functions of Tregs. Treg-cell-mediated suppression is also associated with modulating effector T cell interactions with DCs. Recent findings highlight the importance of TCR expression by Tregs in ensuring their positioning in the secondary lymphoid organs where they intermingle with the DCs and effector T cells^{328,435,436}. Furthermore, Tregs exhibit the ability to prune off peptide-MHC complexes in an antigen-specific manner, therefore removing the ability of conventional T cells to become activated³³¹. TCR engagement has also been shown to play a role in induction of IL-10. Mouse models show that mononuclear phagocytes expressing MHC-II with cognate antigen are important targets for Treg IL-10 mediated suppression^{437,438}.

1.9.9. Tregs and parasites: common goal for homeostasis

Immune suppression by Tregs is essential for preventing an overexuberant immune response and preventing bystander tissue damage. Thus, induction and/or manipulation of Tregs by parasites is a way for parasites to gain an advantage and survive. During malarial infection, increased Treg numbers have been found in both human and mouse infection⁴³⁹ and Tregs have been shown to associate with increased parasite growth in experimental mouse models^{440,441}. Increased parasite load and development of human infection caused by *P. falciparum* are also associated with higher Treg numbers^{442,443}. Tregs are also believed to suppress immune response at the chronic stages of filarial disease. While patients with chronic Onchocerciasis (river blindness) possess high worm burdens, they display little to no signs of dermatitis, associated with antigen specific Tregs⁴⁴⁴. Increased Treg populations are also associated with *Brugia malayi*⁴⁴⁵, *Heligmosomoides polygyrus*⁴⁴⁶, and *Trichinella spiralis*⁴⁴⁷. There is substantial evidence that cellular immunosuppression plays a critical role during *Trypanosoma cruzi* infection, the cause for Chagas disease⁴⁴⁸⁻⁴⁵¹. In mouse models, in addition to IL-10, Tregs have been shown to downregulate

classical activation of macrophages, resulting in a decreased production of TNF⁴⁵². Chagas disease is known to cause cardiomyopathy, and Tregs are believed to decrease tissue damage^{453,454}. Even nematode infections induce Treg expansion⁴⁵⁵, which suggests a role of Tregs in helminth-induced modulation of inflammatory disease. Killing of *Litomosoides sigmodontis* parasites can be induced by Treg depletion⁴⁵⁶ and Tregs are critical in controlling Th2-type responses in chronic *S. mansoni* infection^{457,458}. A similar trend emerges with differing parasitic infection, where a strong anti-parasitic inflammatory response is counterbalanced by suppressive functions that limit tissue damage yet allow for parasite escape.

Mammalian hosts encounter numerous parasites throughout existence, yet in most cases fail to fully clear them despite antigen recognition, contributing to the limited number of effective vaccines against parasites. One driving factor for parasite persistence is the generation of host immunosuppressive functions that counteract effector responses to clear residual parasites. Ongoing co-evolutionary adaptation by both the host and parasite likely led to unique co-habitation mechanisms. Therefore, my focus is on how parasites manage to manipulate host immunity to establish long-lived infections.

1.9.10. Parasite persistence, Tregs, and concomitant immunity

Protective immunity is reliant on CD4⁺ Th1-mediated immunity, characterised by the production of IFN γ and TNF⁴⁹. IFN γ produced by the CD4⁺ Th1 cells activates phagocytes through the IFN γ receptor and STAT1 signaling, which leads to the upregulation of inducible nitric oxide (iNOS) and subsequent conversion of L-arginine into nitric oxide (NO). NO kills or metabolically inactivates the parasites within host phagocytes⁴⁵⁹. TNF downregulates expression of arginase-1, which competes with iNOS for L-arginine^{460,461}. While *Leishmania major* lesions generally heal, a small population (10^2 - 10^4) of parasites continue to be present in healed skin despite the

generation of robust Th1 immunity^{95,462,463}. These persistent reservoirs maintain concomitant immunity against *Leishmania* reinfections^{229,464,465}. At the same time, such long-lived parasitic reservoirs pose a significant risk of disease reactivation in infected individuals^{230,466,467}.

Post lesion resolution, persistent *Leishmania* parasites in the tissue emerge to be vital for the maintenance of protective immunity against reinfection, yet the cellular dynamics which allow for parasite persistence remain incompletely understood^{34,145}. Interestingly, there seems to be two different populations of persistent parasites, harbored within infected tissue macrophages. One population of parasites remains seemingly quiescent, but another population of parasites continues to replicate¹⁴⁵. However, because during the chronic stages the total number of parasites remain unchanged, it is possible to speculate that a state of equilibrium between proliferation and immune responses are established and maintained at this disease stage. This supports the fact that complete parasite clearance results in a loss of long-term immune memory^{34,49,145}. Additionally, these parasites appear to reside in iNOS⁺ host cells, which were largely regarded as cells that can clear parasites through NO production¹⁴⁵. This observation is particularly notable, as it disagrees with the “sanctuary site” theory that proposes that parasites can survive long-term in cells that do not upregulate protective molecules, such as NO. One possibility is that NO preferentially kills metabolically active parasites by inhibiting cell metabolism and targeting amastigotes to a greater extent, as supported by *in vitro* experiments¹⁴⁵. Another explanation is partial resistance to iNOS by the surviving parasites themselves¹⁴⁵. Thus, while persistent *Leishmania* infection is required for maintenance of protective T cell immunity, the possibility of reactivation remains a health concern, prompting further research into cellular mechanisms that allow parasites to persist long-term despite generation of strong CD4⁺ T cell responses *in vivo*.

The immune response is shaped by a delicate balance between effector and regulatory mechanisms that must quickly eliminate pathogens while limiting damage to healthy tissue. Regulatory T cells suppress effector T cell responses and are pivotal for the resolution of inflammation and tissue repair^{259,468}. Tregs seem to play a multifaceted role in leishmaniasis: Treg-driven suppression of Th1 response prevents immunopathology and induces tissue healing, but at the same time prevents complete parasite clearance by the Th1 cells. While Tregs constitute 5-10% of the peripheral blood CD4⁺ T cell population in both mice and humans, natural regulatory T cells, expressing CD25 and CTLA-4 make up as much as 40-50% of the CD4⁺ T cell population in chronic *L. major* lesions in mice^{229,469-471}. Additionally, Tregs are found in lesions from patients suffering from cutaneous leishmaniasis due to *L. major* and *L. braziliensis* infections^{469,472,473}. Increased accumulation of functional Tregs in human chronic biopsy samples compared to acute infection directly indicates the role of Tregs in later stages of infection, as their healing and immunosuppressive functions interfere with parasite clearance. Studies with human *L. guyanensis* also confirmed recruitment of Tregs to the site of infection and demonstrated that Tregs purified from biopsy samples decreased IFN- γ production by T cells stimulated with *L. guyanensis*⁴⁷⁰. Interestingly, PBMCs from patients with asymptomatic *L. major* infection present with significantly higher FoxP3 mRNA and suppressive function of Tregs compared to healed patients⁴⁷⁴, also suggesting that Tregs are involved in suppressing Th1-driven immunopathology and protecting the parasites. Moreover, Tregs from patients with acute and healed infections were Ki-67⁺ and thus undergoing proliferation, and Treg numbers are linked with biological outcomes during leishmaniasis⁴⁷¹. During the first 4 weeks of infection, Tregs were shown to comprise nearly half of total T cells in the mouse dermis, where they inhibited macrophages harboring parasites, produced IL-10 and inhibited IL-12 production that is amenable for optimal parasite growth and

further Treg accumulation²²⁹. At weeks 5-7 there is a decline in the proportion of Tregs, and this is a period of rapid effector T cell expansion, lesion development and parasite clearing²⁴⁶. During lesion healing, tissue repair and resolution of inflammation correlate with the accumulation and expansion of Tregs at the infection site where they play a role in establishing homeostasis²²⁹.

The strongest evidence for the role of Tregs in long-term *Leishmania major* persistence is the that upon in vivo Treg depletion, sterilizing immunity is achieved, while memory is lost²²⁹. CCR5 is preferentially expressed on Tregs, and CCR5 deficiency is associated with an increase in anti-*L. major* effector T cell expansion and IFN γ production within infection sites⁴⁷⁵. Infected CCR5^{-/-} mice displayed higher numbers of parasite-specific effector T cells at the site of infection, yet significantly lower frequency of IL-10-producing cells, suggesting that they lack IL-10 producing Tregs at the lesion site, leading to parasite clearance. Interestingly, the same study demonstrated that expression of CCR5 ligands was actively induced by *L. major* infection and correlated with recruitment of Tregs in infected skin. Regulatory T cells from human lesion sites had an increased proportion of CCR5 as well⁴⁷¹. Ablation of Langerhans cells also led to decrease in Tregs and IL-10, linked with enhanced Th1 response⁴⁷⁶ as well as inactivation of aryl hydrocarbon receptor results in a 30% decrease in Treg population, decrease in IL-10, increase in IFN γ and better resolution of *L. major* infection⁴⁷⁷. In a model where galectin-3 deficiency increases the frequency of peripheral Tregs, severity and *L. major* parasite burden are increased⁴⁷⁸. Together these studies demonstrate a direct positive correlation between Treg recruitment, numbers, and functionality with *L. major* burden. Since it is also demonstrated that *L. major* may induce recruitment of Tregs⁴⁷⁵, it is possible that parasites evolved to utilize the naturally occurring mechanism of Treg-induced homeostasis in order to evade complete clearance by the Th1 cells.

Tregs in the lesions have been shown to produce IL-10 and not IFN γ , while CD4⁺CD25⁻ T cells produce IFN- γ only and not IL-10. In the context of *Leishmania major* infection, IL-10 and TGF β inhibit TNF and IFN- γ production and function³³⁴, induce downregulation of co-stimulatory molecules CD40, CD80, and CD86 induced by the antigen-specific effector cells^{335,336}, inhibit NO synthesis³³⁷, and induce macrophages to secrete anti-inflammatory cytokines. Importantly, this response was demonstrated to be antigen specific. Tregs recovered from healed sites in C57BL/6 mice only produce IL-10 if they are restimulated with *Leishmania* antigen *ex vivo*, arguing that the majority of Tregs infiltrating healed lesions are *Leishmania* specific²²⁹. Transfer of Tregs isolated from healed lesions into wild-type and RAG^{-/-} mice one day prior to *L. major* infection dramatically exacerbate lesion development and parasite growth. In contrast, transfer of effector T cells alone into RAG^{-/-} mice prior to challenge led to T cell expansion and recruitment to the site of infection and resulted in parasite clearance and no latent infection development. Transfer of both Tregs with effector T cells into RAG^{-/-} mice led to a reduction in the number of IFN γ -producing effector cells, an increase in parasite numbers and subsequent establishment of latency, mirroring the course of infection in C57BL/6 mice²²⁹. Importantly, similar observations were reported regardless of the source of Tregs (isolated from healed lesions vs lymph node from naïve mice), indicating that both pre-existing and infection-mediated expanded *Leishmania*-specific Tregs can mediate suppression *in vivo*⁴⁷⁹.

Tregs purified from IL-10^{-/-} mice do not mediate persistence during the healed stage of *Leishmania major* infection²²⁹. IL-10 has been shown to suppress the activated state of macrophages and DCs and make them unresponsive to IFN γ ⁴⁸⁰. In the context of a non-healing strain of *L. major*, in addition to Tregs, a large source of IL-10 is CD4⁺CD25⁻FoxP3⁻ cells^{481,482}.

The increased production of IL-10 during the immune response to this strain leads to uncontrolled parasite division and subsequent tissue destruction that outweigh Treg healing capacity.

Subsequent studies have also demonstrated the role of Tregs during *Leishmania* infection by promoting their in vivo expansion through suppression of Th1-driven parasite control. Inoculation of *L. major* healed mice with heat-killed parasites rapidly increased the number of IL-10 producing Treg numbers and resulted in the expansion of parasite numbers in the previously healed mice²³⁰. Treg numbers also increase with aging in the periphery of mice and in the blood of elderly people, and these Tregs are functional⁴⁶⁷. In experimental model of leishmaniasis, at 24 months post-healing *L. major* infection, aged C57BL/6 mice exhibit spontaneous reactivation of the lesion due to the gradual accumulation of Tregs⁴⁶⁷. This study suggests that although lesions heal, the natural accumulation of Tregs with aging can cause a risk of lesion reactivation in humans. Depletion of Tregs in these mice significantly increased the production of IFN γ by effector T cells. This study also demonstrated that Tregs in aged mice exhibit high IL-10 production in response to *L. major*-infected DCs, as compared to uninfected controls, reinforcing the importance of antigen specificity of Tregs⁴⁶⁷. Thus, these studies provide additional evidence that an equilibrium between effector and regulatory T cells are established in healed skin, with increased Treg numbers tipping this balance towards disease reactivation⁴⁸³. What remains unclear is the dynamic interplay between *Leishmania*-specific Tregs and effector T cells that facilitate parasite persistence, and how antigen recognition enhances Treg recruitment, retention and survival within the healed lesions.

1.9. Intravital microscopy in the study of immunology

Reductionist *in vitro* studies have been invaluable in providing the basis for understanding on generation of immunity against pathogens. However, they fail to recreate the biochemical, physiochemical, and temporal aspects of tissue environments. With the help of two-photon microscopy we can characterize cellular behaviours *in vivo* and understand their dynamics in different immune environments, consequences of which have direct effect on the specificity, breadth, and magnitude of immune response. Indeed, ground-breaking first studies using 2P-IVM in intact⁴⁸⁴ or exhumed lymph nodes^{485,486} demonstrated that naïve T cells travel more vigorously in the T cell zone than reported previously in the *in vitro* studies⁴⁸⁶. This speed increases the chances of encountering an APC presenting cognate antigen, without which a T cell-APC interaction is of short duration. The dramatic change in cell dynamics between APCs presenting cognate antigen and T cells is demonstrated by durable APC:T cell conjugates, which remain in contact for up to 36-48 hours and lead to T cell proliferation, differentiation, and generation of memory T cell responses^{484,485}. The impact of the environment, in particular various environmental cues, similarly dictate the interaction between thymocytes and stromal cells during thymic selection⁴⁸⁷. B cell dynamics have been similarly observed with 2P-IVM, where B cells migrate robustly in the germinal centers in order to encounter a follicular DC with specific antigen⁴⁸⁸. High motility also aids B cells in receiving necessary survival signals, as they compete for both the antigen and the T cell help^{488,489}. As complexity of fluorescence reporters expands, it is now possible to measure signal transduction in living cells and combine the understanding of cell migration patterns and signaling status of cells^{490,491}. As the nature of pathogen most often dictates how adaptive immunity is generated, 2P-IVM has laid the foundation for *in vivo* characterization of immune responses⁴⁹²⁻⁴⁹⁶.

In 1930s Marie Göppert first proposed that it could be possible to excite a molecule to a higher energy state from the ground state with the use of two photons with equal frequencies instead of one⁴⁹⁵. During one photon excitation a fluorophore is excited with a particular wavelength and the emitted light is collected by the camera of the microscope. While indispensable, this method is limited by the resolution and contrast quality, as the entire illuminated sample in and outside the focal plane of interest emits fluorescence. Alternatively, 2P-IVM utilizes two photons of higher wavelengths, or half the energy, and the near simultaneous absorption only occurs at the focal plane⁴⁹⁷. The technology, however, was only developed in 1990 by Wilhelm Denk when he combined a point-scanning microscope with an infrared mode-locked laser⁴⁹⁷. Because the fluorophore of interest is only excited at the focal plane, pinhole in front of the detection unit is no longer necessary. The outcomes of this are significantly reduced phototoxicity and photobleaching of out-of-focus specimens and, therefore, prolonged imaging in a 3D space. Near infrared light possesses a long wavelength and thus has the ability to penetrate deeper into tissues with minimal scattering and can avoid melanocytes and heme molecules in erythrocytes, which possess pigment-containing proteins^{498,499}.

2P-IVM approaches are becoming increasingly accessible with the development of fluorescent proteins and dyes, fluorescent reporter mice, and improvements in 4D analysis software, and have already helped us observe unique in vivo behaviours of immune responses to bacterial, viral, and parasitic infections such as pathogen distribution by phagocytes^{47,500}, swarming responses by neutrophils to damage or injury⁵⁰¹⁻⁵⁰³, and reservoirs of bacteria following infection⁵⁰⁴. 2P-IVM has even highlighted the important functions of macrophages in maintaining homeostasis. Liver-resident Kupffer cells have been shown to sequester dead cells in the liver⁵⁰⁵,

and alveolar macrophages have been demonstrated to engulf inhaled bacteria and shield it from neutrophils, thus preventing further neutrophil recruitment and tissue inflammation⁵⁰⁶.

1.10.1. T cell migration and search strategies in the skin

A single T cell expresses a specific TCR that can only recognize a small range of peptide-MHC complexes. Only one in 10^5 - 10^6 T cells will recognize a given antigen^{507,508}. Therefore, in order to encounter their cognate antigen in vivo, T cells exhibit exquisite and complex migration and search strategies. Naïve T cells first encounter an antigen in the secondary lymphoid organs. Lymph nodes in particular are highly organized organs. They provide a confined and structured environment to facilitate T cell scanning of APCs arriving from other tissues⁵⁰⁹. This structure is necessary because naïve T cells have not encountered their antigen and therefore are not activated to participate in the immune response. Once activated, however, T cells upregulate mechanisms and receptors necessary to modify and direct their migration patterns to enter the periphery and exert effector responses in appropriate tissues.⁵¹⁰ T cell motility is a fundamental requirement for execution of a secondary immune response against intracellular pathogens, such as Leishmania.

Host immunity must quickly detect and respond to foreign antigens upon pathogenic insult, which is largely accomplished by different immune subsets working in concert to enter and scan for infections. In the context of T cell activation, T cells display distinct migratory behaviors that are designed to maximize contact with other immune or stromal cells in various tissues, thereby gathering information about the nature of the infection through cell-cell contacts. While T cell migration is generally thought of as a cell-intrinsic mechanism involving rearrangement the actin machinery, it is now well understood that tissue-derived chemical and physical cues dictate distinct migratory T cell behaviors, and can further modulate their downstream functions⁵¹¹. T cell motility is often described as a “random walk”⁵¹², defined by stochastic movements in random trajectories.

There are two types of random walk used to describe T cell motility: Brownian-type and Levy-type (Figure 5) ^{513,514}. Brownian motion describes diffusive random walks with no general direction or of short persistence. The randomness of movement translates to lack of environmental cues to control direction. Levy walks describe a mix of long trajectories and short random turning, which describes a general explorative behaviour or the result of unique interactions with their immediate environment. At the same time, given proper cues T cells can undergo fully straight migration⁵¹⁵. Upon cognate antigen stimulation, stable APC:T cell contacts are followed by differentiation and proliferation and resumption of T cell motility, largely due to expression of unique chemokine receptors. Chemokine expression in tissues are responsible for the recruitment and positioning of effector T cells⁵¹⁶. The downregulation of secondary lymphoid organ homing receptors CD62L and CCR7 and the reciprocal upregulation of inflammatory chemokine receptor expression is crucial for their migration into peripheral tissues⁵¹⁷. For example, Th1 cells express CCR4, CCR5, CXCR3, and CXCR6, which direct their entry into the skin. At the site of infection Th1 cells can induce further production of chemokines to recruit other cell types to the environment. The site of naïve T cell priming also affects the end location of effector T cells⁵¹⁸. For example, DCs and stromal cells from Peyer's patches in the gut produce retinoic acid, which triggers the expression of $\alpha 4\beta 7$ and CCR9 on T cells⁵¹⁹. The interaction of these molecules with their ligands, MAdCAM and epithelial-derived CCL25, respectively, directs effector T cells to the lamina propria and epithelium of the small intestinal mucosa⁵²⁰. CCR4, CCR5, and CCR10 are implicated in T cell recruitment and retention in the skin⁵²¹. Tregs, similar to CD4 T cells, also downregulate CCR7 and upregulate CCR4, CCR5, and other chemokine receptors that regulate their homing into peripheral tissues. However, this switch appears to occur faster and allows for a

more rapid recruitment into tissue⁵²². Similarly, localized priming endows Tregs with specific tissue-homing adhesion molecules, such as skin-homing E- and P-selectins⁵²³.

In the periphery during inflammation, T cell motility differs significantly from their motility in the lymph node. The framework of most peripheral tissues is characterized by a dense extracellular matrix which changes during inflammation and injury. Unlike in the lymph node, where collagen fibers are coated by fibroblastic reticular cells, T cells in the skin are directly exposed to collagen fibers and ECM components that act as guidance cues for movement. Changes in the ECM directly affect T cell search behaviours. Inflammatory mediators, such as TGF β , IFN γ , and TNF, increase protease secretion and ECM turnover, and loosen ECM framework⁵²⁴. Loose network results in integrin-dependent T cell migration, termed haptokinesis⁵¹¹. The frequency of T cell-APC interaction is high because most T cell recruited to the site of infection will be antigen specific and the site itself will harbor an abundance of antigen. If T cells can follow chemotactic cues to the APC-antigen deposits, they can efficiently locate cognate antigen.

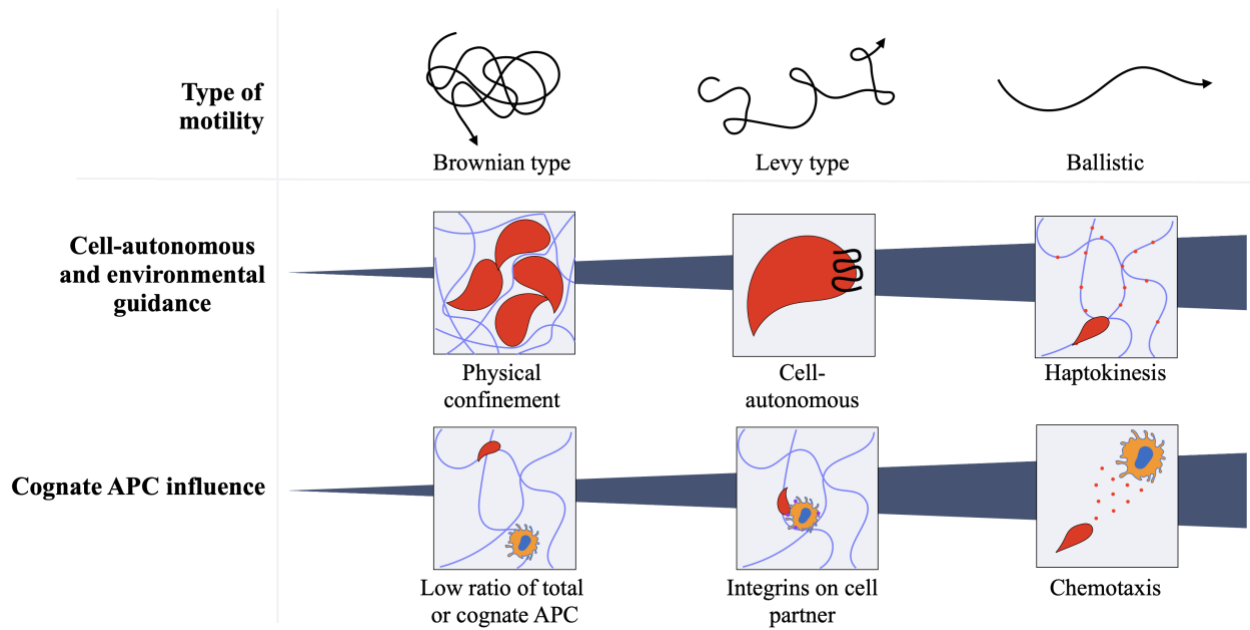


Figure 5. Factors influencing T cell motility.

The various T cell strategic motility behaviours span from Brownian-type normal diffusion, Levy-type mix of long trajectories and short random turning, and ballistic highly directional trajectory. These behaviours can be influenced by a variety of circumstances. Environmental guiding structures, such as 3D confinement, and cell-autonomous properties have an impact on strategic motility.

1.10.2. Parallels in T cell migration in solid tumors and L. major lesions

We can also draw parallels on how T cell migratory behaviors are altered in tissues from studies in solid cancers, which are typically an immunosuppressive environment. Tumor tissue remodeling by regulation of ECM directly inhibits accumulation, activation, and migration of T cells. First, tumors have physical barriers that prevent effector T cells from entering the core of the tumor⁵²⁵. Instead, T cells preferentially migrate in areas of loose collagen which surround the tumor and poorly penetrate dense tumour foci, contributing to their inability to eliminate tumors efficiently^{526,527}. Second, dysregulation of chemokine gradient formation can direct effector T cells away from the tumor parenchyma. For example, stromal cells surrounding pancreatic tumor foci produce CXCL12, which direct CTLs away from the tumor core⁵²⁸. Lastly, a high number of APCs recruited during tumorigenesis provide ineffective targets for T cell search or provide inappropriate signals for T cells reactivation^{529,530}.

T cell extravasation, which is often the first step in T cell trafficking towards tissue, is frequently impeded by endothelial cells' low expression of T cell-specific adhesion molecules, co-stimulatory ligands, or chemoattractants in the context of tumor tissue. Shutting down of T cell-specific chemoattractants may be related to epigenetic silencing or disruptive post-translational changes. Enhancer of zeste homologue 2 (EZH2)-mediated histone H3 lysine 27 trimethylation (H3K27me3) and DNA methyltransferase 1 (DNMT1)-mediated DNA methylation in the ovarian cancer models repress the tumour production of T helper 1 (T_H1)-type chemokines CXCL9 and CXCL10⁵³¹. Importantly, CXCL9 and CXCL10 are known to interact with CXCR3 on Th1 cells and play an important role in Th1 cell trafficking⁵³². CXCL9 and CXCL10 are directly induced by IFN γ in a feedback loop mechanism⁵³³. Antibody blockade of these chemokines drastically decreases average T cell velocity in inflamed skin⁵³⁴ and impedes the search strategy and capacity

of Th1 cells to locate antigen. Importantly, CXCL10 production is suppressed by the presence of *L. major* in host cells through the function of gp63¹⁵⁵. CXCL10 has been suggested to protect against *Leishmania* infection, as susceptible BALB/c mice demonstrate a defect in CXCR3 upregulation^{535,536} and exogenous CXCL10 is protective in both cutaneous and visceral leishmaniasis^{537,538}.

In cases of successful trafficking towards and within the tumor site, T cells will encounter areas of hypoxia. Sustained hypoxia is accompanied by immature vasculature, CD8 T cell evasion and tumor progression⁵³⁹. IL-4 and IL-10 promote M2-like polarization of TAMs and induce CD206 expression⁵⁴⁰, and TAMs have been found preferentially in tumor hypoxic areas, where they accumulate HIF-1 and HIF-2⁵³⁹. HIF-1 α was also revealed to be essential for macrophage-mediated suppression of T cells in hypoxic settings⁵⁴¹, in addition to studies indicating a function for HIF-1 α and HIF-2 α in the enhancement of macrophage angiogenic characteristics. Under hypoxic stress CD4⁺ T cells also upregulate FoxP3 expression through HIF-1 to the Foxp3 promoter region⁵⁴² and tumor hypoxia can also attract Treg cells inside the tumor through secretion of tumor-specific chemoattractants⁵⁴³. Parallels can be drawn to *Leishmania* infection, which is generally characterized by a low-oxygen environment within the infected tissue⁵⁴⁴. During cutaneous leishmaniasis, lesions from human patients contain elevated levels of HIF-1 α and the HIF target *Vegfa*⁵⁴⁵, which promotes lymphangiogenesis during leishmaniasis. It was shown that macrophages experience hypoxic conditions at the cellular level in the skin, and the site of infection VEGF-A and other transcripts which indicate HIF-1 α and HIF-2 α activation were elevated⁵⁴⁶. Taken together, in the context of solid tumors or leishmania lesions, hypoxic conditions lead to the induction of tissue-healing and immunosuppressive mechanisms, which in turn allow for the tumor growth and parasite survival.

1.10. Overall goal:

The fact that *L. major* promastigotes can be engulfed by sandflies even at the healed stages of infection suggests a sophisticated evolutionary mechanism of parasite survival strategy. In my PhD thesis, I unveil the dynamic interplay between effector T cell responses and suppressive Tregs during *Leishmania major* infection and demonstrate that tipping this balance has implications on either sterilizing immunity or re-activation of disease. The overall goal of my PhD thesis is to gain a better understanding of the cellular and molecular mechanisms that lead to the establishment of concomitant immunity towards *Leishmania major*, using a combination of novel mouse models, in vitro co-culture studies and two-photon intravital microscopy approaches.

1.11. Overall hypothesis:

Antigen recognition reinforces Regulatory T cell mediated *Leishmania major* persistence

1.12. Overall rationale:

Concomitant immunity against intracellular parasites is dictated by the strength and breadth of the immune response and parasite-driven escape mechanisms that prevent complete clearance. Infection by *Leishmania major* causes severe skin lesions, and, while strong T cell responses are generated as a result, parasite clearance is incomplete and often leaves a small pool of persistently infected cells. Prior work speculates that a long-lasting balance between effector and regulatory T cells is established after infection, which maintains a small population of surviving parasites. What remains unclear is whether Treg suppression within the healed lesion is driven by antigen recognition and the dynamic interaction between Tregs and effector T cells that support parasite persistence.

Based on my results, I propose a model for suppression of effector T cell functions, which is mediated by an immunodominant *Leishmania*-specific Treg population. My project has three objectives:

Objective 1:

To evaluate whether Th1 cells from the novel PEPCCK-specific TCR transgenic mouse model exhibit strong anti-Leishmania major responses in vitro and in vivo.

Objective 2:

To assess the mechanism of antigen-specific Treg suppressive capability in vitro

Objective 3:

To uncover the role of PEPCCK-specific Tregs in modulating Treg:Th1 balance in chronic L. major lesions

1.13. Significance:

Using a novel TCR transgenic mouse model that allows me to visualize immunodominant antigen-specific T cell responses directly in living tissues, my observations are the first to address whether antigen recognition is required for proper Treg function *in vivo*, a long-standing question in the field.

Chapter 2: Materials and Methods

2.1. Mouse models, housing, and breeding

Male wild type and albino C57BL/6 mice (6–8 weeks old) were obtained from the University of Manitoba Central Animal Care Services (CACS) breeding facility. FoxP3 EGFP, CAG ECFP, and mRFP C57BL/6 mice were purchased from Jackson laboratories (Bar Harbor, ME). PEPCK TCR-transgenic mice on the C57LB/6 background were acquired from an in-house breeding colony from CACS (University of Manitoba). Albino C57BL/6 male mice at 6-8 weeks of age were used for intravital microscopy experiments, while wild type males were used for control T cell and bone marrow-derived macrophage and dendritic cell isolations. All mice were maintained in a specific pathogen-free environment, and all experiments were approved by institutional ethics committee in accordance with the Canadian Council for Animal Care guidelines.

2.2. L. major growth and in vitro and ear infection protocols

Wild-type (WT), GFP, and drRed *L. major* MHOM/80/Friedlin (FV1) were grown in M199 medium supplemented with 20% heat-inactivated FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin (complete M199) in a 27°C parasite incubator. For infection, day-7 stationary-phase promastigotes were washed three times with sterile PBS at 2000g for 15 minutes and counted using an optical microscope at ×100 magnification. C57BL/6 mice were infected in the ear pinna with 10 µL of PBS containing 1×10^6 promastigotes using BD 0.3mL U-100 Insulin syringes 29G x 1/2". Killed parasites were prepared by either autoclaving

2.3. Ear, Lymph node, and spleen processing

For ears, ventral and dorsal sheets were separated, then submerged in 1 mL RPMI containing Liberase TM TL (Sigma) for 90 minutes at 37°C. Following digestion tissues were homogenized

into a single cell suspension with a 1mL syringe plunger on a 40 μ m mesh nylon and depending on experiment either immediately stained for Flow Cytometry directly or cultured for 4 hours in complete RPMI containing 10% FBS (VWR Seradigm), 2 mM GlutaMAX (Gibco), 1mM sodium pyruvate (Corning Cat #25-000-CI), 10mM HEPES (Sigma-Aldrich) and β -Mercaptoethanol and either with 2 μ L/mL cell activation cocktail with Brefeldin A (500x) (BioLegend Cat #423304) or 1 μ L/mL of Brefeldin A Solution (1000x) (Biolegend Cat# 420601) alone. Lymph nodes and spleens were removed and homogenized with a 1mL syringe plunger on a 70 μ m cell strainer and prepared for flow cytometry staining or naïve T cell or Treg isolations.

2.4. *CD4⁺ T cell purification and Th1 culture*

Spleens from wild type or TCR-transgenic PEPCK-specific (PEG) C57Bl/6 mice were excised and mashed with a 40 μ m mesh nylon strainer, washed with PBS, and resuspended at a concentration of 1×10^8 cells/mL in PBS supplemented with 2% FBS (VWR Seradigm cat #1500-500) and 1mM EDTA (Sigma-Aldrich, cat #E7889). Naïve T cells were isolated using Stemcell™ EasySep™ Mouse Naïve CD4⁺ T Cell Isolation Kit (Cat# 19765) and re-suspended at a concentration of 1×10^6 cells/mL in complete RPMI media in a 24 well plate with 25 μ L/mL Dynabeads® Mouse T-Activator CD3/CD28 (Thermo Fisher Scientific Cat# 11456D), 20ng/mL IL-12 (Biolegend Cat# 577002) and 5 μ g /mL anti-mouse IL-4 antibody (Biolegend Cat# 504108). After 48 hours of incubation at 37°C, Dynabeads® were removed from T cells using Stemcell™ EasySep™ Magnet (Cat# 18000). At day 2 cells were counted and resuspended at 0.2×10^6 cells/mL in complete RPMI and 20ng/mL IL-12, 5 μ g /mL anti-mouse IL-4, and 25 ng/mL IL-2 (Peprotech Cat# 200-02). After 48 hours of incubation cells were resuspended in complete RPMI supplemented with 25 ng/mL IL-2 at 0.5×10^6 cells/mL. Day 7 expanded cells were used for all experiments.

2.5. *Dendritic cell and Macrophage cell culture and infection*

Bone marrow–derived macrophages (BMMs) and dendritic cells (BMDCs) were differentiated from progenitor cells obtained from the femur and tibia of a naive C57BL/6 mice. The bones were separated from the surrounding muscles using a bone chisel and the content of the bones was flushed with 5 ml of RPMI 1640 into a polypropylene petri dish using a syringe and a 25-G needle. Single-cell suspensions were created by gently pipetting up and down. Following depletion of RBCs with ACK lysis buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM Na₂EDTA [pH 7.2–7.4]), for BMM differentiation cells were seeded at a density of 25×10^6 /ml in T75 ml flasks in complete RPMI 1640 medium supplemented with 10% FBS (VWR Seradigm), 2 mM GlutaMAX (Gibco), 1mM sodium pyruvate (Corning Cat #25-000-CI), 10mM HEPES (Sigma-Aldrich) and β -Mercaptoethanol with 25ng/mL M-CSF (Biolegend Cat# 574808) for 72 hours at 37°C in a CO₂ incubator. At 72 days adhered cells reached confluency of 80-90% and non-adhered cells were washed off with PBS. BMMs were detached using Accutase® Cell Detachment Solution (Biolegend Cat# 423201) for 15 minutes at 37°C in a CO₂ incubator. Detached BMMS were infected with distinct parasite strains at a cell/parasite ratio of 1:10. After a 6-h incubation (at 37°C), the cells were washed twice (centrifuged at 600 rpm for 5 min) to remove free parasites. For BMDC differentiation, single cell suspension was cultured in 100 x 15mm Petri dish (BD falcon) at 2×10^5 /ml in 10 ml of complete RPMI media containing 20 ng/ml of GMCSF (Peprotech, Indianapolis, IN) at 37 °C. After 3 days, cells were supplemented with 10 ml of freshly prepared supplemented RPMI medium containing 20 ng/ml of GMCSF (Peprotech). On the sixth day of the cell culture, 10 ml of the culture medium was taken away from the growing cells carefully to minimize disturbing the settled cells. The collected culture medium was spinned down for 10 mins at 1000 rpm after which the cell pellets were resuspended in fresh 10ml 20 ng/ml GM-CSF RPMI medium and put back into the Petri dish. On day 8, the BMDCs were ready for use.

2.6. *Regulatory T cell selection protocol*

Spleens from wild type or PEG C57Bl/6 mice were excised and mashed with a 40 μ m mesh nylon strainer, washed with PBS, and resuspended at a concentration of 1×10^8 cells/mL in PBS supplemented with 2% FBS (VWR Seradigm cat #1500-500) and 1mM EDTA (Sigma-Aldrich, cat #E7889). Tregs were extracted using Stemcell™ EasySep™ Mouse CD4⁺ CD25⁺ Regulatory T Cell Isolation Kit II (Cat# 18763) and resuspended at concentration of 1×10^6 cells/mL in complete DMEM media in a 24 well plate with 50 μ L/mL Dynabeads® Mouse T-Activator CD3/CD28 (Thermo Fisher Scientific Cat# 11456D) and 800ng/mL IL-2. After 48 hours of incubation at 37°C, 24 well plate was centrifuged at 300g for 5 minutes and 500 μ L was gently removed and replaced with fresh media supplemented with IL-2. Cells were monitored and 1 ml of media replaced daily. At day 5 Dynabeads® were removed from T cells using Stemcell™ EasySep™ Magnet. Day 7 expanded cells were used for all experiments.

2.7. *Th1 and macrophage co-culture experiments*

Day 6 BMMs were seeded a cell concentration of 0.25×10^6 cells/well in a 24 plate in Complete RPMI containing β -Mercaptoethanol and 25ng/mL MCSF and incubated overnight at 37°C in a CO₂ incubator and allowed adhere to the bottom. In cases where BMMs were used for Immunohistochemistry, sterile glass slides were put at the bottom of the well for BMMs to adhere to. Wild-type or GFP *Leishmania major* parasites were added to culture at a preferred ratio and plates were incubated at 37°C in a CO₂ incubator for 6 hours. After incubation supernatant and non-penetrated parasites were removed. 0.25×10^6 cells/well of either control or PEPCK Th1 in vitro derived day 7 cells were added to each associated well. Cells were allowed to incubate for 18 hours and for experiments evaluating cytokine production, 1 μ L/mL of Brefeldin A Solution

(1000x) (Biolegend Cat# 420601) was added after the 18 hours and incubated for additional 4 hours. Cells were then stained for flow cytometry.

2.8. *Th1, macrophage and Treg co-culture experiments*

Day 6 BMMs were seeded a cell concentration of 0.25×10^6 cells/well in a 24 plate in Complete RPMI containing β -Mercaptoethanol and 25ng/mL MCSF and incubated overnight at 37°C in a CO₂ incubator and allowed adhere to the bottom. In cases where BMMs were used for Immunohistochemistry, sterile glass slides were put at the bottom of the well for BMMs to adhere to. Wild-type or GFP *Leishmania major* parasites were added to culture at a preferred ratio and plates were incubated at 37°C in a CO₂ incubator for 6 hours. After incubation supernatant and non-penetrated parasites were removed. 0.25×10^6 cells/well of either control or PEG Th1 day 7 cells were added to each associated well. In suppression co-culture experiments, prior to co-culture PEG Th1 cells were stained with either Celltracker Blue (CMAC; 20 μ M) or Celltracker Green (CMFDA; 0.5 μ M) to distinguish between Th1 cells and Tregs. Tregs were then added at a preferred ratio (1:1). For some experiments, 1 μ L/mL of anti-IL-10 antibody (Biolegend) was added. Cells were allowed to incubate for 18 hours and for experiments evaluating cytokine production, 1 μ L/mL of Brefeldin A Solution (1000x) (Biolegend Cat# 420601) was added and incubated for additional 4 hours. Cells were then stained from flow cytometry.

2.9. *Flow cytometry staining*

At indicated times, mice were sacrificed and the ears, cervical dLNs, and inguinal non-dLNS were made into single-cell suspensions in complete RPMI medium. Ears were digested using LiberaseTM TL (Sigma, Cat# 5401020001) for 90 minutes at 37°C. The cells were directly stained ex vivo for surface expression of CD4, CD45, CD3 and CD25 and intracellularly for Foxp3 or Tbet by using an eBioscienceTM Foxp3 / Transcription Factor Staining Buffer Set (Cat # 00-5523-

00). In some experiments, the cells were stimulated with Cell activation cocktail with Brefeldin A (BioLegend Cat #423304) and incubated at 37°C for 4 hours prior to flow cytometry staining for IL-10 and/or IFN γ . Phenotypic characterization of BMMs and CD4⁺ T cells were performed using the BD FACSCanto-II and analyzed with FlowJo software (Tree Star).

Target molecule	Conjugated fluorophore	Antibody clone	Target molecule	Conjugated fluorophore	Antibody clone
CD11c	PE	N418	CD4	FITC	GK1.5
CD40	PE	3 23	CD25	PE/CY7	3C7
F4/80	PE/CY7	BM8	CD8a	PE	53-6.7
1-Ab	PE/CY7	AB6-120.1	CD62L	APC/CY7	MEL-14
CD11b	APC	M1/70	CD44	Pacific Blue	IM7
CD86	APC/CY7	GL-1	CD3	APC	17A2
1-Ab	Pacific Blue	AF6-120.1	CD45	APC	30-F11
H-2Kb/H-2Kd	FITC	28 8 8	CD45	PE/CY7	103114
CD80	PE/CY7	16-10A1	CD44	PerCP/CY5.5	1M7
			CD44	BV421	IM7
TIGIT	PE/CY7	G1GD7	CD4	PE/CY7	GK1.5
PD-1	eFluor450	J43			
LAG-3	PE	C9B7W	IFNγ	PE	XMG1.2
GITR	PerCP/CY5.5	DTA-1	IFNγ	APC	XMG1.3
IL-10	PE	JES5-16E3			
FoxP3	PE	3G3			
FoxP3	FITC	3G3			

Table 1. List of fluorochrome-conjugated antibodies used in flow cytometry.

2.10. PEPCK tetramer staining

I-A^b–PEPCK_{335–351} tetramer and the corresponding negative control tetramer (I-A^b–CLIP_{87–101}) were generated at the NIH Tetramer Core Facility, Emory University, Atlanta, GA. Cells were stained with tetramer I-A^b–PEPCK_{335–351} (2 μ g/ml) or I-A^b–CLIP_{87–101} control for 30 min at 37°C. The cells were then stained routinely for surface markers or intracellular cytokines.

2.11. Single-cell immunohistochemistry

After co-culture, T cells were gently washed off and BMMs were fixed with 2.5% Paraformaldehyde. Slides were washed, blocked with Fc blocker (Innovex), 4% mouse serum (ImmunoReagents) and 4% goat serum. The primary antibody used was rat anti-F4/80 (Abcam). Secondary antibodies used were AF568-conjugated goat anti-rat (Abcam) at 1:1000 dilution, and AF488 conjugated chicken anti-GFP (Abcam) at 1:5000 dilution. Slides were stained with Hoechst 33342 (Molecular Probes) for 30 min at 1:2000 dilution and mounted with ProLong Gold (Invitrogen). Images were acquired using the Zeiss AxioObserver confocal microscope and analyzed using ImageJ.

2.12. ELISA Analysis

ELISA supernatant analysis was performed with Eve Technologies Mouse Cytokine Proinflammatory Focused 10-Plex Discovery Assay® Array (MDF10). After normalizing for background cytokine expression, all expression values were expressed as % of maximal responses which was Th1 + *L. major* infected macrophage co-cultures. Expression values were then converted to log2 to describe increase or decrease fold change between the indicated experimental conditions. Values from three independent experiments are shown. Formula used: $\text{Log}_2(\text{experimental value}/(\text{average maximal response} - \text{average background response}))$.

2.13. Live-cell imaging in 3D collagen chambers

Collagen type I was used to recapitulate the three-dimensional (3D) fibrillar networks and were assembled as chambers as previously described^{547,548}. Bovine collagen (PureCol) was used to achieve a final concentration of 1.7mg/mL in each chamber. Cells were labeled with either Celltracker Blue (CMAC; 20μM), Celltracker Orange (CMTMR; 5 μM) Celltracker Green (CMFDA; 0.5 μM) or Celltracker Deep Red (1 μM), washed and embedded into collagen.

Chambers were allowed to solidify for 45 minutes at 37°C / 5% CO₂ and placed onto a custom-made heating platform attached to a temperature controller apparatus (Werner Instruments). A thermocouple device was used to continuously monitor and maintain the chamber temperature at 37°C. A multiphoton microscope with two Ti:sapphire lasers (Coherent) was tuned to between 780 and 920 nm for optimized excitation of the fluorescent probes used. For four-dimensional recordings of cell migration, stacks of 13 optical sections (512 x 512 pixels) with 4 µm z-spacing were acquired every 15 seconds to provide imaging volumes of 48 µm in depth. Emitted light was detected through 460/50 nm, 525/70 nm and 595/50 nm dichroic filters with non-descanned detectors. All images were acquired using the 20X 1.0 N.A. Olympus objective lens (XLUMPLFLN; 2.0mm WD).

2.14. Adoptive transfer protocol and Ear imaging

10-15x10⁶ day 7 control or PEG Th1 were stained with Celltracker Blue (CMAC; 20µM), Celltracker Orange (CMTMR; 5 µM) or Celltracker Deep Red (1 µM), colors alternating between experiments⁵⁴⁸. Cells were washed 3 times with PBS and transferred into infected mice via intravenous injection. Mice were anaesthetized and infected ear was prepared for microscopy⁴⁷. A multiphoton microscope with two Ti:sapphire lasers (Coherent) was tuned to between 780 and 920 nm for optimized excitation of the fluorescent probes used. For four-dimensional recordings of cell migration, stacks of 12 optical sections (512 x 512 pixels) with 4 µm z-spacing were acquired every 15 seconds to provide imaging volumes of 48 µm in depth. Emitted light was detected through 460/50 nm, 525/70 nm and 595/50 nm dichroic filters with non-descanned detectors. All images were acquired using the 20X 1.0 N.A. Olympus objective lens (XLUMPLFLN; 2.0mm WD). Automated 3D tracking of T cell centroids was performed for cell motility analyses with

IMARIS cell tracking software. Further cell track parameters (arrest coefficient and mean displacement) were analyzed in Matlab (Mathworks).

2.15. Lymph node imaging

Dendritic cells were labeled for 15 min at 37°C with Celltracker Green (CMFDA; 2 μ M) prior to injection of 5×10^5 DCs into the footpad of C57Bl/6 mice. 12 hr after DC transfer, mice were given immunomagnetic selected 3×10^6 CD4⁺ naïve T cells prepared from control or PEPCK-specific mice. T cells were stained for 15 minutes with either Celltracker Blue (CMAC; 15 μ M) or Celltracker Orange (CMTMR, 20 μ M) as previously described⁵⁴⁸.

For Intravital microscopy, mice were anaesthetized and the popliteal lymph nodes microsurgically exposed as previously described⁵⁴⁹. Imaging depth was typically 80–200 μ m below the lymph node capsule. A multiphoton microscope with two Ti-sapphire lasers (Coherent) was tuned to between 780 and 920 nm for optimized excitation of the fluorescent probes used. For four-dimensional recordings of cell migration, stacks of 12 optical sections (512 x 512 pixels) with 4 μ m z-spacing were acquired every 15 seconds to provide imaging volumes of 48 μ m in depth. Emitted light was detected through 460/50 nm, 525/70 nm and 595/50 nm dichroic filters with non-descanned detectors. All images were acquired using the 20X 1.0 N.A. Olympus objective lens (XLUMPLFLN; 2.0mm WD). Automated 3D tracking of T cell centroids was performed for cell motility analyses. Further cell track parameters (arrest coefficient and mean displacement) were analyzed in Matlab (Mathworks).

2.16. Statistical analysis

Unpaired Student's t test and Mann-Whitney U test were used for comparisons of datasets with normal and non-normal distribution, respectively, using Prism 9 (GraphPad). Median and p values

from statistical analyses are indicated in each graph. When p values were higher than 0.05, differences were considered as not significant.

Chapter 3: PEPCK-specific Th1 cells exhibit strong anti-Leishmania major responses in vitro and in vivo.

3.1. Introduction

Our collaborators Drs. Zhirong Mou and Jude Uzonna have identified an immunodominant, naturally processed *Leishmania* antigen, derived from the parasite phosphoenolpyruvate carboxykinase (PEPCK), which elicits a strong Th1 CD4⁺ immune response⁵⁵⁰. Using this finding, they have generated a TCR-transgenic PEPCK-specific mouse on the C57Bl/6 background, termed “PEG” mice, where CD4⁺ T cells recognize the PEPCK peptide_{335–351} presented in the context of I-A^b. Using these mice, I was able to visually characterize antigen-specific T cell responses to *L. major* and uncover insights into the immunological mechanisms that regulate resistance during leishmaniasis. Specifically, my goal was to utilize PEG mice to perform both in vitro and in vivo microscopy studies to better understand how T cells evoke anti-*Leishmania* major immunity through cell migration and cell-cell interactions. In this chapter, I performed functional and imaging studies to describe the migratory behavior of PEG T cells, and to determine how specific T cell responses were altered during acute and chronic stages of *Leishmania major* infection in the skin. Specifically, this chapter has 3 aims:

Aim 1: To characterize pro-inflammatory responses, cellular dynamics, and leishmanicidal activity exhibited by PEG T cells in response to *Leishmania major* infection in vitro

Aim 2: To characterize PEG T cell homing and migration within the lymph node and the parasite-challenged ear tissue during *Leishmania major* infected in vivo

Aim 3: To develop an ECFP⁺ PEPCK-TCR mouse model and characterize the cellular dynamics of antigen-specific T cells throughout the course of *Leishmania major* infection in vivo

Significance/Impact: The generation of PEG mice allows for direct visualization and functional analysis of anti-*Leishmania* CD4 T cell responses during infection, which has not been done previously. Aim 1 provides insights into whether there are survival strategies used by parasites themselves and characterizes the migratory capacity of PEG T cells and their ability to engage cell-cell interactions. This aim leads me to uncover the interplay between T cells, macrophages, and *L. major*, which dictates whether the parasite is killed or survives. Aim 2 evaluates the homing capacity and alterations in cellular dynamics of PEG T cells in vivo. Pilot microscopy studies of *L. major*-infected ears showed that residual parasites persist in infected cells at the site of primary infection long after skin is healed. Multiphoton Intravital microscopy (MP-IVM) experiments with naïve PEG cells and *L. major*-infected DCs shows the ability of PEG cells to recognize antigen and form prolonged contacts with DCs, indicating their ability to become activated after antigen recognition in vivo. Evaluating PEG Th1 cell homing capacity and migration responses to the *L. major*-challenged ear tissue demonstrates stark differences in response during acute and healed stages of infection. The objective of Aim 3 is to create internally-labelled PEG cells and evaluate whether they persist in the *L. major*-infected animal and show alterations in their migratory functions. Utilizing flow cytometry, ELISA, and two-photon microscopy, my approach provides a comprehensive view of dynamic anti-*Leishmania* T cell responses in vitro and in vivo.

3.2. Results

3.2.1. Aim 1: To characterize pro-inflammatory responses, cellular dynamics, and leishmanicidal activity exhibited by PEG T cells in response to *Leishmania major* infection in vitro

3.2.1.1. PEG Th1 cells display a strong IFN γ response upon co-culture with PEPCK-pulsed macrophages.

I isolated naïve CD4⁺ T cells from spleens of both wild-type control and PEG mice and activated them for 48 hours via CD3/CD28 bead stimulation in a Th1 polarizing cytokine milieu consisting of IL-12 and anti-IL-4 antibody. After 7 days of active proliferation, I performed phenotypic analysis of both T cell populations and observed no phenotypic changes between the two populations in terms of CD3, CD4, CD25, CD62L and CD44 expression (Figure 6A, B). To validate the specificity of PEG T cells, I co-cultured cells with PEPCK peptide-pulsed bone marrow-derived macrophages (BMM), at various concentrations of peptide (PEPCK_{335–351}) (Figure 6C). Intracellular flow cytometry showed high IFN γ production by PEG T cells but not by polyclonal wildtype control T cells (Figure 6D). At 0.0005 μ M of PEPCK peptide, IFN γ production is 5.31%, and this gradually increased to approximately 76% at 0.5 μ M of peptide. Co-culture with control Th1 cells induced a background IFN γ response of 2% at 0.5 μ M of PEPCK, and similar results were observed when no antigen is present. These results confirm the ability of BMM to present PEPCK peptide_{335–351} in the context of I-A^b and the ability of PEG Th1 cells to recognize this presentation and respond through high IFN γ production.

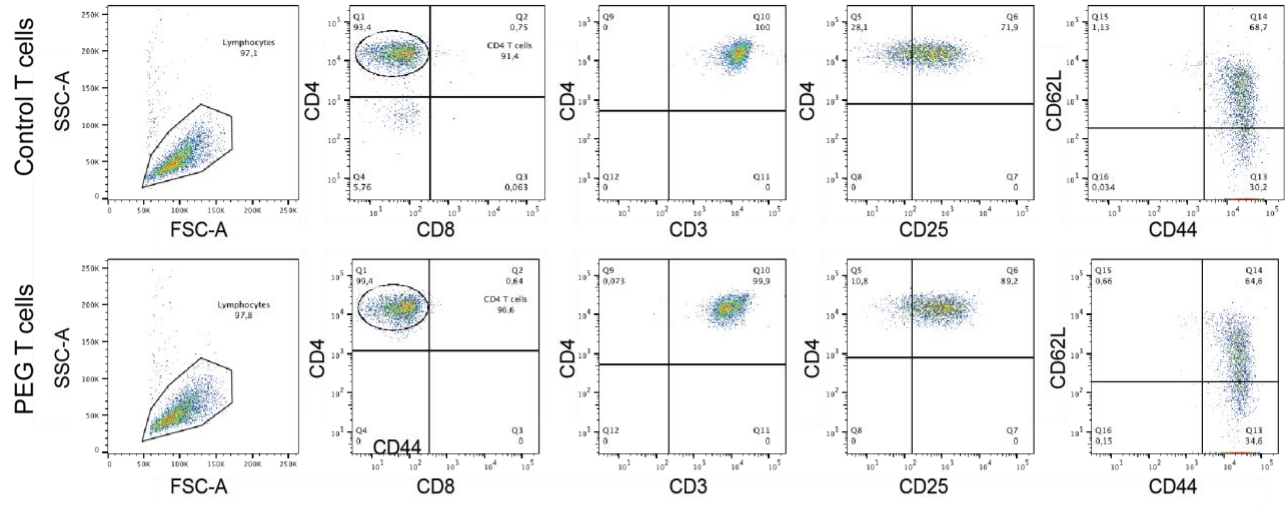
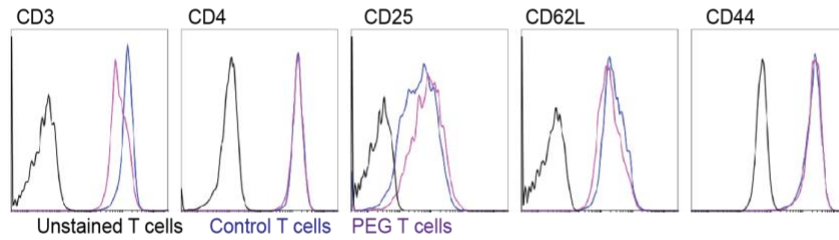
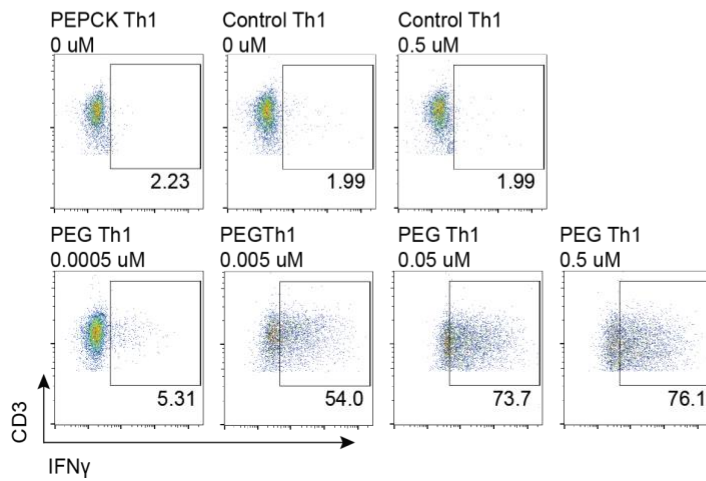
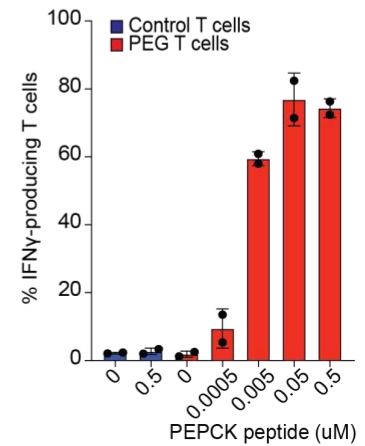
A**B****C****D**

Figure 6. PEG Th1 responses against PEPCK are characterized by high IFN γ production.

- (A) Naïve CD4⁺ T cells were isolated from spleens of wildtype control or PEG C57Bl/6 mice, activated with anti-CD3/CD28 beads and expanded for 7 days in the presence of Th1 polarizing cytokines. Phenotypic analysis of Day 7 T cell was performed with Flow Cytometry.
- (B) Phenotypic analysis of Day 7 Control and PEG Th1 cells as compared to unstained controls.
- (C) Control or PEG Th1 cells were co-cultured with PEPCK335-351-pulsed macrophages and IFN γ production by Th1 cells was measured by Flow cytometry (D). Statistical analysis—unpaired t test. Mean +/- SD. Representative data of three independent experiments.

3.2.1.2. PEG Th1 cells display strong IFN γ responses upon co-culture with *Leishmania major*-infected macrophages.

Next, I evaluated the ability of PEG Th1 cells to respond to *L. major* infection itself. BMM were first infected with *L. major* at 10:1 parasite to macrophage ratio for 6 hours. Next, parasites that did not internalize were washed off and uninfected or infected BMM were cultured with either control or PEG Th1 cells that were previously expanded for 7 days. Intracellular flow cytometry showed 71% of PEG Th1 cells producing IFN γ , as opposed to control T cells producing background level of the cytokine (Figure 7A, B). However, when control and PEG T cells were stimulated with PMA/Ionomycin cell-activation cocktail for 24 hours, both populations responded with equivalently high IFN γ production. To evaluate the dose of parasite infection moving forward, I infected BMM with an increasing ratio of *L. major*, from 0.25:1 to 10:1 and evaluated IFN γ production by PEG cells (Figure 7C, D). As expected, IFN γ production increased with increasing infectious dose, yet it was possible to observe maximal responses at a 0.25:1 ratio, indicating highly sensitive response by PEG Th1 cells. Lastly, I infected BMMs with *L. major* at 10:1 ratio for 6, 24, and 48 hours and cultured them with PEG Th1 cells for additional 18 hours (Figure 7E). Relative IFN γ by PEG Th1 increased with time, indicating that antigen presentation remains high after a few days of infection. These experiments validate the ability of PEG Th1 cells to recognize and respond to *L. major* infection in a sensitive, antigen-specific manner.

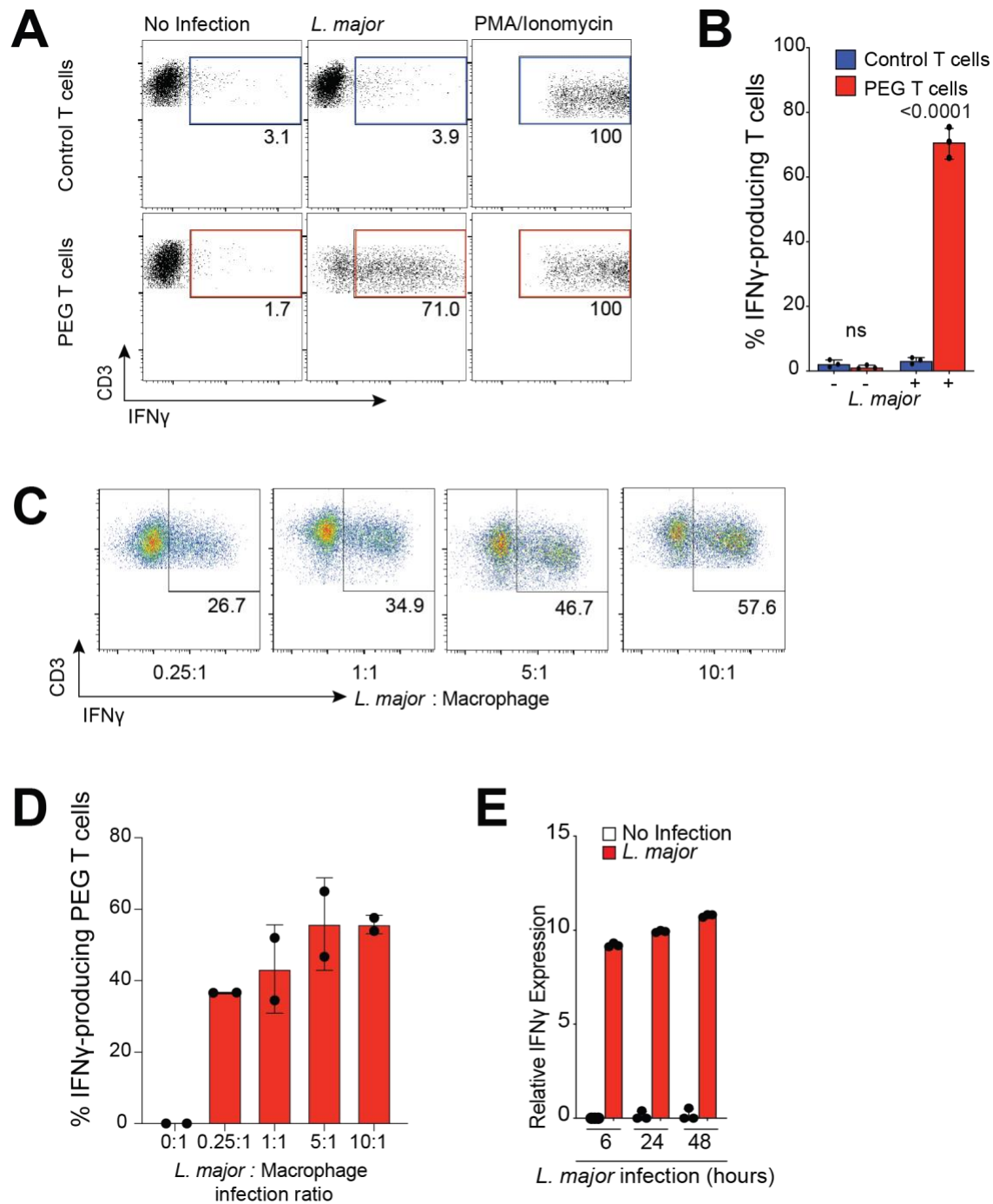


Figure 7. PEG Th1 responses against *Leishmania major* are characterized by high IFN γ production.

(A) IFN γ production was measured by flow cytometry upon co-culture with uninfected or *L. major*-infected macrophages or after stimulation of PMA/Ionomycin.

- (B) Representative graph of four independent experiments is shown. Mean \pm SD. Statistical analysis – unpaired t test, ns = not significant.
- (C) IFN γ production by PEG Th1 cells after co-culture with different *L. major*:macrophage infection ratios were measured with flow cytometry. Red box represents positive IFN γ signal.
- (D) Representative graph of two independent experiments is shown. Mean \pm SD.
- (E) Relative production by PEG Th1 cells was measured by flow cytometry upon co-culture with *L. major*-infected macrophages at 6, 24-, and 48-hours post infection. Mean \pm SD. Data representative of two independent experiments.

3.2.1.3. PEG Th1 cells form prolonged contacts with *Leishmania major*-infected macrophages in a 3D collagen chamber.

To visualize *Leishmania*-infected cells, I utilized the GFP-expressing *L. major* reporter, which allows me to visually track infection in living cells⁵⁵¹. Celltracker blue-labeled BMMs were either left uninfected or infected with GFP⁺*L. major* for 24-72 hours prior to co-embedding with PEG Th1 cells (red) and control Th1 cells (green) in 3D collagen and imaged at 30-minute intervals, as we have done previously⁵⁴⁸ (Figure 8A). I observed that the majority of PEG T cells formed prolonged contacts with macrophages, with aggregates of PEG T cells continuously scanning infected macrophages (Figure 8B-D, direct contacts and contact paths highlighted with yellow arrows). Importantly, control T cells do not stop in response to infection, indicating that PEG T cell arrest is due to antigen-recognition. Notably, I observed an overall reduction in effector PEG Th1 cell migration speeds in the presence of infected macrophages, and a corresponding confined migratory behavior (Figure 8F) compared to control Th1 cells. To further define how *L. major* infection impacted effector Th1 migratory behaviors, mean 3D track velocity was plotted as a function of confinement ratio (track displacement/track length)⁵⁵² (Figure 8F). All cell tracks were divided into four quadrants by setting crosshairs at a mean track speed of 5 $\mu\text{m}/\text{min}$ confinement ratio of 0.4, where T cells displaying a “confined, slow” migratory behavior were defined in quadrant 1 (green shade) and T cells displaying “meandering, fast” migratory behaviors were identified in quadrant 2 (grey shade). Comparison of the relative percentages of confined slow vs meandering fast tracks showed that 90.4% of control cells, compared to only 79% of PEG Th1 cells, exhibited meandering behaviours indicative of no antigen recognition (Figure 8G). These studies confirm that PEG T cells are able to recognize and engage in contacts with parasite-infected macrophages. These results also demonstrate that at least at the early stages of infection, *L. major* does not interfere with the host antigen presentation machinery.

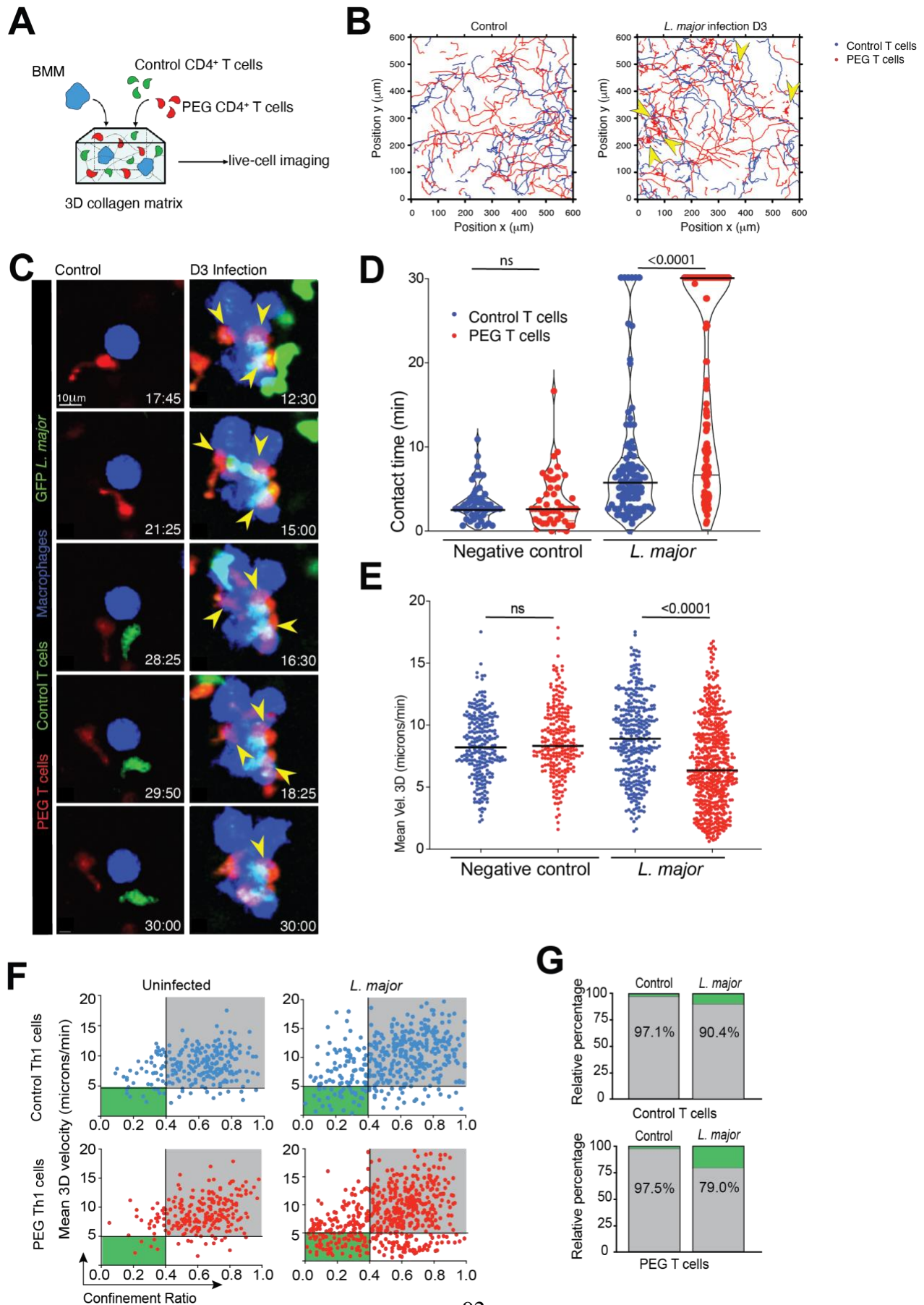


Figure 8. Visualising the dynamic behaviour of PEG Th1 cells in 3D collagen chambers to model their interactions with *L. major*-infected macrophages.

- (A) Control Th1 cells and PEG Th1 cells were stained with cell tracker dyes (CMFDA; green, and CMTMR; red, respectively) and co-cultured with stained uninfected or *L. major*-infected macrophages (CMAC; blue). Chambers were placed on a temperature-controlled imaging platform to maintain temperatures at 37°C and imaged at 30-minute intervals using two-photon microscopy.
- (B) Migratory tracks of control and PEG Th1 populations during a 30-min recording. Yellow arrows point to areas of prolonged Th1: macrophage contact.
- (C) Time series micrograph of co-cultures at indicated times. Time stamps in min:sec represent elapsed time of the recordings. Yellow arrows represent Th1: macrophage contact.
- (D) Real-time contact duration between control (blue dots) or PEG (red dots) Th1 cells and uninfected, or *L. major*-infected macrophages. Each dot represents a single contact. Black lines represent median values. Statistical analysis: Mann-Whitney test, ns = not significant. Data combined from three independent experiments.
- (E) Mean 3D track velocity of control (blue dots) or PEG (red dots) Th1 cells cultured with uninfected, or *L. major*-infected macrophages. Each dot represents a single cell track. Black lines represent median values. Statistical analysis: Mann-Whitney test, ns = not significant. Data combined from three independent experiments.
- (F) Track velocities of control (blue) or PEG (red) Th1 cells cultured with uninfected, or *L. major*-infected macrophages plotted against confinement ratio (F) and relative percentage of confined slow and meandering fast tracks (G). Data combined from three independent experiments.

3.2.1.4. PEG Th1 co-culture with *Leishmania major*-infected macrophages leads to increased production of pro-inflammatory cytokines.

Having confirmed that antigen recognition by PEG T cells leads to their augmented cellular dynamics, stable contacts with BMMs, and IFN γ positivity, I utilized multiplex ELISA to evaluate the cytokine milieu of the PEG: BMM co-cultures with or without *L. major* infection. After 24 hours of co-culture, supernatants were sent to Eve Technologies and ELISA supernatant analysis was performed using the Mouse Cytokine Proinflammatory Focused 10-Plex Discovery Assay® Array (MDF10). IFN γ production in the supernatant was high when PEG Th1 cells were cultured with *L. major* infected BMM, consistent with our flow cytometry analysis (Figure 9). TNF concentration also increased, as expected. IL-2 and IL-6 expression was also elevated, IL-4 increased slightly, while IL-10 and IL-12 production did not show a significant increase in expression. These results indicate that antigen recognition by PEG Th1 cells leads to the generation of a pro-inflammatory environment.

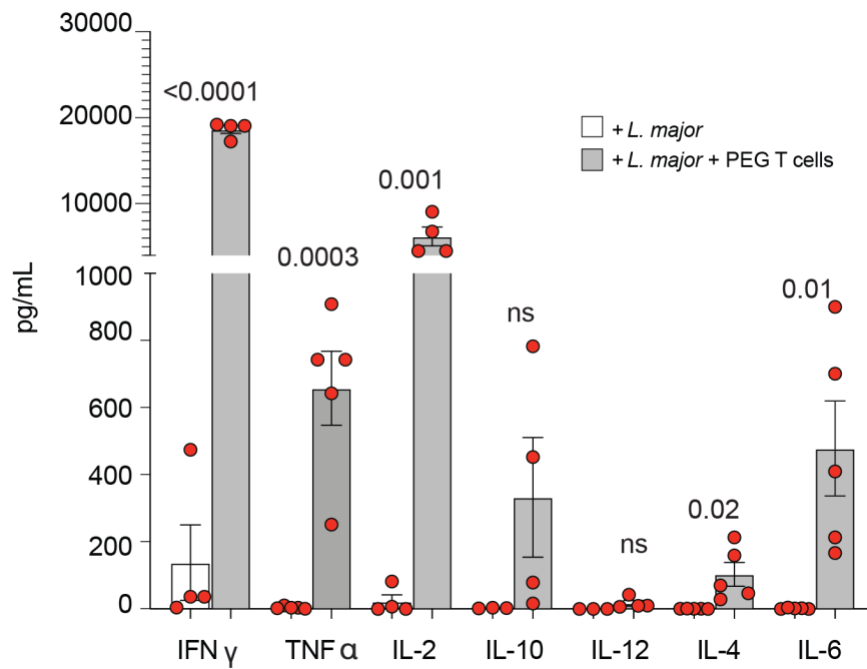
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Figure 9. PEG Th1 co-culture with *Leishmania major*-infected macrophages leads to increased production of pro-inflammatory cytokines.

(A) Cytokine expression after *L. major*-infected macrophages with or without the addition of PEG Th1 cells was measured using multiplex ELISA. Representative data from four independent experiments is shown. Mean \pm SD. Statistical analysis: unpaired t test, ns = not significant.

3.2.1.5. PEG Th1 cells activate macrophages and induce leishmanicidal activity.

Having confirmed the presence of effector function by PEG Th1 cells, I evaluated the extent by which Th1 cells activated macrophages and promoted their leishmanicidal activity. I used the GFP-expressing *L. major* for infections of BMM which have been allowed to attach to sterile microscopy cover slides. After further cell culture alone, or with control or PEG Th1 cells for 24 hours, I washed the T cells off and performed immunohistochemistry staining for F4/80 (macrophage marker), GFP, and DAPI (nuclei) (Figure 10A). Using confocal microscopy, I calculated both the % of infected macrophages out of 100 cells and how many parasites remained per macrophage (Figure 10B, C). Without treatment nearly 80% of macrophages were infected with on average 3-4 parasites, yet some could contain as many as 10-15. Addition of control Th1 cells led to a modest decrease in the percentage of infected BMMs, but no significant decrease in parasites/per macrophage was observed. PEG Th1 cells, however, induced strong leishmanicidal activity. The percentage of infected BMMs decreased to approximately 20% and the majority of macrophages that remained infected harbored on average only one parasite per cell.

Next, I evaluated the effect of *L. major* infection and the addition of PEG Th1 cells on BMM surface markers, with a focus on the antigen presentation machinery. Macrophages were cultured alone or with PEG Th1 cells in the presence or absence of infection. Additionally, I extended the infection time in order to determine whether parasite-mediated modulation of these immune receptors is time dependent. Interestingly, addition of activated PEG Th1 cells in the absence of infection increased the expression of MHC-II over baseline, indicating that the mere presence of T cells induces this upregulation (Figure 10D). No significant downregulation of MHC II was observed after culture with PEG Th1 cells, indicating that suppressive mechanisms of *L. major* were not sufficient to induce substantial suppression of MHC-II cell surface expression. Addition

of PEG Th1 cells substantially increased CD40 expression on infected BMMs (Figure 10E). No significant changes in trends were observed with CD80/86 among different experiments (Figures 10F, G). Overall, these studies indicate that PEG antigen recognition and subsequent IFN γ production leads to macrophage activation and leishmanicidal activity. Our data also indicate that *L. major*-driven immune suppressive mechanisms are not sufficient to overcome Th1 responses.

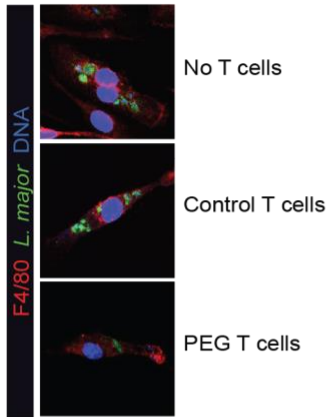
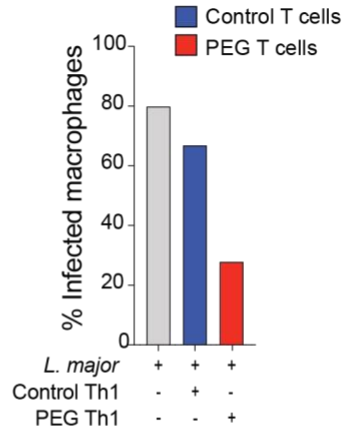
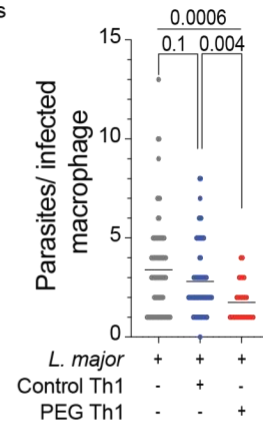
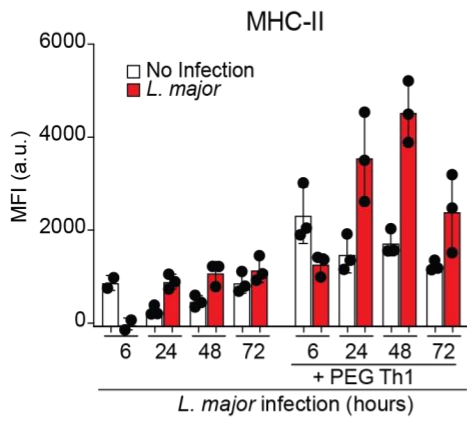
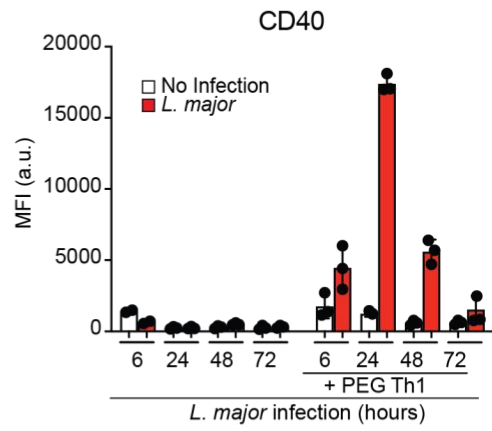
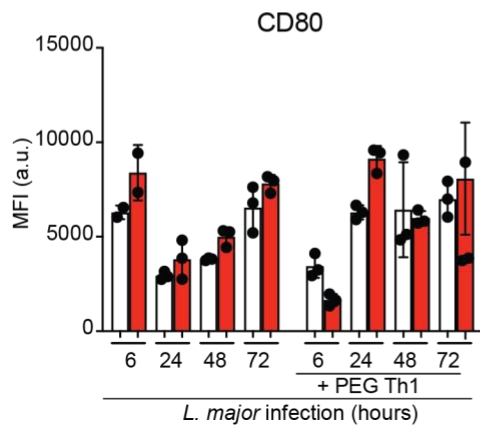
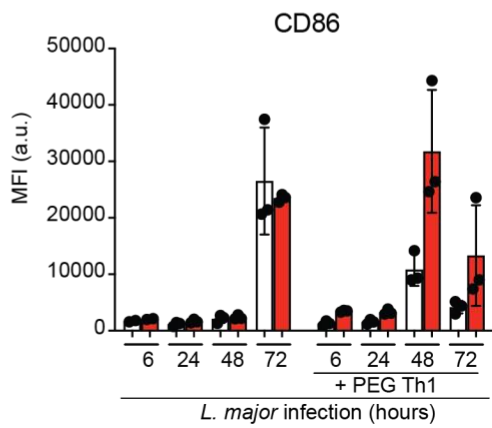
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Figure 10. The impact of PEG Th1 cells on leishmanicidal activity and surface expression of macrophage activation markers.

- (A) Representative micrograph of *L. major*-infected macrophages in the presence or absence of Th1 cells. Blue – DNA, Green – *L. major*, Red – F4/80.
- (B) Percent infected macrophages. Representative graphs of two independent experiments are shown. Statistical analysis: unpaired t test, ns = not significant.
- (C) Parasite numbers per infected macrophage. Representative graphs of two independent experiments are shown. Statistical analysis: unpaired t test, ns = not significant.
- (D) Cell surface MHC-II, CD40 (E), CD80 (F), and CD86 (G) expression on macrophages was measured with flow cytometry over time with (red) or without (white) *L. major* infection in the absence or presence of PEPCK Th1 cells. Mean +/- SD. Representative data of two independent experiments.

3.2.2. Aim 2: To characterize PEG T cell homing and migration within the lymph node and the parasite-challenged ear tissue during Leishmania major infected in vivo

3.2.2.1. Naïve PEG T cells form prolonged contacts with PEPCK-pulsed and L. major-infected DCs in lymph nodes of live mice.

To evaluate whether PEG T cells can become activated in vivo, I performed intravital microscopy directly in the lymph nodes of live mice. Uninfected, PEPCK-pulsed, or *L. major* -infected DCs were stained with celltracker green (CMFDA) and injected into the footpad. 4-5 hours later, naïve control and PEG CD4⁺ T cells were stained with cell tracker blue (CMAC) or cell tracker orange (CMTRM), with colors switched throughout experiments to account for possible fluorophore detection bias of our filter cubes, and injected retro-orbitally into the recipient mice, with 2P-IVM microcopy in the popliteal lymph node performed the next day (Figure 11A, B). Without the presence of antigen, both T cell populations displayed fast average velocity, and the addition of PEPCK peptide caused PEG cells to selectively reduce their migration speeds and arrest more frequently (Figure 11C, D). *L. major* infection of DCs also resulted in more frequent arrest, not significantly different from response to peptide-pulsed DCs. Average velocity of the control cells did not change, however arrest coefficient slightly increased. Overall, these results demonstrate the ability of naïve PEG T cells to engage in stable interactions with DCs displaying cognate antigen. Additionally, our imaging studies again demonstrated that cell-intrinsic *L. major* immunosuppressive mechanisms are not sufficient to overcome PEG cell sensitivity and recognition, at least within the first 24 hours of DC infection.

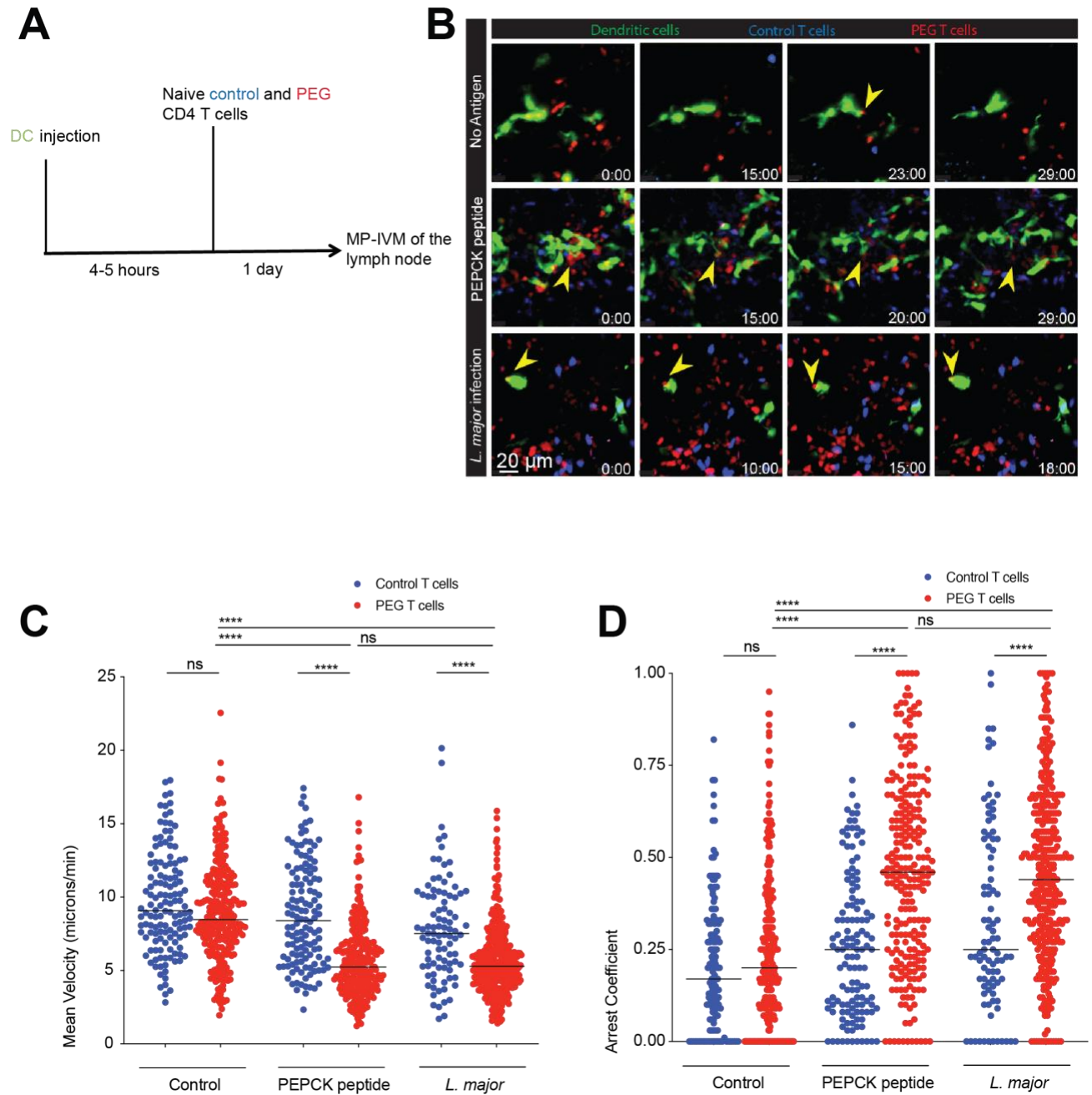


Figure 11. PEG T cell homing and migration within the lymph node.

(A) Experimental design of MP-IVM studies. Control, PEPCK-pulsed, or *L. major*-infected dendritic cells were labeled with Celltracker Green (CMFDA, green) prior to injection of 5×10^5 DCs into the footpad of C57Bl/6 mice. 12 hr after DC transfer, mice were given immunomagnetic selected 3×10^6 CD4⁺ naïve T cells prepared from control or PEG mice. Control or PEG naïve T cells were stained with cell tracker dyes (CMAC blue, and

CMTMR; red, alternating between experiments) and adoptively transferred into recipient mice. Intact lymph nodes were prepared for intravital microscopy 18 hours after T cell transfer.

(B) Representative micrographs of the lymph nodes after adoptive transfer, yellow arrows represent Th1: macrophage contacts. Time stamps in min:sec, scale bar: 20 μ m.

(C) Mean 3D track velocity **(C)** and arrest coefficient **(D)** of the indicated Th1 cells. Blue: control cells, Red: PEG Th1 cells. Each dot represents a single cell track. Black lines represent median values. Statistical analysis: Mann-Whitney test. Representative data of two independent experiments.

3.2.2.2. GFP-*Leishmania major* parasites persist in the ears long after the lesion has been healed.

To visualize anti-*L. major* T cell responses *in vivo*, I developed an *in vivo* imaging approach to image infected cells directly in the ears of anaesthetized mice, as illustrated in Figure 12A. Briefly, wildtype C57Bl/6 albino mice (which are devoid of melanocytes that can interfere with intravital imaging) were injected with 1×10^6 GFP⁺ *Leishmania major* parasites in the ear dermis and the mouse was prepared for intravital microscopy at various time points post-infection. At 12 days post-infection, GFP⁺ cells were observed to be highly motile, which are presumably infected monocytes or neutrophils⁴⁷, as well as sessile infected macrophage-like cells. At 41 days (approximately 6 weeks) post-infection, no motile GFP⁺ cells were observed, and a more defined lesion area was noted. At 84 days (12 weeks) post-infection, a small population of parasite-infected cells can still be observed, despite the ear being healed by week 8. I was able to detect parasites up to 140 days (20 weeks) post-infection. These studies demonstrate my ability to infect, image and analyze *Leishmania* infections directly in skin, and definitively show that *Leishmania major* persists at the site of primary infection long after the skin has healed.

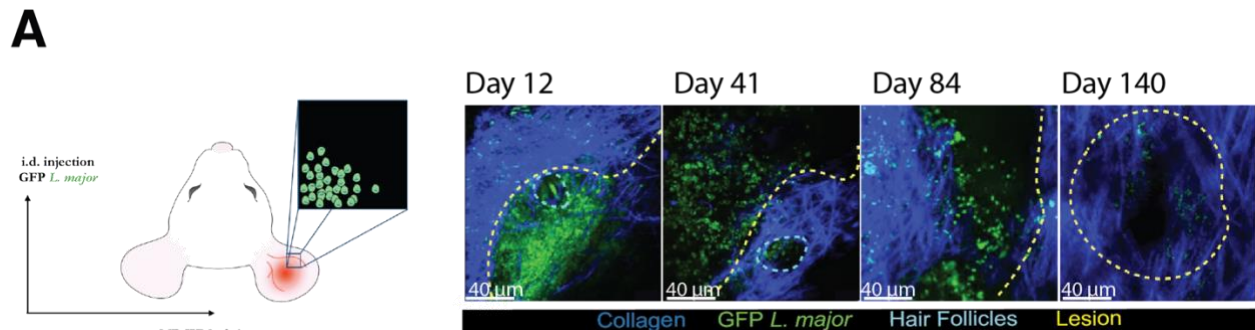


Figure 12. *Leishmania major* parasites persist in the ear pinna.

(A) Albino C57BL/6 mice were infected intradermally in the ear pinna with 1 million GFP *L. major* parasites and intravital microscopy was performed on ear dermis at various time

points. Collagen – blue, GFP⁺ *L. major* – green, yellow dotted line represents lesion area, blue dotted line represents a hair follicle.

3.2.2.3. Adoptively transferred PEPCCK-specific Th1 cells exhibit augmented cellular dynamics in vivo during *L. major* infection.

To visually characterize how effector T cell responses differed during the two phases (acute and healed) of *L. major* infection *in vivo*, 1-1.5x10⁷ day 7 *in vitro* expanded control and PEG Th1 cells were stained with cell tracker dyes (CMTMR or CMAC, colors switched throughout experiments to account for possible fluorophore detection bias of our filter cubes), and adoptively transferred into GFP⁺*L. major* infected mice at weeks 3-5, or 10-15 post-infection, representing acute and healed stages of infection, respectively (Figure 13A). Equal transfer of both populations was confirmed in the spleen of recipient mice by flow cytometry (Figure 13B), and no transferred T cells were found in the contralateral, uninfected ear skin (data not shown). Curiously, fewer cells could be detected localizing to the lesion environment at the later stages of infection (Figure 13C), indicating less inflammation as the lesion heals which contribute to reduced T cell infiltration.

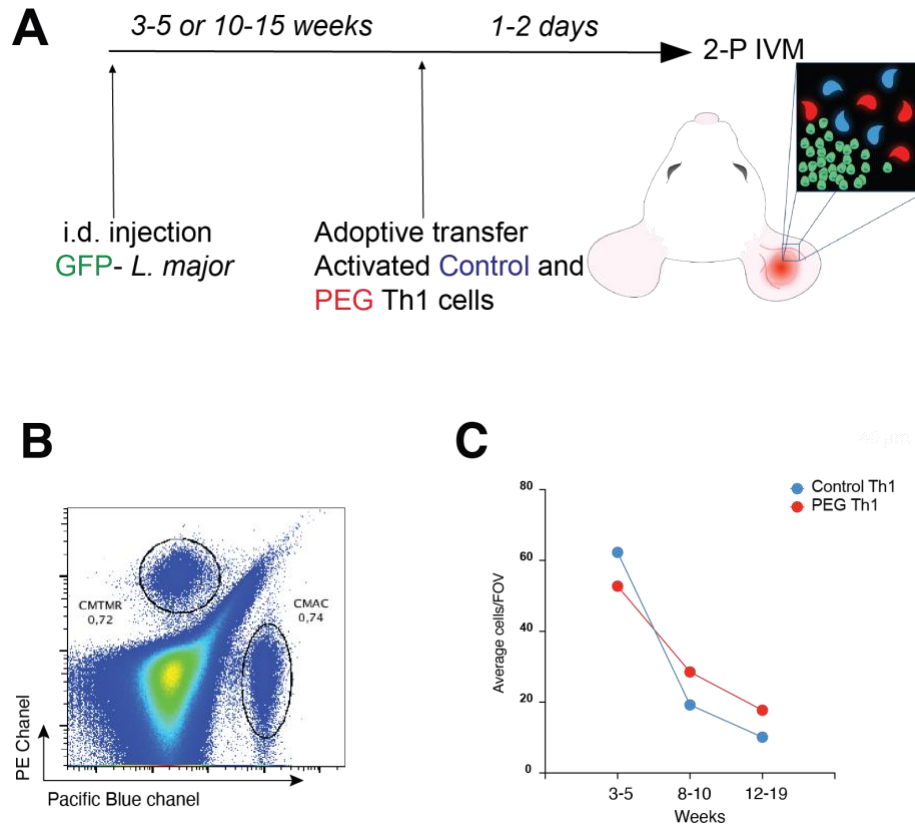


Figure 13. Recruitment of adoptively transferred Th1 cells to the site of infection decreases in the healed stages of infection.

(A) Experimental design of MP-IVM studies. Albino C57Bl/6 mice were infected via intradermal ear injections with 1 million GFP⁺ *L. major* parasites. At various times post-infection, 10-15 million each of control and PEG Th1 cells were stained with cell tracker dyes (CMAC blue, and CMTMR; red, alternating between experiments) and adoptively transferred into recipient mice infected for the indicated time periods. Ears were prepared for intravital microscopy 24-48 hours after T cell transfer.

(B) Flow Cytometry analysis of CMAC (cell tracker blue) and CMTMR (cell tracker red) stained Th1 cells in the spleen posts adoptive transfer and intravital microscopy studies.

(C) Average number of control (blue) and PEG (red) cells in the ear per recording.

Intravital 2P-microscopy was performed at 1-2 days post-T cell transfer (Figure 14A), where both control and PEG T cells were found in infected skin at all stages, corroborating previous studies showing that Th1 cells enter the lesion environment irrespective of their antigen specificity¹⁹⁴. During acute *L. major* infection, transferred control Th1 cells migrated at a mean 3D track velocity of 6.5 $\mu\text{m}/\text{min}$, whereas the mean track velocity of PEG Th1 cells was 5.1 $\mu\text{m}/\text{min}$ and displayed higher cell arrest, indicative of antigen-mediated cell-cell contact formation (Figure 14B, C). Interestingly, adoptively transferred PEG T cells also displayed lower migration speeds compared to control T cells around residual lesions observed in healed skin, suggesting that sufficient cognate antigen recognition facilitated PEG T cell deceleration. To further define how the two phases of *L. major* infection impacted effector Th1 migratory behaviors, mean 3D track velocity was plotted as a function of confinement ratio (track displacement/track length)⁵⁵² (Figure 14D). All cell tracks were divided into four quadrants by setting crosshairs at a mean track speed of 5 $\mu\text{m}/\text{min}$ confinement ratio of 0.4, where T cells displaying a “confined, slow” migratory behavior were defined in quadrant 1 (green shade) and T cells displaying “meandering, fast” migratory behaviors were identified in quadrant 2 (grey shade). Comparison of the relative percentages of confined slow vs meandering fast tracks showed that only 35.2% of control cells, compared to 56.2% of PEG Th1 cells, exhibited constrained behaviours indicative of cognate antigen recognition (Figure 14E). Similarly, PEG cells displayed behaviors consistent with antigen recognition within healed skin. These data suggest that additional structural and immunological constraints modulated effector Th1 responses in healed skin.

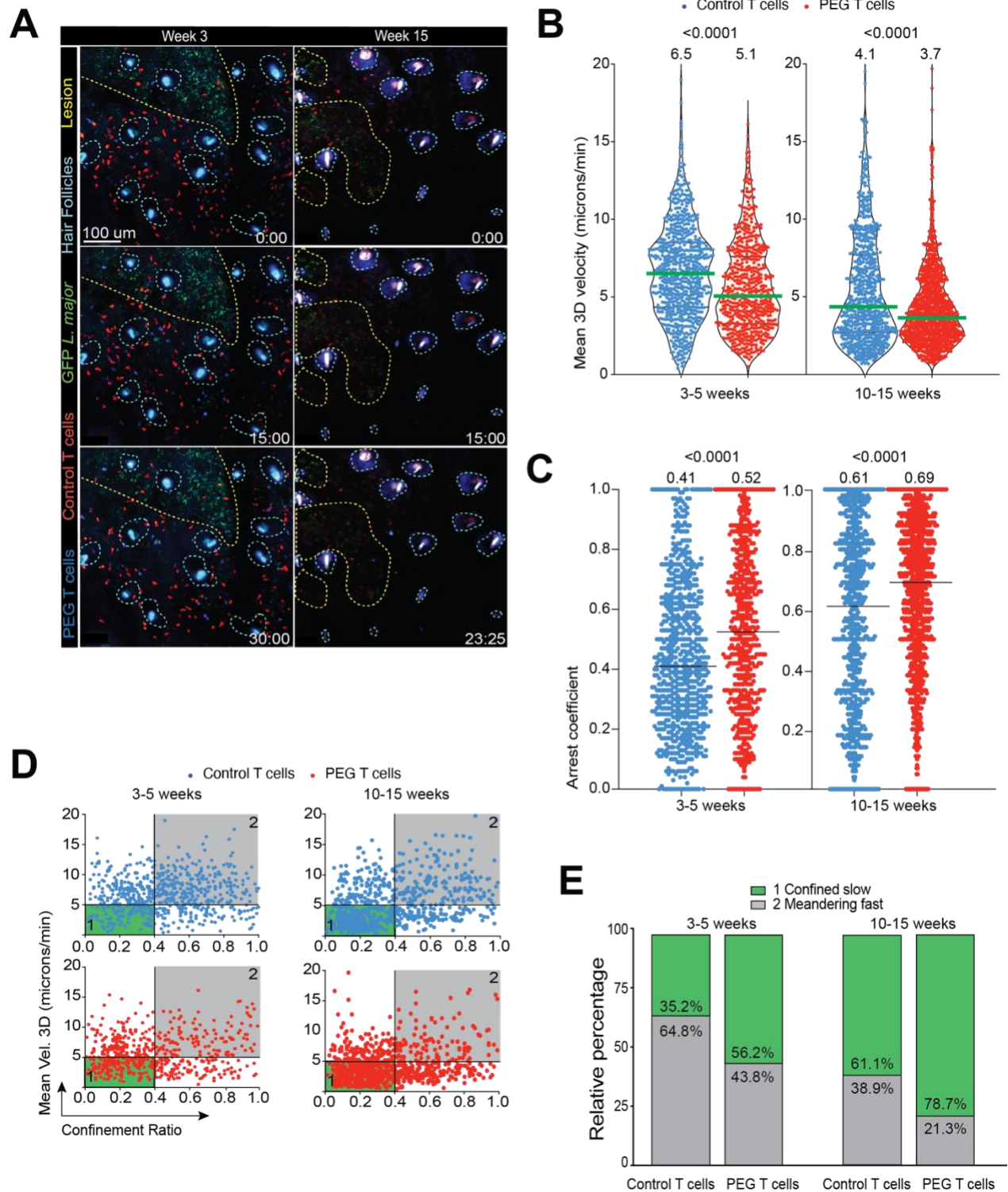


Figure 14. In vivo cellular dynamics of Th1 response against *Leishmania major* infection.

- (A) Representative micrographs of the lesions after adoptive transfer, yellow and blue dotted lines represent the lesion and hair follicles, respectively. Time stamps in min:sec, scale bar: 100 μ m.
- (B) Mean 3D track velocity of the indicated Th1 cells. Blue: control cells, Red: PEG Th1 cells. Each dot represents a single cell track. Green and black lines represent median values. Statistical analysis: Mann-Whitney test.
- (C) Arrest coefficient of the indicated Th1 cells. Blue: control cells, Red: PEG Th1 cells. Each dot represents a single cell track. Green and black lines represent median values. Statistical analysis: Mann-Whitney test.
- (D) Track velocities of Th1 cells plotted against confinement ratio. Blue: control cells, Red: PEG Th1 cells. Each dot represents a single cell track.
- (D) Relative percentage of confined slow and meandering fast cell tracks. Green: confined slow, Gray: Meandering fast. Representative data of nine independent experiments.

3.2.3. Aim 3: To develop an ECFP⁺ PEPCK-TCR mouse model and characterize their cellular dynamics throughout the course of Leishmania major infection in vivo

3.2.3.1. Breeding PEPCK-TCR and CFP mice results in blue-fluorescent PEPCK-specific T cells

Next, I aimed to replicate the endogenous immune response to *L. major* infection. In order to visually track PEG Th1 cells for a prolonged period of time, I bred B6.129(IRC)-Tg(CAG-ECFP)CK6Nagy/J (CK6/ECFP) female mice, which are hemizygous for the CAG-ECFP gene, with PEG males. All tissues from hemizygous CK6/ECFP animals, except for erythrocytes and adipocytes, display fluorescence in all cell types under appropriate lighting conditions. The resulting F1 offspring is heterozygous CAG-ECFP and either PEPCK-specific or polyclonal (Figure 15A). To genotype the offspring, I used a PCR-based analysis. Primers specific for TCR α and TCR β genes were used to amplify genomic DNA from mice to visualize the presence of the PEPCK-TCR genes (Figure 15B). In order to confirm that the presence of the gene translates into

the expression of the TCR on the lymphocyte surface, I performed a phenotypic analysis of the blood samples from the PCR-positive mice and one negative mouse for control using PEPCK335-351:I-A^b tetramer. Approximately 70-80% of the CD4⁺ T cells in the blood samples of transgenic mice bound the tetramer, indicating PEPCK specificity, and fluoresced in the AmCyan channel, indicating expression of ECFP by flow cytometry.

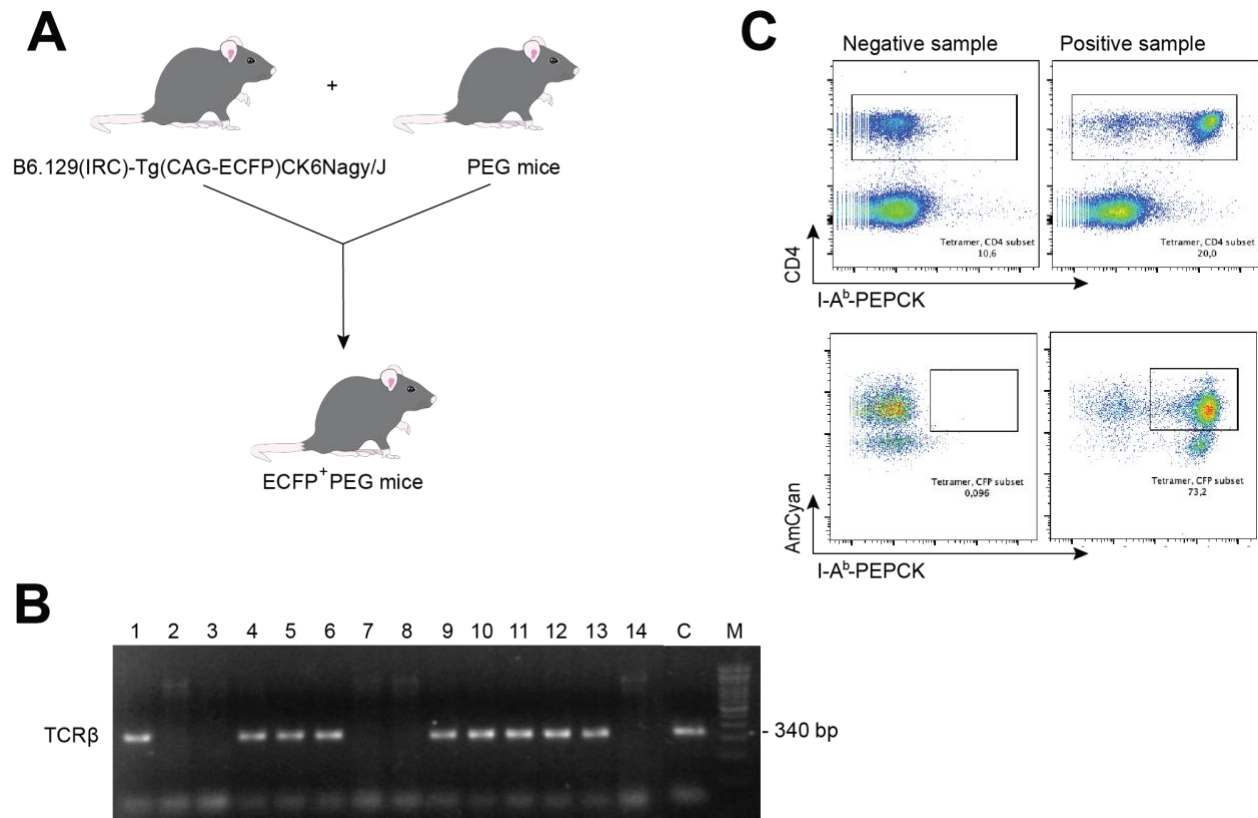


Figure 15. Generating ECFP⁺ PEPCK-TCR mouse model.

(A) Schematic representation of B6.129(IRC)-Tg(CAG-ECFP)CK6Nagy/J (CK6/ECFP) and PEG mouse cross.

(B) PCR analysis of mouse tissue sample for PEG TCR transgene.

(C) Flow cytometry analysis of a blood sample for the expression of the TCR transgene.

3.2.3.2. Adoptively transferred CFP-PEPCK Th1 cells do not persist in the ears of *L. major*-infected mice.

In order to emulate endogenous antigen-specific immune response to *L. major*, I adoptively transferred 1×10^4 naïve ECFP PEG and mRFP control CD4⁺ cells into naïve C57BL/6 males (Figure 16A). The next day I infected the ear dermis of the recipient mice with 1×10^6 GFP⁺ *L. major* parasites and performed intravital microscopy each week starting on the second week post infection. At week 2 I observed a large expansion and recruitment of ECFP PEG T cells (Figure X B, C). Weeks 3 and 4 I observed significantly decreased numbers of these cells, despite the presence of parasites (Figure 16B, C). From weeks 5-9 no cells were observed in the field of view in all 7 animals. At week 9 of experiment the animal facility shut down due to the Covid-19 pandemic and I was ordered to terminate all mice, including the breeding colonies. The breeding and subsequent experiments were not restarted once the university reopened.

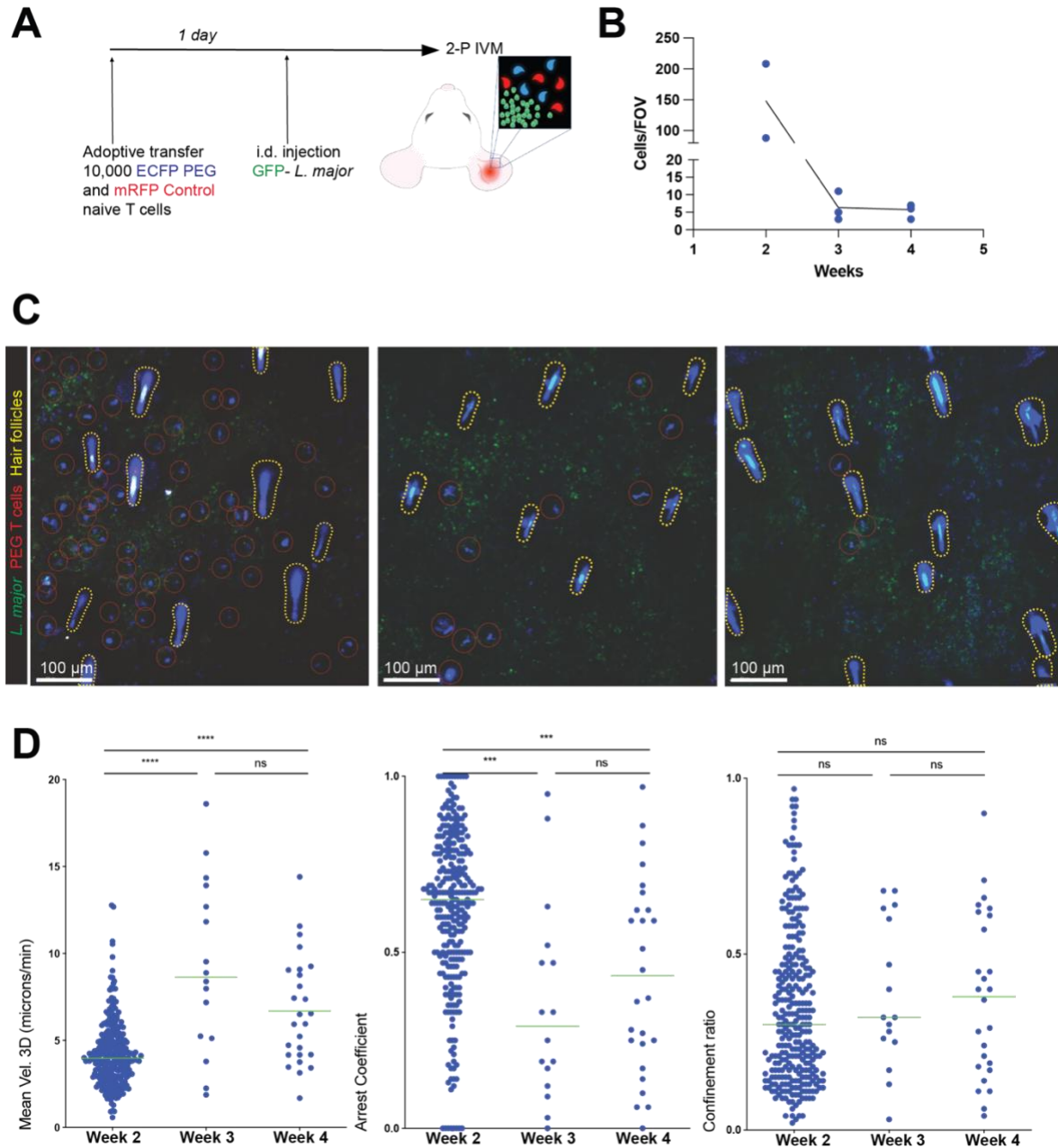


Figure 16. Cellular dynamics of adoptively transferred naïve PEG Th1 cells in vivo.

(A) Experimental design of MP-IVM studies. 1×10^4 naïve ECFP PEG and mRFP control $CD4^+$ cells were adoptively transferred into naïve C57BL/6 males. 24 hours later mice were infected via intradermal ear injections with 1 million GFP $^+$ *L. major* parasites. Ears were prepared for intravital microscopy at various time points after infection.

(B) Average number of ECFP $^+$ PEG Th1 cells per field of view.

(C) Representative micrographs of the lesions after adoptive transfer, yellow and red lines represent hair follicles and ECFP⁺ PEG Th1 cells, respectively. Time stamps in min:sec, scale bar: 100 μ m.

(D) Mean 3D track velocity, arrest coefficient, and confinement ratio of the ECFP⁺ PEG Th1 cells. Each dot represents a single cell track. Green lines represent median values. Statistical analysis: Mann-Whitney test.

3.3. Discussion and Conclusions

Prior to the generation of PEG transgenic mice, no prior study has convincingly characterized expansion, effector function, or migration behaviours of immunodominant, *L. major*-specific CD4⁺ T cells *in vivo*. The immune response to *Leishmania* homolog for activated C kinase (LACK) has been studied on mainly the Balb/c background, and the long-term effects of LACK on the immune response in resistant mice is unknown⁵⁵³, due to the fact that these mice do not survive to reach the chronic infection stage. Additionally, adoptive transfer of naïve ovalbumin (OVA) TCR transgenic CD4⁺ T cells and infection with OVA-expressing *L. major* revealed the generation of both central and effector memory cells⁵⁵⁴, and another study using *Leishmania* expressing 2W peptide, a variant of peptide 55–68 derived from the α chain of mouse I-E^d MHC class II molecule, showed expansion and contraction of CD4⁺ T cells after *L. major* infection¹⁸⁸. However, these peptides are foreign antigens to mice and not expressed on *Leishmania*, and T cell responses to these peptides do not represent natural physiological responses to *L. major*. With PEG mouse model I was able to look at *L. major*-specific and immunodominant responses to visually evaluate the cellular dynamics of antigen-specific responses during *Leishmania major* infection *in vivo*.

My first step was to critically evaluate this novel mouse model and confirm antigen specificity of isolated CD4⁺ T cells, and to visually characterize their motility parameters *in vitro* and *in vivo*.

In vitro expanded PEG Th1 cells are phenotypically similar to their control counterparts and display a strong IFN γ response when encountering their cognate antigen, PEPCK, presented in the context of I-A^b. In order to maximize the chances of encountering and responding to APCs presenting its cognate antigen, T cells exhibit exquisite motility and engage in repeated cell-cell contacts within the body. To mimic the 3D environment in which cellular interactions occur *in vivo*, I used a collagen matrix chamber that provides a 3D network of collagen fibers found in various tissues, including the skin. I co-embedded both T cell populations with either control or *L. major*-infected macrophages and prepared the collagen chambers for two-photon imaging, for the duration of 30 minutes. 2-photon microscopy revealed significantly augmented cellular dynamics of PEG Th1 cells in response to *L. major* infection, indicative of antigen recognition. Having confirmed the ability of PEG Th1 cells to respond to *L. major* infection, I proceeded to evaluate the downstream effects these interactions have on the environment and target cells. I confirmed that co-cultures of PEG Th1 cells and *L. major*-infected create a proinflammatory environment that contribute to macrophage activation and enhanced leishmanicidal activity. These results are in accordance with the literature, where production of both IFN γ has been shown to promote an increase in ROS and nitric oxide (NO) expression to kill intracellular parasites¹⁹¹. PEG cells demonstrate superlative ability to recognize and respond to *L. major*; therefore, they make a highly sensitive tool for antigen recognition *in vivo*.

L. major parasites are known to possess numerous cell-intrinsic immunosuppressive mechanisms. T cells upregulate costimulatory molecules CD40L, which bind CD40 on macrophages and act as a secondary activation signal¹⁹². To counter this, GP63 has been shown to cleave CD4 on T cells, as well as inhibiting MHC-I presentation, physically disabling activation of both CD4 and CD8 T cells¹⁵³. Additionally, early *in vitro* studies showed that *L. major*-infected

macrophages have a reduced capacity to stimulate T cells, possibly due to downregulation of co-stimulation molecules or MHC II, or reduced ability to trigger reorientation of T cell microtubule organizing center^{555,556}. Therefore, I evaluated the capacity of PEG T cells to recognize and respond to *L. major* infection in macrophages and compared these responses to PEPCK peptide-pulsed macrophages, which will lack suppressive mechanisms imposed by the parasite. PEG Th1 cells were able to mount strong IFN γ responses in the presence of infected macrophages, equivalent to their response to the PEPCK peptide. This indicates that cell-intrinsic mechanisms mounted by the parasite from within the macrophage are not sufficient to overcome the highly sensitive PEPCK-specific response by PEG Th1 cells. Studies showing downregulation of MHC-II^{555,556} used LACK as the model antigen in the context of susceptible BALB/c mice. This method is not ideal to evaluate evasion mechanisms by *L. major* as LACK antigen is only expressed at the early stage of infection and the biology of the susceptible mouse model does not represent the establishment of a chronic-surviving parasite niche. I utilized a model where T cells respond to an immunodominant peptide in the context of C57BL/6 mice, which would elicit a substantially stronger response in order to uncover how parasites continue to persist in skin despite robust host immunity generation. In vitro, I saw no difference in the capacity to produce effector cytokines in response to PEPCK peptide or *L. major* infection itself and PEG cells displayed the capacity to activate macrophages and perform strong leishmanicidal activity. These findings led to my conclusion that *L. major* do not possess sufficient immune evasion mechanisms to counteract immunodominant T cell responses, and that other factors must be involved in *L. major* persistence *in vivo*.

Reductionist *in vitro* imaging studies are ideal to address specific questions about cellular dynamics and cell-cell interactions in response to infections, without interference from other cell types. However, they do not recapitulate key processes that occur in the body, such as blood and

lymph flow, innervation, and physiological gas exchange. Thus, I developed and utilized an *in vivo* imaging system in order to study T cell:parasite interactions in their natural environment to further extend my observations derived from *in vitro* studies. It is important to note that two-photon microscopy is the gold standard for imaging cells in living mice, given their ability to penetrate deeper into tissues without causing excessive phototoxicity and photodamage, allowing for repeated tissue imaging over long periods of time. Using adoptive transfer of *in vitro*-activated T cells, I was able to directly compare the behaviour of antigen-specific and nonspecific cells in the infected dermis and saw both completely arrested and dynamic contacts between PEG T cells and infected cells. This spectrum of behaviour could be explained by density of antigen presented at the cell surface or the type of cells presenting, such as monocytes, macrophages, monocyte-derived DCs, or neutrophils. Both control and PEG activated Th1 cells were able to enter the inflamed tissue irrespective of their antigen specificity, as seen in literature before¹⁹⁴. However, I also observed reduction in T cell recruitment as inflammation decreased, despite the fact that parasites were not eliminated.

Another question I addressed in these studies is whether acute and chronic *L. major* lesions were fundamentally altered that restricted T cell entry or reactivation. Based on my *in vitro* co-culture studies that showed no differences in effector T cell responses to infected macrophages in time course studies, I hypothesized that similar T cell responses will be observed in both acute and chronic lesions. My study presented *in vivo* evidence of limitation of antigen specific CD4⁺ T cell response to *L. major*. First, despite the exquisite sensitivity of PEG T cells, a large proportion did not arrest to interact with antigen, suggesting non-uniform accessibility of antigen. The physical structure of the skin, such as granuloma-like highly packed structures and chemokines could account for the heterogeneity. Second, when transferred into mice with healed lesions, T cell

homing ability was much less efficient, and both T cell populations displayed reduced motility profiles at the lesion site suggesting significant structural remodeling and lack of chemoattractants.

Lastly, I aimed to replicate endogenous anti-*Leishmania* response by creating ECFP⁺ PEG T cells in order to be able to observe them for a prolonged period of time. Having organized the breeding in the facility, and genotyping and phenotyping mice for the presence of both the fluorescent reporter and the transgene, I adoptively transferred naïve effector Th1 cells into mice and challenged them with a *L. major* infection. While I did see the clonal expansion of ECFP⁺ PEG T cells at week 2, I observed an overall decrease of cells in the field of view. Unfortunately, this experiment was terminated prematurely due to the outbreak of the Covid-19 pandemic.

Overall, studies described in Chapter 3 sets the stage to utilize PEG TCR transgenic mice to visually characterize immunodominant anti-*L. major* T cell responses *in vitro* and *in vivo*. I found that this approach is a sensitive readout to identify immune evasion mechanisms, priming events in the lymph node during infection, and visualize T cell responses during different phases of *L. major* infection. The main finding was that despite the ability of T cells to recognize infected macrophages *in vitro*, their entry and migratory behaviors in chronic lesions were impaired, and no contribution of PEPCK peptide presentation was observed. I hypothesize that in chronic lesions, there is significant remodeling of the extracellular matrix (as seen as irregular/missing collagen fibers in 2PTM images) that prevents/alters T cell responses in skin. Another possibility is that substantial immunological changes have occurred in the healing phase, with a focus on regulatory T cells. Early studies demonstrated that Treg depletion leads to sterilizing immunity, underscoring their role in suppressing effector T cell responses/behaviors²²⁹ and will be explored in more detailed in Chapter 4 of this thesis.

Chapter 4: Antigen recognition reinforces regulatory T cell suppression of anti-L. major effector T cell responses via IL-10

4.1. Introduction

In infected humans, high presence of FoxP3 positive cells in the lesion is associated with unresponsiveness to treatment and cytokines derived from *Leishmania*-specific Tregs are associated with active disease^{229,470,483,557}. Tregs in healed *L. major* lesions in mice were shown to be *L. major* specific^{229,483}. Therefore, I hypothesized that the accumulation of *Leishmania*-specific Tregs in healed skin restricts effector T cell responses and allows for the low-level infection to persist *in vivo*. However, in studies of *L. major*-specific Tregs, the focus was on the whole polyclonal population, and it is not known whether immunodominance observed with Th1 responses can also translate to Treg function. We discovered that PEG mice have regulatory T cells where a majority recognize PEPCK peptide, giving us the opportunity to examine whether PEPCK-specific Tregs impact effector T cell responses during *L. major* infection *in vivo*, for the first time. My goal was to directly demonstrate whether suppressive responses by anti-Leishmania Tregs are regulated by antigenic stimulation, and whether activation enhanced suppressive function. In this chapter, I first isolated, expanded and performed phenotypic and functional analysis of PEPCK-specific Tregs *in vitro* through co-culture studies with *Leishmania*-infected macrophages. Next, impact of Treg populations on parasite burden in infected macrophages was evaluated. Lastly, I generated a breeding colony by crossing PEG mice with FoxP3-GFP reporter mice in order to obtain PEPCK-specific, GFP-tagged Tregs.

Suppressive mechanisms of Tregs can generally be divided into three categories: secretion of inhibitory cytokines, local competition for growth factors, and cell-cell contact. However, these pathways are not necessarily mutually exclusive. Tregs exhibit numerous mechanisms of

suppression, ranging from limiting T cell expansion in the lymph node to attenuating effector responses at peripheral tissue sites. Tregs can reduce the frequency of stable contacts between dendritic cells and naïve T cells in the lymph node, thereby lowering T cell activation⁵⁵⁸, or remove cognate MHC-II-peptide complexes in an antigen-specific manner through trogocytosis³³¹. CD80 and CD86 co-stimulatory molecules can also be selectively removed during DC-Treg contacts, also reducing T cell activation levels^{330,559}. At the same time, secreted immunosuppressive cytokines interleukin-10 (IL-10) and transforming growth factor-beta (TGFβ)⁵⁶⁰ are implicated as the main players in the effector functions of Tregs. In this chapter I explored the mechanisms by which Tregs execute their functions in vitro, given the substantial suppression of Th1 IFN-γ response to *L. major* by antigen-specific Tregs and examined whether the ability of Tregs to recognize *L. major* antigen enhances their mechanisms of suppression. This chapter has 3 aims:

Aim 1: To develop a protocol to isolate, expand and phenotype PEG Tregs in culture, and directly assess their suppressive capabilities in co-culture systems

Aim 2: To evaluate the cellular mechanism by which Leishmania-specific Tregs suppress effector T cell responses

Aim 3: Assess the role of IL-10 and TGFβ cytokines in the suppression of PEG Th1 function

Significance/Impact: Initial characterization of Tregs described them as having a skewed TCR repertoire towards self-antigen to aid in preventing autoimmunity. However, subsequent studies have described that Tregs can recognize pathogens, and that they play a role in chronic infections. Here, I utilized PEG mice to demonstrate that not only are Tregs able to recognize a foreign PEPCK peptide, but that recognition and subsequent TCR activation led to an enhanced suppressive activity. The ability of Tregs to recognize foreign antigen and the role of this

recognition in disease maintenance is starting to emerge, with studies presented in this thesis being one of the first.

4.2. Results

4.2.1. Aim 1: To characterize PEG Treg phenotype in comparison to control Tregs and evaluate if they are capable of suppressing IFN γ production by PEG Th1 cells in response to PEPCK peptide.

4.2.1.1. Tregs isolated from control and PEG mice retain phenotypic and functional characteristics after in vitro expansion.

CD4⁺CD25⁺ T cells were isolated from spleens of both wildtype and PEG C57Bl/6 mice using a Treg isolation kit and activated for 48 hours via CD3/CD28 bead stimulation supplemented with 2000 U/mL IL-2, as described previously⁵⁶¹. After 7 days of daily media and IL-2 replacements, a phenotypic analysis for CD4, CD25, Foxp3 and CTLA-4 was performed, with antigen specificity measured by PEPCK₃₃₅₋₃₅₁:I-A^b tetramer staining (Figure 17A). I observed no differences in the number of Tregs recovered after expansion between the two populations and no phenotypic differences were observed (approximately 20-fold expansion after 7 days). Tetramer binding of PEG Tregs confirmed PEPCK-specificity.

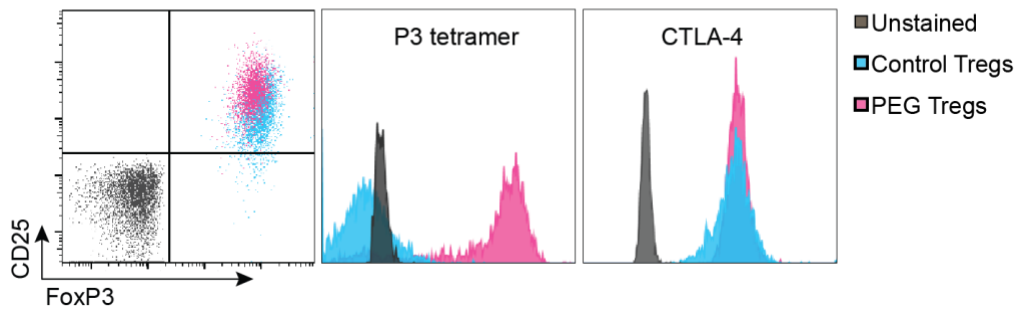
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Figure 17. Phenotypic analysis of Day 7 expanded Tregs from control and PEG mice.

(A) Regulatory T cells were isolated from spleens of wildtype control or PEG C57Bl/6 mice activated with anti-CD3/CD28 beads and expanded for 7 days in the presence of IL-2. Phenotypic analysis of expanded control or PEG Tregs at Day 7 is shown.

Next, I directly evaluated Treg suppressive activity after co-culture with effector PEG T cells and PEPCK-pulsed macrophages. In order to distinguish between Tregs and effector T cells during flow cytometry analysis, I stained effector cells with CMFDA or CMAC celltracker dyes prior to co-culture, which can be detected in the FITC or Pacific Blue channels on the flow cytometer, respectively (Figure 18A, B). After 24 hours of co-culture, IFN γ responses in effector T cells was evaluated as previously described in Chapter 3, but under varying Treg:effector T cell ratios ranging from 0.1:1 to 1:1. A significantly enhanced suppressive activity of PEPCK-specific Tregs were observed compared to control, polyclonal Tregs, indicating that antigenic stimulation increased Treg suppressive activity (Figure 18C-E). Stronger suppressive activity was observed at higher Treg:effector ratios.

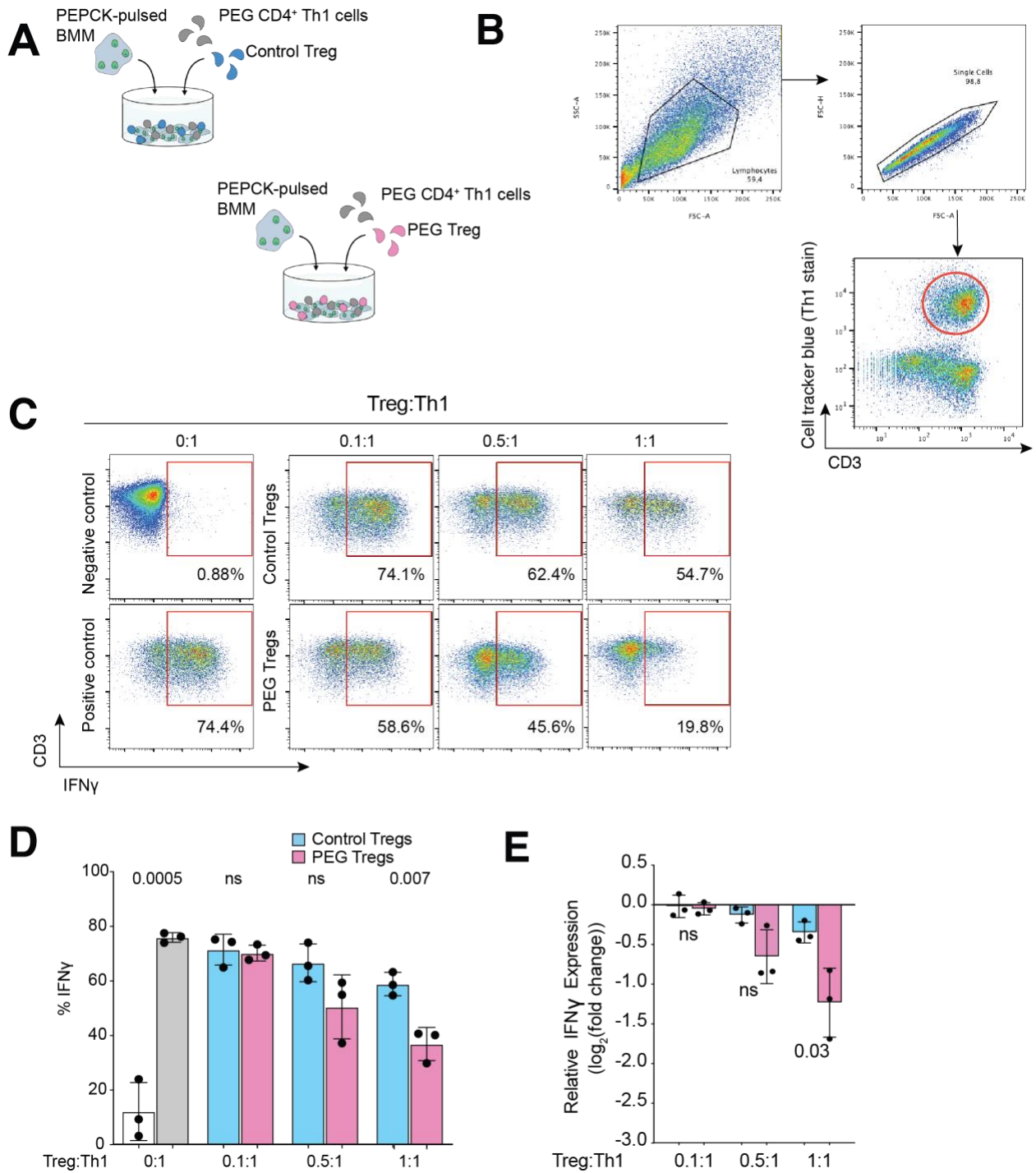


Figure 18. Treg-driven suppression of IFN γ production by PEG Th1 cells.

- (A) Experimental design of the Treg suppression assay. PEG Th1 cells were co-cultured with 0.5 μ M PEPCK335-351 peptide-pulsed macrophages and either control or PEG Tregs for flow cytometry and multiplex cytokine analysis.
- (B) Gating strategy for selecting cell tracker blue-stained PEG Th1 cells from the co-culture with 0.5 μ M PEPCK335-351 peptide-pulsed macrophages and either control or PEG Tregs for flow cytometry.
- (C) IFN γ production by PEG Th1 cells was measured by flow cytometry at various Th1: Treg ratios. (C) Red box: positive IFN γ signal. (D) % IFN γ production and (E) relative IFN γ expression compared to % IFN γ production by PEG Th1 cells without Tregs (gray bar). White bar: PEG Th1 cells cultured with uninfected macrophages (negative control), gray bar: PEG Th1 cells cultured with 0.5 μ M PEPCK335-351 peptide-pulsed macrophages. Blue and pink bars: Control and PEG Tregs, respectively, added to culture with PEG Th1 cells and 0.5 μ M PEPCK335-351 peptide-pulsed macrophages. Data shown are representative of three independent experiments; each dot represents a mean value from three technical replicates. Mean \pm SEM, Statistical analysis: unpaired t test, ns = not significant.

Studies were repeated with *Leishmania major*-infected BMMs (Figures 19A). Consistent with peptide-pulsed macrophages, *L. major*-specific Tregs displayed significantly higher suppressive activity compared to control Tregs (Figures 19B-D).

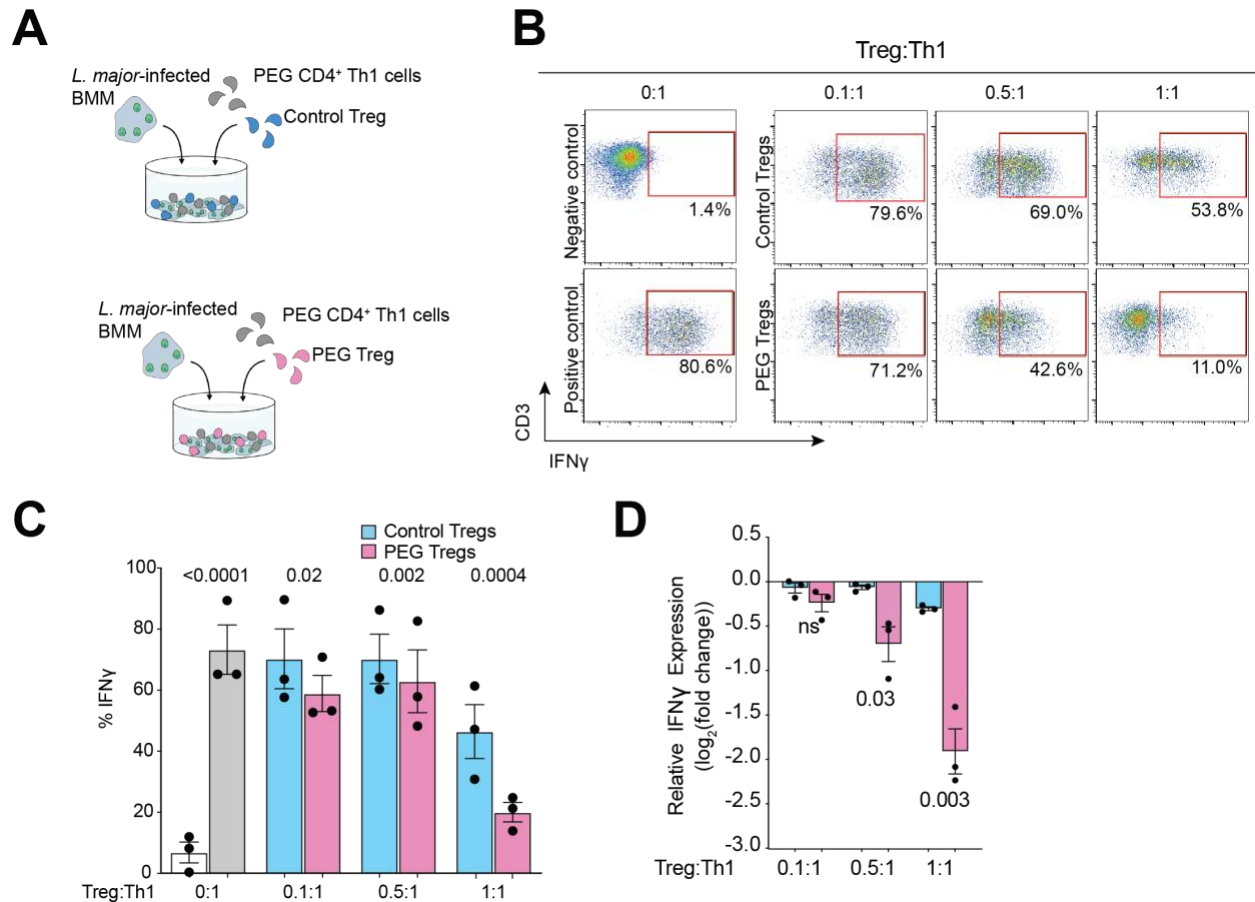


Figure 19. Antigen-specific Tregs suppress Th1 response against *Leishmania major* infection.

- (A) Experimental design of the Treg suppression assay. PEG Th1 cells were co-cultured with *L. major*-infected macrophages and either control or PEG Tregs for flow cytometry.
- (B) IFNγ production by PEG Th1 cells was measured by flow cytometry at various Th1: Treg ratios. (B) Red box: positive IFNγ signal. (C) % IFNγ production and (D) relative IFNγ expression compared to % IFNγ production by PEG Th1 cells without Tregs (gray bar). White bar: PEG Th1 cells cultured with uninfected macrophages (negative control), gray bar: PEG Th1 cells cultured with *L. major*-infected macrophages. Blue and pink bars: Control and PEG Tregs, respectively, added to culture with PEG Th1 cells and *L. major*-infected macrophages. Data shown are representative from three independent experiments; each dot represents a mean value from three technical replicates. Mean +/- SEM, Statistical analysis: unpaired t test, ns = not significant.

Next, the impact of Tregs on leishmanicidal activity by the BMMs was evaluated, with the hypothesis that reduced IFN γ responses translated to reduced parasite clearance. BMMs were seeded onto sterile microscopy cover slides and infected with GFP-expressing *L. major* for 6 hours. Infected macrophages were co-cultured alone or PEG effector Th1 cells in the presence of either control or PEPCK-specific Tregs for another 24 hours. T cells were washed off and I performed immunohistochemistry using F4/80 (macrophage marker) and GFP (GFP signal enhancement) and imaged on a confocal microscope (Figure 20A). Analysis of randomly selected FOV was used to calculate both the percentage of infected macrophages out of 100 cells and the number of parasites remained per macrophage (Figure 20B, C). In the absence of effector T cells, 80% of macrophages were infected with an average 3-4 parasites per cell, with some containing as many as 10-15. The addition of PEG Th1 cells reduced the percent of infected macrophages and the number of parasites per cell, as expected. Both control and PEG Tregs increased the percentage of infected macrophages and the number of *L. major* parasites per cell, but PEPCK-specific Tregs resulted in a significantly increased number of infected macrophages and the number of parasites observed per cell. These studies show that *L. major*-specific Tregs exhibit superior suppressive activity during *Leishmania* infection, and that they can dismantle effector T cell-mediated parasite control.

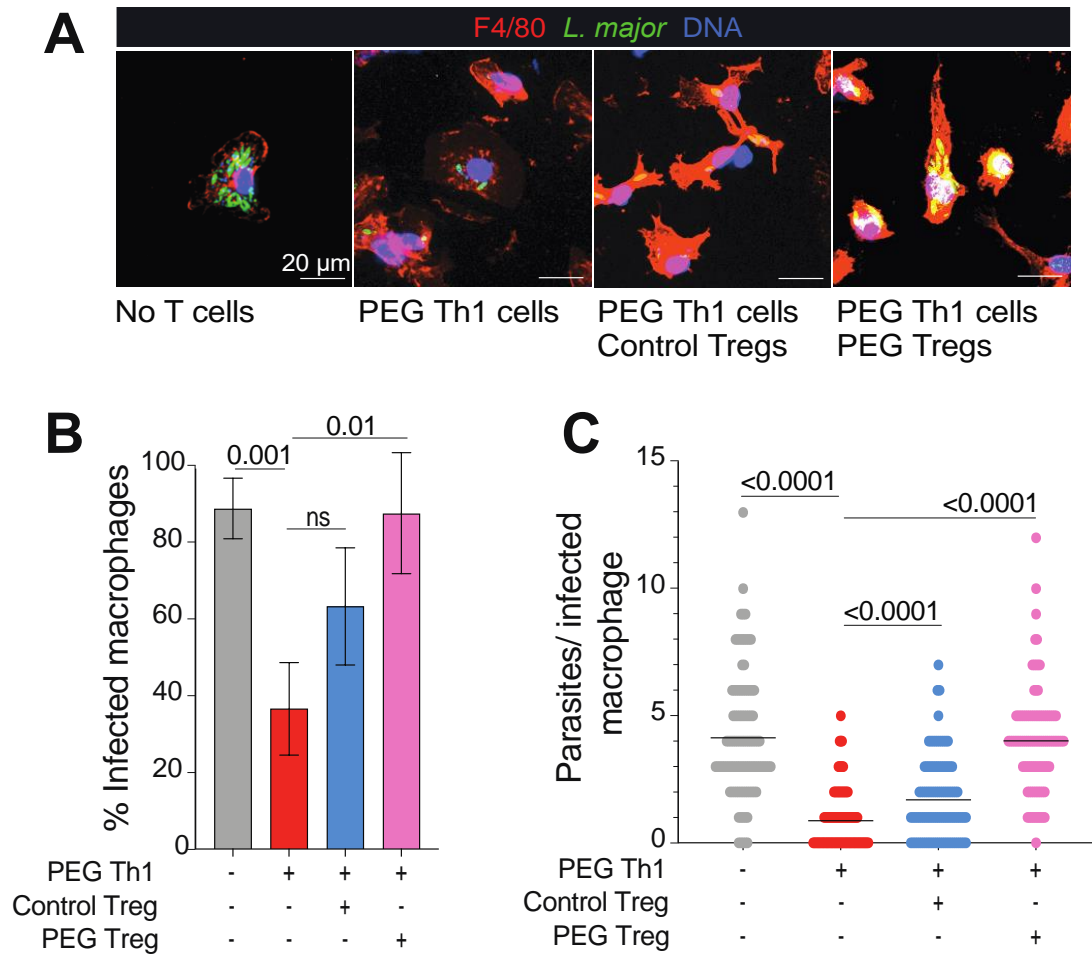


Figure 20. PEG Tregs aid in *Leishmania major* survival.

(A) Representative micrograph of *L. major*-infected macrophages in the presence or absence of Th1 cells and either control or PEG Tregs. Green – *L. major*, Red – F4/80.

(B) Percent infected macrophages and (C) parasite numbers per infected macrophage are shown. Representative graphs of two independent experiments are shown. Gray bar: PEG Th1 cells cultured with uninfected macrophages (negative control), red bar: PEG Th1 cells cultured with *L. major*-infected macrophages. Blue and pink bars: Control and PEG Tregs, respectively, added to culture with PEG Th1 cells and *L. major*-infected macrophages. Statistical analysis: unpaired t test.

4.2.1. Aim 2: To evaluate the cellular mechanism by which Leishmania-specific Tregs augment effector T cell responses

4.2.1.1. PEPCCK Tregs do not modulate T cell:macrophage interactions during Leishmania infection in vitro

As we have demonstrated previously, our live-cell imaging studies in 3D collagen showed that PEG Th1 cells engaged in stable contacts with *L. major* infected macrophages. We hypothesized that PEPCCK-specific Tregs interfere with Tcell:macrophage interactions, thereby reducing effector T cell activation and effector responses, as described in other disease context³³¹. To test this, *L. major*-specific Tregs were co-embedded with infected BMM (red) and effector Th1 cells in 3D collagen and prepared for live cell imaging (Figure 21A, B). As expected, PEG Tregs formed prolonged contacts with infected BMMs, leading to a reduction in their overall mean 3D track velocity (Figure 21C). Surprisingly, the presence of *Leishmania*-specific Tregs did not disrupt effector T cell contacts with infected macrophages (Figure 21D, pink box) but rather reinforced contact durations which often resulted in the formation of large cell clusters consisting of all three cell types (Figure 21B). Notably, I observed an overall reduction in effector Th1 cell migration speeds in the presence of infection, which was consistent with *in vivo* behaviors during the healed stages of *L. major* infection. Tregs increased the proportion of arrested cells equally, regardless of their specificity (Figure 21F, G). Together, observations from multiple imaging experiments indicated that Tregs did not disrupt Tcell:macrophage interactions, and that disrupting Th1:BMM contacts is not the mechanism by which Tregs inhibit effector responses.

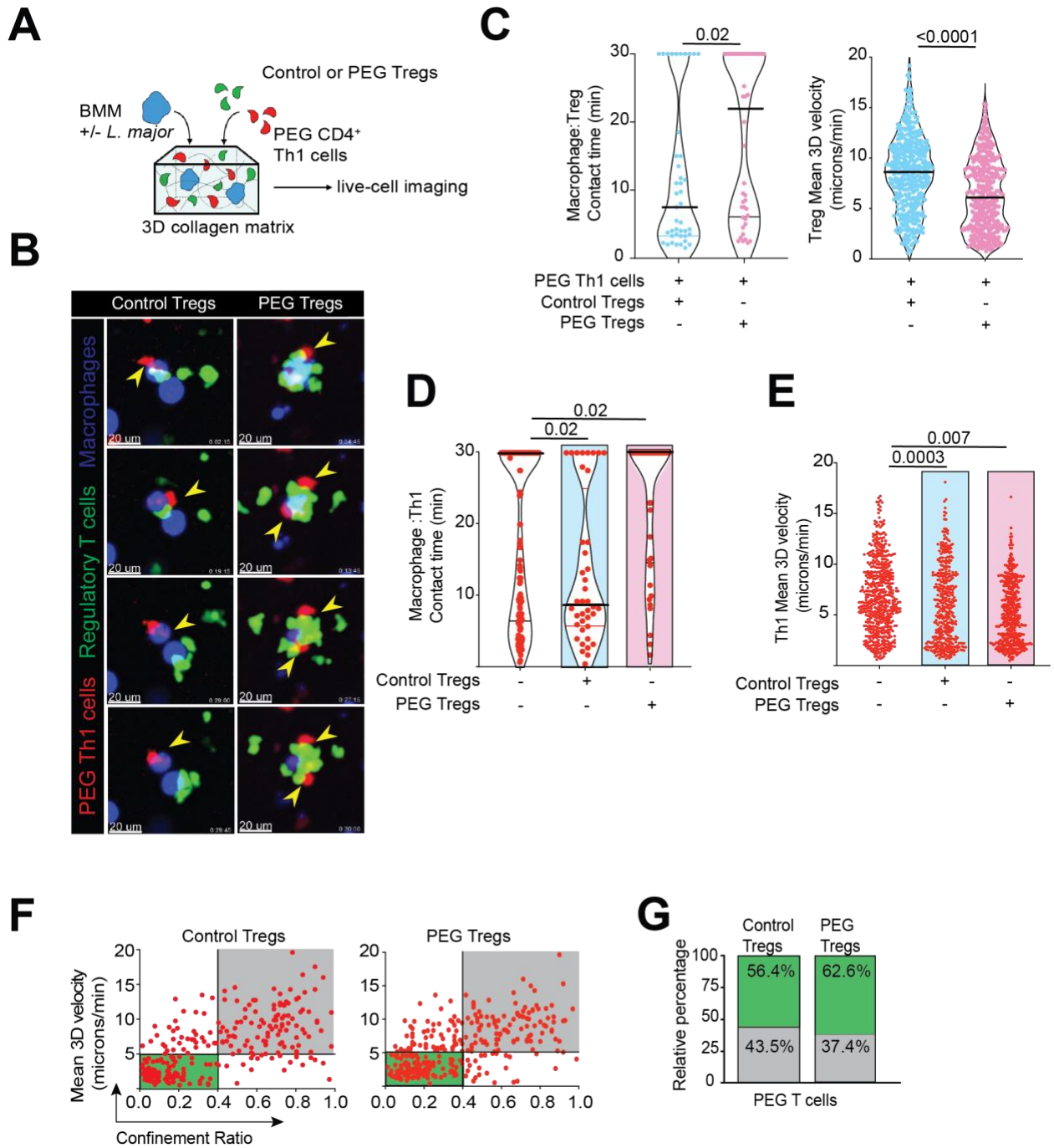


Figure 21. PEG Regulatory T cells do not disrupt prolonged macrophage:PEG Th1 contacts.

(A) Control or PEG Tregs and PEG Th1 cells were stained with cell tracker dyes (CMFDA; green, and CMTMR; red, respectively) and co-cultured with stained *L. major*- infected

macrophages (CMAC; blue). Chambers were placed on a temperature-controlled imaging platform to maintain temperatures at 37°C and imaged at 30-minute intervals using two-photon microscopy.

- (B) A time series micrograph of co-cultures at indicated times. Time stamps in min:sec represent elapsed time of the recordings.
- (C) Real-time contact duration between control or PEG Tregs and macrophages and mean 3D track velocity, each dot representing a single contact. Black lines represent median values. Statistical analysis: Mann-Whitney test. Data combined from three independent experiments.
- (D) Real-time contact duration between PEG (red dots) Th1 cells and macrophages alone or in the presence of control (blue box) and PEG (pink box) Tregs. Each dot represents a single contact. Black lines represent median values. Statistical analysis: Mann-Whitney test, ns = not significant. Data combined from three independent experiments.
- (E) Mean 3D track velocity of PEG (red dots) Th1 cells cultured with macrophages alone or in the presence of control (blue box) and PEG (pink box) Tregs. Each dot represents a single cell track. Black lines represent median values. Statistical analysis: Mann-Whitney test, ns = not significant. Data combined from three independent experiments.
- (F) Track velocities of PEG (red) Th1 cells cultured with *L. major*-infected macrophages in the presence of control or PEG Tregs plotted against confinement ratio (F) and relative percentage of confined slow and meandering fast tracks (G). Data combined from three independent experiments.

4.2.1.1. Tregs do not alter antigen presentation/co-stimulatory receptor expression in Leishmania-infected macrophages

I next explored whether Treg:macrophage interactions altered cell surface expression of receptors involved in antigen presentation, thereby reducing TCR or costimulatory signaling through cell-cell interactions. Specifically, I focused on co-stimulatory molecules CD80/CD86 in regulating effector T cell responses. To test this, I performed flow cytometry analysis to measure MHC-I, MHC-II, CD40, CD80, and CD86 expression on infected macrophages in the presence or absence of Treg populations (Figure 22A). Whereas macrophage activation in terms of upregulation of the aforementioned molecules was observed in the presence of effector T cells alone, no significant differences or trends in the expression profiles in the presence of Tregs were observed. These studies suggest that modulation of antigen presentation/co-stimulation is not a mechanism of Treg-driven immune suppression of effector T cells.

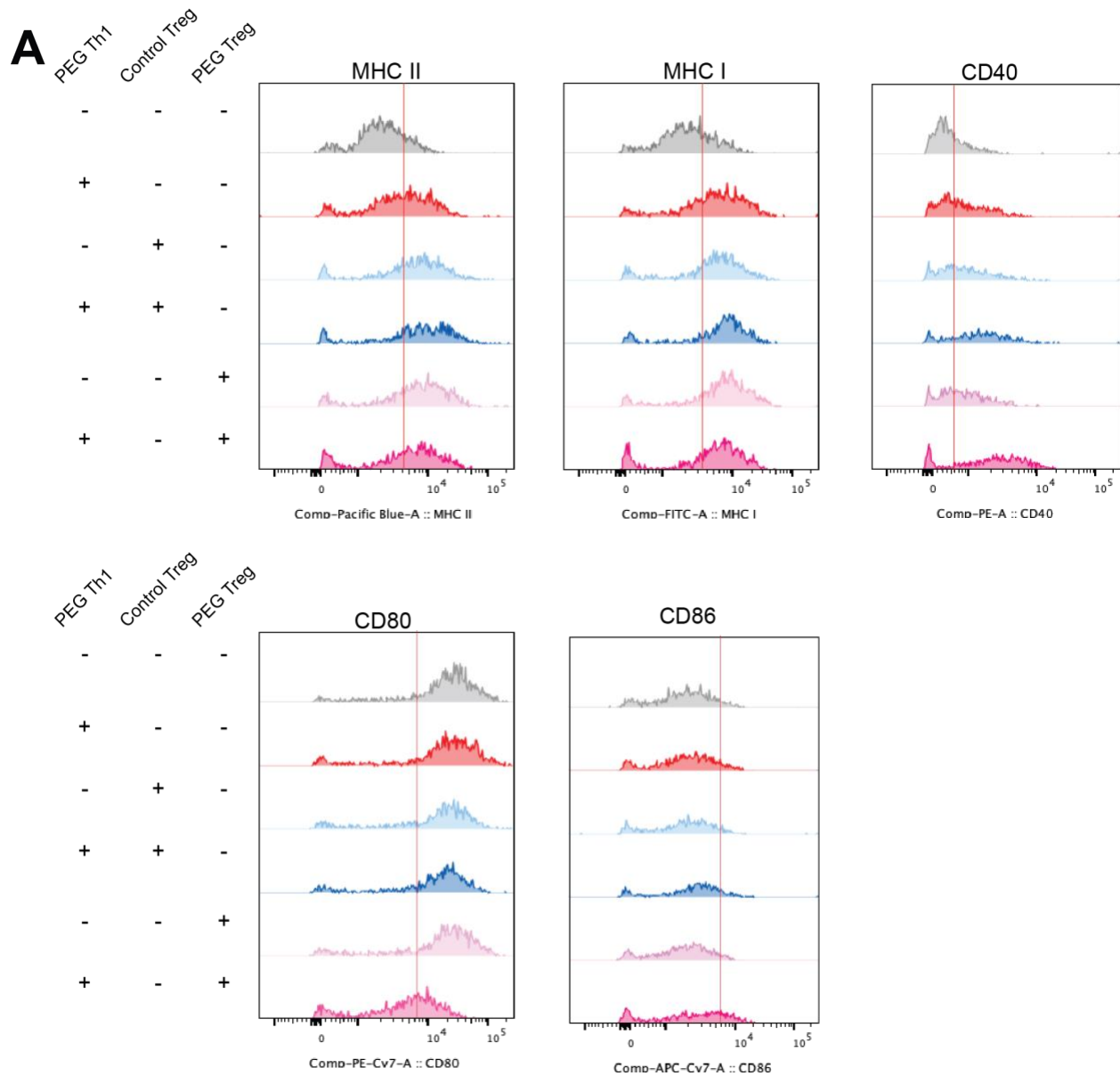


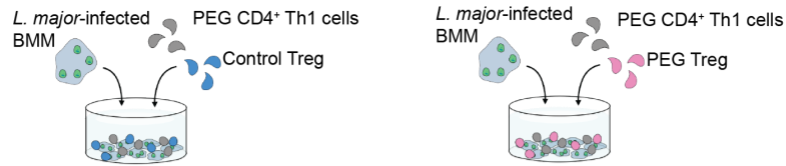
Figure 22. Tregs do not alter antigen presentation/co-stimulatory receptor expression in *Leishmania major*-infected macrophages.

(A) Flow cytometry analysis of *L. major*-infected macrophage surface molecules (gray) and co-culture with PEG Th1 cells (red), with the addition of PEG Tregs (light pink) cells or control Tregs (light blue), or *L. major*-infected macrophages alone with PEG Tregs (dark pink) or control Tregs (dark blue). Representative data from three independent experiments.

4.2.3. Aim 3: Assess the role of immunomodulatory cytokines in the suppression of PEG Th1 function

4.2.3.1 Regulation of inflammatory cytokine production by Leishmania-specific Tregs

To further extend the impact Tregs had on inflammatory cytokine expression during *Leishmania* infection, co-culture studies described in Figure 19 were repeated, and culture supernatants were collected for multiplex ELISA analysis (Eve Technologies) using the pro-inflammatory Discovery Assay®. I observed significantly elevated IL-10 expression in the presence of PEG Tregs compared to control Tregs (Figure 23A, B). At higher ratios of PEG Tregs, IL-10 supernatant concentration was as high as 4000 pg/mL, a 4-fold increase over control Treg cultures. Interestingly, depletion of IL-2 levels in media was observed in the presence of *Leishmania*-specific Tregs, indicating higher IL-2 usage by effector Tregs. Other pro-inflammatory cytokines, such as IL-1b, IL-2, IL-6, IL-12, MCP-1, and GM-CSF were also reduced in the presence of *Leishmania*-specific Tregs. Curiously, IL-4 expression decreased with the presence of control Tregs but increased 1.5x with the presence of PEG Tregs. Lastly, there was no detectable change in TGFβ1, TGFβ2, or TGFβ3 expression in the presence of either control or PEG Tregs. All the changes described were exacerbated with the presence of PEG Tregs, particularly at 1:1:1 ratio, as can be seen with the relative expression analysis of Log₂(Fold change) (Figure 24A). These studies indicate that PEPCK-specific Tregs had a substantial role in regulating cytokine expression profiles by multiple cell types, all consistent with their immunomodulatory role during parasitic infection.

A**B**

Control Tregs
PEG Tregs

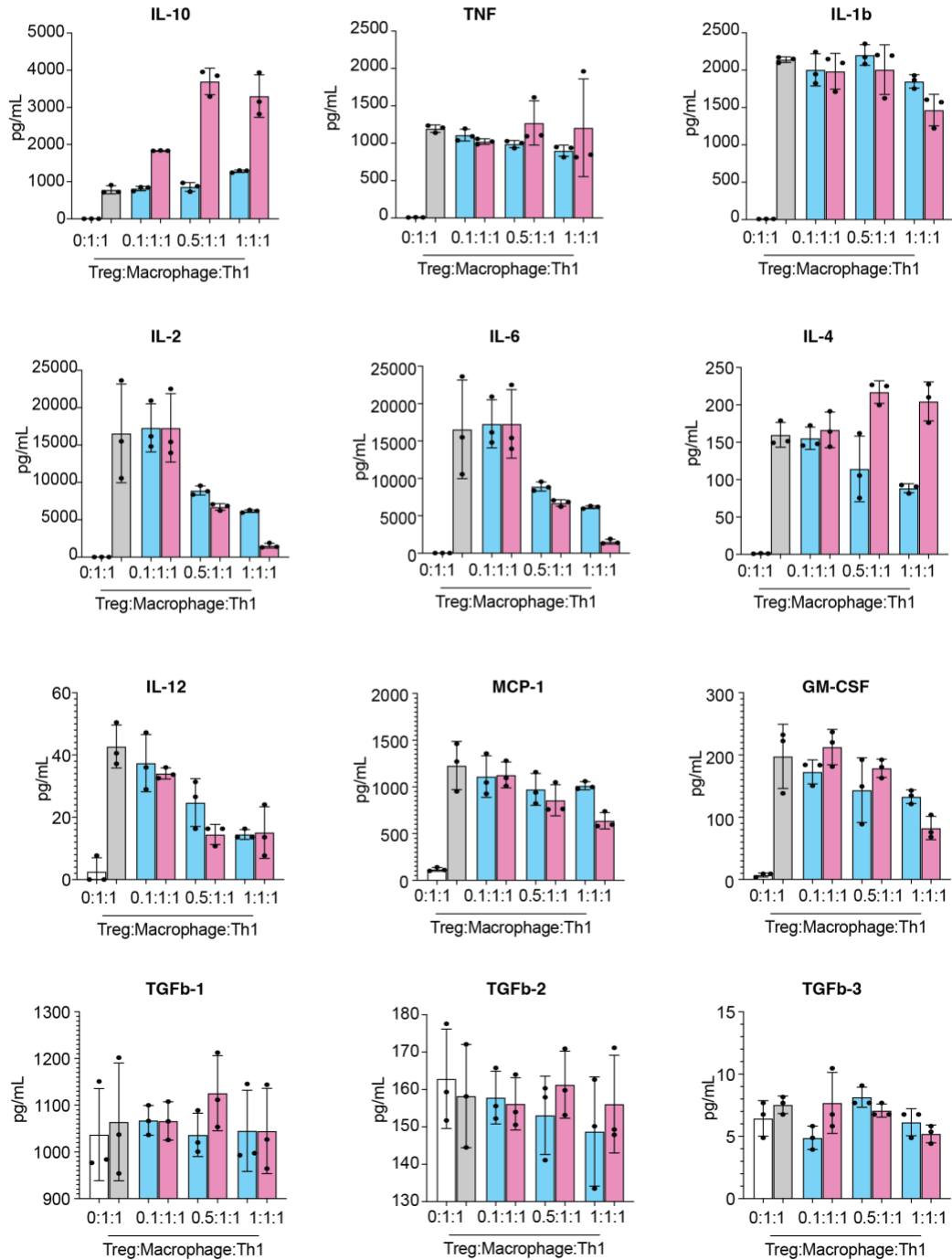


Figure 23. ELISA measurements of pro-inflammatory cytokine panel.

(A) Relative expression of cytokines measured by multiplex ELISA during Treg suppression assays. Control (blue) and PEG (pink) Tregs. Data combined from three independent experiments; each dot represents a mean value from three technical replicates. Mean \pm SEM, Statistical analysis: unpaired t test, ns = not significant.

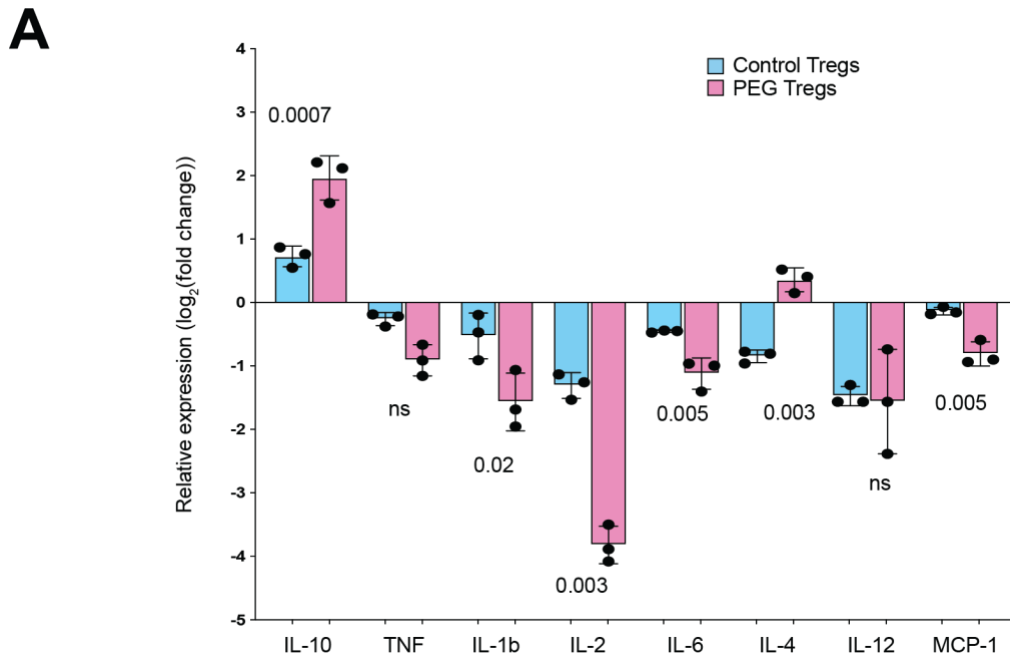


Figure 24. Relative expression of cytokines measured by multiplex ELISA during Treg suppression assays.

(A) Relative expression of cytokines measured by multiplex ELISA during Treg suppression assays. Control (blue) and PEG (pink) Tregs. Data combined from three independent experiments; each dot represents a mean value from three technical replicates. Mean \pm SEM, Statistical analysis: unpaired t test, ns = not significant.

4.2.3.2. IL-10 is the major driver of anti-Leishmania effector T cell responses during infection in vitro.

IL-10 and TGF β are well characterized immunomodulatory factors produced by Tregs that can dampen effector T cell responses. I hypothesized that IL-10 was the main mediator of effector T cell suppression in my co-culture assays, since TGF β was not detected by ELISA. To test this, co-culture experiments were repeated as described above, but in the presence of anti-IL-10 blocking antibody (Figure 25A). 1 μ L/mL of anti-IL-10 antibody was added at the beginning of the macrophage:effector T cell:Treg co-culture for 24 hours, and effector IFN γ production was evaluated by flow cytometry. Neutralization of IL-10 completely restored the capacity of Th1 cells to produce IFN γ (Figure 25B), further cementing the role of IL-10 as a main suppressive cytokine by activated Tregs in my model.

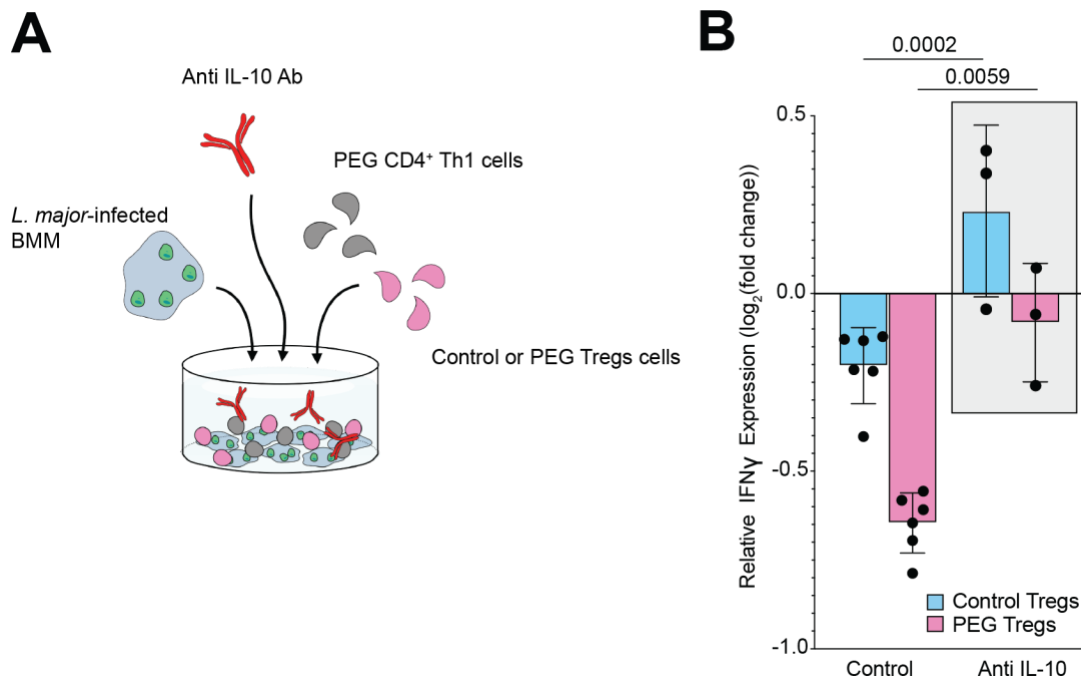


Figure 25. Addition of the blocking anti-IL-10 antibody to the co-cultures restores IFN γ production by PEG Th1 cells.

(A) IFN γ production by PEG Th1 cells in the presence of Control (blue) and PEG (pink) Tregs at 1:1 Th1:infected macrophage ratios. Gray bar: anti-IL-10 antibody was added to the co-culture. Each dot represents a single experimental replicate. Mean \pm SEM. Statistical analysis: unpaired t test. Representative graph of two independent experiments is shown.

To further extend these findings, IL-10 cytokine at varying concentrations were evaluated for their suppressive activity in vitro. Day 7 activated PEG Th1 cells were cultured with *L. major*-infected BMMs and various concentrations of IL-10 and/or TGF β (Figure 26A, experiments performed by Atta Yazdanpanah). Compared to untreated controls, treatment with IL-10 alone resulted in a drastic reduction in IFN γ effector responses in a dose dependent manner. Interestingly, TGF β did not alter IFN γ production, and combinatory treatment with IL-10 and TGF β had no additive effects, as expected (Figure 26B, C). These results confirm our blocking studies that IL-10 is the main regulatory of effector T cell responses in my co-culture model, likely produced by PEPCK-specific regulatory T cells.

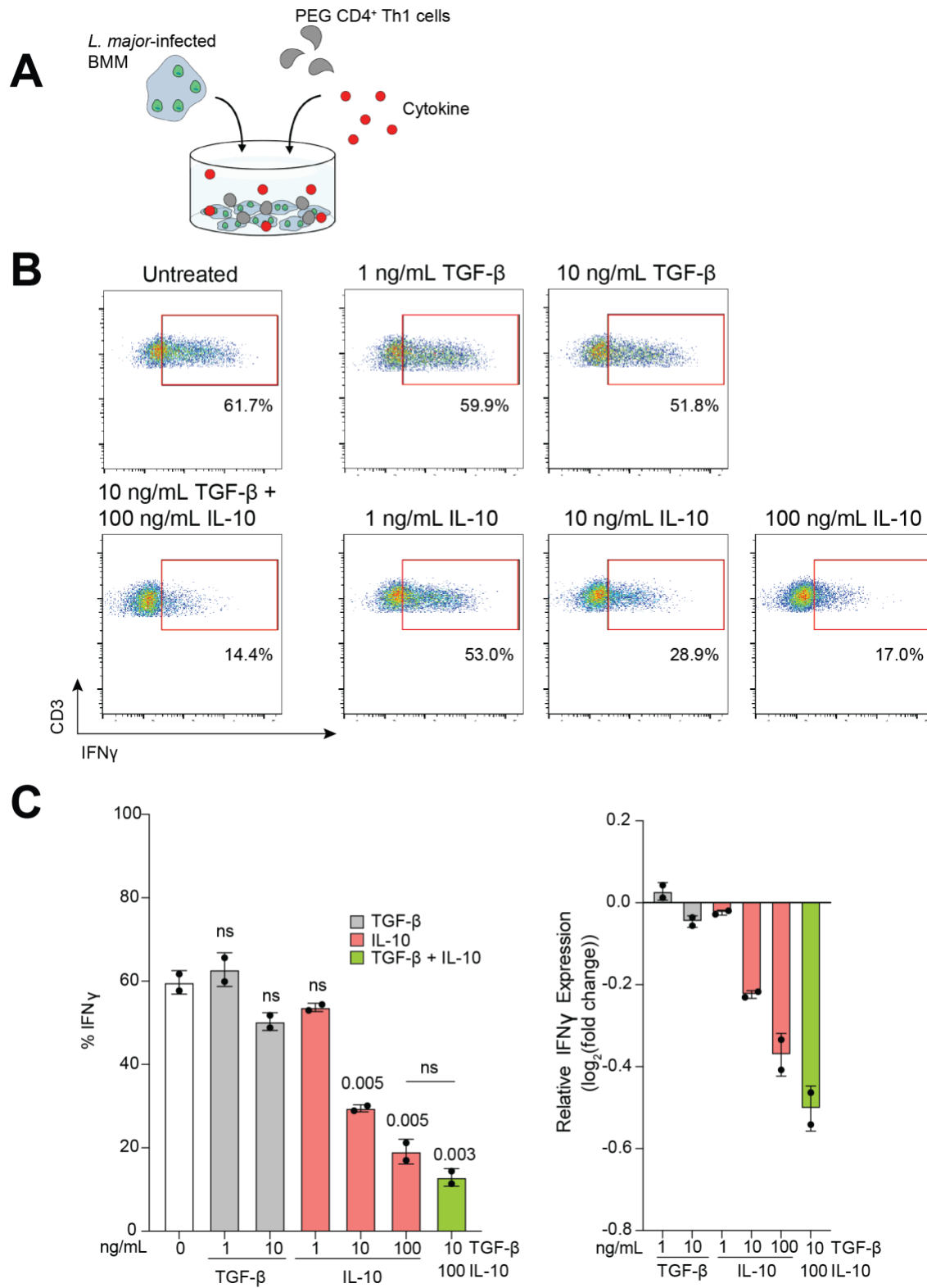


Figure 26. Addition of IL-10 to the co-cultures suppresses IFN γ production by PEG Th1 cells.

- (A) Experimental design. PEG Th1 cells were co-cultured with *L. major*-infected macrophages and either IL-10, TGF- β , or both cytokines.
- (B) IFN γ production by PEG Th1 cells was measured by flow cytometry with addition of suppressive cytokines IL-10 and TGF- β . Red box – positive signal.
- (C) % IFN γ production and relative IFN γ expression compared to % IFN γ production by PEG Th1 cells without suppressive cytokines (white bar). White bar – PEG Th1 cells cultured with *L. major*-infected macrophages. Gray bar: 1 or 10 ng/mL TGF- β was added to culture. Red bar: 1, 10, or 100 ng/mL IL-10 was added to culture. Green bar: 10 ng/mL TGF- β and 100 ng/mL IL-10 was added to culture. Representative data from two independent experiments, each dot represents an experimental replicate. Mean \pm SD, Statistical analysis: unpaired t test, ns = not significant.

4.2.3. The loss of PEPCK TCR in FoxP3-GFP PEPCK Tregs leads to a decrease in suppressive capability

In order to increase the ease of sorting and to obtain the ability to record PEG Treg behaviours with 2-photon microscopy for a prolonged period of time, I generated a breeding colony by crossing PEG mice with B6.Cg-FoxP3^{tm2Tch}/J mice. These "Foxp3^{EGFP}" mice co-express EGFP and the X-linked regulatory T cell-specific transcription factor Foxp3 under the control of the endogenous promoter. The aim was to obtain EGFP fluorescent PEPCK-specific Tregs (Figure 27A). PCR-based analysis with primers specific for TCR α and TCR β genes were again used to amplify genomic DNA from mice to visualize the presence of the PEPCK-TCR genes (Figure 27 B). However, phenotypic analysis of the blood samples from the PCR-positive mice using PEPCK335-351:I-A^b tetramer showed that while around 70-80% of the CD4⁺ T cells in the blood samples of transgenic mice bound the tetramer, indicating PEPCK specificity, all were EGFP negative, indicating their selective elimination in vivo (Figure 27C). Tregs isolated from these mice did not suppress effector responses to the same degree as Tregs from regular PEG mice (Figure 27D). Upon the onset of the COVID-19 pandemic and university closure, these mice were sacrificed, and breeding was not reinstated, as they were not useful to visually track PEPCK-specific Tregs in vivo.

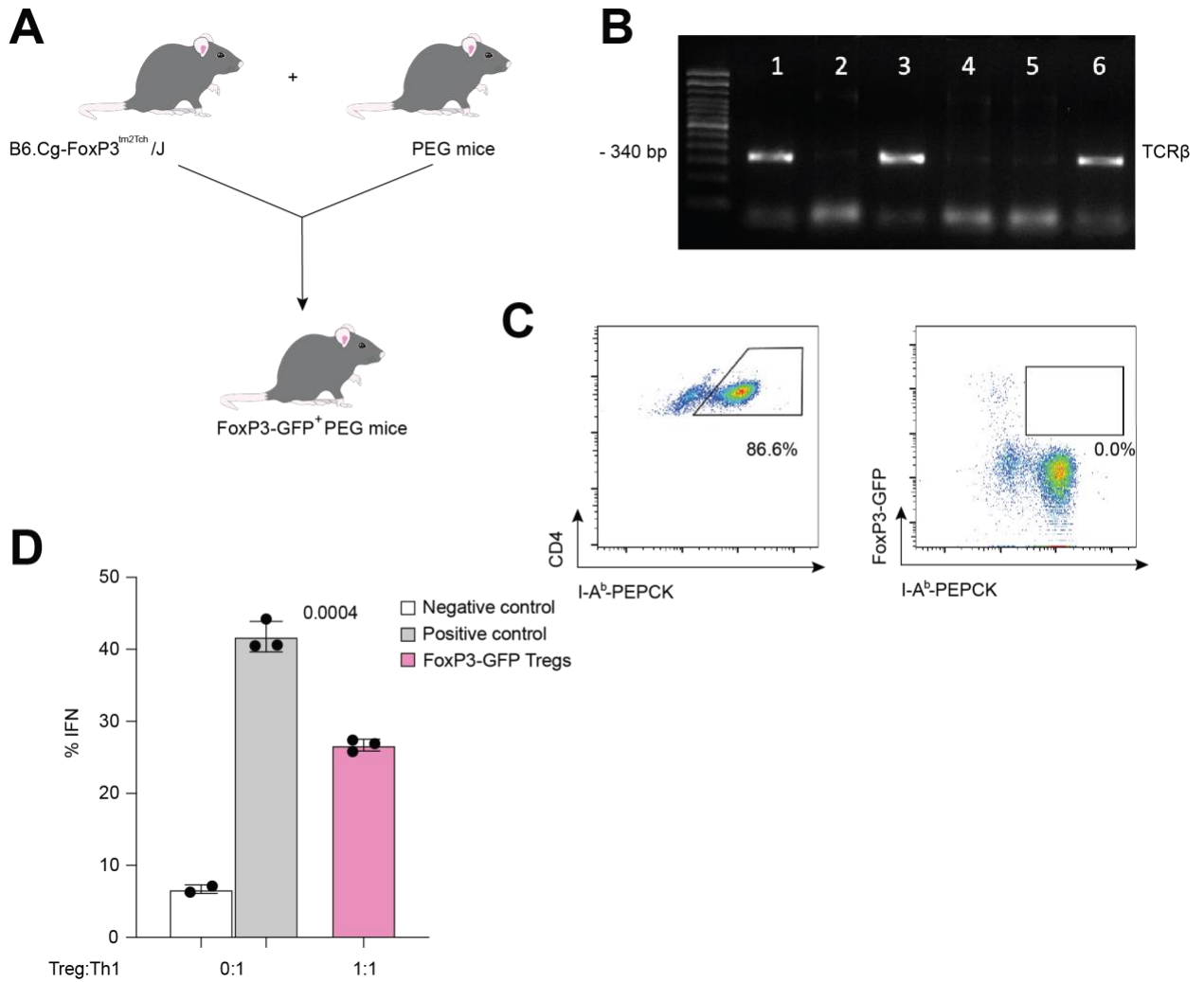


Figure 27. Loss of PEPCK TCR in FoxP3-GFP PEPCK Tregs leads to a decreased suppressive capability by Tregs.

(A) Schematic representation of the mouse cross. PEG mice were crossed with B6.Cg-FoxP3^{tm2Tch}/J mice in order to obtain EGFP-expressing PEG Tregs.

(B) PCR analysis of mouse tissue sample for PEG TCR transgene.

(C) Flow cytometry analysis of a blood sample for the expression of the TCR transgene.

(D) % IFN γ production by PEG Th1 cells without Tregs (gray bar). White bar: PEG Th1 cells cultured with uninfected macrophages (negative control), gray bar: PEG Th1 cells cultured with *L. major*-infected macrophages. Pink bar: FoxP3-GFP PEG Tregs added to the co-culture. Statistical analysis: unpaired t test.

4.3. Discussion and Conclusions

L. major parasites survive in the skin long after the lesion is healed and undergo low rates of replication in the skin macrophages. This ensures low antigen availability and therefore decreases the chances of being recognized by effector T cells, safeguarding long term survival. At the same time, low antigen availability may drive the generation and maintenance of *Leishmania*-specific Tregs. Tregs ensure a healing, immunosuppressive environment that subdues parasite killing. In human patients, high presence of FoxP3 positive cells in the lesion is associated with unresponsiveness to treatment and cytokines derived from *Leishmania*-specific Tregs are associated with active disease^{229,470,483,557}. Therefore, I hypothesized that the accumulation of *Leishmania*-specific Tregs in healed skin restricts effector T cell responses and allows for the low-level infection to persist.

The introduction of TCR-transgenic Tregs first allowed for the direct examination of the role of peripheral self-antigen in controlling Treg homeostasis. OVA-specific⁵⁶² or HA-specific⁵⁶³ Tregs were adoptively transferred into mice expressing the cognate antigen as a tissue-specific protein in the pancreatic islets through the use of rat insulin promoter. Antigen-specific Tregs were demonstrated to proliferate in the pancreatic LNs but not the non-draining LNs. Importantly, the total number of OVA-specific Tregs in the draining LNs was higher in mice expressing OVA as a self-antigen compared to controls. These findings highlighted the importance of antigen recognition in Treg homeostasis. Co-culturing OVA-specific Tregs and dendritic cells presenting OVA leads to Tregs suppressing proliferation and IL-2 production by conventional T cells even with different specificities. Studies with human cells show that Tregs transduced with a TCR recognizing a clotting factor VIII, which stimulates immune responses in hemophilia patients, can suppress factor VIII-specific helper T cells⁵⁶⁴. Likewise, TCR-transduced Tregs specific for

pancreatic islet cell antigens suppress responses by pathogenic T cells with greater potency than polyclonal Tregs in vitro⁵⁶⁵. Combined, these results state that antigen specificity plays a crucial role in Treg homeostatic and suppressive functions.

My first step was to isolate, activate, expand, and phenotype PEG and control Tregs, which was important to confirm the identity of PEG Tregs. Cell counts and phenotypic analysis showed no difference between the cells aside from antigen specificity, which ensured me that these are standard Tregs equivalent to polyclonal controls. Next, I tested the suppressive capability on first the PEG Th1 cells cultured with PEPCK-pulsed BMMs, then on PEG Th1 cells cultured with BMMs infected with *L. major*. Around 50% of T cells in *L. major* lesions were shown to be Tregs, therefore I added Tregs in increasing amounts to the co-cultures, from 0.1:1 to 1:1 Tregs to PEG Th1 cells. Flow cytometry analysis of IFN γ responses in effector T cells after the 24 hour culture and 6 hr BFA treatment showed striking results. While activated Tregs have been shown to have suppressive capabilities regardless of antigen specificity, which I observed as well, antigen recognition by Tregs led to a remarkably stronger suppression of IFN γ . Importantly, while both Tregs were able to arrest the killing of *L. major*, enhanced PEG Treg suppression led to enhanced parasite survival, indicating that decrease of IFN γ results in diminished leishmanicidal activity.

To build on these findings in vivo, I organized breeding FoxP3-EGFP and PEG mice, in order to create EGFP⁺ PEG Tregs and monitor their functions in vivo. Genotyping the tissue samples and phenotyping blood T cells showed the presence of the transgene, however the expression of PEPCK TCR was lost in the FoxP3-GFP population only. Similarly, Tregs from these mice were only able to suppress IFN γ to the levels of polyclonal control Tregs. As the breeding of these mice occurred at the same time as the breeding of ECFP⁺ PEG mice, the termination of the breeding

project also occurred prematurely due to Covid-19 pandemic, without the ability to investigate the reason for the TCR loss. The breeding experiments were not reinstated once the university reopened.

Chapter 5: Modulating Treg:Th1 balance in chronic L. major lesions leads to loss of parasite control in vivo

5.1 Introduction

In Chapter 4, I established that cognate antigen enhanced *Leishmania*-specific Treg function, mainly through production of IL-10. These observations suggest that Tregs help maintain an IL-10 gradient within chronic lesions that prevent complete clearance by effector Th1 responses. Consistent with this, previous studies have demonstrated that depletion of CD4⁺CD25⁺ Tregs or IL-10 signaling blockade resulted in a sterilizing cure in *Leishmania*-infected mice^{229,479}, whereas treatment of healed mice with immunosuppressive drugs or IFN γ signaling blockade resulted in a rapid increase in parasite burden and disease reactivation⁴⁶². These studies formulate a hypothesis that a balance between effector and regulatory T cell responses is established at the site of chronic lesions, and that tipping the balance in either direction can lead to parasite clearance or reactivation in vivo. What remains unexplored is whether Treg suppression of effector T cell response within chronic lesions is driven by antigen recognition, and the dynamic interplay between Tregs and effector T cells that facilitate parasite persistence. In this chapter, I specifically expanded *Leishmania*-specific Tregs in healed mice and measured endogenous T cell responses and disease outcomes by flow cytometry and 2P-IVM. This chapter has two aims:

Aim 1: In vivo expansion of Tregs in chronic lesions and their impact on disease reactivation

Aim 2: To characterize whether *Leishmania*-specific Treg expansion augments effector Th1 responses in healed skin

Significance/Impact: Despite the generation of strong Th1 responses during acute infection, infected cells continue to persist in healed skin, with evidence of ongoing replication¹⁴⁵. The

availability of *Leishmania* antigen in healed skin that can potentially modulate helper T cell pools¹⁸⁸ and/or regulatory T cell functions is unclear, especially in the context of *L. major* persistence. In this chapter I show, for the first time, that Tregs specific for the foreign PEPCK antigen exist in the healed skin and expand in response to PEPCK antigen influx. I show that PEPCK-specific Tregs show signs of antigen recognition in vivo and significantly suppress the capacity of endogenous Th1 cells to produce IFN γ , which can be restored through blocking IL-10. This suppression leads to a loss of lesion control and subsequent inflammation. Taken together, this work provides insights into the establishment of the immunosuppressive environment in the healed *L. major* lesion and argues that the accumulation of *Leishmania*-specific Tregs in healed skin disable effector responses to promote long-term parasite persistence.

5.2. Results

5.2.1. Aim 1: In vivo expansion of Tregs in chronic lesions and their impact on disease reactivation

5.2.1.1. FoxP3-GFP cells infiltrate the active lesion area throughout the course of *L. major* infection.

In order to visualize Tregs in the *L. major*-infected lesions, I expanded on the previously established in vivo imaging approach to visualize infection directly in the ears of live mice by utilizing FoxP3-eGFP reporter mice. To this end, Foxp3-GFP mice were intradermally infected in the ear pinna with 1×10^6 dsRed⁺*L. major* promastigotes⁵⁶⁶ (Figure 28A) and mice were prepared for intravital microscopy at various time points post-infection. As predicted, Tregs were observed in the lesion environment throughout the course of infection and, importantly, accumulated during the healed stages of infection at 10-13 weeks (Figure 28B). Interestingly, while previous experiments show that adoptively transferred Th1 cells generally surrounded the lesion, many

Tregs were localized directly on top of the lesion, likely in continual contact with infected cells. A proportion of Tregs was also observed localized around the hair follicles, indicating their tissue-resident nature.

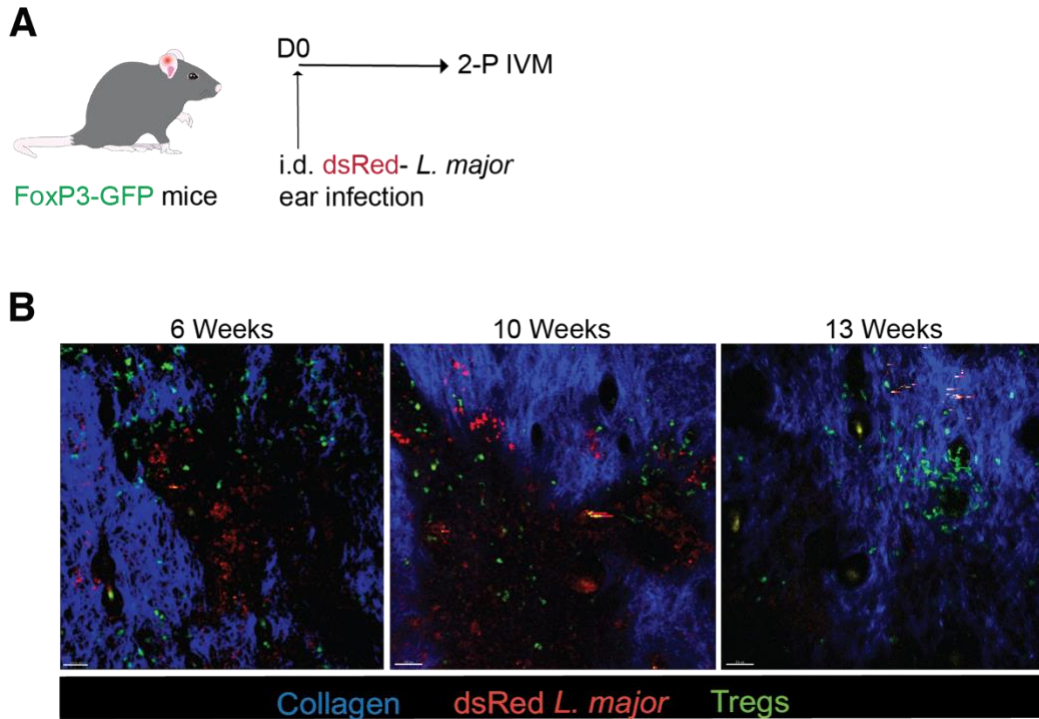


Figure 28. FoxP3-GFP cells infiltrate the active lesion area throughout the course of *L. major* infection.

- (A) C57Bl/6 Foxp3-GFP mice were infected via intradermal ear injections with 1 million dsRed⁺ *L. major* parasites. The ears were prepared for intravital at various time points post infection.
- (B) Representative micrographs of the lesions post infection of two independent experiments. Blue: collagen (SHG), red: dsRed⁺ *L. major*, Green: Foxp3-GFP cells.

5.2.1.2. Inoculation of healed mice with killed *L. major* parasites results in loss of lesion control and induces PEPCK-specific Treg expansion, compared to the *L. major* PEPCK^{-/-} challenge

Having confirmed the feasibility of visualizing infection and FoxP3 cells in the healed lesion, I proceeded to disturb the established homeostasis. Our collaborators have previously shown that Treg expansion leads to reactivation of disease in healed mice²³⁰. I used this approach to mechanistically address how Tregs modulated effector T cell responses to support long-term parasite survival and to address the role of cognate antigen in establishing a suppressive environment in healed skin. At day 60 post ear *L. major* infection, mice were challenged with either PBS, 5x10⁶ heat-killed wildtype *L. major* promastigotes or 5x10⁶ heat-killed PEPCK-deficient *L. major* promastigotes¹⁰⁴ via footpad injection²³⁰. Intravital microscopy of the previously healed ear dermis was performed after 2 weeks (Figure 29A, B). In mice challenged with heat-killed parasites, I observed expansion of Foxp3-GFP⁺ cells, with many found robustly migrating within the primary infection site (visualized by the lack of blue collagen structures; Figure 29B). High lesion score⁵⁶⁷ and significant redness/swelling and an increase parasite burden were observed after challenge with heat-killed wildtype *L. major* but observed to a lesser extent after challenge with PEPCK^{-/-} *L. major* (Figures 29C-E), suggestive of higher parasite replication in the former scenario.

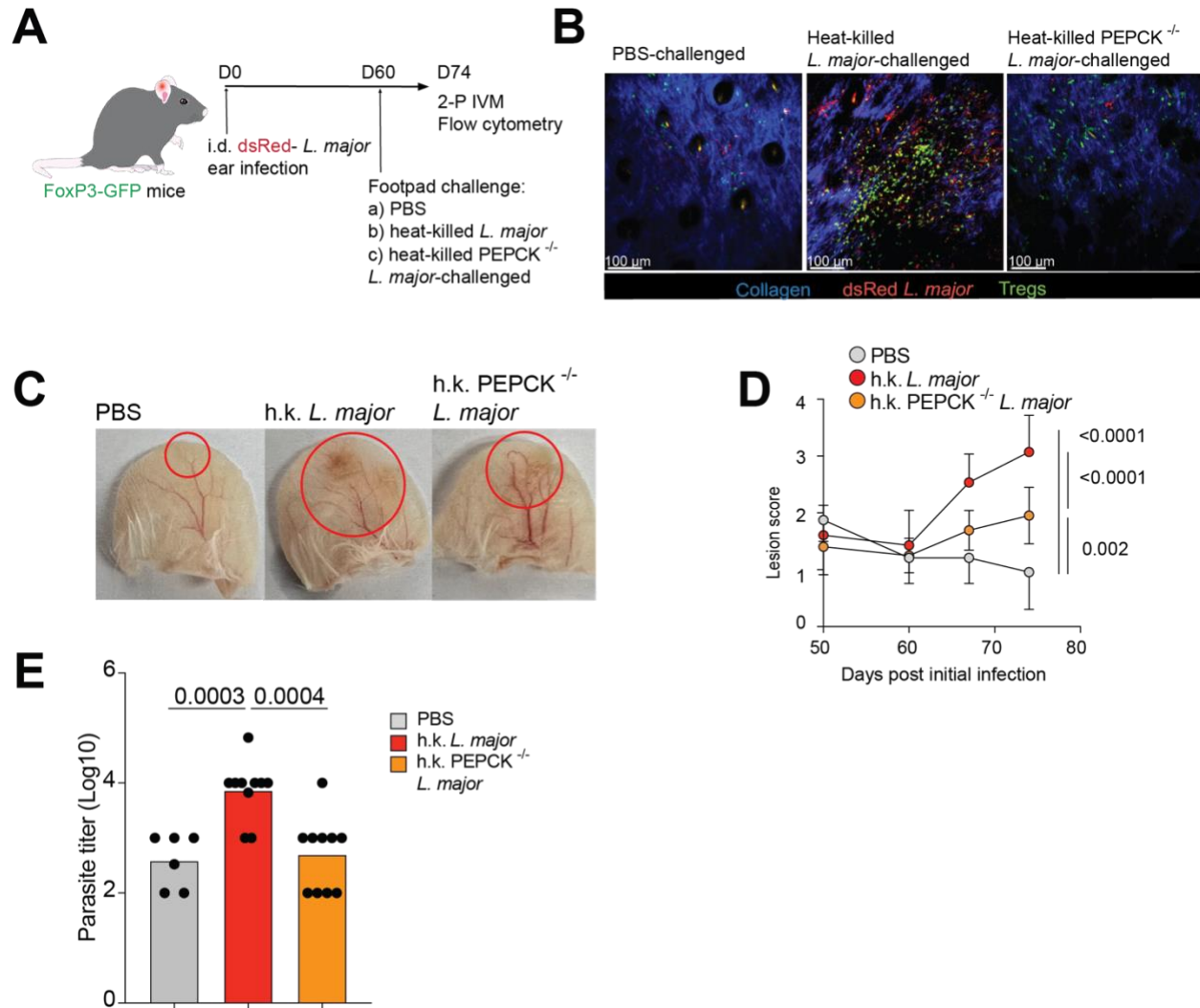


Figure 29. Inoculation of healed mice with killed *L. major* parasites induces lesion reactivation.

- (A) C57Bl/6 Foxp3-GFP mice were infected via intradermal ear injections with 1 million *dsRed*⁺ *L. major* parasites. 60 days post infection, mice were challenged with PBS or 5×10^6 heat-killed wild-type or PEPCK^{-/-} *L. major* parasites into the footpad. The ears were prepared for intravital microscopy or flow cytometry analysis 14 days post challenge.
- (B) Representative micrographs of the lesions post challenge. Blue: collagen (SHG), red: *dsRed*⁺ *L. major*, Green: Foxp3-GFP cells.
- (C) Pictures of albino C57Bl/6 ears 14 days post challenge, red circles highlighting lesion area.

(D) Lesion scores of ears post challenge. Statistical analysis of the last timepoint: unpaired t test. Mean \pm SD. Data combined from three independent experiments.

(E) *L. major* parasite burden in ears post killed parasite challenge. Each dot represents a single mouse ear. Statistical analysis of the last timepoint: unpaired t test.

The observed expansion of Tregs was next confirmed via absolute cell count using flow cytometry (Figure 30A). Cells extracted from the ear dermis and the draining lymph nodes were additionally stained for the PEPCK tetramer, CD44 and CTLA-4. Tregs under all conditions had an equally high expression of CD44 and CTLA-4 (Figure 30B), yet a significant expansion was detected in total and PEPCK-specific Treg numbers in mice challenged with heat-killed wildtype *L. major*. Importantly, control mice harbored PEPCK-specific Tregs, indicating the habitual presence of these cells in the chronic lesions (Figure 30C, D). Lastly, challenging mice with heat-killed PEPCK-deficient *L. major* did not cause a significant expansion in PEPCK-specific Treg population, indicating that Tregs expand in response to their cognate antigen. The inverse of these numbers was observed in the draining lymph nodes, suggesting that Tregs preferentially migrate from the lymph nodes to the ears. Percent PEPCK-specific Tregs out of the total Treg population did not demonstrate significant difference between the experimental conditions (Figure 30E), yet a trend of enrichment in the heat-killed wildtype *L. major* group can be observed in the ears. Contrary to the major enrichment of PEPCK-specific Tregs in the ear, draining and non-draining lymph nodes present with low tetramer-positive population (Figure 30E). This suggests either a local expansion of *Leishmania*-specific Tregs in the ears or an expansion and subsequent migration of Tregs from the draining lymph nodes.

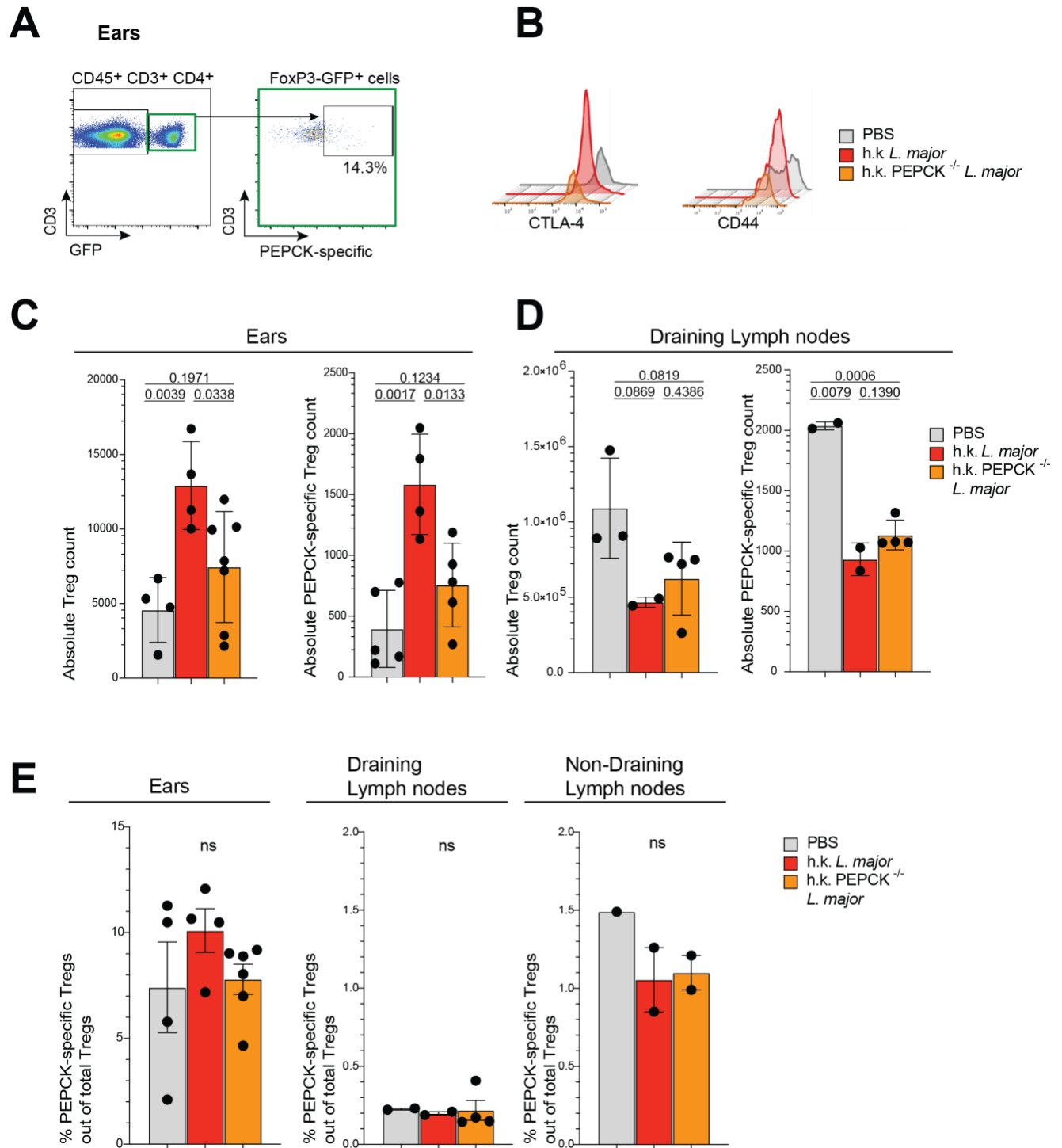


Figure 30. Inoculation of healed mice with killed *L. major* parasites induces Treg expansion.

(A) Flow cytometry gating strategy for endogenous PEPCK-specific Tregs.

(B) Phenotypic analysis of Tregs in lesion skin. Gray: Tregs extracted from PBS-challenged mice, Red and Yellow: Tregs extracted from mice challenged with WT or PEPCK^{-/-} heat-killed *L. major*, respectively.

(C) Absolute cell counts of PEPCK-specific CD45⁺CD3⁺CD4⁺Foxp3⁺ cells in the ears and

(D) draining lymph nodes. Each dot represents a single ear or draining lymph node. Statistical analysis: unpaired t test, ns = not significant. Mean +/- SD. Data combined from two independent experiments.

(E) Percent PEPCK-specific CD45⁺CD3⁺CD4⁺Foxp3⁺ cells out of the total Treg population in the ears, draining lymph nodes, and non-draining lymph nodes. Each dot represents a single ear or draining lymph node. Statistical analysis: unpaired t test, ns = not significant. Mean +/- SD.

5.2.1.3. PEPCK-specific Tregs display signs of antigen-driven recognition in vivo

Next, I assessed the expression of phenotypic markers of Tregs in healed mice challenged with PBS, heat-killed wildtype *L. major*, or heat-killed PEPCK^{-/-} *L. major* promastigotes in order to evaluate whether antigen encounter affects their activation. Transcriptional and phenotypic profiling has revealed that TCR activation in Tregs promotes an effector phenotype characterized by IL-10 production and expression of PD-1^{402,568-570}. In all three conditions of challenge (PBS, h.k. *L. major*, and h.k. PEPCK^{-/-} *L. major*), PEPCK-specific TCR on Tregs have an upregulated expression of PD-1 as compared to non-specific counterparts or non-Treg CD4⁺ T cells (Figures 31A, B), indicating TCR activation. GITR is constitutively expressed on all Tregs and GITR stimulation induces the proliferation of Tregs. The level of GITR expression is usually positively correlated with the immunosuppressive function of Tregs³⁸⁰, similar to LAG3, which is also activated upon antigenic stimulation and is associated with IL-10 production⁵⁷¹, consistent with PD-1. The expression of GITR and LAG-3 is upregulated on all Tregs, and the expression level is higher on PEPCK-specific Tregs (Figure 31C, D). These results indicate the presence of antigen in the system and the ability of Tregs to recognize their cognate antigen in all three conditions. TIGIT, on the other hand, is highly expressed on Tregs³⁸² and TIGIT not only acts as a marker for this Treg cell subset but contributes to the selective suppression of pro-inflammatory Th1 responses³⁸³. All Tregs in lesions of infected mice present with a high expression of TIGIT (Figure 31E).

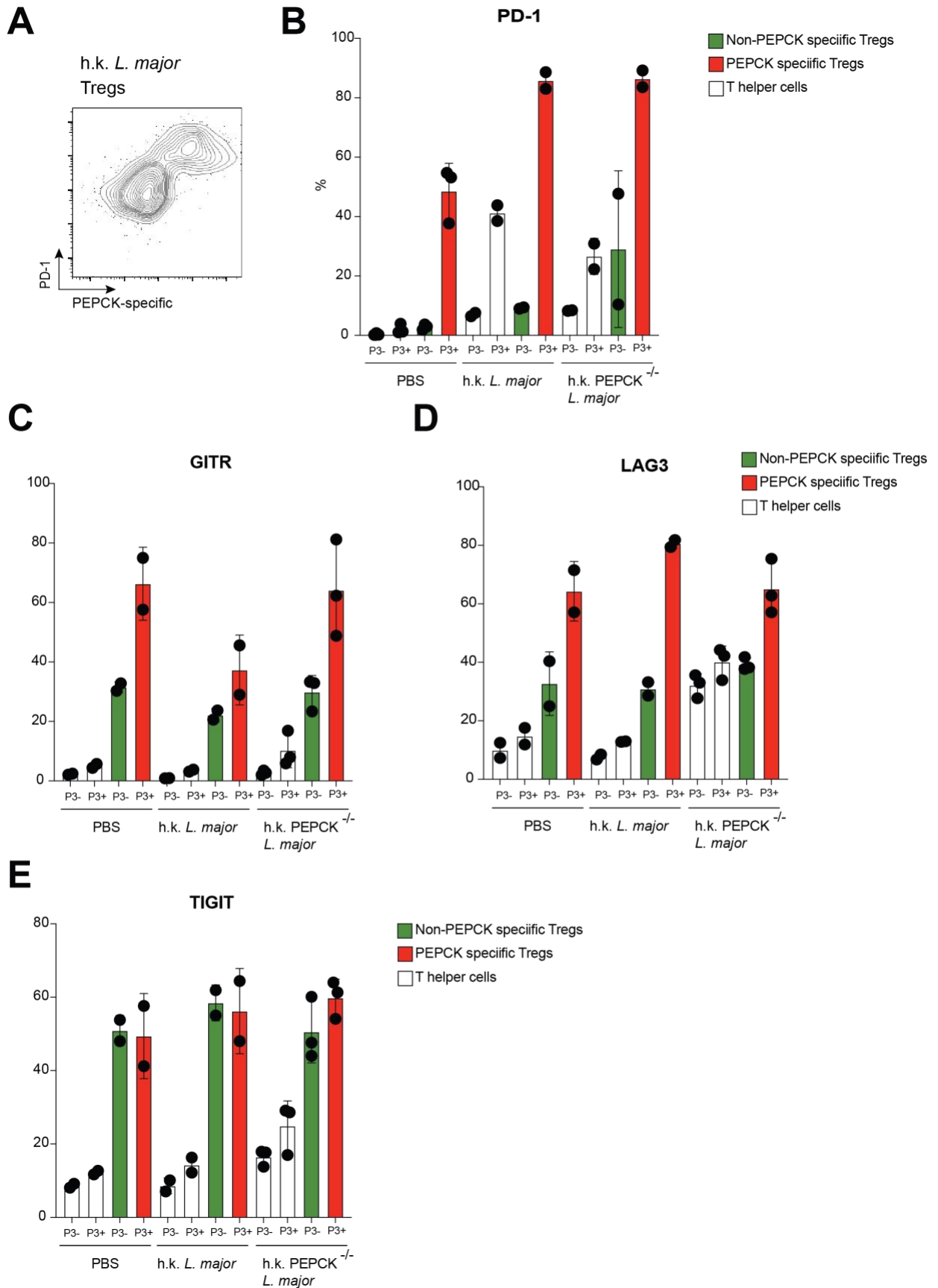


Figure 31. Phenotyping analysis of Tregs expanded from healed mice challenged with heat-killed *L. major*.

- (A) Flow cytometry micrograph of Tregs phenotypes from ears of healed mice challenged with killed *L. major* parasites.
- (B) Phenotypic analysis of Tregs and T helper cells in lesion skin for the expression of PD-1 (B), GITR (C), LAG3 (D), and TIGIT (E). White: helper T cells. P3⁺: PEPCCK-specific. Green and Red: Non-PEPCCK specific and PEPCCK specific Tregs, respectively.

5.2.2. Aim 2: To characterize the effect of Treg expansion on the endogenous Th1 response

5.2.2.1. Expanded Tregs have a high capacity to produce IL-10 while endogenous Th1 cells do not

Next, I confirmed that IL-10 was produced by expanded Tregs at the lesion site and not by effector T cells in the ear pinna. Ears, draining lymph nodes, non-draining lymph nodes, and spleens from healed mice challenged with PBS, heat-killed *L. major*, or heat-killed PEPCCK-deficient *L. major* were digested into a single-cell suspension and activated with a PMA/Ionomycin supplemented with Brefeldin A cell activation cocktail in order to assess the capacity of cells for IL-10 production. CD45⁺CD3⁺CD4⁺FoxP3⁺ cells from all three conditions had a similar capacity to produce IL-10 (Figure 32A), while CD45⁺CD3⁺CD4⁺Tbet⁺ cells did not (Figure 32B, C). These results indicate that Tregs, not Th1 cells, are one of the major sources of IL-10 in healed or reactivated lesions.

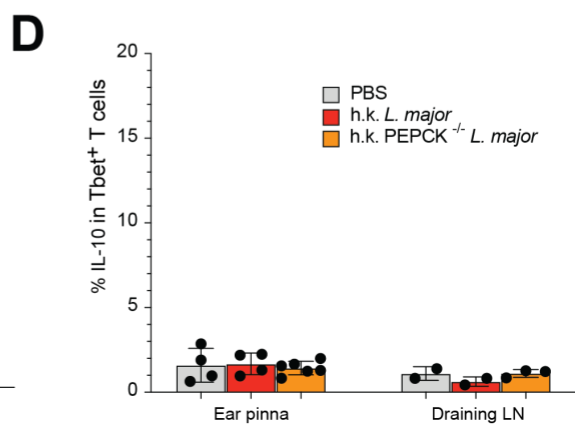
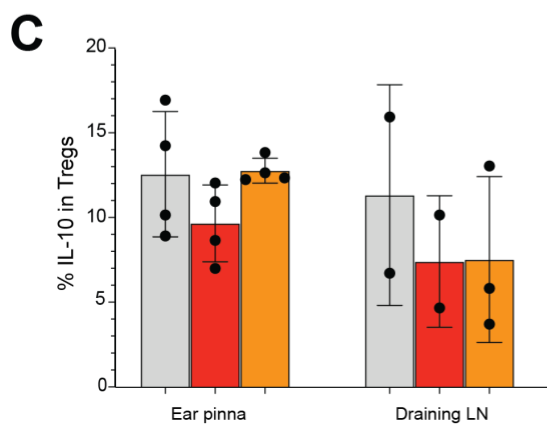
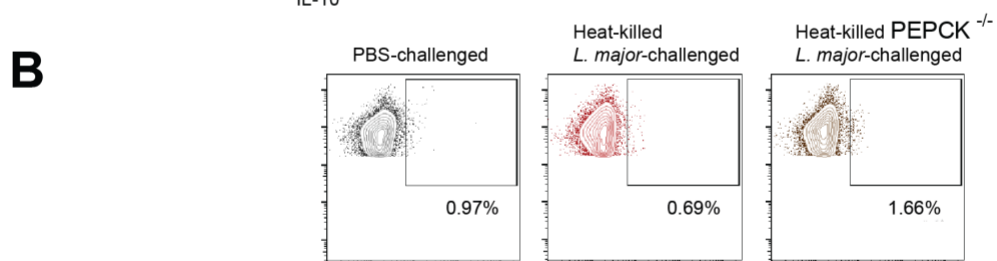
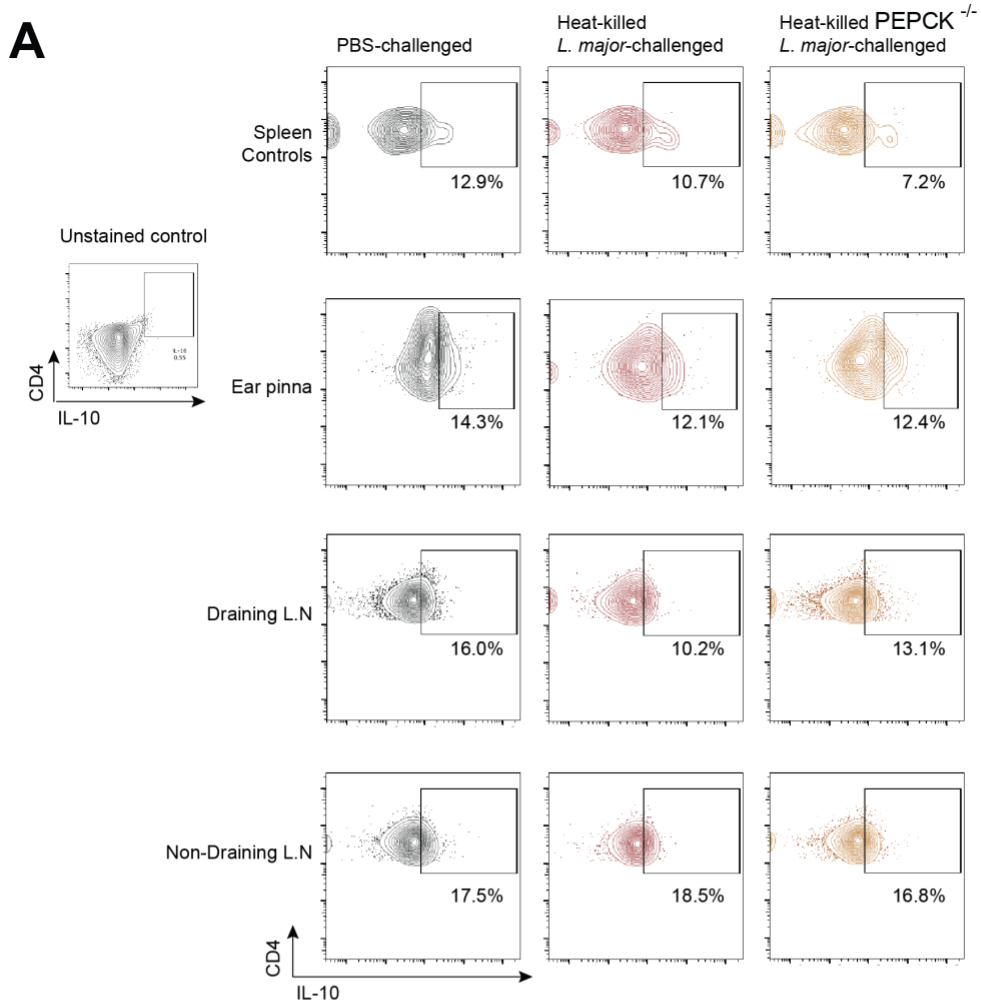


Figure 32. Expanded Tregs have a high capacity to produce IL-10.

- (A) Flow cytometry micrographs of IL-10 production by CD45⁺CD3⁺CD4⁺Foxp3⁺ T cells. Black box: positive signal. Gray: Tregs extracted from PBS-challenged mice, Red and Yellow: Tregs extracted from mice challenged with WT or PEPCK^{-/-} heat-killed *L. major*, respectively.
- (B) Flow cytometry micrographs of IL-10 production by CD45⁺CD3⁺CD4⁺Tbet⁺ T cells in the ear pinna. Black box: positive signal. Gray: Tregs extracted from PBS-challenged mice, Red and Yellow: Tregs extracted from mice challenged with WT or PEPCK^{-/-} heat-killed *L. major*, respectively.
- (C) Percent IL-10 production in CD45⁺CD3⁺CD4⁺Foxp3⁺ and CD45⁺CD3⁺CD4⁺Tbet⁺ cells in lesion ears and draining lymph nodes. Representative data of two independent experiments. Each dot represents a single ear or draining lymph node. Statistical analysis: unpaired t test, ns = not significant. Mean +/- SEM.

5.2.2.2. Treg expansion severely reduces the capacity of endogenous Th1 cells to induce a robust IFN γ response

Prior in vitro experiments indicated that while all activated Tregs have the capacity to suppress IFN- γ production by Th1 cells through IL-10, antigen recognition by Tregs enhances this suppression. Challenge of healed mice with heat-killed wild type *L. major* parasites induced a significant expansion of PEPCK-specific Tregs and loss of lesion control. Therefore, I hypothesized that this increase in antigen-specific Tregs led to a substantial suppression of IFN γ production by endogenous Th1 cells in the ears of live mice, which would explain loss of lesion control in these mice. Single cell suspensions made from ears and draining lymph nodes of mice were treated with PMA/Ionomycin and Brefeldin A in order to characterize the capacity of Th1 cells to produce IFN- γ after treatment. CD45⁺CD3⁺CD4⁺Tbet⁺ cells from healed mice did demonstrate the capacity to produce IFN- γ (Figure 33A, B), indicating that suppression in healed lesions is

incomplete. Interestingly, a substantial suppression of IFN γ was observed when the numbers PEPCK-specific Tregs were increased following a heat-killed wild type parasite challenge (Figure 33A, B) while challenge with PEPCK-deficient *L. major* failed to suppress IFN γ production by endogenous CD4⁺ Tbet⁺ effector T cells in both ears and draining lymph nodes.

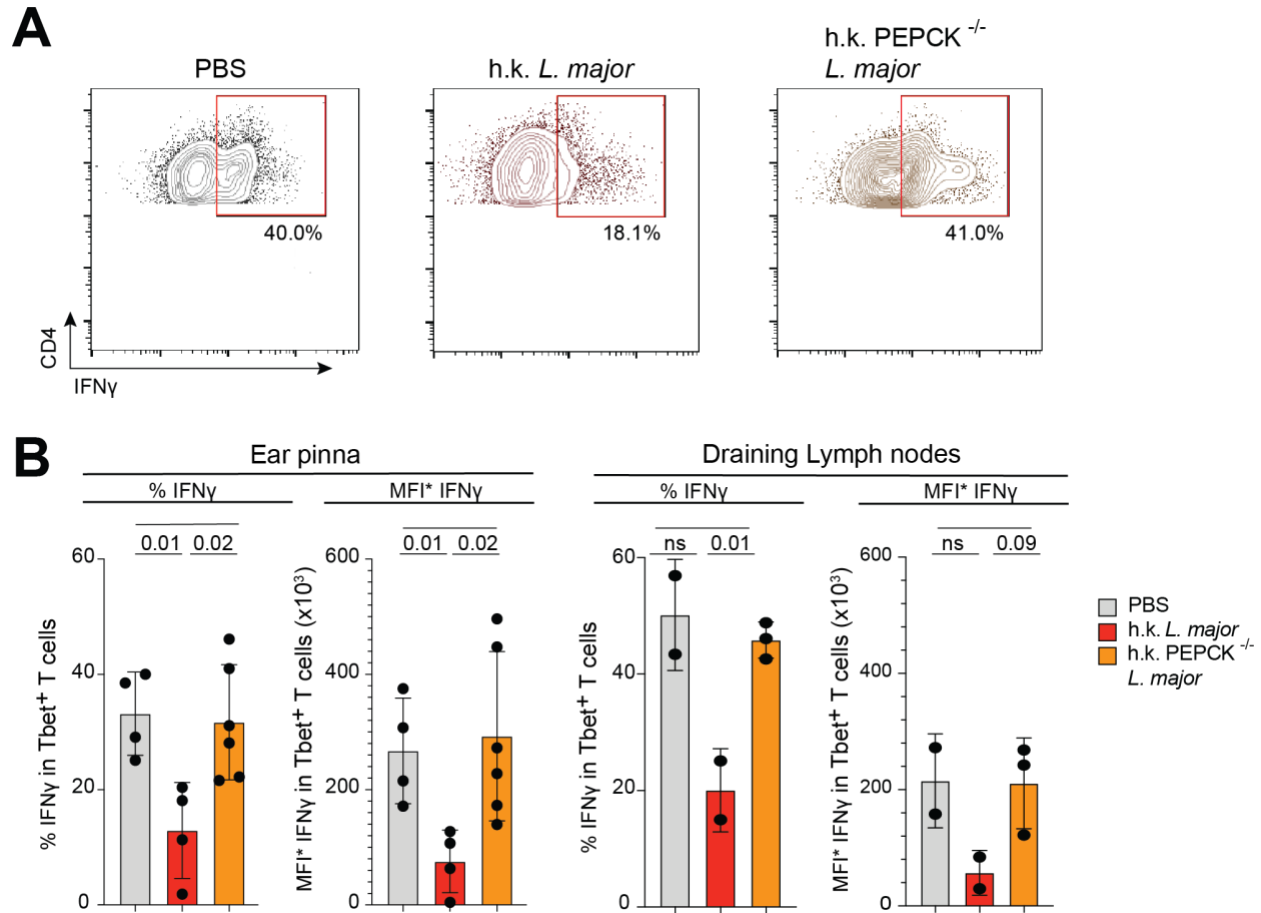


Figure 33. IFN γ response in endogenous Th1 cells after Treg expansion.

(A) IFN γ production in CD45⁺CD3⁺CD4⁺ Tbet⁺ T cells in the ears. Red box: positive signal.

(B) Percent and MFI* (Absolute expression, MFI x %IFN γ ⁺) of IFN γ production in CD45⁺CD3⁺CD4⁺ Tbet⁺ T cells in the ears and draining lymph nodes. Each dot represents a single ear/ draining lymph node. Statistical analysis: unpaired t test, ns = not significant. Mean \pm SD. Representative data of two independent experiments.

5.2.2.3. Treatment with IL-10-blocking antibody restores endogenous Th1 IFN γ production after expansion of Tregs

Given that my co-culture studies indicated that IL-10 mediated strong suppressive function by *L. major*-specific Tregs *in vitro*, and *in vivo* Tregs are one of the main sources of IL-10, I next addressed whether this mechanism was the cause of IFN γ suppression. Healed mice were challenged with either PBS or heat-killed *L. major* promastigotes as before, but additionally some mice were treated with an anti-IL-10 monoclonal antibody given intraperitoneally 2 days prior to sacrifice (Figure 34A). IL-10 blockade completely restored robust IFN γ is essential for IFN γ suppression *in vivo*.

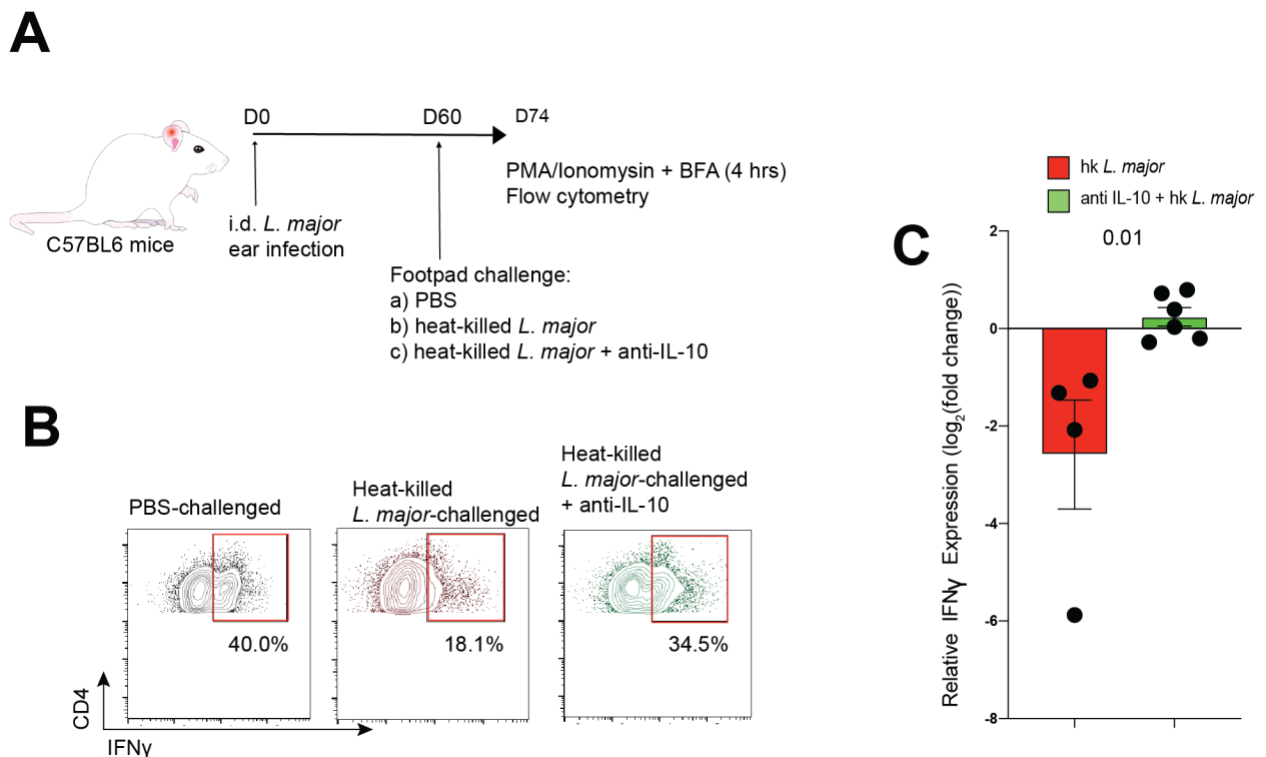


Figure 34. Treatment with anti-IL-10 blocking antibody restores IFN γ production.

(A) C57Bl/6 mice were infected via intradermal ear injections with 1 million *L. major* parasites. 60 days post infection, mice were challenged with PBS or 5×10^6 heat-killed wild-type parasites into the footpad. 2 days prior to analysis, some mice challenged with wild-type heat-killed *L. major* were injected intra-peritoneally with anti-IL-10 antibody. The ears were prepared for flow cytometry analysis 14 days post challenge.

(B) Percent and relative expression of IFN γ production compared too mice challenged with PBS in CD45⁺CD3⁺CD4⁺ Tbet⁺ T cells in the ears and draining lymph nodes. (B) Red box: positive IFN γ signal. (C) Red bar: IFN γ production by Th1 cells form mice challenged with heat-killed wild-type parasites, green bar: treated with anti-IL-10 antibody. Representative data of two independent experiments, mean +/- SEM. Statistical analysis: unpaired t test.

5.2.2.4. Adoptively transferred effector Th1 cells exhibit high speeds in the lesions of mice with expanded Tregs.

To visually characterize how increased Treg numbers altered effector T cell migratory behaviors in vivo, I adoptively transferred differentially labeled control and PEPCCK Th1 cells into the heat-killed *L. major*-challenged healed mice and performed 2P-IVM in the ear dermis 24 hours after cell transfer, as demonstrated in Figure 35A. PEPCCK Th1 cells migrated at lower speeds around the lesion area compared to control Th1 cells, suggesting some antigen recognition (Figure 35B-D), but their overall speeds were higher compared to imaging experiments done in the absence of expanded Tregs in Figure 14 (7.1 $\mu\text{m}/\text{min}$ vs 5.1 $\mu\text{m}/\text{min}$).

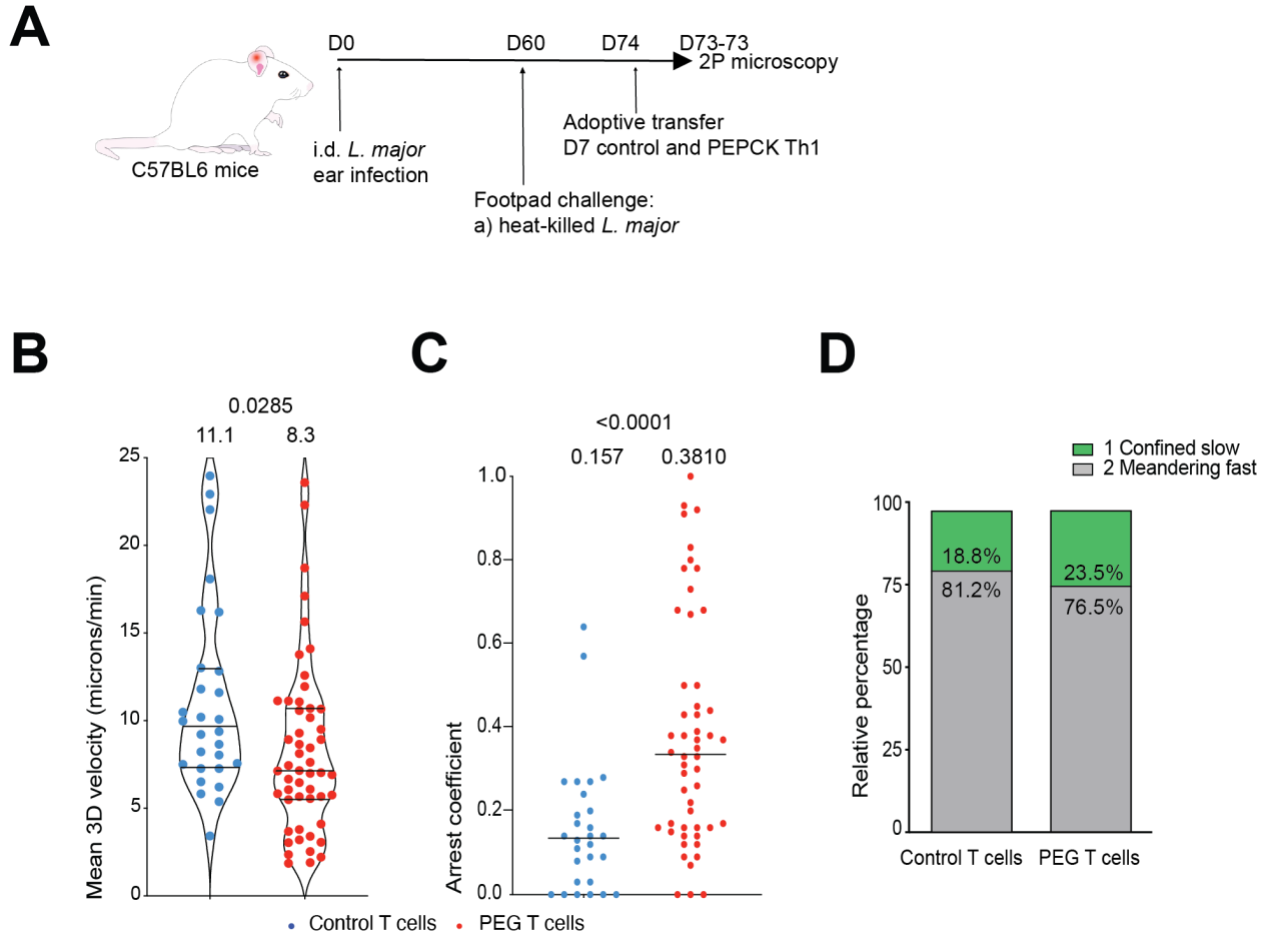


Figure 35. In vivo cellular dynamics of Th1 response against *L. major* infection in ears after Treg expansion.

- (A) C57Bl/6 mice were infected via intradermal ear injections with 1 million *L. major* parasites. 60 days post infection, mice were challenged with 5×10^6 heat-killed wild-type parasites into the footpad. 14 days post challenge, 10-15 million each of control and PEG Th1 cells were stained with cell tracker dyes (CMAC blue, and CMTMR; red, alternating between experiments) and adoptively transferred into recipient mice infected for the indicated time periods. Ears were prepared for intravital microscopy 24-48 hours after T cell transfer.
- (B) Mean 3D track velocity of the indicated Th1 cells. Blue: control cells, Red: PEG Th1 cells. Each dot represents a single cell track. Statistical analysis: Mann-Whitney test.

- (C) Arrest coefficient of the indicated Th1 cells. Blue: control cells, Red: PEG Th1 cells. Each dot represents a single cell track. Green and black lines represent median values. Statistical analysis: Mann-Whitney test.
- (D) Relative percentage of confined slow and meandering fast cell tracks. Green: confined slow, Gray: Meandering fast.

5.3. Discussion and Conclusions

Despite the generation of a robust Th1 response, sterile cure to *L. major* infection is not achieved. To explain this phenomenon, two non-mutually exclusive models have been proposed: the presence of “sanctuary sites” where parasites are found in specific tissue niches that are resistant to immune-mediated clearance, and the immunomodulation effects imposed by infiltrating Tregs at the primary lesion site. The “sanctuary site” theory speculates that host cells, such as alternatively-activated macrophages lacking iNOS expression, harbor parasites long-term⁵⁷². A continual low influx of inflammatory monocytes could contribute to host reservoir longevity⁵⁷³. However, a more recent study demonstrated that persistent *L. major* parasites also reside within iNOS⁺ host cells¹⁴⁵. iNOS⁺ host cells have the capacity to kill parasite and, therefore, cannot be defined as “sanctuary” cell. Accumulation of regulatory T cells and immunosuppressive cytokine IL-10 that function to dampen host immunity to help maintain parasite persistence is another explanation for the lack of cure. This chapter provides direct evidence that Tregs actively suppress effector T cell responses in healed skin, which is further enhanced by *L. major* antigenic stimulation and is mediated by IL-10 production.

Skin Treg cell pool is dominated by thymic Tregs, which contribute 80% of skin Tregs and are central to maintaining tissue homeostasis. Tregs are also crucial players in maintaining tolerance

to the skin microbiota and promoting maintenance of hair follicles⁵⁷⁴. In mice, Tregs were shown to accumulate in skin after wounding and deletion of Tregs resulted in IFN γ -dependent accumulation of pro-inflammatory macrophages and attenuated wound closure³¹⁸. In lungs of mice, Tregs have been shown to preferentially mediate tissue repair as well⁴³³. Thus, it is possible that Tregs present in the *L. major* lesion are there to induce wound healing and collagen restructure through suppression of IFN γ and pro-inflammatory phagocyte polarization, therefore inducing tissue remodeling and confining T cell migration. The dominant source of Tregs in the lesion remains an outstanding question. Skin-resident Tregs can be observed in the lesion through microscopy by their localization to the hair follicles, yet both skin-resident and recruited Tregs express FoxP3-GFP and are indistinguishable. Some evidence suggests that a source of antigen-specific Tregs may actually be the draining lymph node. Infected CCR5^{-/-} mice displayed higher numbers of parasite-specific effector T cells at the site of infection and significantly lower frequency of IL-10-producing cells. This evidence suggests that CCR5-directed chemotaxis is responsible for the recruitment of IL-10 producing Tregs to the lesion site⁴⁷⁵. My data supports this hypothesis, as after challenge of healed mice with heat-killed *L. major*, numbers of PEPCCK-specific Tregs decrease in the lymph node and increase in the ear, indicating directional movement. The same study demonstrated that expression of CCR5 ligands is actively induced by *L. major* infection and correlates with recruitment of Tregs in infected skin; this suggests that the natural function Treg recruitment and wound healing may be a mechanism *L. major* further induces in order to avoid complete clearance by Th1 cells.

Antigen specificity of skin Tregs is poorly understood. Treg selection in the thymus has been shown to require high affinity MHC class II-self peptide complexes, suggesting Tregs should only be specific for self-antigen. As a consequence of their development in the thymus, Tregs are

believed to recognize a wide array of self-antigens and be recruited during acute infection due to these antigens being released from the bystander tissue damage⁴³⁰. However, given that skin is the largest human organ and interfaces with the external environment, it is also constantly subject to microbial insult whether it is commensal or pathogenic in origin. Using the FoxP3-GFP mouse model I showed a rapid increase in numbers of Tregs to the site of infection with 2-photon microscopy and, importantly, I saw that a substantial proportion of these Tregs is specific for a foreign antigen – PEPCK. Similarly, in murine model of *Leishmania* infection Tregs accumulating at the chronic lesion were shown to be able to recognize parasite-derived antigen⁵⁷⁵. Crucially, PEPCK-specific Tregs have a much higher suppressive capability compared to control polyclonal Tregs *in vitro* and exposure of healed animals to PEPCK antigen from killed parasites results in expansion of antigen-specific Tregs, loss of protective immune responses, and subsequent lesion reactivation. This study also brings back to light the importance of preventing the induction/activation of regulatory T cells during vaccination.

Prior findings utilizing *L. major* parasites that express a secreted chimeric protein consisting of the 2W peptide and the *L. donovani* 3' nucleotidase/nuclease, and a pMHCII tetramer-based approach showed very few Tregs within the parasite-specific T cell population at 3- or 8-weeks post infection¹⁸⁸, suggesting that the majority of Tregs in the skin may not be parasite-specific. The major difference in approach is that I measured endogenous responses to an immunodominant, naturally processed PEPCK protein that is essential for *L. major* replication *in vivo*, using tetramer staining of isolated cells *ex vivo*. While 2W:I-A^b-specific Foxp3⁺ Tregs are present in other non-leishmania models⁵⁷⁶, it could be that differences in the environment may not generate Tregs specific to 2W in the context of *L. major* infection. Alternatively, given that PEPCK is immunodominant, a population of PEPCK-specific Tregs may be larger than those of other antigen

specificities. An extended look into other antigen specificities would expand our knowledge on the importance of Treg antigen recognition.

My data demonstrates that out of the CD4⁺ T cells present in the healed lesion, Tregs are a much greater source of IL-10 compared to Th1 cells. *In vivo* blockade of IL-10 also resulted in stronger effector responses. However, in other models of *L. major* infection, the source of IL-10 has been reported to be non-regulatory T cells. In the context of the non-healing strain of *L. major* NIH/Seidman (Sd), Th1 cells produced IL-10 along with high IFN γ compared to the FV1 strain⁴⁸¹. While some IL-10 was still produced by Tregs, and their removal did exacerbate the disease even further⁴⁸², the non-healing phenotype stemmed from the IL-10 production by the CD25⁻Foxp3⁻ subset. Similarly, both Foxp3⁺ and Foxp3⁻ CD4⁺ T cells produced IL-10 upon infection in IL-4 α ^{-/-} BALB/c mice⁵⁷⁷. Th1 cells specific for the chimeric protein 2W also produce IL-10 in response to *L. major* expressing 2W¹⁸⁸. In the scenario presented in this chapter, where *L. major* infection does heal and an equilibrium is established between Tregs and Th1 cells, Tregs, and not Th1 cells, were one of the main sources of IL-10. Taken together, these studies highlight the importance of disease strain and environmental context, as the plasticity of helper T cells allows them to adjust their function based on the environmental cues. In this scenario where lesions do heal, high IL-10 production by a relatively limited number of parasite-specific Tregs may be sufficient to enforce an immunomodulatory setting during persistent *Leishmania major* infection and the removal of immunodominant Tregs may be sufficient to facilitate improved parasite clearance.

Lastly, a small portion of parasites continues to divide and stimulate Th1 responses in the presence of Tregs. Endogenous Th1 cells do have the capacity to produce small amounts IFN γ in healed mice. My data indicates that a balance between the opposing forces is established, where

Th1 cells continuously clear the parasites yet full clearance is prevented by Tregs. An increase in PEPCCK-specific Treg population thus significantly suppressed Th1 function and, therefore, led to an increase in parasite burden and loss of lesion control. Adoptive transfer of effector T cells into this condition led to fascinating observations. First, differences in antigen recognition were observed, where PEPCCK Th1 cells displayed relatively lower 3D velocity and higher arrest coefficient compared to control counterparts. However, both cell population localized to the outside of the lesion, displayed enhanced 3D velocities, and the vast majority of the cells displayed meandering fast behaviours indicative of the overall lack of antigen recognition. The spectrum of T cell motility is generated by the combination of cell-intrinsic locomotion (such as rate of actin polymerization), chemical information of the environment, and physical guidance from the environment. The relative importance of each parameter varies and can be dictated by activation status of the T cell, density of cognate antigen-presenting cells, and the organization of stromal environment. Under healed conditions, I observed low velocity of activated Th1 cells, due to anti-inflammatory environmental cues and severely altered stromal network. Rapid Treg expansion and parasite number increase leads to host cell rupture, subsequent bystander cell and collagen network destruction, and increase in danger signals and inflammation. Tissue inflammation is correlated with increased T cell velocity, explaining high velocities seen in T cells in this model. The meandering behaviours correlate with low confinement, and low antigen recognition. Newly recruited Th1 cells are therefore unable to produce IFN γ and clear the parasite due to high levels of IL-10 and inability to locate antigen. Further studies should investigate whether these cells can eventually establish lesion control and eventually overcome immunosuppressive effects imposed by Treg.

Chapter 6. Discussion

It has long been recognised in the scientific community that cutaneous *L. major* infection in both mice and humans results in a strong Th1 cell response that is essential for identifying and removing infected cells. Dr. Uzonna was the first to describe a number of immunodominant peptides that are the target of CD4⁺ T cell responses, and that these responses confer substantial protection during infection. Nevertheless, a small but active population of persistently infected cells continues to exist at the site of initial infection. Parasites in healed skin appear to establish an equilibrium with the surrounding environment to maintain a constant pool of persistently infected cells, and when protective T cell immunity is compromised, surviving parasites have the capacity to reactivate disease. It has long been unclear why and how *L. major* persists in vivo, as well as why the host is unable to completely eliminate the parasites. In order to understand what maintains the survival of residual parasites in healed skin, it is imperative to determine if chronically infected cells continue to display *L. major* antigen and, if so, whether continued Th1 responses are counterbalanced by immunosuppressive forces. Since *L. major*-specific regulatory T cells have been discovered at chronic lesion sites, significant evidence has accumulated for the involvement of Tregs in this process. However, it is unknown how the distribution and effector functions of Tregs relate to host T cell responses in vivo. Furthermore, whether antigen-specificity of Tregs primes suppressive functions against foreign antigen has not been thoroughly explored, in both *Leishmania* and other immunology fields.

In this thesis, I describe the use of a new TCR transgenic mouse model to directly visualise the interplay between anti-parasitic effector and suppressive T cell responses and address a gap in knowledge of how Tregs help promote parasite persistence. Using this novel mouse model as a sensitive tool to evaluate both antigen-specific effector and suppressive T cells responses in

combination with two-photon microscopy, I demonstrate that *Leishmania*-specific Tregs have a considerably stronger suppressive activity compared to polyclonal Tregs. I show that this suppressive mechanism is mediated by IL-10 production and not by severing cell-cell contacts or altering antigen presentation. I show that disease reactivation can occur when Th1 responses are impaired by expansion of *Leishmania*-specific Tregs, which is also depended on IL-10. It is noteworthy that the strong effector Th1 response and improved parasite control were restored in the absence of Treg expansion that recognized the immunodominant peptide PEPCK. Together, my data suggests a stochastic model of *Leishmania major* persistence in the skin, in which the presence of *Leishmania*-specific Tregs modulates cellular and molecular factors that influence parasite numbers. This work provides new insights into a balance between effector and regulatory T cells at the chronic lesion site and provides direct evidence for the role of antigen in driving suppressive functions during persistent infection.

Graphical abstract

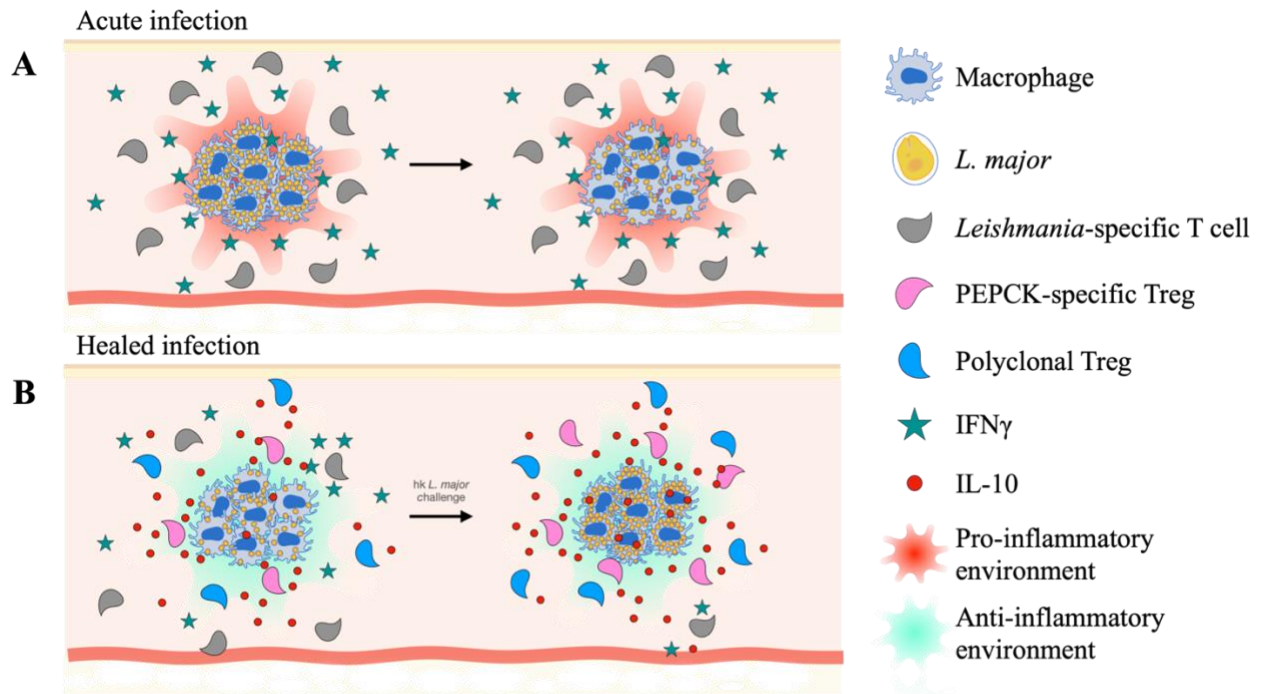


Figure 36. Graphical abstract representation of the cellular dynamics of immune evasion during *Leishmania major* infection.

- (A) During acute stages of *L. major* infection, inflammation-induced cues recruit a large population of *Leishmania*-specific Th1 cells to the site of infection. Antigen recognition leads to arrested cellular dynamics and a robust IFN γ production, which activates macrophage parasite hosts to perform leishmanicidal activity, clearing most parasites.
- (B) At the healed stages of infection, parasite and PEPCCK-specific Tregs become accumulated at the site of infection. Through production of IL-10, they suppress a large proportion of Th1 responses and induce tissue healing. A small pool of the dividing *L. major* becomes recognized by Th1 cells, yet the majority is quiescent and inaccessible by effector responses. If healed mice become challenged with heat-killed *L. major* at a site distal to the original lesion, the influx of antigen induces an expansion in PEPCCK-specific Tregs. PEPCCK specific Tregs, through an increased production of IL-10, significantly suppress remaining IFN γ production, which leads to uncontrolled parasite division and lesion reactivation.

6.1. Cellular dynamics of effector Th1 cells in acute and chronic L. major lesions

In this thesis I visually characterized effector T cell migratory behaviours of the immune responses during acute and healed stages of *Leishmania major* infection. No earlier investigation has successfully evaluated the expansion, effector function, and search strategies of immunodominant *L. major*-specific T cells in vivo in healed lesions prior to the generation of PEG transgenic mice. Preceding microscopy studies in Balb/c mice used CD4⁺ T cells specific for the *Leishmania* homologue of receptors for activated C kinase (LACK) epitope. These mice succumb to leishmaniasis prior to reaching the healed stage and LACK is not expressed throughout *L. major* lifecycle. Therefore, it has not been feasible to evaluate effector immune response in healed ears. In contrast, PEPCK is expressed at both promastigote and amastigote forms of the parasite. Thus, the generation of PEPCK TCR transgenic mouse model on the resistant C57BL/6 background has provided an opportunity to investigate *L. major*-specific and immunodominant responses in mice that have the capacity to heal the infection in order to uncover the mechanisms that allow for a small pool of parasites to survive despite a robust immune response.

6.1.1. T cell migration dynamics during the acute stages of L. major infection

In agreement with previous microscopy studies¹⁹⁴, I observed that control and PEG Th1 cells have the capacity to enter the site of infection irrespective of antigen specificity. T cell search strategy is directed by inflammatory cues, inflammation-induced loose collagen network, and T cell activation status. Acute *L. major* lesion is a highly inflammatory environment and in vitro activated adoptively transferred T cells exhibit high migratory speed in vivo when searching for antigen. Only those T cells that encounter cognate antigen display low motility and high confinement and in contrast to control Th1 cells, a large proportion of PEG Th1 cells arrests around *L. major*⁺ cells. However, despite the exquisite sensitivity of PEG T cells, a large proportion did

not arrest to interact with infected cells, suggesting non-uniform accessibility of antigen. This spectrum of behaviour could be explained by density of antigen presented at the cell surface or the type of APCs present, as low antigen density would not induce cell arrest. Alternatively, the physical configuration of the skin, such as granuloma-like highly packed structures of *L. major* lesion could also account for the heterogeneity in responses observed. However, despite these limitations, the majority of parasites are eventually eliminated. Studies by Muller et al demonstrated that paracrine expression of IFN γ by Th1 cells can diffuse and activate macrophage up to 80 microns away from the site of T cell:APC interaction¹⁹³. Therefore, parasite-harboring macrophages may not need direct contact with Th1 cells to become activated, as long as IFN γ -producing T cells are located in the vicinity.

6.1.2. T cell migration dynamics during the healed stage of L. major infection

In vitro-activated Th1 cells displayed less efficient homing ability to healed lesions. Cells that were able to localise to the site of infection also displayed remarkably reduced motility profiles. The fact that both cell types displayed low speeds regardless of antigen specificity suggests that reduction in velocity is not due to antigen recognition, but rather because of the altered local microenvironment. Shift to an immunosuppressive state and induction of healing mechanisms may explain the change in cellular dynamics.

During inflammation, the ECM stromal networks become loose due to an increased protease activity and a constant ECM turnover⁵⁷⁸. As the lesion heals, ECM networks undergo a change in composition, which could limit access to parasites. In the context of *Leishmania* infection, only one study has examined the ECM composition throughout the chronic stages of infection²⁵². During tissue repair in the event of a successful pathogen elimination, newly synthesized collagen type III is gradually replaced by the prevalent collagen type I throughout the

tissue remodeling stage. *L. amazonensis*-parasitized macrophages in BALB/c mice, however, present with a significantly more pronounced deposits of collagen type III when compared to their control group. Although it is unknown how these structural alterations affect T cell migration and trafficking in the context of *L. major* infection, the granulomatous formation of the lesion may mimic the tumor microenvironment, where the tumor is also enveloped by remodeled ECM that prevents T cells from penetrating the tumor mass^{527,579}.

Severe immunological changes brought on by *L. major* may also impact T cell responses in addition to the structural remodeling of the tissue. My data shows that adoptive transfer of naïve PEG cells into mice prior to infection, thus increasing the pool of parasite-specific cells, does not lead to sterilizing immunity. Therefore, generation of a strong effector Th1 immune response is insufficient to achieve complete parasite clearance. Sterilizing immunity can only be achieved in cases of Treg depletion²²⁹, which implicates Tregs as key cells in protecting *L. major* from elimination.

6.2. Antigen recognition drives Regulatory T cell responses

Early studies have demonstrated strong evidence for the role of Tregs in suppressing anti-*Leishmania* responses. RAG^{-/-} recipient mice of lesion-derived CD4⁺ CD25⁻ cells become highly resistant to *L. major* infection and rapidly control parasite growth. Mice transferred with Tregs alone are highly susceptible to infection. Transfer of Th1 cells together with Tregs suppressed the ability of Th1 cells to control infection, and the parasite burden and lesion progression mirrors the course of C57BL/6 mouse infection^{229,575}. This suppression is achieved whether Tregs were derived from healed lesions or lymph nodes of naïve mice⁴⁷⁹, which indicates the existence of endogenous pool of Tregs capable of recognizing *L. major*. Work in this thesis corroborates prior

findings of *L. major*-specificity of Tregs in lesions and expands on these studies by highlighting immunodominance of Tregs.

6.2.1. Immunodominance of Tregs in the context of *L. major* infection

Other studies have demonstrated that C57BL/6 mice infected with PEPCCK^{-/-} *L. major* display both low levels IFN γ production during in vitro recall response and significantly impaired production of IL-10¹⁰⁴. While this may be due in part to the attenuated phenotype of the parasite itself, the lack of PEPCCK can also fail to stimulate both the inflammatory Th1 and the immunosuppressive Treg cells, reducing the number of cytokine-secreting immunodominant cells from the start. My data shows that Tregs specific for the immunodominant *L. major* peptide PEPCCK display strong suppressive activity *in vitro* and *in vivo* through IL-10 secretion. My findings offer convincing evidence that, by dampening effector Th1 responses, the recruitment of PEPCCK-specific Tregs is a key factor in creating an immunosuppressive environment in healed lesions. I show that infected skin has higher levels of PEPCCK-specific Tregs than cervical lymph nodes and supports a model where Tregs in healed *L. major* lesions originate from the draining lymph nodes^{229,475}. Challenging healed mice with heat-killed *L. major* expands the immunodominant PEPCCK-specific Treg population and leads to suppression of IFN γ production and loss of lesion control. Removal of PEPCCK antigen does not lead to the expansion of PEPCCK Tregs and lesion control is not lost to the same extent. Collectively, my data suggests that immunodominance also translates to Treg population and a small, but highly immunosuppressive PEPCCK-specific Treg pool plays a major role in parasite persistence.

6.2.2. Evaluation of antigen specificity of Tregs in other models of persistent infection

Antigen-specific Tregs are not unique to *Leishmania*, as Tregs have been shown to be specific for microbial peptides, such as *Mycobacterium tuberculosis* (Mtb)⁵⁸⁰. Pulmonary infection

with *M. tuberculosis* induced a robust expansion of highly activated Tregs recognizing an immunodominant Mtb epitope in the lymph nodes of mice. The time of effector T cell recruitment to the lung is crucial for establishing immune control, and Mtb-specific Tregs were shown to have a detrimental effect and slow the rate at which protective T cell responses were recruited⁵⁸¹. In the *Salmonella enterica* serotype Typhimurium-infected mice Tregs have been shown to suppress Th cells⁵⁸², and samples from typhoid volunteers exhibit *S. enterica* serotype Typhi-specific Tregs with upregulated activation molecules HLA-DR and LFA-1⁵⁸³. More recently, MHC-II tetramers specific for two epitopes expressed by the rJ2.2 strain of house hepatitis virus were utilized to identify virus-specific Tregs that were recruited during infection and regulated effector response⁵⁸⁴. The availability of appropriate detection mechanisms and models for Tregs specificity in other models will surely uncover impactful insights into the role of pathogen specific Tregs in maintaining persistent infections.

6.3. Evaluating mechanisms of Treg suppression during L. major infection

Tregs have a variety of suppressive strategies, from restricting T cell activation and proliferation in the lymph node to dampening effector responses in the peripheral tissues. Tregs gain competency during TCR stimulation and there is no obligatory requirement for further antigen recognition or TCR signalling. However, subsequent TCR activation leads to enhanced function. Work presented in this thesis demonstrates that indeed, while all activated Tregs do exhibit baseline suppression, TCR engagement significantly increases suppressive capability.

6.3.1. Contact-dependent suppressive function by Tregs

Studies of Treg-DC interactions using antigen-specific Tregs from TCR transgenic mice demonstrate that the primary mechanism of Tregs in this context is to prevent the formation of stable interactions between antigen-specific naïve cells and antigen presenting DCs^{558,585}. Even

when DCs are simultaneously pulsed with two different peptides, suppression by Tregs is entirely antigen-specific³³¹. Strong binding of Tregs and their capacity to incapacitate DC function suggests that Tregs are removing their cognate antigen from the APC surface through trogocytosis⁵⁸⁶⁻⁵⁸⁸. During DC-Treg interactions, CD80 and CD86 co-stimulatory molecules can also be selectively removed, which further lowers T cell activation levels^{330,559}. I speculated that the mechanism of physically disrupting contacts would translate to Th1:macrophage interactions and explain the strong suppression of IFN γ production by PEG Th1 cells. However, while I also observed the importance of PEG Treg-BMM presenting PEPCK contacts on suppression of PEG Th1 function, PEG Tregs neither disrupted cell-cell interaction dynamics between effector Th1 and infected macrophages *in vitro*, nor did they alter antigen presentation or CD80/86 expression. Uptake of PEPCK-MHC II complex was observed by neither the phenotypic analysis of MHC-II expression on BMMs after *in vitro* co-culture with Treg, nor by their ability to maintain stable contacts with PEG Th1 cells. This unexpected finding can be explained by the fact that Treg:DC interactions primarily result in suppressing immune responses in the lymph node, Treg:DC interactions in inflamed tissue result in dampened Th1 activation and inflammation to suppress bystander tissue damage and to promote tissue healing. My results support the conclusion that disrupting Th1:BMM contacts was not a mechanism of suppression employed by Tregs in my model and that other mechanisms were involved in this process.

6.3.2. Cytokine-dependent immunosuppression

Corresponding antigen recognition increased PEG Treg suppressive activity through elevated IL-10 production. *In vitro* co-cultures of *L. major*-infected BMM, PEG Tregs, and PEG Th1 cells showed significantly elevated quantities of IL-10 in the supernatant, as compared to cultures with control Tregs. Blocking IL-10 with neutralizing antibodies completely restored IFN γ

production and adding IL-10 alone to PEG Th1 and *L. major*-infected BMMs suppressed T cell responses. I also observed the importance of IL-10 in Treg suppressive function in vivo, when injection of IL-10 neutralizing antibody into *L. major*-healed mice challenged with heat-killed *L. major* parasites completely negated the detrimental effects of PEPCK-specific Treg expansion. It is likely that, similar to previously shown effector Th1 cells exerting protective effects through IFN γ release to diffuse into the tissues¹⁹³, increased concentration of Treg-derived IL-10 diffuses into the surrounding environment and exerts suppressive bystander effect. It is probable that IL-10-mediated control of CD4⁺ T cells operates in combination with its capacity to enhance M2 features in dermal macrophages, which amplifies regulatory functions and allows long-term parasite persistence⁵⁸⁹⁻⁵⁹¹.

I demonstrate that the majority of IL-10 in healed lesions was produced by regulatory T cells and not Th1 cells, and that in vivo IL-10 inhibition led to more potent effector responses. However, other studies show evidence that under different context, IL-10 can be produced by Th1 cells as well. Studies with *L. major* NIH/Seidman (Sd) non-healing strain showed that Th1 cells produced IL-10 along with high IFN γ ⁴⁸¹. The development of a non-healing phenotype in mice required IL-10 production by the CD25⁻Foxp3⁻ subpopulation, however the ablation of IL-10-producing Tregs also made the condition worse⁴⁸². Foxp3⁻CD4⁺ T cells in IL-4 $\alpha^{-/-}$ BALB/c mice also produce IL-10 upon infection⁵⁷⁷. Lastly, 2W-specific Th1 cells also produce IL-10 in response to *L. major* expressing the chimeric protein 2W¹⁸⁸. I found that Tregs, not Th1 cells, were the main source of IL-10 during chronic *Leishmania major* infection in C57BL/6 mice. In my model, elevated IL-10 production by a relatively small number of Tregs that are specific for the parasite *Leishmania major* may be sufficient to maintain an immunomodulatory environment and the fact

that Th1 cells do not also produce IL-10 leads to lesion healing. My findings imply that enhanced parasite clearance may be enabled by the targeted elimination of immunodominant Tregs.

Conclusion

My thesis shows that *Leishmania major*-specific Regulatory T cell populations are directly involved in restricting anti-*L. major* Th1 responses in the skin. This is the first report showing that Treg responses against the immunodominant *Leishmania* antigen PEPCK₃₃₅₋₃₅₁ exhibit strong suppressive capabilities, and that their accumulation in healed lesions is what drives parasite persistence in the skin. Mechanistically, loss of parasite control in healed lesions is facilitated by IL-10 mediated suppression of IFN γ responses by endogenous effector Th1 cells that are present at healed lesion sites. My study also visually characterizes how effector Th1 migratory responses are altered over the course of *L. major* infection, and how Treg expansion further impacts their effector T cell immunity in vivo. Future studies will investigate antigen specificity of healed and chronic lesions in humans and identify targeted strategies in order to clear the parasite and avoid inflammation-induced pathology.

Chapter 7: Unanswered questions and future directions.

In my PhD thesis I evaluated the cellular dynamics of *L. major*-specific Th1 cell population at acute and healed stages of infection. Dynamic behaviours of Th1 cells in healed skin suggest a fundamentally altered lesion environment. I was able to detect a population of Tregs which are specific for the immunodominant *L. major* peptide PEPCK and demonstrated that these Tregs directly impede anti-*L. major* Th1 effector function through heightened production of IL-10. Additionally, I was able to prove that an increase in this population leads to loss of lesion control through suppression of IFN γ . However, outstanding questions remain, which can be addressed in future studies described below. The altered extracellular matrix of healed *L. major*-infected skin should be characterized to determine whether it is able to impact T cell dynamics and access to antigen, similarly to the tumor microenvironment. Additionally, the anti-inflammatory status of healed skin in vivo can be characterized and correlated to the in vitro data provided in my work. Lastly, the impact of Tregs on the host cell activation status in vivo has not been addressed, in particular whether the increased presence PEPCK-specific Tregs are able to induce healing type in host macrophages or newly recruited monocytes, or, alternatively, whether PEPCK-specific Treg ablation can alleviate their suppressive status.

7.1. To evaluate extracellular matrix alterations in experimental *L. major* in the ear dermis of resistant mice and their impact on T cell migratory behaviours

In this study, I evaluated the cellular dynamics of effector T cells at acute and healed stages of *L. major* infection and uncovered significant augmentation of response when the lesion is healed. Both control and PEG T cell populations displayed surprisingly slow and confined behaviours despite the parasites not being cleared. These changes may be due to the significant remodeling of ECM and further experiments examining these changes would provide a direct

explanation of the altered dynamics. Prior study examining ECM remodeling during *L. amazonensis* infection evaluated ECM remodeling in the footpad of infected BALB/c mice using histopathology²⁵². The authors divided the footpad lesion tissue section of each animal into two fragments. One fragment was processed for paraffin embedding, cut, and stained with either Haematoxylin-eosin for the integrity of the tissue, Masson's trichome for dermis restoration, picrosirius red for the arrangement of type I and type III collagen, or Gomori's method or Weiger's recoscin-fuchsin after monopersulphate compound for tissue fibers. Other fragments were embedded in OCT for immunohistochemistry and stained for ECM proteins with anti-fibronectin and anti-laminin. Applying these methods and expertise in histopathological evaluation on my model of *L. major* infection would undoubtedly provide significant insight into the exact changes of ECM that occur throughout the course of infection. In this study, collagen type III surrounded parasitized cells, and other studies have demonstrated that T cells embedded in type I collagen matrix display robust migration, while varying 3D collagen composition directly impacts T cell migration behaviours⁵⁷⁹. Similarly, I hypothesize that *L. major* infection will also result in parasitized cells surrounded by impenetrable type III collagen given that many *L. major*⁺ cells in vivo appear to be inaccessible by T cells. Given this finding, I would recreate these collagen conditions with the in vitro 3D collagen system and directly prove the impact of ECM remodeling on Th1 cell migratory behaviours.

Additional experiments utilizing immunohistochemistry to assess the distribution of adoptively transferred T cells surrounding the lesion environment would also be important. Preliminary IHC experiments performed in our lab provided little insight into the distribution of T cells surrounding the lesion and highlighted the technical difficulties in this approach. The complexity of this experiment involves the relatively small area of the lesion itself, compared to

the whole ear, which leads to a need to process a high number of tissue samples and materials in order to find the few samples that contain the lesion and the transferred T cells. Additionally, the rarity of a sample containing *L. major* increases as numbers of parasites and localized T cells decrease with the healed lesion. Nevertheless, given the removal of technical restrictions, evaluating the relatively proximity of PEG Th1 cells to parasites vs control Th1 cells at acute and chronic stages of infection would provide valuable results. Given the fact that a large proportion of adoptively transferred PEG cells displayed slower 3D velocities during acute stages of infection, I hypothesize that a large proportion of these cells would be in proximity to parasites. However, I predict that 3D velocity of PEG cells will not correlate to their proximity to *L. major* at the healed stage, rather only a few cells will have access to the parasites, but the majority will be restricted by the dense network of ECM. Given no correlation between the ECM and migratory patterns of T cells, it would also be important to examine the pro- or anti-inflammatory signatures of the ear tissue itself.

7.2. Identifying biomarkers of disease using spatial transcriptomics on acute and healed *L. major* lesion samples

Both adoptively transferred control and PEG Th1 cells exhibited significantly augmented 3D behaviours at the healed stages of infection. Additionally, prior data demonstrated recruitment of Tregs to the lesion site, which significantly suppress Th1 effector functions through the production of IL-10. ELISA analysis of in vitro co-cultures containing Tregs, PEG Th1 cells, and macrophages demonstrated that addition of Tregs augments the cytokine environment towards the anti-inflammatory state and it would be beneficial to directly evaluate the distribution of these cytokines in the tissue samples of both infected mice and humans using spatial transcriptomics. For immunohistochemistry analysis, fresh frozen tissue samples would be sectioned and placed in

the Capture Areas on the Visium Spatial Gene Expression slide, rinsed in PBS and blocked with PBS containing BSA and Triton X before staining with primary anti-*Leishmania* and anti-FoxP3 antibodies and prepared for confocal imaging. Tissue samples would then be permeabilized to release mRNA, which will bind spatially barcoded nucleotides present on the capture area. cDNA will be produced from the captured mRNA with reverse transcription. The second strand of cDNA would be synthesised, denatured, and pooled for downstream processing, library preparation and amplification. cDNA will then be fragmented and processed to create a complete sequencing-ready library with PCR. A Gene Expression library is sequenced using standard short-read sequencers, and data is processed and visualized using 10x Genomics software. By combining IHC staining parasites and surrounding Tregs with 10x Genomics, I would not only be able to identify which cellular functions of Tregs are upregulated or downregulated in the whole infected tissue from in healed samples, but also in the areas proximal vs distal to the infected cells themselves. Prior data suggests direct impact of Tregs on creating immunosuppressive environment, yet the direct distribution, proximity, and exact Treg products have not been assessed before.

7.3. To identify the major source of IL-10 and access the phenotype of L. major infected cells in healed lesions after L. major infection in vivo.

A large proportion of my in vitro experiments focused on identifying how Tregs alter the nature of T cell:macrophage interactions, and whether antigen recognition by Tregs is essential for this process. My results have demonstrated that Tregs were able to significantly decrease effector T cell responses when co-cultured with the PEPCCK-pulsed or *L. major*-infected macrophages, and that antigen recognition by PEPCCK-specific Tregs played an important role in enhancing suppression. Tregs are known to secrete suppressive cytokines TGF- β and IL-10, as well as

sequester IL-2, which is necessary for effector T cell function⁵⁹². ELISA experiments to quantify the production of these cytokines, as well as levels of IFN γ and TNF α , in both control and PEG Treg co-cultures with infected macrophages and PEG Th1 demonstrated strong evidence for the IL-10 driven suppression, while TGF- β seemed to not play a significant role. When Tregs were able to recognize antigen, IL-10 levels in the supernatant were elevated and suppression was enhanced. An important outstanding question is whether Tregs themselves produce more IL-10 or can they induce Th1 cells and/or macrophages to produce the cytokine. Technical challenges did not allow for accurate IL-10 intracellular staining in the in vitro culture, therefore utilizing IL-10 reporter mouse models would be a possible future direction to investigate. Several IL-10 reporter mice have been published⁵⁹³ in the literature and could be utilized as macrophage and control Treg cell donors. However, a cross between the PEG mice and IL-10 reporter mice would need to be thoroughly tested, as my prior experiments demonstrated that it is possible to lose the TCR transgene when PEG mice were crossed with FoxP3-GFP mice. Given that standard C57BL/6 mice did have FoxP3-GFP Tregs expressing the PEPCCK-specific TCR, it is likely that in PEG mice the TCR transgene was disrupted by the GFP-insertion, perhaps due to its location.

IL-10 reporter mice would additionally provide powerful insights for the in vivo biology of the host macrophage population. Tregs are known to induce IL-10 production in macrophages and induce their M2-like healing phenotype³³⁸, which also creates a permissive environment for *L. major* amastigotes. In my studies I focused on the Treg:Th1 dynamics and demonstrated Tregs, not effector Th1 cells, were larger producers of IL-10. However, the capacity to produce IL-10 is not unique to the Treg population, therefore the relative contribution of other cell types in healed lesions, particularly the host macrophages, is worthy of investigation. Another important question worthy of insight is the macrophage polarization status, particularly after the Treg population is

expanded through heat-killed *L. major* challenge. Future studies could assess host cell polarization by identifying the cytokines they produce and assessing their antigen presentation capacity.

7.4. To investigate selective PEPCK-specific Treg ablation as a treatment option to achieve sterile cure in leishmaniasis patients

While PEPCK³³⁵⁻³⁵¹ may not be a viable target for ablation, as it may not be presented on human HLA alleles, Treg specificity for PEPCK protein itself is a powerful target for selective ablation in both leishmaniasis and other infections where PEPCK is expressed.

Total Treg ablation in order to induce sterile immunity is dangerous treatment due to increase in proinflammatory functions in response to parasites and commensal bacteria in the lesion. Breach in skin due to leishmania infection exposes underlying tissues to invasion by microbiota, which has been shown to promote increased disease in experimental models of cutaneous leishmaniasis^{594,595}. For instance, *S. epidermidis* colonization of germ-free (GF) mice recovers ulcer development in *Leishmania major* infected mice, and *Leishmania major* infection results in smaller lesions in GF mice than in conventional animals⁵⁹⁶. Increased pathology of the lesion due to increased colonization of commensal bacteria is accompanied by excessive inflammation and higher expression of pro-inflammatory cytokines^{594,595,597}. As one of Treg functions is maintenance of homeostasis, Tregs actively suppress disproportionate effector T cell activation and subsequent infection-induced pathology. While depletion of Tregs would lead to increase in parasite clearance, it would also cause detrimental effects on the lesion pathology and, subsequently, higher bacterial burden in the skin due to a larger area of exposure.

7.5. To evaluate the Treg:Th1 interactions and Treg antigen specificity in other models of disease utilizing PEG mice

PEPCK₃₃₅₋₃₅₁ peptide is 100% conserved in all *Leishmania* species⁵⁵⁰. Cross reactive anti-PEPCK B cell responses were identified with humans and dogs infected with *L. donovani* and *L. infantum*, respectively. I-Ab-PEPCK₃₃₅₋₃₅₁ tetramer reagent has also identified *Leishmania*-specific CD4⁺ T cell responses in mice infected with *L. donovani* and *L. mexicana*. Not only could this target protein be good target for vaccination strategies, but PEG mice could also be used to study the immunodominant responses in species causing mucocutaneous leishmaniasis. Moreover, PEPCK from *Schistosoma* and *Mycobacterium tuberculosis* induce strong T cell responses in mice⁵⁹⁸⁻⁶⁰⁰, PEPCK from *Mycobacterium bovis* is associated with its virulence^{598,600}, *Trypanosoma brucei* relies on PEPCK to differentiate into the procyclic form⁶⁰¹, and other kinetoplastids rely on PEPCK for metabolic activities⁶⁰². Concomitant immunity, particularly with phagolysosomal infections, is a common hurdle against vaccination in many microbial infections. Secondary *L. major* infection is rapidly controlled in healed mice, yet sterilizing immunity leads to loss of protection. Similar observations exist with Mtb, where ongoing infection protects against a secondary challenge in macaques⁶⁰³. In areas with endemic schistosomiasis, infection prevalence rises rapidly through childhood and declines thereafter, with this peak only repeated if mass drug administration occurs⁶⁰⁴. This suggests that adult worms protect the host against the larval stages for their own benefit⁶⁰⁵. Taken together, numerous infections exhibit a similar trend of host protection against reinfection, likely through constant but suppressed immune stimulation and many parasites share similar immunogenic molecules. While it is unlikely that all infections share the PEPCK-specific Th1 and Treg dynamics, the immunodominant responses to disease and the forces that restrain them are worthy of investigation.

Additionally, the Uzonna group has recently identified another naturally processed peptide DLD₆₃₋₇₉, derived from *Leishmania* dihydrolipoyl dehydrogenase (DLD) protein⁶⁰⁶, which is also conserved in both amastigote and promastigote stages of all *Leishmania* species. DLD-specific CD4⁺ Th1 cells undergo expansion, contraction, and stable memory phase. DLD induces stronger IFN- γ responses than PEPCK, yet the frequency of DLD-specific CD4⁺ T cells is much lower than PEPCK-specific at acute stages of infection but significantly higher 18 weeks post infection, suggesting that DLD-specificity may be more important in maintaining infection-induced immunity compared to PEPCK-specific responses. Given the availability of DLD-specific TCR transgenic mice, an investigation of DLD-specific T cell dynamics in vivo is now feasible. If DLD-specific Tregs exist in this model, it would be important to assess their role in maintenance of concomitant immunity. Additionally, it would be of significant interest to assess the suppressive capability of DLD-specific Tregs on PEPCK-specific Th1 cells and vice versa, further exploring the role of Treg antigen recognition on the suppressive function during an inflammatory response.

Significance and Impact of thesis project

My research provided a mechanistic understanding regarding the establishment of concomitant immunity and uncovered survival strategies employed during *Leishmania major* infection. For the first time, I visualized these responses at both the acute and healed stages of infection and described the dynamic balance between Treg:Teff that dictates the outcome of anti-*Leishmania* T cell responses. A better understanding of T cell immunity can be translated into the development of viable vaccines and identify new therapy targets. Observations from these studies can also shed light on immunity against other intracellular pathogens as well. Finally, I identified the role of antigen recognition in augmenting Treg functions, a question which remains largely

unanswered in the field. I provided a molecular and visual mechanism of Treg suppression and their dynamics between T cells and macrophages. My findings will have a significant impact in other fields as well, as regulatory T cells are implicated in numerous diseases, such as autoimmunity and cancer. This is only possible because of the availability of a new PEPCK-Tg mouse model through our collaboration with Dr. Uzonna's team and has led to highly impactful observations.

While it is widely accepted that regulatory T cells play a major role in concomitant immunity and parasite survival based on previous studies^{229,483,607,608}, their interplay with effector T cells during acute and healed *Leishmania* infections has largely remained unanswered. A better understanding of the cellular and molecular mechanisms that regulate the balance between Tregs and effector T cells provided in this work unveiled important insights into why some parasites can persist. Finally, while Tregs are known to act in both antigen-dependent and antigen-independent manner, a novel aspect of my research proposal is the ability to define the requirement of antigen recognition by regulatory T cells to invoke their suppressive functions *in vivo*. Whether or not Tregs need to see their antigen in order to increase their suppressive functions has remained controversial, and my studies provided direct evidence that addresses this long-standing question. Strategies targeted against only a small population of Tregs which exert anti-*Leishmania* suppressive activity could, however, prove beneficial, particularly when applied in healed lesions. The selective depletion of *Leishmania*-specific Tregs may represent a viable option to improve parasite clearance while keeping beneficial Treg population in skin intact.

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