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Étude de la régulation des lymphocytes T à mémoire virtuelle en réponse à l'interleukine 4 lors d'infestation helminthique

Investigation of the regulation of virtual memory T cells in response to interleukin 4 during helminth infection

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2020 Nobel Prize in Chemistry for "the discoveries one of gene technology's sharpest tools: the CRISPR/Cas9 genetic scissors"...
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In addition, If You're Searching for Someone to Change Your Life, Look in the Mirror.
.....

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仅以此献给中华人民共和国成立 74 周年!!!

青丝落，银丝长。

扶光掩盖脸颊上的沧桑，

扶摇抚平回忆里的伤疤。

愿台上人金榜题名，

不忘台下故人旧识。

. . . . . .

吾把青丝换功名，

奈何功名垂银丝。

青丝去，功名迟，

少年归来满银丝。

. . . . . .

Abbreviations

ACD	Asymmetric cell division
APCs	Antigen presenting cells
ATP	Adenosine triphosphate
BCR	B cell receptor
BM	Bone marrow
CCR	CC chemokine receptor
CD	Cluster of differentiation
CDR	Complementary determining region
CLR	C-type lectin receptor
Cre	Cre recombinase
CSP	Circumsporozoite protein from *Plasmodium yoelii*
CTLA	Cytotoxic T-lymphocyte-associated protein
CXCR	CXC chemokine receptor
DALY	Disability-Adjusted Life Year
DAMP	Danger associated molecular pattern
DC	Dendritic cells
DE	Differentially expressed
DNA	Deoxyribonucleic acid
DP	Double positive
EEC	Early effector cells
Eomes	Eomesodermin
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FOXO	Forkhead box O
GATA-3	GATA-binding protein 3
GF	Germ-free
GzmA	Granzyme A
GzmB	Granzyme B
GzmM	Granzyme M
H-2D/K	Classical MHC class I antigens subclasses
HIV	Human immunodeficiency virus
Hp	*Heligmosomoides polygyrus*
IAV	Influenza A virus

IBD	Inflammatory bowel disease
ID	Inhibitor of DNA binding
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-4c	IL-4-antibody complexes
IL-4Rα	IL-4 receptor α chain
ILC	Innate lymphoid cells
i.n.	Intranasal
iNKT	Invariant natural killer T cells
i.p.	Intraperitoneal
i.t.	Intratracheal
ITK	IL-2-inducible T cell kinase
i.v.	Intravenous
KIR	Killer-cell immunoglobulin-like receptor
KLRG1	Killer-cell lectin receptor G1
KO	Knockout
L3	Stage 3 larvae
LN	Lymph node
Luc	Luciferase
MAIT cell	Mucosal-associated invariant T cells
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MPEC	Memory precursor effector cells
MuHV-4	Murid gammaherpesvirus 4
NK	Natural killer
ns	Not significant
NTD	Neglected tropical diseases
ORF	Open reading frame
OVA	Ovalbumin
PAMP	Pathogen associated molecular pattern
PBS	Phosphate-buffered saline
PCA	Principal-component analysis
PD	Programmed cell death
PFU	Plaque-forming unit

pi	Post-infection
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptor
PLZF	Promyelocytic leukemia zinc finger
RAG	Recombinant-activating gene
RNA	Ribonucleic acid
RLK	Receptor like kinase
s.c.	Subcutaneous
SEA	Soluble egg antigens
SEM	Standard error of the mean
Siglec	Sialic acid-binding immunoglobulin-type like lectin
SLEC	Short-lived effector cells
Sm	*Schistosoma mansoni*
SP	Single positive
SPF	Specific pathogen free
STAT	Signal transducer and activator of transcription
STH	Soil-transmitted helminths
T-bet	T-box expressed in T cells
T_{CM}	Central memory T cells
TCR	T cell receptor
TEC	Tyrosine kinase expressed in hepatocellular carcinoma
T_{EM}	Effector memory T cells
TGF-β	Transforming growth factor β
Th	T helper
T_{IM}	Innate memory $CD8^+$ T cells
TLR	Toll-like receptors
T_N	Naive T cells
TNF	Tumor necrosis factor
Treg	Regulatory T cells
TRA	T cell receptor alpha locus
TRB	T cell receptor beta locus
T_{RM}	Tissue-resident memory T cells
TSLP	Thymic stromal lymphopoietin
T_{TM}	True memory $CD8^+$ T cells
T_{VM}	Virtual memory $CD8^+$ T cells

WHO	World Health Organization
WT	Wild type
γc	Common gamma chain
ω-1	Omega-1 (secreted protein from *S. mansoni* eggs)

Résumé - Summary

Résumé

Les infections à helminthes sont très répandues et restent un problème de santé publique mondiale non négligeable. Les helminthiases consistent le plus souvent en des pathologies chroniques et insidieuses, qui ont un impact sérieux sur la santé humaine et animale et mettent en péril la stabilité du développement socio-économique. La complexité générale du cycle de vie des parasites rend difficile la mise au point d'un contrôle efficace des infections helminthiques. De plus, la variété des réponses immunitaires induites chez les hôtes infectés augmente la diversité des complications résultant des infections helminthiques et des co-infections avec des pathogènes hétérologues, et une meilleure compréhension de l'immunité helminthique est une étape importante vers le développement de stratégies de contrôle durables. Classiquement, l'immunité de type 2 au cours de l'infection par les helminthes assure la médiation de l'équilibre complexe entre l'éradication des helminthes, la réparation des tissus après les dommages dus à la migration des parasites et la régulation immunitaire induite par les produits sécrétoires des helminthes au cours de l'infection persistante. Généralement, les lymphocytes T $CD4^+$ comme les lymphocytes T helper 2 (Th2) polarisées sont recrutés pour contribuer à la réponse immunitaire, alors que le rôle des lymphocytes T $CD8^+$ reste sous-estimé. En effet, bien que les helminthes puissent induire des lymphocytes T $CD8^+$ effecteurs, ces cellules ne contrôlent pas facilement l'infection parasitaire. Cependant, il a été démontré que les réponses immunitaires de type 2 induites par les helminthes sont associées à des changements significatifs dans le compartiment des lymphocytes T $CD8^+$, ce qui pourrait avoir un impact sur la régulation de l'immunité contre les helminthes ainsi que sur la réponse aux co-infections virales.

Dans cette thèse, nous avons étudié une population particulière de lymphocytes T $CD8^+$ appelée "lymphocytes T à mémoire virtuelle" (T_{VM}) et sa réponse à l'interleukine (IL-) 4, une cytokine canonique de type 2 associée à l'infection par les helminthes. Les T_{VM} sont des lymphocytes T $CD8^+$ à mémoire non conventionnelle qui se développent en l'absence d'expérience d'antigènes étrangers. Des travaux antérieurs ont montré que l'IL-4 induite par les helminthes peut provoquer l'expansion des T_{VM}, directement via la signalisation dans les lymphocytes T $CD8^+$, et entraîner une protection accrue contre une infection virale concurrente. Ici, en utilisant un modèle de souris spécifiquement déficientes pour la réponse à l'IL-4 spécifiquement dans les lymphocytes T $CD8^+$ périphériques, nous avons également démontré que l'expression de la chaîne du récepteur α de l'IL-4 sur les lymphocytes T $CD8^+$ périphériques est nécessaire pour le maintien des T_{VM}. Nous avons ensuite développé une approche de séquençage "*single-cell*" de l'ARN pour décrire la signature de l'expression génique des T_{VM} dépendant de l'IL-4 lors d'une infection helminthique dans les tissus lymphoïdes périphériques. De manière inattendue, nous avons observé que la signature génétique des T_{VM} dépendant de l'IL-4 incluait le récepteur de surface CD22, un régulateur canonique de l'activation des cellules B. Nous avons en outre démontré que l'expression de CD22 observée est limitée aux T_{VM} induits par l'IL-4. Le séquençage ARN

en « bulk » des T_{VM} $CD22^{+}$ et $CD22^{-}$ a démontré que les T_{VM} $CD22^{+}$ présentent non seulement un phénotype activé, mais qu'ils régulent également de manière significative des récepteurs inhibiteurs supplémentaires. Ensuite, les analyses de séquençage du TCR ont fortement suggéré que si les T_{VM} $CD22^{+}$ conservent un répertoire TCR diversifié, leur région déterminante complémentaire (CDR) 3 est enrichie en caractéristiques physico-chimiques auto-réactives. Enfin, nous avons démontré que le CD22 agit comme un récepteur inhibiteur qui régule négativement l'activation des effecteurs T_{VM}. Nous présentons ici une nouvelle découverte par laquelle l'infection helminthique peut déterminer l'activation des T_{VM} dépendants de l'IL-4 via l'induction d'un programme contre-inhibiteur pour réguler leur activation.

Summary

Helminth infections are highly prevalent worldwide and remain a non-negligible public health problem. Helminthiases mostly consist in chronic and insidious pathologies, which seriously impact human and animal health and endanger stable socio-economic development. The general complexity of the parasite lifecycle results in difficulties in developing an efficient control of helminth infections. Moreover, the variety of the immune responses induced in the infected hosts increases the diversity of complications resulting from helminth infections and coinfections with heterologous pathogens, and a better understanding of helminth immunity is an important step towards the development of sustainable control strategies. Classically, type 2 immunity during helminth infection mediates the complex balance between helminth eradication, tissue repair after damage due to parasite migration, and immune regulation induced by helminth excretory-secretory products during persistent infection. Commonly, $CD4^+$ T cells, such as polarized T helper 2 (Th2) cells, are recruited to contribute to the immune response, while the role of $CD8^+$ T cells remains underappreciated. Indeed, although helminths can induce effector $CD8^+$ T cells, these cells do not readily control the parasite infection. However, there are evidence that helminth-induced type 2 immune responses are associated with significant changes in the $CD8^+$ T cell compartment, which could impact on the regulation of helminth immunity as well as the response to viral coinfections.

In this thesis, we have studied a particular population of $CD8^+$ T cells named "virtual memory T cells" (T_{VM}) and its response to interleukin (IL-) 4, a canonical type 2 cytokine associated with helminth infection. T_{VM} are unconventional memory $CD8^+$ T cells, which develop in absence of foreign antigen experience. Previous work has shown that helminth-induced IL-4 can cause the expansion of T_{VM}, directly via signaling in $CD8^+$ T cells, and resulting in an enhanced protection against a bystander viral infection. Here, using a specific mouse model impaired for IL-4 response specifically in peripheral $CD8^+$ T cells, we further demonstrated that the IL-4 receptor α chain expression on peripheral $CD8^+$ T cells is required for T_{VM} maintenance. We then developed a single-cell RNA sequencing approach to describe the IL-4-dependent T_{VM} gene expression signature upon helminth infection in peripheral lymphoid tissues. Unexpectedly, we observed that the gene signature of IL-4-dependent T_{VM} included the surface receptor CD22, a canonical regulator of B cell activation. We further demonstrated that the observed CD22 expression is restricted to IL-4-induced T_{VM}. Bulk-RNA sequencing on $CD22^+$ and $CD22^-$ T_{VM} demonstrated that $CD22^+$ T_{VM} not only display an activated phenotype, but also significantly upregulate additional inhibitory receptors. Then, TCR sequencing analyses strongly suggested that while $CD22^+$ T_{VM} conserve a diverse TCR repertoire, their complementary determining region (CDR) 3 were enriched in self-reactive physico-chemical characteristics. Finally, we provided evidence that CD22 acts as an inhibitory receptor, which negatively regulates T_{VM} effector activation. We provide here a new discovery by which helminth infection can determine the activation of IL-4-dependent T_{VM} via the induction of a counter-inhibitory program to regulate their activation.

General preamble

General preamble

The work presented in this thesis has focused on how virtual memory $CD8^+$ T cells (T_{VM}) are regulated by helminth-induced IL-4. We have investigated the influence of helminth infection on $CD8^+$ T cells after CD8α-specific IL-4Rα deletion in peripheral $CD8^+$ T lymphocytes by studying the activation, maintenance, and expansion of T_{VM}. We have made important observations by defining the IL-4-induced gene signature of T_{VM} expanding during helminth infection using state-of-the-art single cell-based and bulk RNA sequencing, as well as TCR-sequencing to study not only transcriptomic changes and the diversity of the T cell repertoire but also the physico-chemical characteristics of the CDR3 of the identified populations of IL-4-induced T_{VM}. Importantly, we identified CD22 as a signature receptor specific to IL-4-induced T_{VM} and propose a model by which CD22 takes part in a tightly regulated control of T_{VM} activation and expansion during helminth infection and potentially viral coinfections.

The following introduction aims to present the different theoretical concepts and recent reports that could help the understanding of our work. Hence, chapter 1 describes important helminth infections in humans, their epidemiology and existing experimental mouse models. Chapter 2 describes the main features of anti-helminth immunity and the bystander regulation by helminth infection. To describe the diversity of conventional vs. unconventional memory $CD8^+$ T cell responses, the first section of chapter 3 presents the dynamics of the conventional response to foreign antigens from activation to memory formation and the main described sub-populations. Then, unconventional memory T cells are described, including lymphopenic-induced memory $CD8^+$ T cells, innate memory $CD8^+$ T cells and T_{VM}, with a focus on recent studies investigating their origin, maintenance, and responses during helminth infection and/or their bystander functions.

Introduction

1. Helminths

1.1 Taxonomy

The term “helminth” is not associated with any particular phylogenetic classification. Helminths are described as complex multicellular eukaryotic invertebrates with tube-like or flattened bodies exhibiting bilateral symmetry mainly belonging to the unrelated Nematoda, Platyhelminths or Acanthocephala phyla (Cox, 2009). Species from the Nematoda and Platyhelminth phyla are either free-living or parasitic worms (including parasites of plants or vertebrates). The term “helminth” is sometimes used as a synonym of “worm” but, although there is no clear consensus, it is usually restricted to parasitic worms. Their common particularity as pathogens is that their life cycle is not usually completed in a single individual. Indeed, with some limited exceptions, they do not proliferate within their final host as opposed to other pathogens such as protozoans, bacteria or viruses that have a strong potential of *in situ* exponential population growth. Eggs or larvae are rather externalized to complete the life cycle and infect a new host.

Nematoda are commonly called “roundworms” referring to their long, narrow and threadlike body, basically like a tube within a tube (Kiontke and Fitch, 2013). All nematodes go through four larval stages during their life cycle, and a new synthesized cuticle can replace the old one at the end of each larval stage (Kiontke and Fitch, 2013). Along with arthropods, they belong to the Ecdysozoa clade which share the common characteristic of moulting during growth (Aguinaldo et al., 1997). Parasitic nematodes count a wide diversity of important pathogens of humans or other vertebrates such as *Ascaris spp.*, *Anisakis spp.*, whipworms (e.g. *Trichuris spp.*), pinworms (e.g. *Enterobius spp.*, *Oxyuris spp.*), hookworms (e.g. *Ancylostoma spp.* and *Necator spp.*), filarial worms (e.g. *Onchocerca spp.*), *Trichinella spp.* or *Strongyloides spp.*. Infections with hookworms, whipworms, *Ascaris spp.* or *Strongyloides stercoralis* are referred to as “soil-transmitted helminthiasis” (STH) because transmission occurs through contact with water, soil or food contaminated with fecal material (Jourdan et al., 2018). In STHs, adult worms live in their host’s intestine and eggs excreted in the feces contaminate soil. Eggs hatch and develop in larval stages in the environment without the requirement of intermediate host to reach an infective stage. It forms a direct cycle of infection and transmission. Infection by *Ascaris lumbricoides* or whipworms occurs by faeco-oral transmission, while *Strongyloides* and hookworm larvae can actively penetrate intact skin (Jourdan et al., 2018). Infection by filarial worms, *Anisakis spp.*, or *Trichinella spp.*, are not directly transmitted via the contaminated environment but are arthropod- or food-borne parasites. Filarial adult worms living in lymphatic vessels (*Wuchereria bancrofti*), serous cavities or subcutaneous tissue (*Onchocerca volvulus*) release larvae (microfilariae) which have to be taken up by an arthropod vector during a blood meal to be transmitted to another host. *Trichinella spp.* adult worms deliver larvae in the intestinal mucosa. After migration through lymphatic and blood

vessels, these larvae encyst in muscles and will infect a new host upon ingestion (Gottstein et al., 2009). *Anisakis spp.* have an indirect cycle, which depends on predation involving fish and marine mammals, and are therefore transmitted via seafood.

Because of their flattened shape, **Platyhelminths** are also called flatworms. Two main groups of parasitic worms of vertebrates are found in this Phylum: **Cestoda** and **Trematoda**.

Cestodes (*Taenia spp.*, *Echinococcus spp.*) are hermaphrodite segmented worms. These helminths are characterized by an indirect life cycle with production of encysted larvae (metacestode) in muscles or other tissues of the intermediate host and transmission to the final host via feeding on these tissues.

Within **Trematoda**, the order Digenea contains the most important parasitic species. Digenea have indirect development with an asexual reproduction phase in their intermediate host, usually an invertebrate such as a gastropod. Infectious larvae (cercariae) are released from intermediate host and can encyst on aquatic weeds or in the tissues of a second intermediate host (e.g. a fish) (Fürst et al., 2012a). In these cases, infections may be acquired by consumption of aquatic products (foodborne trematodiases). Concerning the members of the genus *Schistosoma*, cercariae are released in the water from a freshwater gastropod and actively penetrate the skin of their final host. The majority of members of the order Digenea are hermaphrodites, however schistosomes have separated genders with a clear dimorphism.

Finally, **Acanthocephala** (thorny- or spiny-headed worms) are parasites with complex life cycles including an encysted stage in an arthropod intermediate host. Transmission depends on predation and adult worms are hooked to the intestinal wall of their final host.

1.2 Epidemiology

Helminths infecting humans are widely distributed around the world but the highest prevalence is found in sub-Saharan Africa, the Middle East, Asia, South and Central America and the Caribbean (Herricks et al., 2017). Given their high prevalence in developing countries, the majority of helminthiases are considered as Neglected Tropical Diseases (NTDs) prioritized by the World Health Organization (WHO). So far, helminthiasis still account for the vast majority of NTDs cases (World Health Organization, 2021). Therefore, the WHO made a map for 10 years on these infections and established specific strategies aiming at their prevention, control and even eradication (World Health Organization, 2021), of which efforts to control helminthiasis have been implemented including preventive chemotherapy with anthelmintic drugs as well as water safety, sanitation and hygiene improvement and vector control (Campbell et al., 2018, 2016; Gabrielli et al., 2011).

Helminths may induce direct damage by their activity: intestinal obstruction, volvulus and intussusception by *Ascaris lumbricoides*, even appendicitis, cholecystitis, pancreatitis, and gastric ascariasis after invading orifices (Al Amin and Wadhwa, 2023; Jourdan et al., 2018); lymphedema/elephantiasis or blindness with filarial worms (Nutman, 2013); hypertension, ascites and digestive tract varices caused by schistosome egg-induced granuloma and fibrosis and bile duct hyperplasia (Colley et al., 2014; Coltart and Whitty, 2015; Elbaz and Esmat, 2013; Marchese et al., 2018); biliary obstruction and cholangitis in foodborne trematodiases (Fürst et al., 2012b; Keiser and Utzinger, 2009) or cysticercosis in the central nervous system due to development of *Taenia solium* in brain (Coyle et al., 2012; Gonzales et al., 2016; I.O. and E.V., 2014), and indirect damage by the host immune response against it: prolonged inflammation of the intestine and villous atrophy by *Strongyloides* and *Trichinella*; Loeffler syndrome by type 1 hypersensitivity reaction by *S. stercoralis*; inflammation of the colon that leads to blood loss and rectal prolapse by *Trichuris* (Al Amin and Wadhwa, 2023). Certain helminth infections are also associated with development of cancers (e.g. *Schistosoma haematobium*, *Clonorchis sinensis*, *Opisthorchis viverrini*) (Brindley et al., 2015; Fried et al., 2011; IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2012; Oikonomopoulou et al., 2014; Scholte et al., 2018; van Tong et al., 2017). In addition, more insidious effects associated with low burden chronic infections cannot be overlooked (Pisarski, 2019). These aspecific disabling morbidities are usually the consequences of an altered nutritional status (Scott and Koski, 2021; Shea-Donohue et al., 2017) linked to gut inflammation, diarrhea, intestinal bleeding and parasites feeding on tissues leading to weakness and impaired development. Helminth infections thus have a profound impact on population health status based upon both their high prevalence and debilitating consequences. The “Disability-Adjusted Life Year” (DALY) parameter helps summarize and quantify this impact by integrating both mortality and morbidity. Calculated as the sum of years of life lost due to premature mortality and years of healthy life lost due to disability, DALY expresses the impact of a disease or injury on health status when compared to an ideal health status (Devleesschauwer et al., 2014). For helminthiases, grade of disability depends on infection intensity and associated symptoms or sequelae (Pullan et al., 2014).

The most prevalent helminth species belong to the STH group with *A. lumbricoides* (roundworm) infecting around 804 million people and inducing 1 million DALYs followed by *T. trichiura* (whipworm) and hookworms with approximatively 477 and 472 millions of cases and 0.5 and 4 million DALYs, respectively, and the *S. stercoralis* (threadworm) infects up to 100 million people (Herricks et al., 2017; Jourdan et al., 2018; Vos et al., 2015) (Figure 1). The majority of cases occurs in Asia, mostly India and China, with a prevalence that can reach up to 50% or more in some Asian countries (Malaysia or the Philippines) or certain islands of Oceania. Sub-Saharan Africa and Latin America also have high absolute numbers of cases and/or high prevalence (Herricks et al., 2017; Pullan et al., 2014).

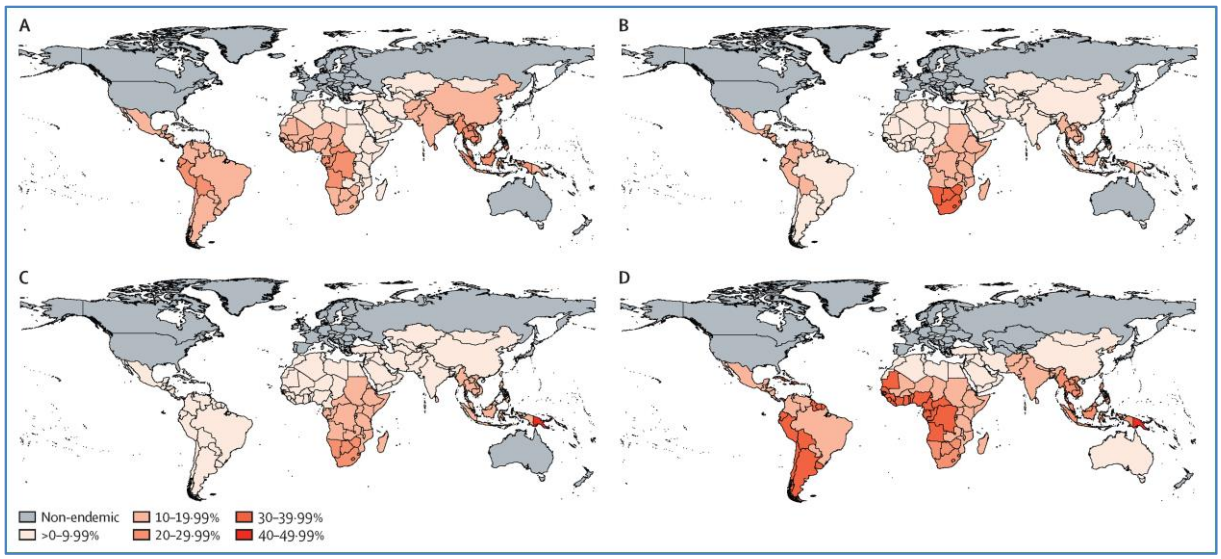


Figure 1. Prevalence by global regions of (A) *Ascaris lumbricoides* (for 2010), (B) *Trichuris trichiura* (for 2010), (C) hookworm (*Necator americanus* and *Ancylostoma duodenale*; for 2010), and (D) *Strongyloides stercoralis* (for 2011). From (Jourdan et al., 2018).

Schistosomiasis is also a highly prevalent disease with 290 million cases reported around the world and 3.06 million DALYs (Herricks et al., 2017). Estimates show at least 251.4 million people required preventive treatment in 2021 and the deaths due to schistosomiasis are currently estimated at least 11,792 globally per year (World Health Organization, 2023). The main species of schistosomes infecting humans are *S. mansoni*, *S. japonicum*, *S. mekongi*, *S. guineensis* and related *S. intercalatum* (intestinal and hepatic schistosomiasis) and *S. haematobium* (uro-genital schistosomiasis) (McManus et al., 2018; World Health Organization, 2023). Ninety percent of schistosome infections occur in sub-Saharan Africa, but schistosomiasis is also present in the Arabian Peninsula, South-east Asia and South America (McManus et al., 2018) (Figure 2). Schistosome species have an important specificity for their intermediate host, therefore their distribution correlates with that of the freshwater snail species that sustain their asexual replication (Gryseels et al., 2006).

Foodborne trematodiases comprise infections with liver flukes (*C. sinensis*, *Fasciola hepatica*, *F. gigantica*, *O. felineus*, *O. viverrini*), lung flukes (*Paragonimus spp.*) and intestinal flukes (*Echinostoma spp.*, *Fasciolopsis buski*, *Heterophyes spp.*, *Metagonimus spp.*, *Gymnophalloides seoi*) (Fürst et al., 2012b; Toledo et al., 2012), concern 80 million people and induce 3.63 million DALYs (Herricks et al., 2017). The geographical distribution of these infections, which are mostly found in East Asia and South America, can be linked to diet habits such as raw fish, crustaceans or vegetables contaminated with the parasite larvae (Fürst et al., 2012a; WHO Foodborne disease burden Epidemiology Reference Group (FERG), 2021). However *Fasciola spp.* and *Paragonimus spp.* are more broadly distributed with cases reported in North America, Europe and Africa (Fürst et al., 2012a; Keiser and Utzinger, 2009).

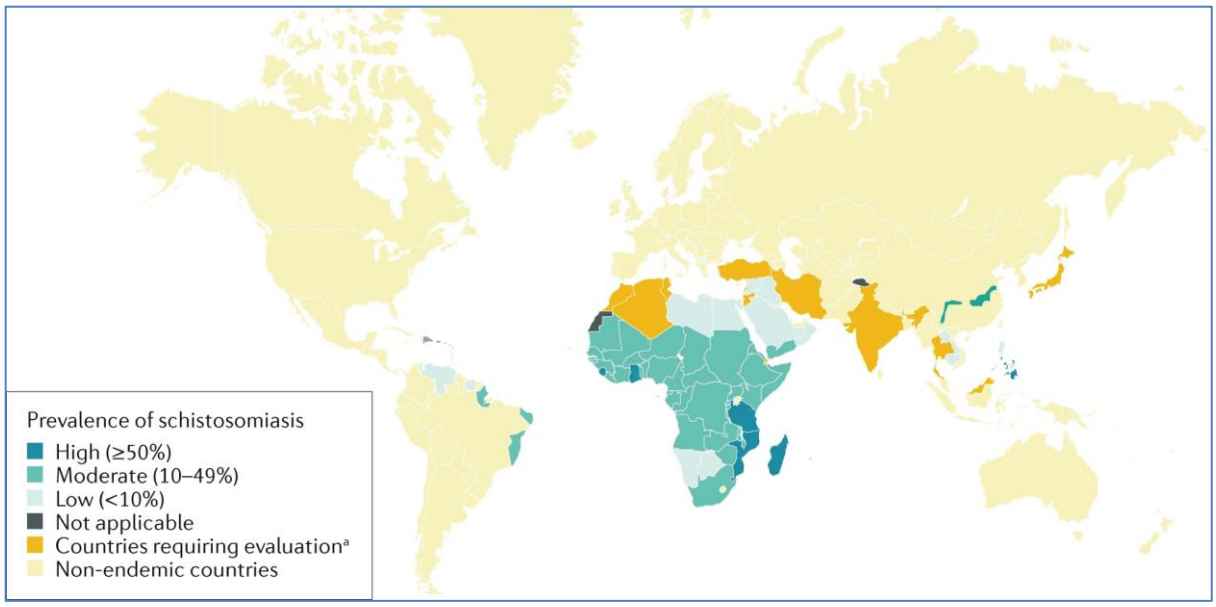


Figure 2. Worldwide distribution of schistosomiasis in 2012. Figure adapted from Map: Distribution of schistosomiasis, worldwide, 2012, WHO. From (McManus et al., 2018).

Lymphatic filariasis is a mosquito-borne infectious disease that affects 44 million people and induces 2.02 million DALYs, mostly in sub-Saharan Africa and South and South-east Asia (Herricks et al., 2017). The majority of cases are caused by infection with *W. bancrofti* but two other filarial nematodes (*Brugia malayi* and *B. timori*) may also be the source of the infection (Taylor et al., 2010). Another species of filarial nematodes, *O. volvulus*, is responsible for onchocerciasis affecting 17 million people with 1.18 million DALYs, mostly in sub-Saharan Africa (Herricks et al., 2017).

Infections with cestodes are much less frequent but can have severe consequences. Indeed, while infection with adult worms is not associated with major complications, development of cystic larval stages in host tissues may have substantial health impacts. *T. solium* cysticercosis and cystic echinococcosis concern one and 0.8 million people and induce 0.34 and 0.18 million DALYs, respectively (Herricks et al., 2017). *T. solium*, *E. granulosus* and *E. multilocularis* were ranked as the top three foodborne parasites based on multiple criteria including incidence, disease severity or trade relevance (Pascal Boireau, 2014) and therefore pose both human and veterinary medical challenges. *T. solium* is mostly found in sub-saharan Africa, Asia and Latin America (Devleesschauwer et al., 2017), *E. multilocularis* distribution is limited to the Northern Hemisphere while *E. granulosus* is more widely distributed (Deplazes et al., 2017). Unlike infections with other helminths, cestode infections remain a public health concern in developed countries (Devleesschauwer et al., 2017; Gottstein et al., 2015), probably due to the important health risks and difficulties of treatment. In 2015, the WHO Foodborne Disease Burden Epidemiology Reference Group identified *T. solium* as a leading cause of deaths from food-borne diseases, resulting in a considerable total of 2.8 million DALYs (World Health Organization, 2022). Furthermore, data indicate that the presence of *E. multilocularis* in red foxes is spreading through

Europe with a prevalence that can reach more than 10% in the most affected European countries such as Estonia, Latvia, Lithuania, France, Switzerland or Germany (Gottstein et al., 2015; Oksanen et al., 2016).

Trichinella spp. is present all over the world, Antarctica being the only continent where this parasite has not been reported (Pozio and Darwin Murrell, 2006). However, the vast majority of human cases occur in Europe (mostly central and eastern countries) (Darwin Murrell and Pozio, 2011). Trichinellosis has a much lower global burden than other foodborne helminthiasis, with 500 DALYs (Devleesschauwer et al., 2015).

1.3 Investigating anti-helminth immune response: experimental mouse models

Helminths include various genera from different Phylae with a wide diversity of life cycles, going from direct transmission between hosts to complex life cycles including intermediate host species, sometimes multiple. This diversity is also reflected in the variety of organs impacted by helminth infections. Together with the complexity of the life cycle which renders of helminths, these parasites are multicellular organisms and the tools for studying helminths *in vitro* as well as potential mutagenesis to investigate host-parasite interactions are very limited as opposed to viruses or bacteria (White and Artavanis-Tsakonas, 2012). Despite such difficulties, important advances are being continuously made in uncovering full parasite genomes, which should open new avenues for genome manipulation in the future (www.wormbase.org).

An important majority of helminths target the gastro-intestinal tract and infect their host orally (STH or food-borne helminthiasis); however infection can also occur by direct penetration of the skin or even *via* mosquito bites. The most used helminth experimental models in laboratory rodents will be presented in the following sections.

1.3.1 Cestodes

Cestodes have a strict indirect life cycle. Eggs eliminated in the faeces of the final host are ingested by the intermediate host (arthropod or vertebrate, depending on the species) and larvae (oncospheres) develop into metacestodes after tissue migration. Ingested metacestodes lead to the formation of adult worms in the intestine of the final host. Alternatively, when humans ingest eggs of *T. solium* or *Ecchinococcus spp.* they develop metacestodes in various tissues.

The human parasites *Hymenolepis diminuta* and *H. nana* also productively infect mice. Both the intestinal adult and metacestode stages are studied. The intestinal adult stage is achieved by oral gavage

of metacestodes and metacestodes can be studied either following ingestion of eggs or by direct injection of metacestodes (Ito et al., 1988; McKay et al., 1991, 1990).

Taenia solium is responsible for human cysticercosis, and mouse models include the intraperitoneal or intracranial injection of *Mesocestoides corti* or *T. crassiceps* metacestodes (Alvarez et al., 2010; Cardona et al., 1999; Terrazas, 2008). The particularity to reproduce asexually in the metacestode stage is used in biological models: *T. crassiceps* larvae injected in peritoneal cavity cause long-lasting infection and reproduce by asexual budding. An early type 1 immune response at the infection site is shifted to a mixed type 1/type 2 response with production of both IFN-γ and IL-4 (Toenjes et al., 1999; Toenjes and Kuhn, 2003).

Intraperitoneal injection of protoscoleces extracted from *E. multilocularis* or *E. granulosus* hydatid cysts leads to formation of cysts and is used to study cystic echinococcosis (Ma et al., 2016; Wang et al., 2016; Zhang et al., 2018). *E. granulosus* induce an initial type 2 immune response which changes to a more mixed type 1/2 later on (Baz et al., 2006; Dematteis et al., 2003, 1999; Mourglia-Ettlin et al., 2011).

1.3.2 Trematodes

Studies of immune responses against trematodes have mostly focused on *Schistosoma spp.* parasites. *S. mansoni* is the species most studied within the genus *Schistosoma*. As a trematode, it has an indirect life cycle with a freshwater snail as its intermediate host. Eggs are excreted in the faeces of the final host and hatch upon contact with water. The ciliated larvae, called miracidia, is released and penetrate the intermediate host snail. The larvae then develop into sporocysts, which in turn produce new daughter sporocysts by asexual reproduction. Four to 5 weeks after infection, the snail begin to release large numbers of cercariae in response to sunlight, the larval infective stage for vertebrates. Cercariae penetrate the final host's skin, transform into schistosomula and reach the circulation within 48 hours. They then migrate first through the lung and then within the arterial circulation to reach the portal system and the mesenteric vessels. There, adults live for up to 30 years as a mating pair, the long and thin female being grasped within the gynaecophoric canal of the shorter but larger male. Egg laying begins 4 to 6 weeks after infection. Eggs are laid in the mesenteric vein and burrow their way through the intestinal wall to reach the lumen and be excreted in the faeces. However, a significant proportion of eggs are swept away by the blood flow and get trapped in the liver where they elicit a strong type 2 granulomatous inflammation (Allen and Maizels, 2011; Gryseels et al., 2006; Walker, 2011) (Figure 3). Inflammation as well as tissue damage and remodeling elicited in reaction to the worms' eggs are responsible for the main clinical signs including diarrhea, hematochezia, hepatomegaly, splenomegaly, ascites or hematuria (Colley et al., 2014). In the first weeks of infection, the immune response is dominated by a type 1

response however with onset of egg laying, it is surpassed by the emergence of a strong type 2 immune response. Indeed, the eggs are strong inducers of type 2 immune responses (Grzych et al., 1991; Pearce et al., 1991; Pearce and MacDonald, 2002; Vella and Pearce, 1992). More precisely, the secreted glycoprotein omega-1 (ω-1) from *S. mansoni* eggs and also found in soluble egg antigens (SEA) mixture, is one of the main driver of type 2 immunity (Everts et al., 2009; Steinfelder et al., 2009). After a peak at around 8 weeks post-infection, the type 2 immune response is then downmodulated.

S. mansoni cercariae successfully infect mice percutaneously and complete the entire life cycle. *S. mansoni* infection of laboratory mouse is well-described and has previously been used as a model for human pathology (Fallon, 2000). To study the immune response restricted to *S. mansoni* eggs, eggs collected from infected livers can also be used for direct immunization. A first sensitization by intraperitoneal (i.p.) injection of eggs, followed by an intravenous injection 14 days later leads to a robust formation of granulomas in the lung (Joyce et al., 2012). Injections of SEA or ω-1 can also be used to induce strong type-2 immune responses in mice (Everts et al., 2009).

1.3.3 Nematodes

Several mouse models can be used to study the immune response against parasitic nematodes in mice. Among worms establishing enteric infections, hookworm-related *Nippostrongylus brasiliensis* and intestinal *Heligmosomoides polygyrus* are commonly used in *in vivo* studies in mice. Besides, other nematodes infecting humans are also patent in mice (*Trichinella spiralis* or *T. pseudospiralis*, *Ascaris suum* or *A. lumbricoides*, *Trichuris muris*, *Brugia malayi*) or rodent species exist (*Trichuris muris*, *Strongyloides venezuelensis*, *S. ratti*, *Litosomoides sigmodontis*) and are also used in experimental models. *N. brasiliensis* is a gastro-intestinal parasite of rodents (naturally infecting rats), similar to the hookworms *A. duodenale*, *A. caninum*, or *N. americanus*, with an extensive migratory phase. *N. brasiliensis* eggs are excreted in the faeces and release a free-living larva that needs a period of maturation in the environment before becoming infective: following hatching, the L1 larvae molt twice to produce the highly mobile and infective L3 stage. Infection occurs through skin penetration; larvae reach blood vessels rapidly (from 11h post-infection) and are transported to the lung where a third molt produces L4 larvae. Larvae then cross the alveolo-capillary barrier, causing lung hemorrhages and chronic lesions leading to emphysema. They migrate up the respiratory tract, are coughed and swallowed to ultimately reach the intestine. After a fourth moult, adult worms mate and the females release eggs beginning from day 6 post-infection (Camberis et al., 2003) (Figure 3). Experimental infection of mice is usually performed via subcutaneous (s.c.) injection of L3 larvae. As opposed to rats, infected mice naturally clear *N. brasiliensis* infection by day 9-11 post-infection, with the exception of parasite strains adapted to the mouse which can persist longer periods. Nevertheless, full migration is completed, and a strong systemic type 2 immune response is induced.

H. polygyrus is a mouse gastro-intestinal helminth used to model hookworm infections. Its lifecycle differs from that of *N. brasiliensis* by being exclusively enteric. Experimental infections are performed by gavage of infective L3 larvae. Importantly, as a mouse parasite, it can establish chronic infection in susceptible mice, even though a great variation in susceptibility exists between mouse strains (Reynolds et al., 2012). Eggs are excreted for months in the faeces and hatch in the environment. As for *N. brasiliensis*, larvae moult twice before becoming infective L3. After faceo-oral transmission, L3 encyst in the duodenal submucosal layer by day 1 or 2 post-infection and molt twice. Adults return to the intestinal lumen at day 8-10 to mate, and egg laying begins around day 14 (Camberis et al., 2003; Johnston et al., 2015). The immune response during *H. polygyrus* infection is first dominated by a strong type 2 immune response (Grencis et al., 1994; Mohrs et al., 2005) before development of a regulatory response (Finney et al., 2007; Setiawan et al., 2007).

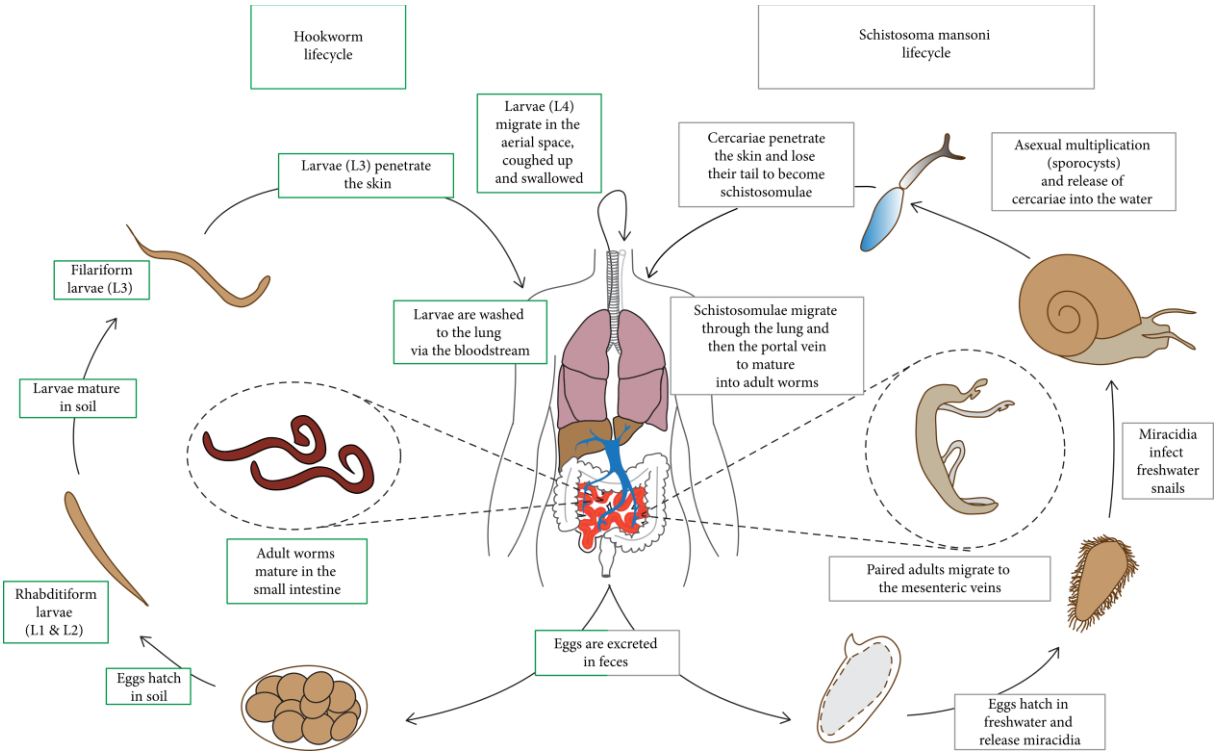


Figure 3. Graphical representation of the life cycles of hookworms (human: *Necator americanus* or *Ancylostoma duodenale*; mouse: *Nippostrongylus brasiliensis*) and *Schistosoma mansoni*. From (Rolot and Dewals, 2018).

2. Immune response to helminths

2.1 Orientation of the immune response to helminths

Helminth infections may present multiple challenges to the host immune system for several reasons. First, the far larger size of helminth parasites than that of immune cells makes it cannot be directly phagocytized. Instead, killing of helminth larvae has been shown to be achieved *in vitro* by accumulation of neutrophiles and/or eosinophils around the developmental stages of the worms present in the tissue (Buys et al., 1981; Patnode et al., 2014). Secondly, since the antigens from different stages of the parasite life cycle (eggs, larvae or adult worms) may be different, a differential immune response over the course of infection may be necessary. Additionally, eradication of the parasite is greatly limited due to establishment of long-term chronic infections by helminths, the persistence mechanisms often develop to dampen unsuccessful excessive inflammation and collateral damages. Furthermore, numerous helminths are characterized by multi-organ migration stages, which are often accompanied by tissue damage. Immune responses aimed at containing parasites and/or maintaining tissue integrity are therefore required. Hence, immune against helminth infections makes a balance between parasite containment and/or elimination (control) and minimization of collateral damages associated with inflammation (tolerance of persistent parasite).

As illustrated in figure 4, the orientations of immune responses are classified based on $CD4^+$ T lymphocyte polarizations, each orientation being characterized by typical cytokines and transcription factors and associated with particular pathological processes.

Type 1 immunity is mostly based on the release of pro-inflammatory and cytotoxic factors that are typically induced by rapidly replicating intracellular pathogens, like viruses or bacteria. Instead, and despite their wide diversity, helminth infections are consistently associated with type 2 immunity which major characteristics are anti-inflammatory and wound-healing properties. As discussed later, the type 2 immune response is mostly associated with protection against helminth infection. A protective role for type 2 immune responses was also found against venoms (Marichal et al., 2013; Palm et al., 2013). However, similar responses may also be deleterious when they develop against normally harmless substances and mediate allergic pathologies (Holgate, 2012).

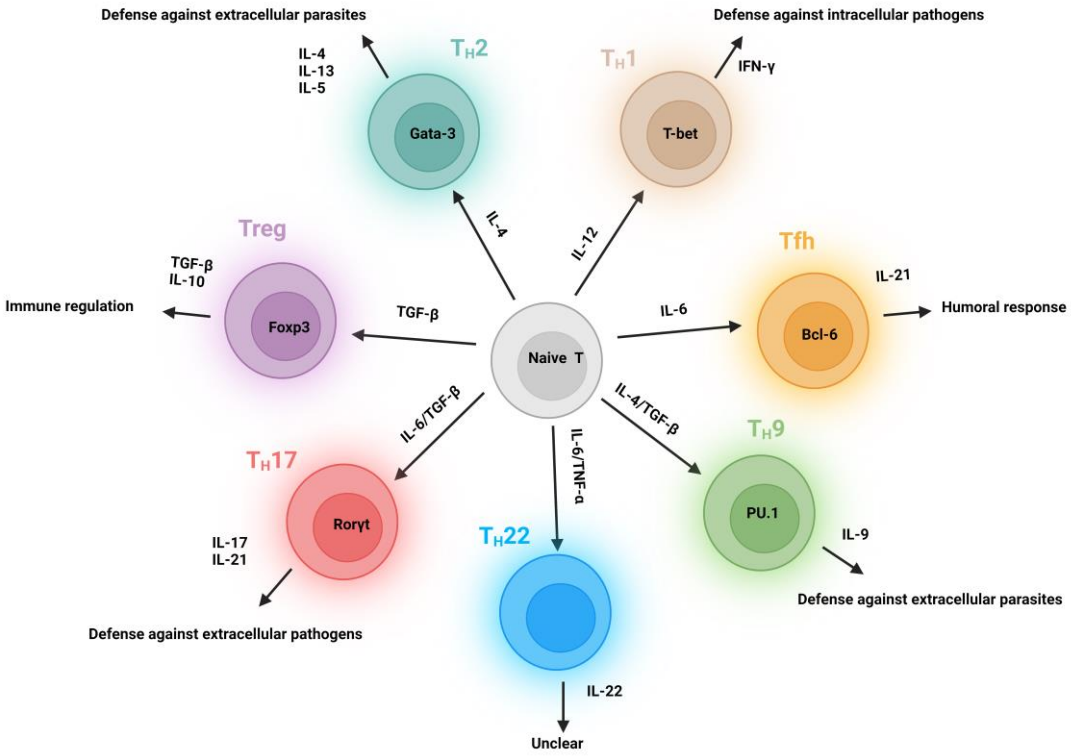


Figure 4: Polarization of T helper cell responses. Summary of the different orientations that $CD4^+$ T cell can adopt during activation by their cognate antigens, in response to cytokine environment. Each subtype of $CD4^+$ T cells expresses a specific transcription factor, produces a particular set of cytokines and displays specific effector functions. Adapted from (Bonelli et al., 2015; Corripio-Miyar et al., 2022).

In type 2 immunity, $CD4^+$ T helper lymphocytes are committed towards a Th2 activation state characterized by the expression of transcription factor GATA-binding protein 3 (GATA-3) and signal transducer and activator of transcription (STAT)-5 and -6 and by the production of type 2 cytokines interleukin (IL)-4, IL-13, IL-5 and IL-9. The type 2 immune response also involves secretion of immunoglobulin (Ig)E and IgG1 by B lymphocytes and recruitment and activation of innate immune cells such as eosinophils, alternatively activated macrophages (aaMφ), basophils, mast cells or group 2 innate lymphoid cells (ILC). These elements will also produce type 2 cytokines, participate in initiation and maintenance of type 2 immune responses and, through their effector functions, induce typical pathological changes associated with type 2 immunity (e.g. smooth muscle contractility, mucus hypersecretion, tissue remodeling).

2.2 Initiation of the immune response to helminths

An immune response against a pathogen is induced upon recognition of typical molecular patterns by **pattern recognition receptors (PRR)**, amongst which membrane-bound toll-like receptors (TLR) or C-type lectin receptors (CLR). PRRs, harbored by innate immune cells, recognize various sets of foreign or abnormal molecular patterns associated with infection with different classes of

microorganisms. Recognition of these pathogen- or danger-associated molecule patterns (PAMPs or DAMPs) by innate immune cells will lead to production of signals that will tailor the immune response. These molecular features, their signaling pathways and the characteristics of the response they induce are well described for type 1/type 17 responses against viral, bacterial or fungal infections but much less is known about induction of type 2 immune responses, especially during helminth infections (Iwasaki and Medzhitov, 2015). Nevertheless, several PRRs have been implicated in the recognition of helminth products and molecular patterns from both parasite surfaces and excretory-secretory (ES) products are susceptible to be recognized by PRRs (White and Artavanis-Tsakonas, 2012). Products from various helminth species such as *S. mansoni*, *T. spiralis*, *N. brasiliensis* or *L. sigmodontis* may notably signal through TLR2, TLR3 or TLR4 (Aksoy et al., 2005; Pellefigues et al., 2017; Rodrigo et al., 2016; Van der Kleij et al., 2002; Zhang et al., 2018). Shared motifs in helminth glycans are not commonly found in vertebrates. They constitute targets for the host immune system and have been identified to drive type 2 immune responses (Okano et al., 1999; Prasanphanich et al., 2013; White and Artavanis-Tsakonas, 2012). Thus C-type lectin receptors (CLRs) have been suggested to represent major PRRs in the case of helminth infections (Vázquez-Mendoza et al., 2013). In accordance with this, numerous molecular patterns from helminth carbohydrates are recognized and internalized by CLRs, notably dendritic cell (DC)-specific intercellular molecule-3-grabbing non-integrin, macrophage galactose-type lectin or mannose receptor and trigger immune signals (Deschoolmeester et al., 2009; Everts et al., 2012; Hussaarts et al., 2014; van Die et al., 2003; van Liempt et al., 2007; van Vliet et al., 2005).

As one of the main types of **professional APCs**, conventional DCs establish the bridge between the innate and the adaptive immune responses. The activated and polarized lymphocytes induced by DCs via producing costimulatory signals and presenting captured foreign antigens to adaptive cells specifically recognize the pathogen. Thus, DCs not only play a role in activation of the adaptive immune response, but they also shape this response depending on the signals they receive and are thereby a key factor in its adequacy to the risks presented by a particular pathogen. In order to successfully induce a T cell response, DCs recognize, process and present foreign antigens taken up from their environment, while upregulating class II major histocompatibility complexes (MHC) and expressing costimulatory molecules (CD40, 80 and 86) and pro-inflammatory cytokines, to drive T helper lymphocytes polarization. DCs clearly participate in the polarization of T lymphocytes during helminth infections, since it has been shown that the Th2 response against several helminth infections is impaired in mice devoid of $CD11c^+$ cells (Phythian-Adams et al., 2010; Smith et al., 2012, 2011). Moreover, DCs stimulated with helminth products induce Th2 differentiation *in vitro* or *in vivo*, indicating that DCs are sufficient to drive Th2 polarization (Balic et al., 2004; MacDonald et al., 2001; Smith et al., 2012; Whelan et al., 2000). On the contrary, the type 2 innate immune response is not affected by $CD11c^+$ cells depletion (Smith et al., 2012). Surprisingly, helminth products mostly fail to induce DCs classical maturation such as upregulation of CD40, 80 or 86 (MacDonald et al., 2001; Whelan et al., 2000).

Helminth products seem to make DCs resistant to conventional type 1 stimulation and induce priority Th2 polarization. For example, they have a striking inhibitory effect on type 1 polarizing capacities of DCs, countering LPS-induced pro-inflammatory activation (Kane et al., 2004; Rodríguez et al., 2015; van Liempt et al., 2007) and suppressing IL-12 production from DCs (Balic et al., 2004; Cervi et al., 2004; Kane et al., 2004). It is thought that in absence of Th1 priming signals, DCs spontaneously induce Th2 differentiation. Consistent with an inhibitory role of helminth products on DCs, CLR detection of ω-1 from *S. mansoni* SEA is not sufficient to polarize towards a type 2 immune response but needs its RNase activity. After internalization, depending on its glycosylation, ω-1 impairs protein synthesis depending on RNase in DCs (Everts et al., 2012). In addition of response to helminths by DCs themselves, polarization of Th2 cells by DCs is associated with activation of several pathways, in which notably NF-κB1 pathway, STAT-5, IRF4, upregulation of Notch ligand Jagged-2 or OX40L (Artis et al., 2005; Gao et al., 2013; Ito et al., 2005).

During tissue migration, helminths damage the host tissue, and these injured barrier sites such as epithelial cells release cytokines named **alarmins** that act as potent inducers of type 2 immune responses. Moreover, induction of type 2 immune responses is typically observed in tissue or cellular damaging circumstances: physical wounds, exposition to allergens or venoms enzymes (Kheradmand et al., 2002; Palm et al., 2013), inert particles (Mishra et al., 2011) or adjuvants (alum) (Gause et al., 2013; Kool et al., 2011). Early events in the induction of anti-helminth responses include the secretion of the **epithelial cytokines thymic stromal lymphopoietin (TSLP)**, **IL-33** and **IL-25** which are involved in initiating type 2 immunity including stimulation of ILC2 and Th2 $CD4^+$ lymphocytes to produce type 2 cytokines (Fort et al., 2001; Ito et al., 2005; Schmitz et al., 2005). The relative importance of these cytokines in type 2 immune responses depends on the helminth species involved and on the localization of the response (Harris and Loke, 2017), in addition evidence indicates that they may have redundant or synergistic roles (Vannella et al., 2016).

IL-33 is released during cellular necrosis, and can also be secreted by living cells such as macrophages (Mirchandani et al., 2012; Ohno et al., 2009). Signalization *via* its receptor IL-33R/T1/ST2 has been shown to play a role in inducing an optimal type 2 immune response (Humphreys et al., 2008; Schmitz et al., 2005; Townsend et al., 2000). IL-33 is an actor in the protection against several helminth infections (Ajendra et al., 2014; Hung et al., 2013; Scalfone et al., 2013) but could also be involved in exacerbation of type 2 pathologies associated with helminth infections (Du et al., 2013; Yu et al., 2015). Many cell types from both the innate and adaptive immune system express IL-33R and respond to IL-33 stimulation (Lott et al., 2015). Moreover, conditioning of DCs by IL-33 has been shown to induce Th2-like and Treg polarization in $CD4^+$ T cells (Matta et al., 2014; Rank et al., 2009). Nonetheless, typical consequences of IL-33 *in vivo* injection, such as eosinophil infiltration, goblet cell hyperplasia and airway hyperreactivity, are T cell/B cell independent, since they also occur in recombinant-

activating gene (Rag)-deficient mice (Kondo et al., 2008). In line with these observations, the protective role of IL-33 in helminth infection has been shown to be mediated by group 2 innate lymphoid cells (ILC2) that express high levels of IL-33R (Bouchery et al., 2015; Neill et al., 2010) and macrophages (Yang et al., 2013).

IL-25 (or **IL-17E**) is involved in early production of type 2 cytokines (Fort et al., 2001) and is essential for protection against several nematode (Fallon et al., 2006; Owyang et al., 2006; Pei et al., 2016; Price et al., 2010; Zaiss et al., 2013a) or trematode infections (Muñoz-Antoli et al., 2016) independently of the presence of lymphocytes (Fallon et al., 2006). Initially, IL-25 was shown to be expressed by Th2 cells (Fort et al., 2001) but more recent data comprehensively identified tuft cells, an intestinal epithelial cell subset, as the main source of IL-25 (Gerbe et al., 2016; Howitt et al., 2016; Von Moltke et al., 2016). Tuft cells have been shown to constitutively produce IL-25 but not IL-33 or TSLP. Furthermore, during *N. brasiliensis* infection, tuft cells appear to be the main source of IL-25 in the intestine. At steady state, IL-25 secreted by intestinal tuft cell induces the production of IL-13 by ILC2, which in turn maintains intestinal tuft cells numbers. This homeostatic mechanism is amplified by helminth infections, leading to increased production of IL-13 by ILC2 and intestinal tuft cell accumulation. It has been shown that through production of IL-25, tuft cells are the central actor of the induction of type 2 immune response and the protection against intestinal nematodes (Gerbe et al., 2016; Von Moltke et al., 2016). Thus, it has been proposed that similarly to PRR-dependent activation of innate immune cells, tuft cells could be triggered by detection of parasite presence *via* their taste receptors (Howitt et al., 2016). Although the short chain fatty acid succinate produced during infection with the protozoan *Tritrichomonas spp.* is sufficient to induce tuft cell expansion in C57BL/6 mice but not in BALB/c (Nadjsombati et al., 2023), it however remains unknown how helminths do trigger tuft cells activation (Nadjsombati et al., 2018; Schneider et al., 2018).

TSLP secretion by epithelial cells is induced by mechanical injuries or protease detection (Kouzaki et al., 2009; Oyoshi et al., 2010). Mast cells and basophils are also a potential source of TSLP during type 2 immune responses (Sokol et al., 2008; Soumelis et al., 2002). It is a central factor in induction of type 2 immune responses during allergic airway inflammation (Zhou et al., 2005) but its requirement for induction of type 2 immune responses during helminth infections is highly variable, from essential during *T. muris* infection (Taylor et al., 2009) to dispensable against *H. polygyrus* (Massacand et al., 2009). The main target of this cytokine are DCs (Liu et al., 2007). Signaling of TSLP on DCs involves the transcription factor STAT-5 (Bell et al., 2013), induces OX40L and prevents expression of type-1 polarizing cytokines interferon(IFN)-γ and IL-12p40. OX40L expression by DCs trigger Th2 cells differentiation only if IL-12 is not present. Thus through increased OX40L expression and decreased IL-12 production, TSLP-activated DCs allow commitment of T cells towards Th2 activation (Ito et al., 2005). TSLP has been shown to be dispensable during *H. polygyrus* and *N. brasiliensis* infections, since

they both secrete products with equivalent effects (Massacand et al., 2009). There is also evidence for a direct action of TSLP on $CD4^+$ T cells for induction of Th2 cells (Omori and Ziegler, 2007).

Other molecules such as uric acid or extracellular ATP are associated with tissue damages and are potent inducers of type 2 immune responses (Kool et al., 2011; Kouzaki et al., 2011).

ILC2s are innate lymphoid cells sharing many characteristics with Th2 cells, but devoid of TCR. Thus, their activation mainly results from their response to cytokines independently of antigen direct recognition. ILC2s can be activated by alarmins to secret large amounts of type 2 cytokines IL-5 and IL-13. The expansion of ILC2s is a hallmark of helminth infections (Nausch and Mutapi, 2018). Interestingly, rIL-25 treatment of *N. brasiliensis*-infected Rag-deficient mice renders this susceptible strain resistant through an action on ILC2s (Fallon et al., 2006; Price et al., 2010). However, expulsion of *N. brasiliensis* requires the presence of both lymphocytes and IL-25 signaling on ILC2s in the absence of treatment with rIL-25 (Neill et al., 2010). Furthermore, IL-25 and IL-33 expression during *H. polygyrus* infection helps worms expulsion in wild-type (WT) but not Rag-deficient mice (Zaiss et al., 2013a). Interestingly, while induction of IL-4 production by IL-25 required the presence of lymphocytes, IL-25 or IL-33 treatment of Rag-deficient mice still induces significant IL-5 and IL-13 production as well as histological changes (Fort et al., 2001; Moro et al., 2010). These data indicate that ILC2s play a central role in the induction of the type 2 immunity against helminths but may not be sufficient to mediate critical effector functions leading to worm expulsion.

A role in the induction of the type 2 immune response was also proposed for other immune and nonimmune cell types. In response to *N. brasiliensis* infection, neurons from the submucosal plexus secrete neuromedin U which is a strong and fast inducer of type 2 cytokines production by ILC2s and has been shown to play a role in efficient parasite expulsion (Cardoso et al., 2017; Klose et al., 2017). Mast cells are essential for induction of type 2 immune responses after *H. polygyrus* or *T. muris* infection, with the ability to respond to extracellular ATP and a link to production of IL-25 and IL-33 stimulating IL-13 production from ILC2s (Hepworth et al., 2012; Shimokawa et al., 2017). Basophils may also represent an early source of IL-4 (Gessner et al., 2005). Indeed, besides the classical IgE-dependent activation, they also respond to IL-33, TSLP, proteases or IL-18 stimulation (Kondo et al., 2008; Phillips et al., 2003; Siracusa et al., 2011; Yoshimoto et al., 1999). Like IL-33, IL-18 precursor is present in epithelial cells and can be released from dying cells. IL-18 is also expressed by macrophages and dendritic cells (Dinarello et al., 2013). Furthermore, eosinophils, basophils and mast cells might act as APCs (Kambayashi and Laufer, 2014).

The initiation of type 2 immunity involves an intricate network of interactions that is summarized in figure 5. The multiple paths leading to type 2 immunity induction reflect the variety of stimuli resulting

in the initiation of such response. Identifying the key factors involved in the initiation of type 2 immune responses during helminth infection is a complex task, mainly due to the redundancy within the immune system (Allen and Maizels, 2011). As an example, *S. mansoni* ω-1 has been identified as the main driver of the Th2 response against *S. mansoni* eggs. However, despite an impaired capacity to condition DCs to induce Th2 differentiation *in vitro*, ω-1-depleted SEA was still able to induce type 2 immune responses *in vivo*, highlighting the existence of more complex mechanisms *in vivo* (Everts et al., 2009). Furthermore, induction mechanisms are likely to vary depending on helminth species and tissues involved.

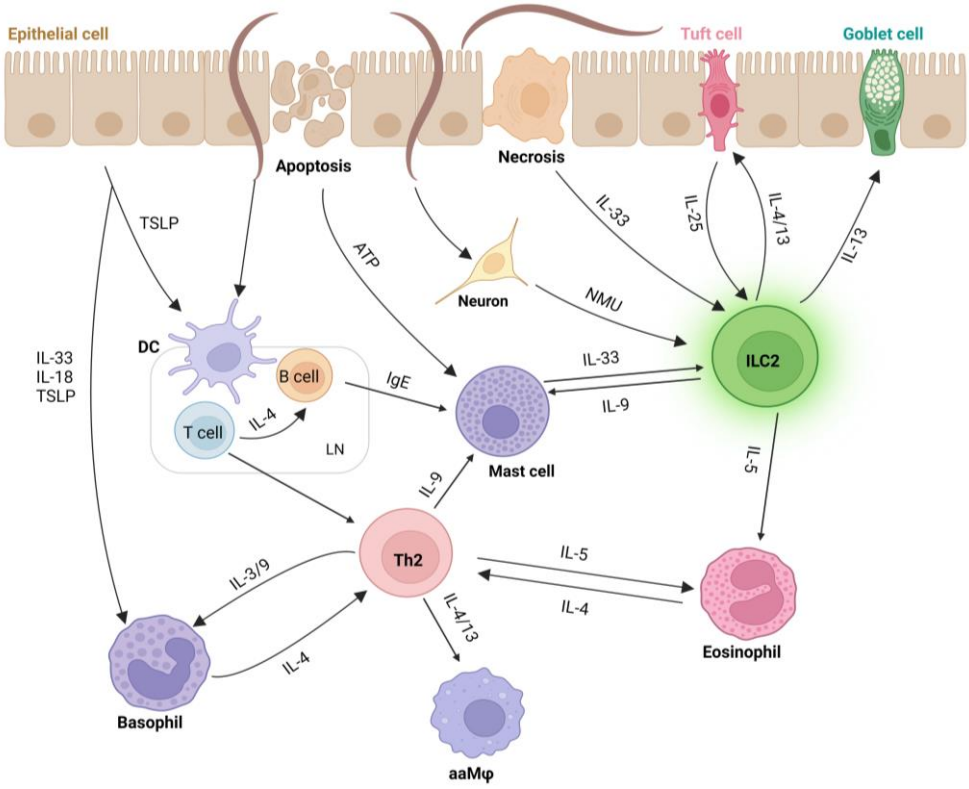


Figure 5: Induction of type 2 immunity against helminths. Helminth infections can trigger several pathways leading to initiation of type 2 immunity. DCs play a central role in the initiation as they can directly detect helminth molecules, migrate to draining lymph nodes and instruct $CD4^+$ T cells to adopt a Th2 phenotype. DCs capacity to stimulate Th2 differentiation is also influenced by alarmins such as TSLP. Th2 cells secrete large amounts of type 2 cytokines IL-4, IL-13 and IL-5 and sustain B cells isotype switch towards production of IgE and IgG1. Alarmins induce activation of other type 2 immune mechanisms. ILC2, which produce IL-5, 13 and 4, are stimulated by IL-33 released by damaged epithelia, IL-25 released by tuft cells or neuromedin U secreted by neurons, the latter two being able to sense helminth products. Other cell types such as mast cells or basophils also respond to alarmins (extracellular ATP, TSLP or IL-33) and are an early source of IL-4. ATP, also released during tissue damage, stimulates mast cells to produce IL-33, further activating ILC2. Mast cells degranulation is also stimulated by IgE binding or IL-9 secreted by both ILC2 and Th2 cells. IL-5 from ILC2 and Th2 cells attract and expand eosinophils that in turn (like basophils) secrete IL-4 to sustain Th2 responses. IL-4 and 13 promote differentiation of macrophages in alternatively activated macrophages (aaMφ). Cytokine and immune mediators secreted by all these cells promote parasite expulsion and wound healing. (DC, dendritic cell; ILC2, type 2 innate lymphoid cell; aaMφ, alternatively activated macrophage; TSLP, thymic stromal lymphopoietin; NMU, neuromedin U; IL, interleukin; LN, lymph node.) From (Gause et al., 2013; Hammad and Lambrecht, 2015; Harris and Loke, 2017).

2.3 Anti-helminth effector functions

Anti-helminth immune responses are aimed at eliminating helminths (either by killing or expelling them) to reach sterile immunity whenever possible or, more frequently, to keep a low parasite burden. Besides, anti-helminth immune responses must deal with tissue damage. Canonical **type 2 cytokines IL-4 and IL-13** are central players of maintenance and amplification of type 2 immune responses as well as mediation of effector functions. Signaling of both IL-4 and IL-13 is dependent on expression of IL-4Rα (CD124), either in combination with common γ chain (γc, IL-2Rγ or CD132) to form type I IL-4 receptor or with IL-13Rα1 to form type II IL-4 receptor and is transduced via phosphorylation of STAT-6 (Figure 6). The common γ chain and therefore type I IL-4 receptor is mainly expressed on hematopoietic cells and specifically binds IL-4. Non-hematopoietic cells express high levels of IL-13Rα1 and only low levels of γc, therefore mostly expressing the type II IL-4 receptor that can bind both IL-4 and IL-13. Myeloid cells express both types of IL-4 receptor (Junttila, 2018). IL-13 also binds IL-13Rα2 with high affinity. IL-13Rα2 can act as a secreted decoy receptor to neutralize IL-13 but also has a role as a cell surface receptor. Signaling of IL-13 on IL-13Rα2 induces activation of *TGFB1* promoter and promotes fibrosis (Fichtner-Feigl et al., 2006). Thus, while these cytokines have overlapping functions, they also mediate specific functions. As proven in different settings using IL-4-antibody complexes (IL-4c) treatment or mice deficient for IL-4, IL-13, STAT-6, and/or IL-4Rα, protection against helminth infections requires the correct development of type 2 immune responses with a central role for IL-4 and IL-13 (Bancroft et al., 1998; Brunet et al., 1997; Finkelman et al., 2004; Herbert et al., 2004; McKenzie et al., 1998; Urban et al., 1998, 1995).

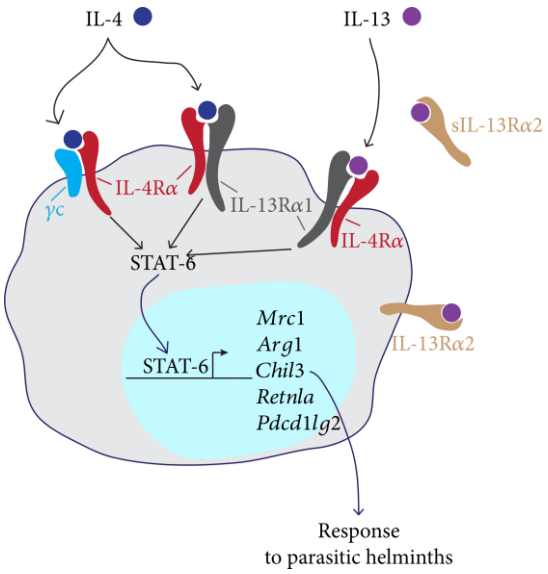


Figure 6. IL-4Rα-dependent alternative macrophage activation during helminth infection. Type 2 innate and adaptive immune cells produce the cytokines IL-4 and IL-13 after exposure to parasitic helminths. In the laboratory mouse, these cytokines induce aaMφ, which are characterized by the upregulation of signature genes. IL-4Rα, IL-4 receptor alpha chain; γc, common gamma chain; IL-13Rα1, IL-13 receptor alpha 1 chain, IL- 13Rα2, IL-13 receptor alpha 2 chain (sIL-13Rα2, secreted form); STAT-6, signal transducer and activator of transcription 6; *Mrc1*, mannose receptor (CD206); *Arg1*, arginase 1; *Chil3*, chitinase-like 3 (Ym1); *Retnla*, resistin-like molecule alpha (Relm-α), *Pdcd1lg2*, programmed cell death 1 ligand 2 (PD-L2). From (Rolot and Dewals, 2018).

Cells from both innate and adaptive immune systems cooperate in a complex network to sustain the induction, maintenance and effector functions of the type 2 immune response (Figure 5). Each contributing cell type produces cytokines and inflammatory mediators to activate and/or enhance other immune cell functions, and instruct non-haematopoietic cells such as epithelial or smooth muscle cells to mediate effector functions. While Th2 cells are seen as the central actor of type 2 immune response, innate immune cells do not only initiate Th2 differentiation, but they also directly participate in protective effector functions, some of which can be mediated even in the absence of lymphocytes (Gause et al., 2013; Kondo et al., 2008).

Different strategies are developed by the type 2 immune response to defeat helminth infection, including the so-called "weep and sweep" response, impairment of parasite fitness, wound repair (and parasite encapsulation) and regulation of excessive inflammation.

In the digestive tract, parasite clearance is facilitated by the **"weep and sweep"** response, which includes increase of epithelium permeability, mucus production, epithelium turn over and smooth muscle contractility to sweep out the worms present in the lumen. The responsiveness of intestinal epithelial cell to type 2 cytokines IL-4 and -13 is crucial in this response (Gerbe et al., 2016; Herbert et al., 2009; Urban et al., 2001). IL-13 particularly has an important impact on worm expulsion, it stimulates goblet cell hyperplasia and drives overproduction of mucus (McKenzie et al., 1998). Increase of permeability of intestinal epithelium, induced by type 2 cytokines, also contributes to the "weep and sweep" response (Finkelman et al., 2004; Madden et al., 2004). Through secretion of immune mediators containing proteases, mast cells mediate the degradation of tight junctions and modify epithelium permeability (McDermott et al., 2003). Mast cells are critically involved in the expulsion of certain helminth species (Knight et al., 2000; Sasaki et al., 2005). IL-13-driven increase in intestinal epithelial cells proliferation and migration along crypts during *T. muris* infection is associated with increased resistance to the parasite. This process is especially relevant for a parasite such as *T. muris* which lives partially buried in the intestinal epithelium (Cliffe et al., 2005). IL-4 and IL-13 increase the contractile capacity of smooth muscle cells favoring intestinal helminth expulsion (Horsnell et al., 2007; Zhao et al., 2003). Interestingly, during helminth infection smooth muscle cells interact with other component of type 2 immunity and impact on goblet cell hyperplasia and type 2 cytokines production (Horsnell et al., 2007).

Type 2 cytokines stimulate the intestinal epithelium to produced effector molecules that **impair parasite fitness**. While Muc2 is physiologically produced in the intestine, Muc5ac is only produced during inflammation. When Muc5ac is produced, the porosity of the mucus network is decreased and affects worm viability in *T. muris* infection (Hasnain et al., 2011). Modification in the composition of mucus can increase the barrier between luminal worms and the intestinal epithelium, impairing the

capacity of worms to feed on epithelial cells (Bansemir and Sukhdeo, 2001). However, Muc5ac appears to directly act on the parasite to alter its viability (Hasnain et al., 2011). Resistin-like molecule (Relm)-β is expressed by goblet cells in response to IL-4 and IL-13 and interferes with a worm's chemosensory organs hampering its nutrition, fecundity and viability (Artis et al., 2004; Herbert et al., 2009). Impairment of worm fitness is also mediated by arginase (Arg)-1, secreted by aaMφ. Arg-1 metabolizes L-arginine and produces L-ornithine which helps in trapping *H. polygyrus* larvae by directly impacting their motility (Anthony et al., 2006; Esser-von Bieren et al., 2013).

Eosinophils, neutrophils and macrophages are able to mediate an **antibody-dependent cellular cytotoxic reaction**. Antibody-coated worms are recognized by the Fc receptor of these cells and mediate release of toxic molecules which directly kill larvae by secretion (O'Connell et al., 2011; Venturiello et al., 1995, 1993). Release of extracellular traps by these cells has also been proposed to help with the killing process by trapping larvae (Bonne-Année et al., 2014). The role of eosinophils in protection is not straightforward, although a role for protection has been supported by some evidence (Huang et al., 2015; Shin et al., 1997; Vallance et al., 2000), it may not be absolutely required and, on the contrary, may sometimes protect larvae (Gebreselassie et al., 2012).

The type 2 immune response is associated with **wound healing** (Gause et al., 2013; Seno et al., 2009) which is essential to maintain tissue integrity during helminth infection. Type 2 immune response mediated tissue healing is especially important against damaging migrating larvae (Chen et al., 2012) or to avoid bacterial contamination from the intestine (Herbert et al., 2004). In addition, production of toxic enzymes may need to be contained by the formation of granulomas to protect the healthy surrounding tissue (Herbert et al., 2004). Wound healing critically depends on IL-13 through induction of fibrosis (Fallon, 2000; Fichtner-Feigl et al., 2006). Macrophages respond to IL-13 and secrete various effector molecules involved in fibrosis. During metabolization of L-arginine, Arg1 secreted by alternatively-activated macrophages (aaMφ) produce proline, a component of collagen (Wynn, 2004). Matrix metalloproteinases regulate matrix degradation. Other aaMφ-derived products, such as TGF-β and Relm-α act directly on fibroblasts to stimulate collagen formation (Gieseck et al., 2018). It is noteworthy that direct IL-4Rα signaling on fibroblasts may be critical for fibrosis (Gieseck et al., 2016). Insulin-like growth factor 1 expressed by aaMφ has also been implicated in restoration of tissue integrity (Chen et al., 2012).

Amphiregulin has been shown to be important for epithelium integrity (Monticelli et al., 2011). This epidermal growth factor-like molecule can be secreted by various cell types (basophils, mast cells, neutrophils, ILC2, $CD4^+$ T cells) in a context of type 2 immune responses and is a factor of *T. muris* resistance (Zaiss et al., 2006; Zaiss et al., 2015) and *H. polygyrus* expulsion (Minutti et al., 2017).

Thus, the type 2 immunity is mostly associated with increased protection against helminth infection. However, some helminth-associated pathologies are linked to a deleterious aspect of the type 2 immune responses. Unlike in nematode infections, protection against *S. mansoni* infection is not dependent on parasite expulsion. *S. mansoni* adult worms persist for years within their host (Pearce and MacDonald, 2002) and mice with higher survival rates can harbor a number of worms similar to more susceptible strains but show decreased egg-induced inflammation (Herbert et al., 2008; Pesce et al., 2009; Vannella et al., 2014). Thus, protection against schistosomiasis rather depends on the control of egg-induced inflammation. The type 2 immune response, notably *via* signalization through IL-4Rα, is essential for survival during schistosomiasis through its capacity of down-regulating the tissue-damaging type 1 inflammatory immune response, supporting the suitable formation of granulomas around eggs that sequester toxins and promoting wound healing to ensure tissue integrity (Brunet et al., 1997; Herbert et al., 2008, 2004; Jankovic et al., 1999). However, type 2 immune responses and in particular IL-13 also lead to hepatic fibrosis during chronicity, which is an important complication in schistosomiasis and is responsible for major symptom (Chiaramonte et al., 1999; Ramalingam et al., 2008). When IL-13, but not IL-4, signaling is impaired, an important reduction of hepatic fibrosis is associated with increased long-term survival (Fallon, 2000; Ramalingam et al., 2008). However, given the counter-regulatory role of type 1 and type 2 immune response on each other, blocking some arms of the type 2 immune response led to deleterious increase in type 1 immune response. However, blocking both IL-13 and IFN-γ controls deleterious inflammation (Ramalingam et al., 2016).

In some cases, a type 1 rather than a type 2 immune response is beneficial for the host. The immune response is skewed towards a type 1 immune response during the first weeks of *S. mansoni* infection, while the increased resistance to reinfection by *S. mansoni* cercariae is associated with the strongly polarized type 1 immune response (Wynn et al., 1996). A type 1 immune response also mediates protection against larval stages of *T. crassiceps* infection (Rodríguez-Sosa et al., 2004).

2.4 Helminth-induced immune modulation

2.4.1 Modulation of the anti-helminth responses: mechanisms of persistence

Helminth infections are mostly associated with the development of **immunoregulatory mechanisms**, which prevent both parasite clearance, and the development of immunopathology. Thus, helminths are in general tolerated by the immune system, allowing persistence of the parasites for long periods of time. Such strong immunoregulatory environment can have important consequences on the outcome of the parasite infection and disease development. Indeed, asymptomatic but chronically infected patients with helminths harbor a strong regulatory response, which is absent in patients who develop overt pathology in response to worm infection. Furthermore, development of pathology is associated with a strong and

more effective type 2 immune response leading to a lower worm burden (McSorley and Maizels, 2012). In other words, regulatory immune responses may be beneficial for both the parasite and its host. Elimination of helminths by chemotherapy often rescued immune responsiveness, thus proving evidence for an active role of helminth in the suppression of immune responses (Grogan et al., 1996; Passeri et al., 2014; Sartono et al., 1995).

With the need for controlling potential excessive amplification of type 2 immune response, leading to immunopathology, helminths actually often elicit a "modified" type 2 immune response. This response combines type 2 with regulatory immune elements and is associated with chronic infections. Helminths can, for example, induce expression of IL-10 and transforming growth factor-β (TGF-β), playing an important role in the control of inflammation and in the downregulation of type 2 (as well as type 1) immune responses, to impair helminth elimination (Worthington et al., 2013; Wynn et al., 1997). TGF-β has been shown to promote expansion of $Foxp3^+$ regulatory T cells (Tregs) during helminth infection, while Tregs are in turn a source of anti-inflammatory functions and their expansion is correlated with long-term persistence of helminth infections and control of immunopathology (D'Elia et al., 2009; Taylor et al., 2009; Turner et al., 2011). Strikingly, some helminths produce TGF-β mimics resulting in amplifying Treg responses (Johnston et al., 2017). DCs are involved in the induction of Tregs, some helminth signaling pathways on DCs can lead to their ability to promote Tregs while some DC subpopulations appear to specifically induce Tregs (Rodríguez et al., 2017; Smith et al., 2011; Van der Kleij et al., 2002). Induction of co-inhibitory molecules CTLA-4 or PD-1 on T cells is also associated with dampening Th2 cell responses and reduced helminth control (McCoy et al., 1997; van der Werf et al., 2013). Development of an anergic phenotype in $CD4^+$ T cells can also be mediated by repeated stimulation of these cells indicating a physiological response of immune cells to chronic antigen stimulation rather than any specific effect of the pathogen (Taylor et al., 2009).

Likewise, regulatory elements can be triggered by helminths at the level of innate immunity as well. *H. polygyrus* elicits production of IL-1β, which decreases IL-25 and IL-33 production early during infection, to impair parasite expulsion (Zaiss et al., 2013b).

Besides induction of regulatory mediators of the immune response, helminth products actively obstruct the immune response that they elicit: induction of immune cell apoptosis (Guasconi et al., 2012; Serradell et al., 2007), degradation of immunoglobulins (Berasain et al., 2000), alteration of antigen presentation competence of macrophages (Robinson et al., 2012), blocking of cytokine release and activation pathways, including small RNA delivery (Buck et al., 2014; Osbourn et al., 2017) or inhibition of mast cell degranulation (Melendez et al., 2007).

2.4.2 Bystander effects of the immune regulation induced by helminths

Persistence of chronic helminth infection means the existence of immunoregulatory mechanisms. Helminths have evolved several mechanisms discussed above that downregulate the immune response allowing their persistence but also avoiding deleterious consequence of excessive and prolonged type 2 immune responses on the host. These mechanisms drew a lot of attention as they can also influence bystander effects notably by enhancing the control of excessive and harmful inflammation. Figure 7 illustrates the diversity of effect helminth infection may have on bystander immunity, either beneficial or deleterious.

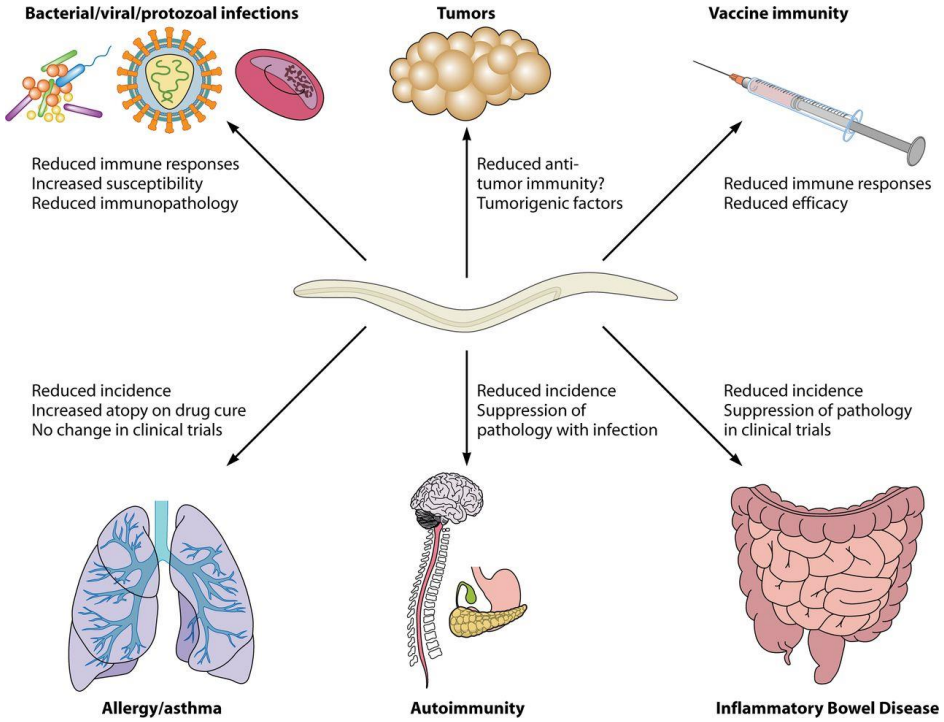


Figure 7. Immunoregulatory effects of helminths on bystander responses. Helminths can suppress a wide range of bystander immune responses, including those of both immunopathogenic and protective natures. Coinfection with helminths suppresses antibacterial, antiviral, and antiprotozoal immunity, leading to increased susceptibility and attenuated immunopathology or, in some cases, exacerbated pathology due to higher infection burdens. Antitumor immunity may be suppressed by helminth infections, which may also release directly carcinogenic factors, potentially leading to increased numbers of malignancies in infected individuals. Vaccine efficacy is compromised by helminth infections due to suppressed immune responses. Immunopathologies such as asthma, autoimmune diseases, and inflammatory bowel diseases are all reduced in prevalence in areas where helminth disease is endemic, and direct effects of helminth infections on the suppression of disease have been shown in clinical trials for inflammatory bowel diseases. From (McSorley and Maizels, 2012).

Helminth infections have been shown to have beneficial effects on various types of immunopathology. Increased prevalence of immune dysregulation observed in developed countries, correlating with improved sanitary situation, led to the formulation of the **"hygiene" or "old friends" hypothesis** several years ago. These concepts suggest that microorganisms and parasites exposure plays a crucial role in the proper development and balance of the immune system, and that reduction in

microbial exposure is one of the parameters explaining the increased prevalence of allergy or autoimmune disorders in developed countries (Lambrecht and Hammad, 2017; Maizels, 2020; Murdaca et al., 2021; Stiemsma et al., 2015; Strachan, 1989). According to these hypotheses, prevalence of allergy has risen in urbanized area where helminth infections have declined, which supported the idea that helminth-host interactions could be valuable for balancing the regulation of the immune system (Ayelign et al., 2020; Bohnacker et al., 2020; Flohr et al., 2009; Gazzinelli-Guimaraes and Nutman, 2018). Experimental data in animal models confirmed the relationship between helminth infection status and immunopathology and uncovered some mechanisms involved. Studies have implicated various helminth species in the protection against allergy, inflammatory bowel disease (IBD)-like colitis, type 1 diabetes or experimental autoimmune encephalomyelitis (EAE, modeling multiple sclerosis) (Maizels, 2022, 2020; McSorley and Maizels, 2012; Vacca et al., 2020; White et al., 2020a). Protection is often correlated with worm burden (Smits et al., 2007) and is sometimes associated with a specific life cycle stage (He et al., 2010; Mangan et al., 2006; Smith et al., 2007). Immune mediators involved in the protection depend on the context and include IL-10 and TGF-β signaling (Ince et al., 2009; Kitagaki et al., 2006), Treg (White et al., 2020b; Yang et al., 2007), B cells (B cells can also secrete IL-10) (Mangan et al., 2006), DCs (Matisz et al., 2017), macrophages (including aaMφ) (Espinoza-Jiménez et al., 2017) or ILC2 (McSorley et al., 2014). Interestingly, numerous helminth products, either excretory/secretory products or recombinant molecules, successfully recapitulate protection induced by infection and could therefore be used as safer therapeutic drugs (Du et al., 2011; Khudhair et al., 2022; Langdon et al., 2022; McSorley et al., 2012; Schnoeller et al., 2008; Vacca et al., 2020; White et al., 2020b). Furthermore, adoptive transfer of immune cells like macrophages (Ziegler et al., 2015), T cells (Grainger et al., 2010) or dendritic cells (Matisz et al., 2017), treated with helminth-derived products has proven efficient in recapitulating the protection induced during infection. Effects of helminth on inflammatory disorders could also be mediated by altering the intestinal microbiota. In recent years, studies unraveled the important role of microbiota in the development of the immune system. Modification of commensals composition dysregulated the crosstalk with the host and impact homeostasis of several system, including the immune system and favored the development of chronic inflammatory diseases (Belkaid and Hand, 2014). Supporting these observations, alterations of the composition of gut microbiota and prevention of the expansion of deleterious and pro-inflammatory bacteria species by helminth infections reduced the development of allergic asthma or IBD (Ramanan et al., 2016; Zaiss et al., 2015).

However, despite the substantial amount of convincing data from animal models, observation of a clear protective effect of helminth infections or helminths products on the development of immunopathology in human is not straightforward. Epidemiological data do not always confirm an inverse correlation between infection and immunopathology status. Atopy, as studied by skin test reactivity to allergens, is the parameter which is the most consistently negatively correlated with helminth infections (Flohr et al., 2009). However, the trend is less clear when considering allergy. Some

studies found a negative correlation between helminth infections and development of allergy, while other did not (Briggs et al., 2016; Feary et al., 2011; Wammes et al., 2014). Yet, effects of helminth infections appear highly variable depending on the species involved, some of them, in particular *A. lumbricoides*, were even associated with increased risk of developing asthma (Leonardi-Bee et al., 2006). Still, several data support the idea of a beneficial role for helminth infection on immunopathology. For example, patient suffering from multiple sclerosis who naturally acquire helminth infections show lower relapse rate (Correale and Farez, 2007). Studies of population in endemic area for helminths indicate that deworming might be associated with an increased inclination to develop immunopathology, as indicated by increased skin test reactivity to allergen or increased level of autoantibody (Flohr et al., 2010; Mutapi et al., 2011; Van Den Biggelaar et al., 2004). However effect on clinical allergies was either null or contradictory (Almeida et al., 2012; Flohr et al., 2010; Lynch et al., 1997).

Besides, the role of helminths in inducing inflammatory diseases cannot be overlooked. Migration of *A. lumbricoides* larvae in the lung affect tissue integrity and multiple exposures can be responsible of an asthma phenotype (Nogueira et al., 2016). Similarly, chronic trichuriasis in mice is associated with colitis that shares several features with Crohn's disease (Briggs et al., 2016). Thus, helminths might represent a cause of inflammatory disease as well as a protective agent. Furthermore, some helminth infections are also linked to carcinogenesis (Pastille et al., 2017). Therefore, extreme precautions must be taken when considering the use of helminths as therapy.

Nevertheless, treatment of inflammatory disorder with helminth products in mice yielded promising results and led to the investigation of the therapeutic use of helminth live infections or helminth products in humans. Again, this has brought conflicting results. Some studies showed an improvement in clinical signs or lesions, particularly the usage of *T. suis* ova had positive outcomes in IBD or multiple sclerosis (Fleming et al., 2011; Summers et al., 2005a, 2005b; Yordanova et al., 2021). But again, other studies fail to assign a protective role to helminths (Bager et al., 2010; Daveson et al., 2011; Feary et al., 2010; Voldsgaard et al., 2015). A better understanding of how helminths manipulate the host immune response and identifying specific helminth molecules with immunoregulation potential are required for future use of helminth products as a therapy.

Immune responses required to control extracellular helminths or intracellular microorganisms are strikingly different and are characterized by mutual inhibition. Helminth infections elicit strong type 2 and regulatory immune response, typically associated with a suppression of type 1 immune responses and could therefore negatively impact protection against **coinfections** with microbial pathogens. As distribution of helminths often overlap that of major microbial threats, identifying the role of helminth infection in resistance to micro-organisms is crucial (Salgame et al., 2013).

Although sometimes contradictory and dependent on helminth species considered, epidemiological data indicate an association between helminth infection and microbial infection like *Mycobacterium tuberculosis*, human immunodeficiency virus-1 (HIV) or malaria (Salgame et al., 2013). Some studies associated helminth infection with development of active tuberculosis (Elias et al., 2006) while other did not (Chatterjee et al., 2014). However, helminth infections impair immune response against *M. tuberculosis* that could be restored by deworming (Babu et al., 2009; Babu and Nutman, 2016; Resende Co et al., 2007). Similarly, association of helminth infection and *Plasmodium* infection is inconsistent in the literature. *S. mansoni* and hookworm infection were often linked to increase prevalence and malaria clinical signs (Degarege and Erko, 2016). *A. lumbricoides* and *T. trichiura* however have been shown to decrease the risk of development of cerebral malaria (Abbate et al., 2018). Schistosomiasis has been shown to increase the probability of HIV infection and was associated with increased viral loads (Downs et al., 2017), although a slower development of adverse HIV outcomes was also described in people coinfected with schistosomes (Colombe et al., 2018). Yet, eradication of ongoing helminth infections in HIV-positive people might have a little but beneficial impact on viral load and $CD4^+$ T cell counts (Means et al., 2016).

Again, variability of data obtained from human epidemiological studies, besides reflecting differences in methodologies, might be explained by the complexity of the interactions between helminths, microorganisms, and their host. Helminth infections can affect host resistance to microorganisms in different ways. On the one hand, inhibition of protective type 1 immunity can lead to decrease control of pathogen propagation (Helmby et al., 1998). Lesions induced by helminths can also promote host colonization by other pathogens. For example, urogenital schistosomiasis is associated with increased prevalence of HIV infection (Downs et al., 2011). On the other hand, regulatory responses elicited by helminths can be beneficial to lessen pathologies induced by inflammatory responses against micro-organisms (Furze et al., 2006). Type 2 immune response may also be beneficial to return to tissue homeostasis after microbial infection (Blériot et al., 2015). Effects of helminth infections on subsequent microbial infection is also dependent on the timing of coinfection (Salazar-Castañón et al., 2018). For example, in the mouse model, a beneficial role of helminth infection for protection against infection by mycobacteria was observed when coinfection occurred early after *N. brasiliensis* (5 days post-infection) and resulted in increased immune response and clearance of bacterial infection (Du Plessis et al., 2013). Likewise, type 2 immunity potentially impairs early stage of HIV infection, including viral entry and replication (Bailer et al., 2000; Creery et al., 2006; Denis and Ghadirian, 1994). Another confounding factor is the coexistence of multiple parasite infections. Abbate and colleagues (2018) showed that presence of *T. trichiura* in patients with hyperparasitemia malaria was associated with a reduced risk to develop cerebral malaria. However, when patients were coinfected with hookworms and *T. trichiura*, the protection conferred by the later was reduced, although infection with hookworms alone had no effect on cerebral malaria occurrence (Abbate et al., 2018). Mouse models

also showed variability in the effect of helminth infections on the outcome of microbial infections and highlighted that this variability is potentially dependent on mouse genetic background, timing of coinfection, identity of helminth and micro-organism involved (including difference between species of the same micro-organism genus) and inoculation route (Babu and Nutman, 2016; Petrellis et al., 2023; Salazar-Castañon et al., 2014). Helminth identity, and therefore its life cycle particularities, could influence the outcome of coinfections. First they may migrate through different organs and consequence of coinfection could be different if both players share the same niche or not (Moriyasu et al., 2018). Then, they are all characterized by a particular immune response dynamic, potentially including early type 1 immune response (which can limit micro-organism proliferation but can also be accompanied with increased inflammatory pathology) followed by type 2 and regulatory immune responses of variable strength (Babu and Nutman, 2016; Salazar-Castañon et al., 2014). Thus, uncovering specific mechanisms that link helminth infections to modifications of the immune response against concurrent or subsequent micro-organisms is required to better understand how coinfection could be beneficial or detrimental for the host.

Furthermore, interaction of helminths with the host immune system might have prominent effects on efficacy of **vaccination**. Indeed, history of infection was shown to greatly affect vaccine response. Reese and colleagues (2016) developed a model of sequential infection of laboratory mice, including herpesviruses, influenza virus and helminths mimicking common infection history that humans could undergo. They showed that the response to a yellow fever virus vaccine was altered by this previous sequential exposure to pathogens (Reese et al., 2016). Adding to these observations, helminth infections have been associated with impairing vaccine efficacy. Unless dewormed before immunization, *H. polygyrus* or *S. mansoni* infected mice failed to develop protection against *Plasmodium* infection after immunization with plasmodium antigens or infection followed by treatment (Laranjeiras et al., 2008; Su et al., 2006). Interestingly, *H. polygyrus* similarly impact efficiency of DNA-based vaccine but did not impair protection conferred by irradiated sporozoites (Noland et al., 2010). *S. mansoni*, but not *H. polygyrus*, infection impaired the protection induced by BCG vaccination against *M. tuberculosis* infection (Elias et al., 2005; Rafi et al., 2015). Hepatitis B vaccination was also impaired in *S. japonicum* infected mice (Chen et al., 2012). However, results from Guan and colleagues indicated that different life cycle stages of the same parasite could have distinct influence on vaccine response (Guan et al., 2013). Furthermore, some helminth products were also used as vaccine adjuvant and increased type 1 immune response (Jiang et al., 2014). Still, these results might have important repercussion on vaccine efficacy in helminth-endemic regions and might explain lower efficacy observed in developing countries (Cherian et al., 2012). Consistent with that, human studies showed that deworming before vaccination ameliorates response to vaccination against tuberculosis (BCG) (Elias et al., 2008), tetanus (Nookala et al., 2004) or cholera (Cooper et al., 2001).

In addition to the effects introduced above, helminths can also **influence anti-viral immunity** (Petrellis et al., 2023). Viruses are typical inducers of type 1 immunity that is crucial for their control, including cytotoxic $CD8^+$ T cell effector responses. This immune response can be considered as the integration of different components which can all be modified by bystander infections, like helminths (Stelekati and Wherry, 2012). Recent studies in mice highlighted that both innate and adaptive components of the anti-viral immune response can be impacted by helminth infections. Using *H. polygyrus* infection and *S. mansoni* egg exposure, it was shown that helminth-induced type 2 immune response elicited reactivation of latent γ-herpesvirus infection through binding of STAT-6 to a viral gene controlling the switch between lytic and latent state. In this process, both IL-4 production and inhibition of IFN-γ were required. Furthermore, aaMφ were shown to be more permissive to γ-herpesvirus (Reese et al., 2014) and murine norovirus (Osborne et al., 2014) replication. Besides, intestinal tuft cells were recently described as the main cellular target of murine norovirus and IL-4 and IL-13 have been shown to stimulate proliferation of tuft cells (Gerbe et al., 2016). In addition, type 2 cytokines production induced during helminth infections could enhance susceptibility to murine norovirus infection through expansion of tuft cells (Wilen et al., 2018).

$CD8^+$ T cells are a hallmark of the adaptive immune response against viruses, and the impact of coinfection with helminths remains underappreciated. Osborne and colleagues (2014) showed that type 2 immune response induced by *T. spiralis* or *H. polygyrus* infection impaired $CD8^+$ T cell responses against a subsequent viral infection with a murine norovirus, both in term of cell number and poly-functional effector functions. Interestingly, anti-viral response was not only impaired locally (in the intestine) but also in distant organs such as the lung, as response against influenza infection was also reduced (Osborne et al., 2014). This modulation of adaptive anti-viral immune response involved the chitinase-like molecule YM1 that is highly expressed by aaMφ during helminth infection. These mechanisms are summarized in Figure 8. Interestingly, another study reported that *H. polygyrus* and influenza A virus (IAV) coinfection resulted in reduced virus-specific $CD8^+$ T cell responses in mice (King et al., 2017). Moreover, mice coinfected with *H. polygyrus* and West Nile virus (WNV) resulted in the exacerbation of intestinal lesions, bacterial translocation, and compromised WNV-specific $CD8^+$ T cell responses, overall leading to increased mortality in a STAT-6- and tuft cell-dependent mechanism (Desai et al., 2021b). In addition, co-infection during patent *L. sigmodontis* infection increased susceptibility to IAV, significantly increasing weight loss and clinical signs of infection as well as increasing IAV titer in mice (Hardisty et al., 2022).

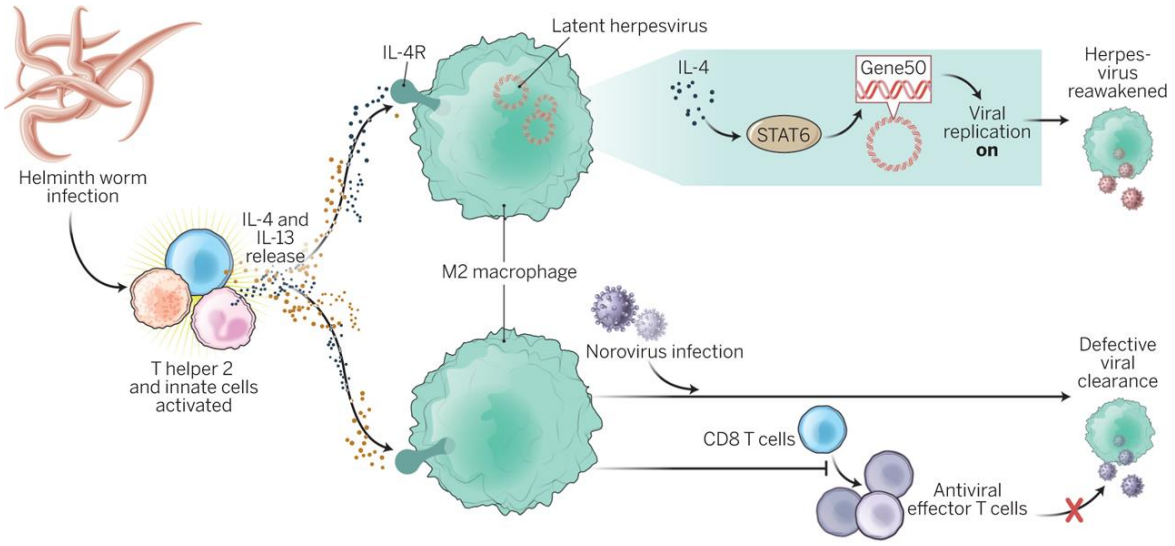


Figure 8: Helminth-induced type 2 immune response alter anti-viral immune response. Top: Activation of STAT-6 transcription factor by IL-4 signaling pathway binds to γ-herpesvirus reactivation transactivator ORF50 and stimulate reactivation of latent infection (Reese et al., 2014). **Bottom**: Alternatively-activated macrophages express high levels of the chitinase-like molecule Ym1 which directly inhibits $CD8^+$ T cell proliferation during viral infection (Osborne et al., 2014). From (Maizels and Gause, 2014).

Although several studies indicate that helminth infections are detrimental for anti-viral protection, other studies rather highlighted a protective role. Notably, *S. mansoni* chronic infection was associated with increased protection against pulmonary IAV infection and Pneumonia of mice (PVM) infection (Scheer et al., 2014). *H. polygyrus* infection protected against respiratory syncytial virus (RSV) infection in mice, where co-infected mice showed lower viral load and reduced lung inflammation. Here, signalization through IL-4Rα was not required for protection, neither was adaptive immune response but it was dependent on the presence of intestinal microbiota and type I IFN. The authors proposed that invading microbiota through *H. polygyrus* induced gut wall lesions induced a systemic anti-viral type I IFN response to control RSV infection (McFarlane et al., 2017). In another study, the protection induced by *T. spiralis* against IAV infection was strikingly different. Indeed, this protection was observed late during viral infection as an enhanced recovery rate in coinfected group. Here, no difference in lung viral load was observed between groups, instead protection was likely linked to decrease inflammation, independently of IL-10 production (Furze et al., 2006). Other data on the effect of filarial cystatin on RSV infection confirmed that helminth products are potent regulator of virus induced excessive inflammation without affecting infection itself. In this study, modulation was mediated by macrophages (Schuijs et al., 2016). In addition, a recent study from the host laboratory found that helminth exposure could expand a subpopulation of memory-phenotype $CD8^+$ T cells, named virtual memory $CD8^+$ T cells (T_{VM}) in an IL-4-dependent manner. BALB/c mice exposed to helminth products, or infected with various helminth species developed an enhanced early $CD8^+$ T cell-mediated response against Murid gammaherpesvirus 4 (MuHV-4) (Rolot et al., 2018). Moreover, T_{VM} expanding in response to *H. polygyrus* were also found to confer antigen-specific protection against *Listeria monocytogenes*

infection (Lin et al., 2018). The main effects of helminth infection on concurrent viral infections as studied in mouse models are summarized in Figure 9.

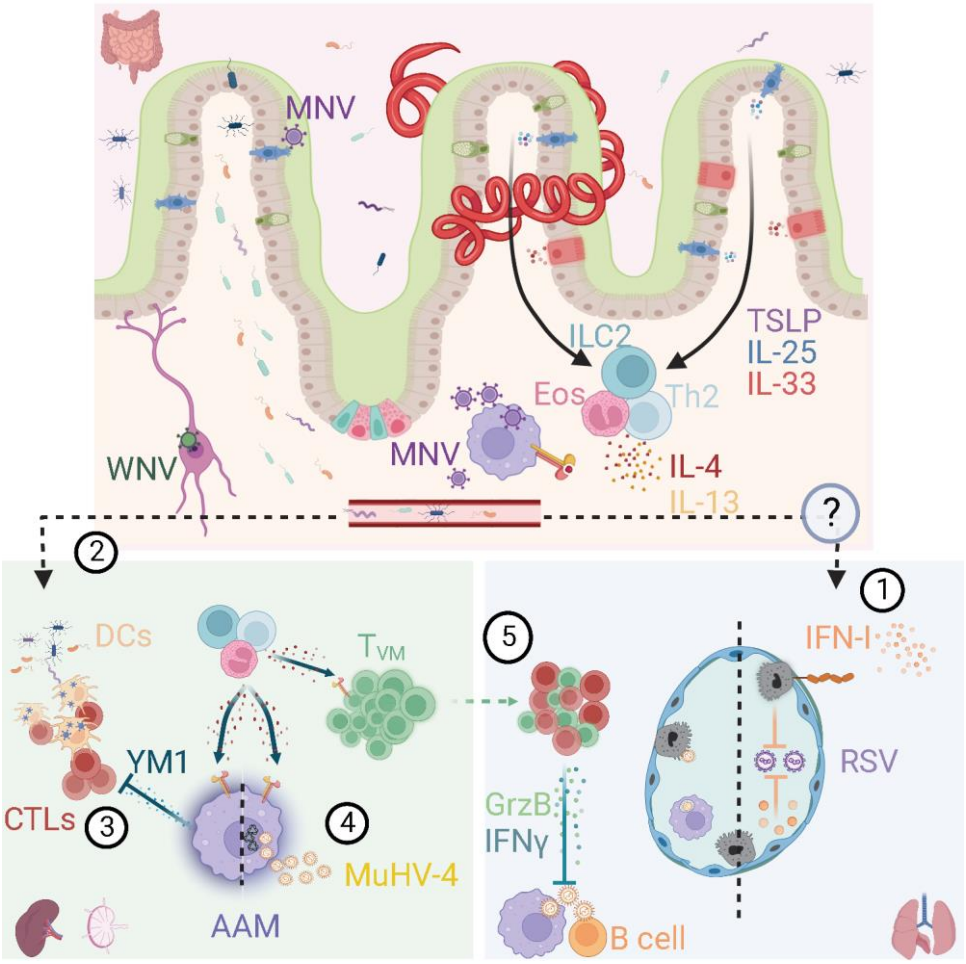


Figure 9. Mechanistic insights on how helminths shape the response to viral infections. Helminth sensing in the gut is followed by the release of the alarmins TSLP, IL-25 from tuft cells and IL-33 from stressed epithelial cells and the subsequent production of IL-4 and IL-13 from activated immune cells. The resulting type 2 immune response in the gut causes changes in the microbiome affecting antiviral immunity at distal sites. Microbiota-dependent upregulation of type I interferons (IFN-I) and interferon stimulated genes in the gut and lung results in a better control of respiratory syncytial virus (RSV) (1). Bacterial translocation increases the susceptibility to West Nile virus (WNV), a flavivirus that infects enteric neurons, due to the impaired activation of cytotoxic T lymphocytes (CTL) by dendritic cells (DCs) in the spleen (2). Type 2 cytokines IL-4 and IL-13 drive the alternative activation of macrophages (AAM). The production of chitinase-like protein YM1 by AAMs impairs the activation and proliferation of murine norovirus (MNV)-specific CTLs in the secondary lymphoid organs (3). Direct IL-4 signaling in AAMs latently infected with MuHV-4 promotes the viral reactivation (4). Simultaneously, IL-4 signaling in $CD8^+$ T cells causes the expansion of **virtual memory T cells (T_{VM})** in secondary lymphoid organs that contributes to the immunity against murid gammaherpesvirus 4 (MuHV-4), resulting in increased virus-specific CTLs producing granzyme B (GrzB) and interferon γ (IFNγ) (5). ILC2: group 2 innate lymphoid cells, Th2: $CD4^+$ T helper 2 cells, Eos: eosinophils. Based on (Petrellis et al., 2023).

These data illustrate how helminth infections can act in various steps of both innate and adaptive mechanisms of anti-viral immune responses, even in distant organs, with either beneficial or detrimental effects for the host (Desai et al., 2021a), which is summarized in Figure 10. Of course, numerous other mechanisms already discussed in previous sections could influence the outcome of viral infection in

helminth-infected host. For example, helminth products could increase viral replication *in vitro* as it is the case for SEA on cell culture of hepatitis C virus (Bahgat et al., 2010). Helminth products can also condition DCs that become "refractory" to induction of type 1 immune response (Everts et al., 2009; Metenou et al., 2012). Proliferation of effector T cells might also be compromised by helminth-induced expression inhibitory costimulation molecules like expression of PD-L2 on aaMφ (Huber et al., 2010). Finally, modification of the host's microbiota by the presence of helminth could impact several parameters including immune system balance (Belkaid and Hand, 2014), epithelium composition (Wilen et al., 2018) or, by its interaction with incoming virus, alter establishment of viral infections (Pfeiffer and Virgin, 2016).

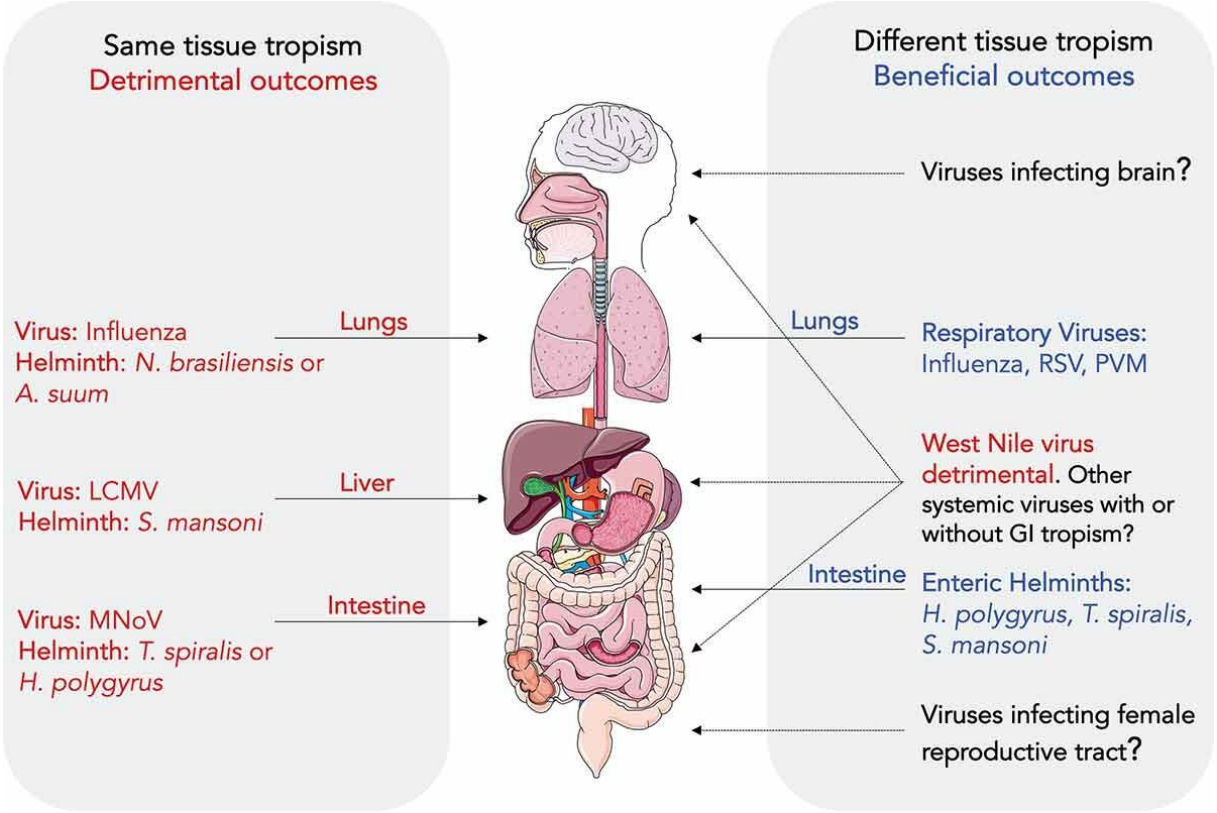


Figure 10. Tissue tropism of helminths and viruses can modulate coinfection outcome. (**Left**) Helminths and viruses that infect the same tissue can result in detrimental outcome for the host. For example, *N. brasiliensis* or *A. suum* and influenza infection of lungs; *S. mansoni*/LCMV infection in the liver; *T. spiralis*/MNoV or *H. polygyrus*/MNoV infection in the small intestine. (**Right**) Helminth and virus infection of different tissues can have beneficial effects. For example, helminths in their enteric phase such as *H. polygyrus*, *T. spiralis and S. mansoni* protect against respiratory viruses including influenza, RSV and PVM. However, *H. polygyrus* coinfection with WNV in the GI tract was detrimental to the host. The effect of enteric helminths on other systemic viruses that do not have tropism for the GI tract is unknown. From (Desai et al., 2021a).

3. Virtual memory CD8$^+$ T lymphocytes

Various facets of T cell-mediated immunity are continuously being elucidated. Typically, T lymphocytes can be divided into two major types, namely ***conventional*** **and** ***unconventional*** **T cells** (Roberts and Girardi, 2008). Conventional CD4$^+$ and CD8$^+$ T lymphocytes express an αβ T cell receptor (TCR), are present in the peripheral blood, lymph nodes, and tissues, and recognize processed antigenic peptide presented within the grooves of major histocompatibility complex (MHC) molecules on other cells *via* the ability of their collectively diverse TCRs (Roberts and Girardi, 2008). Classically, naive single-positive CD8$^+$ thymocytes (CD8 SP) egress to the periphery to form mature naive CD8$^+$ T cells (T_N) after positive and negative selection in the thymus. Then, they can differentiate into effector cells and form "true" antigen-experienced memory cells or "conventional memory" cells via undergoing cognate antigen recognition, for instance after infection or immunization. Conventional memory CD8$^+$ T cells can be further divided in three major subsets: effector/memory T cells (T_{EM}), central memory T cells (T_{CM}) and resident memory T cells (T_{RM}).

Unconventional T lymphocytes include mucosal-associated invariant T cells (MAIT cells), invariant natural killer T cells (iNKT cells) and γδ T cells. These unconventional T cells express a (semi-)invariant restricted TCR repertoire. Whereas MAIT and iNKT cells recognize specialized MHC-I molecules (CD1d) presenting non-protein epitopes, some invariant γδ T cells can directly recognize antigens independently of MHC-I presentation in a fashion more dependent on conformational shape of intact protein or non-protein compounds (Roberts and Girardi, 2008). Unconventional T lymphocytes must not be confused with **unconventional memory CD8$^+$ T cells**, which are memory-phenotype CD8$^+$ T cells developing in a foreign antigen-independent manner (Jameson et al., 2015; Thiele et al., 2020; White et al., 2017). Besides the fact that unconventional memory CD8$^+$ T cells have not been activated by foreign antigen encounter, they share several common features with conventional memory T cells. Unconventional memory CD8$^+$ T cells are αβ T cells, have undergone normal TCR rearrangement, are present in the thymus and the periphery, highly express CD44, but in contrast to conventional memory T cells, downregulate the α4 integrin chain (CD49d) (Haluszczak et al., 2009). This section will present the current knowledge on how conventional and unconventional memory T cells differentiate.

3.1 Conventional memory CD8$^+$ T cells

3.1.1 Dynamics of CD8$^+$ T lymphocyte responses

"Adaptive memory" develops when immune cells such as B or T lymphocytes experience an immune response to a specific foreign pathogen (or an antigen) and differentiate into long-lived antigen-specific immune cells, which are able to respond more quickly and efficiently against re-encounter with the same

antigen. Immunological memory is supported by the persistence, after resolution of infection, of memory T and B cells, which have undergone epigenetic modifications that enable them to respond more effectively to their cognate antigen, when compared to their naïve counterparts. As part of the adaptive immune system, $CD8^+$ T lymphocytes are activated upon specific recognition of epitopes from foreign (non-self) molecular patterns (antigens). In secondary lymphoid organs, specific interactions between the unique T-cell receptor (TCR) from a $CD8^+$ T lymphocyte and the MHC-I-peptide complex presented by an APC (signal 1), along with costimulatory signals (signal 2) associated with an inflammatory context (signal 3), induce the activation of a naive $CD8^+$ T cell into an effector $CD8^+$ T cell. The broad diversity of TCR receptors in the naive population of $CD8^+$ T cells allow them to specifically recognize and react to an exceptionally wide variety of antigens. Antigens from intracellular pathogens such as viruses are processed into antigenic peptides loaded to an MHC-I molecule and exported to the surface of the infected cell, allowing the presentation of the MHC-peptide complex to specific effector $CD8^+$ T cells. Although MHC-I are dedicated to the presentation of endogenous antigens, cross-presentation can occur in DCs to allow presentation of exogenous antigens in a context of MHC- I (Joffre et al., 2012). Antigen-specific activation of $CD8^+$ T cells results in the production of a greatly enlarged population of effector cells with the same antigenic specificity towards the pathogen, thanks to two mechanisms: clonal expansion and acquisition of functional and phenotypic effector features (Blattman et al., 2002). A mouse possesses about 25 million naive $CD8^+$ T cells, among which about 100 to 1000 cells are specific to a given antigenic peptide (Jenkins and Allen, 2010). Upon antigen recognition and activation, specific cells undergo clonal expansion and can produce up to 10 million effector $CD8^+$ T cells in a week (expansion phase, Figure 11), which function to eradicate invading pathogens (Smith et al., 2018). Effector $CD8^+$ T cells are also called cytotoxic T cells, as their principal function is to recognize and kill infected cells. Beside their cell-killing role (i.e. through secretion of cytotoxic molecules such as granzymes and perforin or apoptosis-induction through Fas ligand), effector $CD8^+$ T cells also secrete inflammatory cytokines such as IFN-γ and tumor necrosis factor (TNF)-α. Following elimination of the pathogen, 90 to 95% of the expanded $CD8^+$ T cells pool undergo apoptosis (contraction phase, Figure 11) (Cui and Kaech, 2010) and a population of memory $CD8^+$ T cells persists (memory phase, Figure 11). Memory T cells possess long-term survival potential and self-renewal capacities retaining a multipotent state with high proliferative and effector potential (Kaech and Wherry, 2007). As for naive T cells, memory T cells have a stem cell-like behavior by their ability to regenerate effector population diversity and provide immune-competence during secondary responses (Buchholz et al., 2016; Graef et al., 2014). They are more numerous than the initial naive population (Blattman et al., 2002) but are also characterized by altered distribution pattern (Gebhardt et al., 2009; Masopust et al., 2014; Sallusto et al., 1999) and enhanced effector capacities following TCR stimulation compared to naive T cells (Wolint et al., 2004). Thus, memory T cells can give rise to a faster and stronger effector response against a secondary encounter with the same pathogen (antigen). However, recent evidence indicates that memory T cells may also respond to inflammatory stimuli (including cytokines) in absence of cognate antigen,

which may lead in some circumstances to innate-like properties and control of pathogens in an antigen non-specific manner (referred to as "bystander") (Chu et al., 2013; Mehlhop-Williams and Bevan, 2014).

3.1.2 Diversity within CD8$^+$ T cell responses

Activation and clonal expansion of CD8$^+$ T cells do not produce a homogeneous population of effector cells and transition to memory is not a random process (Figure 11). On the contrary, all activated CD8$^+$ T cells during the acute phase of the infection (expansion phase) do not have an equivalent potential to participate to the memory pool. By focusing mainly on the expression of IL-7 receptor α (CD127) and killer cell lectin-like receptor G1 (KLRG1), short-lived effector cells (SLEC: CD127-KLRG1+) and memory precursor cells (MPEC: CD127+ KLRG1-) can be differentiated within the clonally expanded population (Cui and Kaech, 2010; Kaech et al., 2003). Interestingly, effector and memory fate are not strictly separated as evidence indicates that memory-precursor cells are able to produce (at least at one point during their ontogeny) the effector molecules IFN-γ or granzyme B (Bannard et al., 2009; Harrington et al., 2008).

Diversity of effector population might represent a continuum of intermediate states between terminally differentiated SLEC and memory precursors. Thus, there are a range of cells with high effector capacities but short half-life and cells with lower effector capacities but deeply increased life time (Cui and Kaech, 2010). Memory precursor cells can further generate several subsets of memory cells (Arsenio et al., 2015; Obar and Lefranois, 2010; Plumlee et al., 2015). Memory CD8$^+$ T cells are usually divided in cell subsets based on the expression of specific combination of surface receptors and ligands, their longevity, as well as their effector, proliferative and migration characteristics. Central memory T cells (T_{CM}: CD62L$^+$ CCR7$^+$), as quiescent memory T cells, are located in secondary lymphoid tissues with producing IL-2 but with low levels of cytotoxic proteins like granzymes and perforin; effector memory T cells (T_{EM}: CD62L$^-$ CCR7$^-$) migrate through nonlymphoid tissues and/or inflammatory sites with capability of rapid cytotoxicity by producing IFN-γ and tumor necrosis (TNF) but not IL-2, which could convert into T_{CM} and acquire properties of self-renewal and long-term homeostatic maintenance; and tissue-resident memory T cells (T_{RM}: CD62L$^-$ CCR7$^-$ CD103^{hi} CD69^{hi} CD27^{low}) are present within the non-lymphoid tissue and do not recirculate (Gebhardt et al., 2009; Halle et al., 2017; Jameson, 2021; Jameson and Masopust, 2018; Masopust et al., 2014, 2010; Mueller et al., 2013; Muroyama and Wherry, 2021; Reinhardt et al., 2001; Sallusto et al., 1999; Wherry et al., 2003). Although memory precursors within the circulation have potential to give rise to T_{RM}, only a selection of memory precursor cells can commit to the T_{RM} cell lineage and adopt tissue residency upon stochastic tissue entry and subsequent encounter of local micro-environmental factors, such as TGFβ and IL-15 (Kok et al., 2022). T_{EM} and T_{RM} act as a first line of defense against secondary infections as they can express immediate lytic functions (Masopust et al., 2014), as opposed to T_{CM} that do not show direct

effector functions but rather present longer half-life and higher proliferative potential (Bachmann et al., 2005). T cells are produced at various stages of the development and are facing different immune environments and challenges throughout the life, from fetal to adult life. Thus, the development T cells starts in a more sterile environment of the uterus to a foreign antigen-rich environment after birth up to the adult life. During such periods, naive T cells can differentiate towards several differentiation stages of naïve and memory T cells (Davenport et al., 2020), before differentiation into SLEC and memory precursors to show further immune response to foreign antigens. Thus, with the description of an increasing number of memory T cell subsets, it has been proposed that memory differentiation and/or acquisition may be better represented as a continuum (Jameson and Masopust, 2018; Newell et al., 2012).

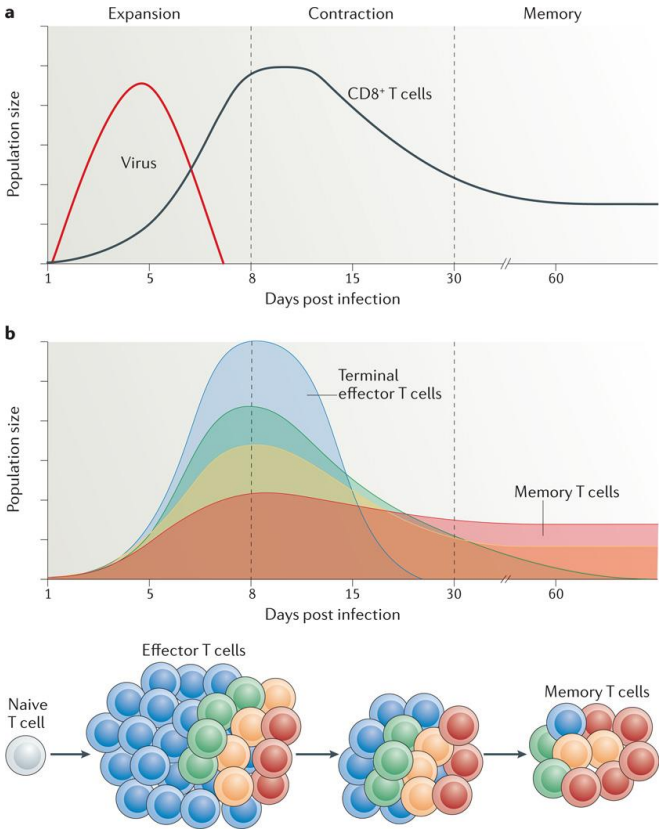


Figure 11. Kinetics of a T cell response and distribution of memory cell potential. a. During an acute viral infection, antigen-specific T cells rapidly proliferate (expansion phase) and differentiate into cytotoxic T lymphocytes that mediate viral clearance. Most of these cells die over the next several weeks (contraction phase). **b.** The pool of effector T cells can be separated into multiple diverse subsets. Some cell-surface markers correlate with distinct effector and memory T cell fates: terminal effector T cells (shown in blue) are KLRG1^{hi}IL-7Rα^{low}CD27^{low}BCL-2^{low}, and long-lived memory (and memory precursor) cells (shown in red) are KLRG1^{low}IL-7Rα^{hi}CD27^{hi}BCL-2^{hi}. However, other T cell subsets with intermediate differentiation states also exist that have mixed phenotypes as depicted by the yellow and green populations. Over time, there may also be some interconversion between these subsets. From (Kaech and Cui, 2012).

3.1.3 Actors of memory formation and maintenance

Several models were proposed concerning the mechanisms leading to the functional diversity of the T cell response. Despite the existence of the theory "one cell, one fate" suggesting that fate of T cells is already predetermined in naive cells, naive CD8$^+$ T cells were shown to have a "stem-like" feature. Indeed, a single naive CD8$^+$ T cell has been able to generate a phenotypically and functionally diverse offspring containing both effector and memory cells (Gerlach et al., 2010; Stemberger et al., 2007). However, optimal response still requires recruitment of several naive cells of the same specificity as individual cell does not always fully translate its diverse potential upon activation (Buchholz et al., 2016).

It is still unclear which and how elements of the immune response are integrated in the fate decision process and several models are proposed such as strength and duration of TCR stimulation, inflammatory cytokines, transcriptional regulations, metabolic switches and uneven segregation of lineage-determining factors during division (Cui and Kaech, 2010). Prolonged exposure to antigens is not required for effective activation of $CD8^+$ T cells (Van Stipdonk et al., 2001), but strong cumulative signals from antigen stimulation (depending on binding affinity to TCR and duration of TCR stimulation), costimulation and inflammation are important favoring factors for the expansion associated with increased short-lived fate (Joshi et al., 2007). Importantly, increased activation of T cells following excessive inflammation or TCR/costimulation signal may be associated with a deficient memory formation as more cells acquire terminally differentiated states (Kaech and Cui, 2012). Integration of the overall strength of the signals received by $CD8^+$ T cells is proposed to respond to one of the following models. First, in the "decreasing-potential" model, those signals are continuously integrated after initial priming, prolonged signals promoting terminally differentiated effector cells. Second, the "progressive differentiation" or "signal-strength" model suggests that those signals are integrated during initial priming and influence the phenotype at later timepoints. Besides, the "asymmetric cell fate" model proposes that asymmetric cell division generates one cell that will adopt an effector fate and the other a memory fate (Buchholz et al., 2016; Kaech and Cui, 2012). Although these models propose that fate decision occurs following antigen stimulation, with all naive $CD8^+$ T cell having the same differentiation potential, recent data indicate a diversity even within naive $CD8^+$ T cells with a partially "pre-programmed" fate that is influenced by the life stage at which the cell was produced (Davenport et al., 2020; Smith et al., 2018). Based on this model, it is proposed that fetal T cells mainly maintain tolerance to harmless antigens, such as food antigens that are transferred across the placenta. Then, neonatal T cells mainly tolerate commensal organisms and offer immune defense against dangerous microorganisms, like microbiota establishment in gut and some foreign antigens, but have reduced immunological memory due to lower numbers and more TCR-restricted clonotypes (Carey et al., 2017; D'Arena et al., 1998; Le Campion et al., 2002; Rudd et al., 2013, 2011a; Schönland et al., 2003). With age advancing, naive T cells continuously respond to foreign antigens during primary infections and reinfections, and naïve T cells in adults have TCRs displaying higher avidity to MHC-peptide complexes presenting specific foreign antigens, while tolerate self-antigens (Davenport et al., 2020). During newborn and infant stages, development of T cells shows rapid proliferation, enhanced tissue access, and cross-reactive TCR repertoires. However, adult T cells have increased memory potential and mature effector functions. Hence, each naive $CD8^+$ T cell could have differential potential to develop into only effector or final memory cells. To add another level of complexity, recent reports suggested that effector $CD8^+$ T cells can dedifferentiate into long-lived memory cells (Herndler-Brandstetter et al., 2018; Youngblood et al., 2017).

Several **transcription factors** have been shown to influence the fate decision process in $CD8^+$ T cells. The T-box factors T-bet (T-box expressed in T cells) and Eomes (eomesodermin) act in concert to regulate the SLEC/MPEC fate (Cui and Kaech, 2010). Reports highlighted that both of them participate into the formation of the effector response with partially redundant roles (Intlekofer et al., 2005; Pearce et al., 2003; Sullivan et al., 2003) and the presence of at least one of them was essential to avoid uncontrolled and deleterious type 17-like activation of $CD8^+$ T cells (Intlekofer et al., 2008). T-bet expression peaked during acute infection, and it was associated with efficient formation of effector cells and declined after resolution of infection (Takemoto et al., 2006; Wiesel et al., 2012). Eomes expression was upregulated during the effector phase but increased further during the memory phase. In term of memory formation, it appeared that Eomes has a crucial role for optimal formation of T_{CM} (Banerjee et al., 2010). Study found that Eomes promotes memory precursor formation early after activation, mediating survival of low-affinity memory precursor by directly driving expression of Bcl2, and promotes the clonal diversity of the memory $CD8^+$ T cell pool (Kavazović et al., 2020). On the contrary, overexpression of T-bet was shown to be responsible for defective T_{CM} formation (Intlekofer et al., 2007). However, both T-bet and Eomes enhanced IL-2/IL-15Rβ (CD122) expression, allowing cells to better respond to IL-15, which is important for the maintenance of memory cell population (Intlekofer et al., 2005). Study found that the induction of Eomes instead of T-bet cannot rescue T-bet deficiency in $CD8^+$ T cells during acute lymphocytic choriomeningitis virus (LCMV) infection, in which expansion and differentiation of SLEC is dependent on T-bet (Fixemer et al., 2020). According to the role of inflammation in promoting SLEC formation at the expense of memory formation, IL-12 enhanced T-bet but repressed Eomes expression in effector $CD8^+$ T cells (Joshi et al., 2007; Takemoto et al., 2006). This mechanism appeared to be dependent on mTOR kinase activity. Indeed, when mTOR activity was blocked, IL-12 treatment of $CD8^+$ T cells induced Eomes expression and formation of memory precursors (Rao et al., 2010). Other inflammatory cytokines like type I IFN are implicated in T-bet upregulation and efficient production of effector cells (Wiesel et al., 2012).

Other transcription factors like B lymphocyte-induced maturation protein-1 (Blimp-1), inhibitor of DNA binding (ID) 2 and STAT-4 are associated with terminal differentiation of SLEC, while B-cell lymphoma (BCL) 6, ID3, T cell factor (TCF) 1, STAT-3 and *forkhead* box O (FOXO) 1 are implicated in the maintenance of memory cells. These transcription factors regulate each other in a complex network of interactions (Kaech and Cui, 2012). Another study found that Runx3 establishes long-lived memory cytotoxic T lymphocytes-associated chromatin accessibility during TCR stimulation, and drives transcription that induces differentiation of true memory precursor $CD8^+$ T lymphocytes and represses differentiation of terminal effector cytotoxic T cells (Wang et al., 2018).

Although maintenance of memory $CD8^+$ T cells is independent of antigen or tonic stimulation from self-antigen /MHC (Murali-Krishna et al., 1999), it requires cytokine signals. Both **IL-7 and IL-15** have

been involved in the optimal $CD8^+$ T cell memory formation and maintenance by cooperatively regulating survival and proliferation of these cells (Becker et al., 2002; Buentke et al., 2006; Judge et al., 2002; Ku et al., 2000; Osborne et al., 2007; Schluns et al., 2000). IL-7 sustains survival of memory $CD8^+$ T cells. As previously discussed, CD127 is used to distinguish SLEC and MPEC fate as $CD127^+$ $CD8^+$ T cells better survive when transferred in uninfected host than their $CD127^-$ counterparts (Kaech et al., 2003). CD127 is downregulated in the early time of the infection when a population of early effector $CD8^+$ T cells (EEC; $KLRG1^-$ $CD127^-$) is observed. EECs conserve the ability to give rise to both SLEC and MPEC (Obar et al., 2011). At the peak of the immune response, to majority of $CD8^+$ T cells express low levels of CD127 but following resolution of infection, CD127 expression is upregulated within the population of activated cells, which can be explained by the fact that cells expressing higher levels of CD127 will better survive the contraction phase (Cui and Kaech, 2010; Kaech et al., 2003; Schluns et al., 2000). Concerning IL-15, it was shown to be essential to sustain memory $CD8^+$ T cell proliferation and homeostatic survival (Goldrath et al., 2002; Ku et al., 2000; Raeber et al., 2018). Excess of IL-15 in IL-15 transgenic mice or by exogenous administration of IL-15 or IL-15/IL-15Rα complexes (briefly, IL-15 complexes) leads to vigorous proliferation of memory $CD8^+$ T cells in vivo (Fehniger et al., 2001; Rubinstein et al., 2006; Stoklasek et al., 2006). IL-7 and IL-15 have a complementary role, and differential migratory capacities of memory T cell subsets may allow them to reach their niche if either of these signals are available (Judge et al., 2002; Jung et al., 2016). Other cytokines signaling through the common gamma chain (γc), like IL-2 and IL-21, have also been implicated in the maintenance of memory $CD8^+$ T cells (Mitchell et al., 2010; Zeng et al., 2005).

Effector and memory $CD8^+$ T cells are most identified by their high expression of CD44, whereas naive $CD8^+$ T cells express low levels of CD44 and referred to as $CD44^{low}$ $CD8^+$ T cells. While CD44 is widely acknowledged in mice to define T cells in which TCR signaling has been activated, and CD44 expression is maintained in memory T cells. In addition, there are several other surface molecules (CD122, CXCR3, Ly6C or CD49d) or transcription factors (T-bet and Eomes) which are also differentially expressed between naive and effector/memory $CD8^+$ T cells (Jameson et al., 2015).

3.2 Unconventional memory $CD8^+$ T cells

Besides conventional memory T cells, some populations of $CD8^+$ T cells with foreign antigen-inexperience exist in naive conditions and have characteristics of memory $CD8^+$ T cells. Among those characteristics, these cells express a αβ-TCR and upregulate memory markers such as CD44, CD122 and CXCR3. However, as opposed to true memory T cells, they downregulate the expression of the integrin α4 chain (CD49d), and they were named unconventional memory $CD8^+$ T cells. Although many terms were used to describe these particular cell populations, we summarize below the main populations based on how and in which tissues they were identified.

3.2.1 Lymphopenia-induced memory CD8$^+$ T cells

T lymphocyte development includes negative (elimination of cells with high self-peptide/MHC affinity) and positive (survival of cells with lower but significant affinity to self-peptide/MHC) selection in the thymus. At steady state, survival of resting naive CD8$^+$ T cells still depends on the presence of low affinity self-peptide/MHC signals, known as a "tonic" signal, along with homeostatic IL-7 stimulation (Sprent and Surh, 2011; Sun et al., 2019; Tanchot et al., 1997). Such interactions ensure that the cell compartment is maintained with constant cell numbers at the steady state. Hence, adoptive transfer in a T lymphocytopenic environment (induced notably by irradiation, Rag deficiency, or T cell antibody-based depletion) would induce the homeostatic proliferation of donor CD8$^+$ T cells to balance the T cell pool (Bell et al., 1987; Ernst et al., 1999; Rocha et al., 1989). Since the CD8$^+$ T cells with memory phenotype have been found before birth in human (Byrne et al., 1994; Szabolcs et al., 2003), germ-free mice, and antigen-free mice (Dobber et al., 1992; Huang et al., 2005; Vos et al., 1992), various models by exposing mouse CD8$^+$ T cells to lymphopenia or a mimic lymphopenia environment have demonstrated that T_N can be provoked into proliferation in the absence of TCR engagement with foreign antigen and undergo conversion into memory T cells with upregulated CD44, CD122, and Ly6C (but not CD69 or CD25), and rapid IFN-γ production when restimulated by anti-CD3 antibody. These features resemble the characteristics of conventional memory CD8$^+$ cells (Clarke and Rudensky, 2000; Ernst et al., 1999; Goldrath and Bevan, 1999; Hamilton et al., 2006; Jameson, 2002; Kieper and Jameson, 1999; Murali-Krishna and Ahmed, 2000; Sprent and Surh, 2011; Surh and Sprent, 2008). Also, increasing evidence suggest that these cells are generated in direct response to self-antigens and homeostatic signals driving cell proliferation in absence of foreign-antigen experience (Haluszczak et al., 2009; La Gruta et al., 2010; Sprent et al., 2008; Surh et al., 2006; Takada and Jameson, 2009). To distinguish lymphopenia-induced memory CD8$^+$ T cells from other foreign antigen-induced memory T cells, the expression of α4-integrin (CD49d), a component of the homing receptors VLA-4 and LPAM, was used (Haluszczak et al., 2009). Foreign antigen-induced memory CD8$^+$ T cells highly express CD49d, while lymphopenia-induced memory CD8$^+$ T cells downregulate CD49d expression, usually lower than CD49d expression in T_N (Haluszczak et al., 2009). However, lymphopenia-induced memory CD8$^+$ T cells have elevated expression of CCR7 and CXCR5 (Cheung et al., 2009) (Table 1, Figure 12). It is still not clear whether the differential expression level of CD49d can be used as a unique marker to reliably discern conventional and unconventional memory T cell subsets.

3.2.2 Innate memory CD8$^+$ T cells

A unique population of memory-phenotype CD8$^+$ T cells was initially discovered in the thymus of mice lacking the TEC kinase IL-2-inducible T cell kinase (ITK) or receptor like kinase (RLK). These memory phenotype single-positive CD8 T cells (SP8) cells arise in mice having an altered TCR

signaling and were referred to as innate memory $CD8^+$ T cells (T_{IM}) (Berg, 2007). In particular, ITK deficient and RLK/ITK double deficient mice are almost devoid of conventional SP8 thymocytes and contain a large number of SP8 cells that express consensus lineage markers indicative of T_{IM} cells ($CD44^{hi}$, $CD122^+$, and $Eomes^{hi}$) (Viano et al., 2022). These cells develop in the thymus and acquire an effector phenotype in absence of foreign antigen encounter and share some characters of conventional memory T cells (Andreotti et al., 2010; Bendelac et al., 1994; Berg, 2007; Dutton et al., 1998; Kawachi et al., 2006; Kurepa et al., 2003; Salmond et al., 2014; Urdahl et al., 2002). The origin of T_{IM} developing in the thymus is dependent on IL-4 (Lee et al., 2011). Several studies have demonstrated that particular T cells produce IL-4 in the thymus, which promotes the development of $Eomes^+$ T_{IM} (Broussard et al., 2006; Gordon et al., 2011; Jordan et al., 2008; Lee et al., 2010; Min et al., 2011; Weinreich et al., 2010, 2009). In particular, data showed that PLZF (promyelocytic leukemia zinc finger) expression is essential for the development of invariant NKT (iNKT) cells in the thymus (Kovalovsky et al., 2008; Savage et al., 2008). PLZF is also critical for development of a subset of γδ T cells, like 'γδNKT' cells, which express PLZF and NK receptors and can produce IL-4 (Felices et al., 2009; Lees et al., 2001). Not only the total number of $PLZF^+$ iNKT cells but also their differentiation state affects $CD8^+$ T cell memory development (Jameson et al., 2015). A model of lineage differentiation prevails, where T-bet, GATA-3, and RORγt, together with PLZF, direct a thymic multipotent precursor to NKT1 ($PLZF^{low}$), NKT2 ($PLZF^{hi}$), and NKT17 ($PLZF^{int}$) effector fates, with the potential to produce IFN-γ, IL-4, or IL-17, respectively (Constantinides and Bendelac, 2013; Engel and Kronenberg, 2014; Y. J. Lee et al., 2013). Since T-bet transcription factor directs differentiation and IFN-γ secretion by NKT1 cells, the development of NTK1 cells can be blocked in the absence of T-bet, which favors the development of NKT2 and NKT17 cells. Then, the expanded NKT2 cells provide more IL-4 at steady state facilitating the development of T_{IM} (Jameson et al., 2015; Lee et al., 2013). Thus, T_N are exposed to IL-4 derived from thymic PLZF+ thymocytes and convert into T_{IM} (White et al., 2017) (Figure 12).

In various gene deficient mice (deficient for genes like *Itk*, *Klf2*, *Id3*, *Ly9*, *Tbet*) (Jameson et al., 2015), it was suggested that expanding $PLZF^+$ NKT cells regulate the development of T_{IM} in the thymus via production of IL-4. Interestingly, the frequency of iNKT cells was shown to differ between various inbred mouse strains (Hammond et al., 2001; Rymarchyk et al., 2008). Indeed, BALB/c mice have 3–5 times higher numbers of $PLZF^+$ cells in the thymus compared to C57BL/6 mice, with the majority of them being iNKT (essentially NKT2) in BALB/c mice (Lee et al., 2011). Thus, T_{IM} account for a substantial percentage of total SP8 thymocytes in BALB/c mice (Broussard et al., 2006; Lee et al., 2015; Weinreich et al., 2010).

Compared to conventional and lymphopenia-induced memory $CD8^+$ T cells, T_{IM} highly express Eomes but not T-bet, while lymphopenia-induced memory $CD8^+$ T cells express both T-bet and Eomes (Lee et al., 2013; Sosinowski et al., 2013; Weinreich et al., 2010, 2009). Eomes is essential for the

development of all the phenotypic and functional memory-like characteristics of IL-4-induced T_{IM}, which include upregulated expression of CD44, CXCR3, CD122, and the ability of rapid production of IFN-γ (Weinreich et al., 2010, 2009). IL-4 signaling through STAT6 induced Eomes expression (Oliver et al., 2012; Ventre et al., 2012) but reduced expression of NKG2D (a receptor initially identified on NK cells, but which can co-stimulate $CD8^+$ T cells) and CCL5 (Ventre et al., 2012). The phenotype of the described T_{IM} is summarized in Table 1. However, it remains unclear whether the IL-4-dependent conversion of developing $CD8^+$ T cells into T_{IM} within the thymus is stochastic or requires additional unidentified stimuli. Since T_{IM} differentiation is mainly thought to arise from SP8 thymocytes after thymic positive selection by responding to self-peptide/MHC, it is experimentally difficult to identify whether TCR signal is essential for conversion of naive T cells into T_{IM}.

3.2.3 Virtual memory $CD8^+$ T cells (T_{VM})

In contrast to T_{IM} developing in the thymus, memory-phenotype $CD8^+$ T cells have also been identified in the periphery in specific-pathogen-free (SPF) and germ-free (GF) mice (Haluszczak et al., 2009). These cells have a phenotype similar to lymphopenia-induced memory $CD8^+$ T cells and T_{IM}, such as $CD44^{hi}CD122^{hi}CD49d^{low}$ (Haluszczak et al., 2009; Sosinowski et al., 2013), and were termed virtual memory $CD8^+$ T cells (T_{VM}). T_{VM} form the majority of memory-phenotype $CD8^+$ T cells in unimmunized mice and around 10–20% of total $CD8^+$ T cells in the peripheral blood and lymphoid organs of C57BL/6 mice (Chensue et al., 2013; La Gruta et al., 2010; Nikolich-Zugich Kristin Renkema et al., 2014; Rudd et al., 2011b; Sosinowski et al., 2013). Haluswak and colleagues (2009) identified antigen-inexperienced memory phenotype $CD8^+$ T cells in both SPF but also in germ-free mice, suggesting T_{VM} differentiate in absence of microbial antigens. Based on these observations, three major hypotheses of have been proposed to explain T_{VM} differentiation. First, the differentiation into T_{VM} could occur purely on a stochastic basis. Second, the lymphopenic environment in newborns would induce differentiation of the first wave of thymic emigrants into T_{VM} (Akue et al., 2012; Davenport et al., 2020). Third, the T_{VM} are generated from relatively highly self-reactive T cells that receive strong homeostatic TCR signals at the periphery (Drobek et al., 2018; White et al., 2016).

Interestingly, $CD8^+$ T cells in the fetal stage have been shown to have a more inherent propensity to develop into T_{VM} (Smith et al., 2018). Using a fate-mapping system of tamoxifen-inducible Cre ($CD4^{CreERT2}$) in Rosa-tdTomato mice, "timestamping" experiments including transfer of newborn thymus in adult mice before timestamping revealed that newborn-derived $CD8^+$ T cells underwent more proliferation and preferentially developed into T_{VM} when compared to adult-derived $CD8^+$ T cells (Smith et al., 2018). These results argued against the first hypothesis. In addition, as neonatal mice are deficient in T cells which may support lymphopenia-induced proliferation to explain T_{VM} differentiation, these results highlighted lymphopenic environment in newborns is not required for the formation of T_{VM}.

Rather, $CD8^+$ T cells of early developmental origin have a cell-intrinsic propensity to become T_{VM}, and this fate bias occurs independently of environmental factors experienced in the periphery (Smith et al., 2018). In addition, another study showed naive T cells with higher levels of CD5 expression, a marker of self-reactivity (Mandl et al., 2013), are more likely to convert into T_{VM} in the periphery (White et al., 2016). The propensity of self-reactive T cells to develop into T_{VM} was further suggested using a CD8.4 knock-in mouse model (transgenic mice in which T cells carry a CD8 molecule formed by the extracellular portion of CD8α fused to the intracellular part of CD4 to increase Lck-mediated TCR activation), and Vβ5 transgenic mice with fixed TCRβ chain (Drobek et al., 2018; Miller et al., 2020; Paprckova et al., 2022). Importantly, T_{VM} were shown to mediate effector functions, through increased ability to respond to cognate antigens when compared to their naïve counterparts (Lee et al., 2013), but also through bystander mechanisms in an IL-15-dependent manner (White et al., 2016). T_{VM} cells were also described as a subset of "antigen-naïve", semi-differentiated $CD8^+$ T cells. Indeed, while expressing CD44 which is rapidly upregulated on TCR-mediated stimulation, CD49d is part of the very late antigen-4 (VLA-4, α4β1) integrin complex and its upregulation requires more sustained and robust TCR stimulation from cognate antigens (Hussain and Quinn, 2019; Quinn et al., 2020). Further phenotyping identified that T_{VM} could upregulate the chemokine receptors CCR2, CCR5 and CXCR5, when compared to naive T cells (Hou et al., 2021) (Table 1, Figure 12). The expression of CXCR5, a marker increasingly associated with $CD8^+$ T cell stemness (Hashimoto et al., 2018; He et al., 2016; Im et al., 2016; Leong et al., 2016), supports the description of "antigen-naive" and "semi-differentiation". Although T_{IM} and T_{VM} shared some phenotypic characteristics, T_{IM} seem to express increased levels of CD49d relative to the T_{VM} in the periphery (Sosinowski et al., 2013). However, it remains not possible to distinguish T_{IM} from T_{VM} based on CD49d expression once the cells are outside the thymus (White et al., 2017).

3.3 Human T_{VM}

Studies in the mouse model enabled the characterization of T_{IM} and T_{VM} populations in more details, whereas the existence of the human equivalents of both populations is yet to be clarified. However, recent data reasonably suggest that human T_{VM} exist. A first study identified $CD8^+$ T cells expressing either KIR receptors or the inhibitory NKG2A receptor with an EMRA phenotype (effector memory cells re-expressing CD45RA, $CD45RA^+CCR7^-$) in cord blood, intact from viral infection or placental pathology (Warren, 2006). Then another study found are a substantial number of T_{VM}-like cells in spleen samples obtained from pre-term infants, with Eomes expression and rapid IFN-γ production upon restimulation (Min et al., 2011). Also, PLZF is highly expressed in both thymic and splenic human fetal $CD4^+$ T cells, which potentially provide good conditions for T_{IM}/T_{VM} expansion (Lee et al., 2010; Min et al., 2011). Recent studies further described a human cell population that has phenotypical and functional similarities to mouse T_{VM} (Jacomet et al., 2015; White et al., 2016). The notable difference is

that CD5 expression on human T cells decreases as the T cell undergoes differentiation (Herndler-Brandstetter et al., 2011), and T cells that express the lowest levels of CD5 are the most sensitive to IL-15 and have the highest expression of CD122 (Geginat et al., 2003). These $CD5^{low}CD122^{hi}$ cells increased expression of Nur77, suggesting a higher basal TCR–MHC self-affinity, NKG2A, both Eomes and T-bet, and were $CD45RA^{+}KIR^{+}$ (Intlekofer et al., 2005; Jacomet et al., 2015; Kasakovski et al., 2021; White et al., 2017, 2016) (Table 1). However, Pieren *et al.* defined the human T_{IM}/T_{VM} in two subsets, one $NKG2A^{+}CD45RA^{+}TIGIT^{-}CD226^{+}$, another is $KIR^{+}CD45RA^{+}TIGIT^{+}CD226^{-}$ (Pieren et al., 2021). RNA-seq has been performed to characterize T_{IM}/T_{VM} in human, and showed difference between these two subsets related to the expression of the genes of transcription factors *Helios*, *Cd122* and *Tigit* (Li et al., 2022; Pieren et al., 2021). The subset displaying a $TIGIT^{+}Helios^{+}NKG2A^{+}CCR7^{-}CD27^{-}CD28^{-}$ phenotype showed high cytotoxic ability (perforin and granzyme B), a less diverse TCR repertoire and regulatory functions in autoimmune diseases (Li et al., 2022; Viano et al., 2022). In addition, expression of IL-15Rβ on T_{VM} is significantly higher than that on naive T cells in young adults, and this expression increased significantly with age (Quinn et al., 2020). Analogous to mice, human naive T cells show a remarkable retention of function with age, while human T_{VM} cells suffer a profound loss of proliferative capacity (Quinn et al., 2018).

About the role of human T_{IM}/T_{VM} during infection, recent studies have highlighted interesting findings. First, IL-15 was increased during hepatitis A virus (HAV) infection, which was associated with TCR-independent activation of memory $CD8^{+}$ T cells. Interestingly, non-HAV-specific $CD8^{+}$ T cells upregulated NKG2D and displayed innate-like cytotoxicity associated with liver injury in acute hepatitis A (Kim et al., 2018; Kim and Shin, 2019). Later, Seo *et al* found that IL-15 upregulates CCR5 expression on bystander-activated $CD8^{+}$ T cells and enhances CCR5-mediated migration, which correlates with inflammation in patients with acute hepatitis A (Seo et al., 2021). During HIV-1 infection, dendritic cell (DC) activation and the resulting IL-15 production was shown to drive bystander activation of $CD8^{+}$ T cells (Bastidas et al., 2014; Younes et al., 2016). Another study found that a population with T_{VM} characteristics negatively correlated with HIV DNA and positively correlated with circulating IFN-α2 and IL-15 in patients undergoing antiretroviral therapy. In these patients, T_{VM} constitutively expressed high levels of cytotoxic granule components, including granzyme B, perforin and granulysin, to control HIV replication (Jin et al., 2020). Moreover, Hu *et al* reported that the levels of HIV-1 DNA and cell-associated unspliced viral RNA correlated negatively with $CCL4^{-}CCL5^{+}CD8^{+}$ terminally differentiated effector memory cells, in which a T_{VM} subset was enriched and showed superior cytotoxicity potentially driven by T-bet and Runx3 (Hu et al., 2022). Moreover, HIV reactivation is effectively suppressed by T_{VM} cells through KIR-mediated recognition (Jin et al., 2020; Quinn and Hussain, 2020; Rolot and Dewals, 2020), and dependent on CCL5 secretion *in vitro* (Hu et al., 2022).

Thus, although unconventional memory $CD8^+$ T cells have been identified in human, it remains difficult to demonstrate that the identified population in human match mouse T_{IM}/T_{VM}. More studies are required to clarify the phenotype, origin and maintenance in human and other species than mice.

Table 1. Phenotype of conventional and unconventional memory CD8+ T cells

	Antigen inexperienced					**Antigen experienced**			
	Naïve CD8$^+$T cells	**Memory phenotype CD8$^+$ T cells**							
Cell types	mouse T_N	mouse Lymphopenia-induced memory CD8$^+$ T cells	mouse T_{IM}	mouse T_{VM}	human T_{VM}	mouse T_{CM}	mouse T_{EM}	mouse T_{RM}	human Effector memory Re-expressing CD45RA
Key markers	CD3$^+$, CD8$^+$ CD44^{low} CD49d^{low}	CD3$^+$, CD8$^+$ CD44^{hi} CD49d^{low}	CD3$^+$, CD8$^+$ CD44^{hi} CD49d^{low}	CD3$^+$, CD8$^+$ CD44^{hi} CD49d^{low}	CD3$^+$, CD8$^+$ CD45RA$^+$ KIR^{hi} and/or NKG2A^{hi}	CD3$^+$, CD8$^+$ CD44^{hi} CD49d^{hi}	CD3$^+$, CD8$^+$ CD44^{hi} CD49d^{hi}	CD3$^+$, CD8$^+$ CD44^{hi}	CD3$^+$, CD8$^+$ CD45RA$^+$
Additional markers	CD62L^{hi} CCR7^{hi} Eomes^{low} CD122^{low} CD127^{hi} CXCR3^{low} CX3CR1^{low}	CD62L^{hi} CD122^{hi} CXCR3$^+$ CCR7$^+$ CXCR5$^+$ Ly6c$^+$ CD122$^+$ CD69^{low} CD25^{low}	CD62L^{hi} CD122^{hi} Eomes^{hi} CXCR3^{hi} NKG2D$^-$ T-bet$^-$ CD5$^+$	CD62L^{hi} CCR7^{low} Eomes^{hi} CD122^{hi} CD127^{hi} CXCR3^{hi} CX3CR1^{low} NKG2D$^+$ T-bet$^+$ CCR2$^+$ CCR5$^+$ CXCR5$^+$ CD5$^+$	CD45RO$^-$ CCR7^{low} Eomes^{hi} CD27^{low} CD5^{low} CD122^{hi} NUR77^{hi} T-bet$^+$	CD62L^{hi} CCR7^{hi} Eomes^{hi} CD122^{int} CD127^{hi} CXCR3^{hi} CX3CR1^{low} T-bet^{low}	CD62L^{low} CCR7^{low} Eomes^{low} CD122^{low} CD127^{low} CXCR3^{hi} CX3CR1^{hi}	CD62L$^-$ CCR7$^-$ CD103^{hi} CD69^{hi} CD27^{low} CD49a$^+$ CD127^{hi} CXCR3^{hi} CX3CR1$^{low/int}$ T-bet^{low} EOMES$^{-/low}$ (depending on tissue)	CD27$^-$ CCR7$^-$ CD127^{low}

From (Hou et al., 2021; Tabinda Hussain and Quinn, 2019; Martin and Badovinac, 2018; White et al., 2017).

3.4 Origin and maintenance of T_{VM}

Cytokines are important in $CD8^+$ memory T-cell homeostasis (Prlic et al., 2002). Studies indicated signaling through common γ (γc) chain cytokines, of which **IL-2**, **IL-4**, **IL-7** and **IL-15**, are thought to drive and maintain unconventional memory T cells. An early study indicated that homeostatic expansion produced T cells that expressed memory markers only transiently and then reverted to a naive state (Goldrath et al., 2000). However, later experiments indicated that the memory phenotype of homeostatically proliferating cells is stable (Ge et al., 2002; Murali-Krishna and Ahmed, 2000; Tanchot et al., 2002). These phenotypic and functional changes do not actually replenish the naive T-cell pool but leads to the production of memory-like cells without encountering any foreign antigen, such as unconventional memory T cells.

Homeostatic proliferation elicited by a combination of self-pMHC and **IL-7** typically expand at a slow rate and is readily observed for naive $CD4^+$ and $CD8^+$ T cells upon adoptive transfer into syngeneic normal hosts that have been made acutely lymphopenic, e.g., by irradiation as well as in T/B-cell deficient Rag-knockout mice (Ernst et al., 1999; Goldrath and Bevan, 1999; Labrecque et al., 2001; Schluns et al., 2000; Surh and Sprent, 2008; Tan et al., 2001), followed by acquisition of the memory phenotype. Also, these studies have proved more evidence that lymphopenia-induced proliferation is severely diminished in the absence of either MHC or IL-7 for normal polyclonal T cells. These findings highlighted that IL-7 concentration increases with T cell depletion (Fry and Mackall, 2001) and lymphopenia-induced proliferation is driven by increased availability of IL-7, which amplifies the weak TCR signaling resulting from contact with self-pMHC ligands (Surh and Sprent, 2008, 2005).

Besides IL-7, it is now clear that homeostatic proliferation of naive T cells can be driven by elevated amounts of other γc cytokines, especially **IL-2** and **IL-15**. In line with the view that IL-2 and IL-15 differentially favor the generation of effector cells and central memory cells, respectively (Manjunath et al., 2001). Early study in IL-15-deficient mice found that IL-15 is required for sustained lymphopenia-induced proliferation of $CD8^+$ T cells (Sandau et al., 2007). Further study demonstrated irradiation-induced lymphopenia enhances T-cell memory formation via IL-15 activation of the fork head-box family of transcription factor (FOXO1)/Eomes memory and ULK1/autophagy-related gene-7 (ATG7) autophagy pathways, and via IL-15 activation of the mitochondrial remodeling (Xu et al., 2016). Importantly, such proliferation/expansion can also occur in the absence of lymphopenia. At least for $CD8^+$ memory T cells, the homeostatic expansion can be supported by either IL-15 or IL-7 (Becker et al., 2002; Goldrath et al., 2002; Schluns et al., 2002; Tan et al., 2002). In CD25(*Il2ra*)-deficient, CD122(*Il2rb*)-deficient, or CD132(*Il2rg*)-deficient hosts, donor naive T cells, including TCR transgenic cells, underwent a massive rate of proliferation after adoptive transfer, which is comparable to foreign antigen-specific T cell responses. In this context, the proliferating donor cells rapidly acquired the

characteristics of effector cells or central memory ($CD62L^{hi}$) cells, in presence of high amounts of IL-2 or a mixture of both IL-2 and IL-15, together with recognition of self-pMHC ligands presented by the host antigen presenting cells (APCs) or by adjacent T cells (Cho et al., 2007; Ramsey et al., 2008; Surh and Sprent, 2008). In addition, deficiency of suppressor of cytokine signaling-1 (SOCS-1) in $CD8^+$ T cells resulted in increased responsiveness to IL-15 and self-ligand, resulting in expansion, which consist predominantly of memory-phenotype $CD8^+$ T cells (Davey et al., 2005; Ramanathan et al., 2006). Moreover, IL-15 trans-presentation by $CD8\alpha^+$ dendritic cells (DCs) to naive $CD8^+$ T cells was shown to induce T_{VM} differentiation (Sosinowski et al., 2013). In conclusion, while homeostatic proliferation is ongoing in lymphoreplete mice, the degree of proliferation is dictated by the number of naive $CD8^+$ T cells with sufficiently high affinity for self-antigens in presence of peripheral IL-15 (White et al., 2016).

Other cytokines, such as the **type I and II interferons**, **IL-12** and **IL-18**, also enhance memory $CD8^+$ T cell survival and/or proliferation, which could be mediated through the induction of IL-15 (Tough et al., 2001). Type I interferon has been reported to the enhance (Zhang et al., 1998) or diminish (Zhang et al., 2002) $CD8^+$ memory T cell proliferation, depending on dose. More importantly, type I interferon signaling in $CD8^+$ T cells has been shown to drive Eomes expression and thereby regulate the function and homeostasis of T_{VM} (Martinet et al., 2015). In addition, a study found that only when low-affinity self-pMHC complexes are available, the lack of IFN-γ receptor signaling in $CD8^+$ T cells promotes the generation of IFN-γ-producing memory-phenotype T cells without affecting cell numbers (Sercan et al., 2010).

As mentioned above, **IL-4** produced by $PLZF^+$ thymocytes can drive the generation and development of T_{IM} in the thymus. In addition, high concentrations of peripheral IL-4 is sufficient to expand T_{VM} as observed in Nedd4-family interacting protein 1 knock-out mice, which showed overexpression of IL-4 in the periphery an increased T_{VM} (Kurzweil et al., 2014). A pivotal role of IL-4 to maintain and expand T_{VM} in secondary lymphoid tissues was further confirmed in IL-$4^{-/-}$ and IL-$4R\alpha^{-/-}$ of both BALB/c and C57BL/6 background (Akue et al., 2012; Park et al., 2016; Renkema et al., 2016). Similarly, IL-4 signaling stimulation can induce T_{VM} expansion and likely results in the conversion of naive T cells rather than proliferation or recruitment of T_{VM} cells in a model of helminth antigen exposure (Rolot et al., 2018). Interestingly, IL-4 can rescue $CD8^+$ T cells from apoptosis via Jak3-dependent activation of STAT1, STAT3, and STAT5, together with low-level transcription of SOCS genes (Acacia de Sa Pinheiro et al., 2007). Also, IL-4 is sufficient to drive Eomes expression and the $CD8^+$ innate-like lymphocyte phenotype through cooperation between STAT6- and Akt-dependent pathways, in settings of attenuated TCR stimulation in peripheral $CD8^+$ T cells (Carty et al., 2014). In addition, a recent study found that Eomes directly drives expression of the anti-apoptotic protein Bcl-2 to mediate the survival of low-affinity memory precursor (Kavazović et al., 2020). Hence, although the exact mechanism by

which IL-4 drives T_{VM} expansion is yet to be elucidated, IL-4 could also induce an anti-apoptotic mechanism to maintain the memory-phenotype T cells.

Although T_{VM} in the periphery was found both in BALB/c and C57BL/6 mice, the contribution of IL-4 and IL-15 to promote their maintenance is differential based on the mouse strain background. By comparing the levels of T_{VM} depending on IL-4 and IL-15, Tripathi *et al.* have demonstrated that T_{VM} cells in BALB/c mice rely more on IL-4 than IL-15, while it is opposite in C57BL/6 mice (Tripathi et al., 2016).

Cytokines, such as IL-4 and IL-15, can maintain the survival of T_{VM}, and this is mainly associated with the induction of Eomes. Recently, Istaces *et al.* uncovered the molecular mechanisms at the epigenetic level. Innate memory SP8 acquired only a portion of the active enhancer repertoire of conventional memory cells, in which Eomes is recruited to Runx3-bound regions and induces epigenetic changes in enhancer regions (Istaces et al., 2019). BRG1, as one of epigenetic regulators, is critically involved in the program and contributes the acquisition of memory features by $CD8^+$ T cells under steady-state conditions and upon *in vivo* injection of IL-4c (Istaces et al., 2019). In addition, deletion of the histone methyltransferase DOT1L in the T cell lineage strongly increased the number of T_{VM} by controlling TCR signaling, and by regulating a network of other transcriptional and epigenetic regulators, like EZH2 (Kwesi-Maliepaard et al., 2021, 2020). The information on maintenance of unconventional memory $CD8^+$ T cells are summarized in Figure 12.

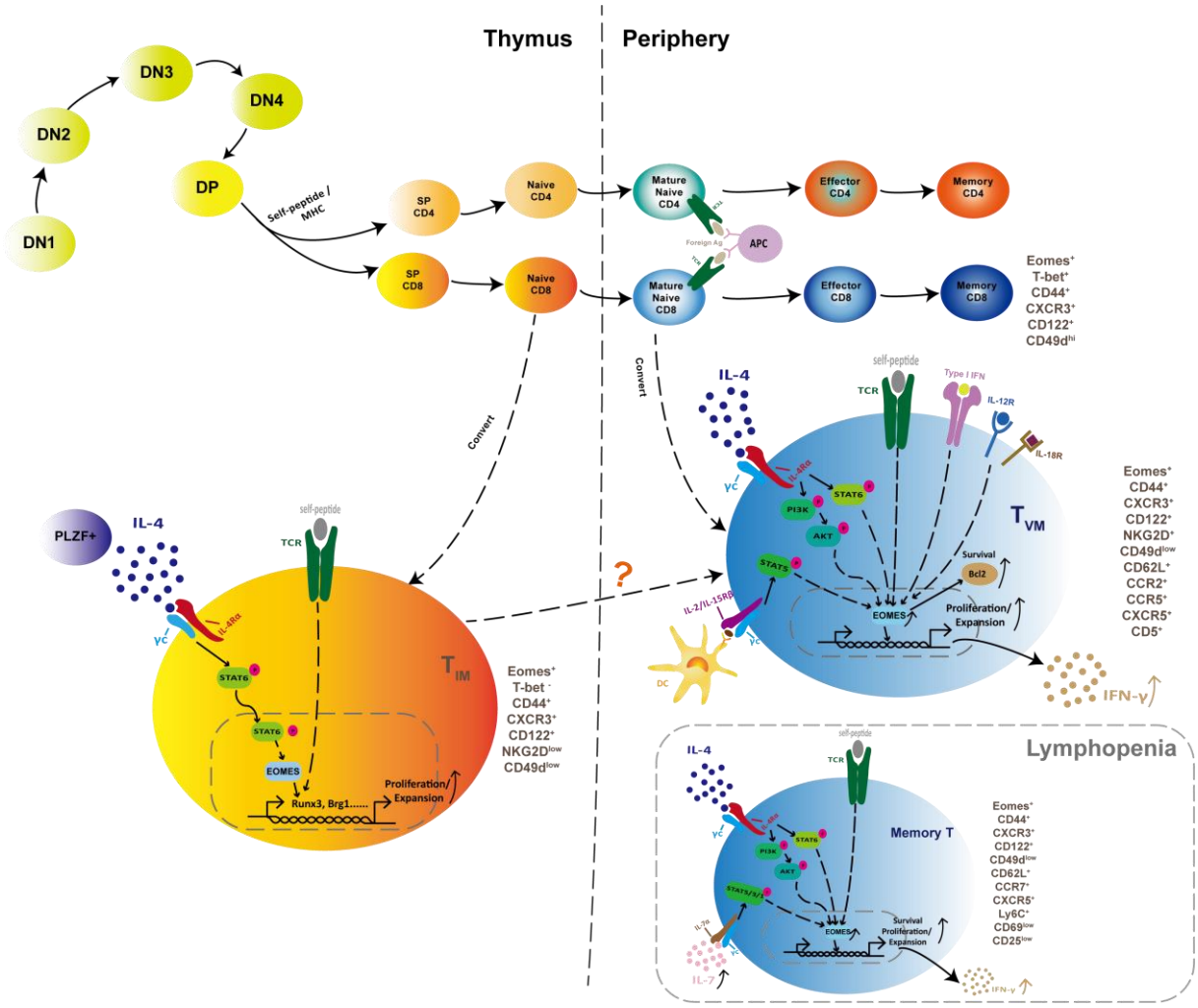


Figure 12. Origin and maintenance of unconventional memory CD8$^+$ T cells. Lymphopenia-induced memory CD8$^+$ T cells can be maintained/expanded by IL-7 or IL-4. T_{IM} can be converted from naive CD8$^+$ T cells in the thymus and maintained/expanded by PLZF$^+$ cells-producing IL-4. T_{VM} could be converted by mature naive CD8$^+$ T cells in the periphery, mainly maintained/expanded by IL-4/ IL-15, or Type I IFN signals (and/or together with IL-12/IL-18 signals). In this process, the signaling regulating expression of Eomes is important, together with a low affinity TCR recognition of self-antigens presented by MHC-I. However, it is still not clear if T_{IM} could develop into T_{VM}. No reference showed IL-13Rα1 on CD8$^+$ T cells. The main phenotype of these cells is showed here. DN, double negative; DP, double positive; SP, single positive; APC, antigen-presenting cells; γc, common γ chain.

According to some studies mentioned above, it has been suggested that naive CD8$^+$ T cells could convert into unconventional memory T cells in some conditions, like T_{IM} (White et al., 2017) and T_{VM} (Rolot et al., 2018). In these conditions, we would expect that naive and T_{IM}/T_{VM} should share the same TCR repertoire. However, studies found T_{IM}/T_{VM} use distinct TCR repertoire than T_N cells (Drobek et al., 2018; Miller et al., 2020). Drobek *et al.* found that the enhanced binding of the tyrosine kinase Lck to the chimeric CD8.4 coreceptor, in which stronger TCR signaling upon antigen recognition than wild-type (WT) mice, could significantly expand the T_{VM} compartment (Drobek et al., 2018). However, these memory cells just represented a minority of the CD8$^+$ T cell population, indicating that enhanced TCR signaling was not sufficient to enable all cells to assume a memory phenotype and implying that TCR

specificity for self is still an important parameter in T_{VM} generation (Truckenbrod and Jameson, 2018). Indeed, they found that only T cells expressing T_{VM} TCR clones formed a significant T_{VM} population, whereas T cells expressing naive TCR clones formed a homogenous naive population. Using Vβ26 transgenic C57BL/6 mice to fix the TCRβ sequence, Miller *et al.* studied the complementarity determining region 3 (CDR3) of TCRα chains from T_N or T_{VM}. They found that the CDR3α sequences expressed by T_{VM} are highly recurrent and significantly distinct from CDR3α sequences of naive $CD8^+$ T cells, and demonstrated that T_{VM} display a significant level of **self-specificity** driving their memory phenotype (Miller et al., 2020). Moreover, they found that T_{VM} clones upregulated Eomes in the thymus, which identified polyclonal thymic precursors of T_{VM} cells. These findings suggest that T_{VM} differentiation is triggered in the thymus, but requires a subsequent consolidation phase that can only be conferred in the periphery (Miller et al., 2020). Another interesting study found that C57BL/6 mice deficient of *Themis*, a T cell lineage-restricted protein regulating the threshold between positive and negative selection of T cells in the thymus (Fu et al., 2013, 2009; Gascoigne et al., 2016; Johnson et al., 2009; Lesourne et al., 2009), expanded large population of T_{VM} cells expressing $V\alpha3.2^+$ TCR, with increased responsiveness to IL-15 (Prasad et al., 2021). Furthermore, mice deficient for DOCK2, which contributes to cellular signaling events by activating small G proteins, are more sensitive to TCR stimulation with low-affinity antigens and have increased numbers of T_{VM} cells (Kwesi-Maliepaard et al., 2021; Mahajan et al., 2020). Together, these studies suggested a differential TCR repertoire in T_{VM} distinguish them from naive $CD8^+$ T cells and could govern their generation and development.

The clear association between self-reactivity and differentiation of T_{VM} is challenging. Moreover, the combination of a hyperresponsive differentiation state with the expression of highly self-reactive TCRs suggests that T_{VM} cells could be less self-tolerant than naive T cells and might represent a risk for inducing autoimmunity. Interestingly, Drobek *et al.* found that T_{VM} retain self-tolerance phenotypic traits and less able to induce autoimmune diabetes compared to true memory cells, while they has a similar to lower ability to drive experimental diabetes compared to naive T cells (Drobek et al., 2018; Truckenbrod and Jameson, 2018). Generally, T_{VM} have impaired upregulation of the high-affinity IL-2 receptor chain CD25 and express low CD49d, meaning low-affinity to self-antigens. These observations might suggest that T_{VM} activation is tightly controlled by potential counter-inhibitory mechanisms that are yet to be uncovered. Supporting such hypothesis, an interesting report identified a subset of $CD8^+$ T cells expressing inhibitory Fc receptor (FcγRIIB, CD32) that controls antigen-specific responses (Morris et al., 2020). Thus, more studies are required to clarify the regulation and the somehow contradiction in self-reactive but self-tolerant T_{VM}.

3.5 T_{VM} during inflammation and infection

By definition, T_{VM} have not encountered foreign antigens. However, several studies found that T_{VM} display an enhanced capability to respond to their cognate antigen when compared to their naive $CD8^+$ T cell counterparts. Below, we review the main understanding of T_{VM} functions during inflammatory and infection settings.

Tomasz Sosinowski *et al.* reported that mice transferred with T_{VM} obtained from naive OVA(SIINFEKL)-specific OT-I mice were better protected against *Listeria monocytogenes*–expressing whole OVA (Lm-ova) challenge when compared to mice transferred with either naive OT-I cells or untransferred controls (Sosinowski et al., 2013). Thus, T_{VM} have a unique potential of protection during infection and distinct from antigen-experienced memory cells. Later, Lee *et al* reported that T_{VM} can provide potent antigen-specific protective immunity against *L. monocytogenes* infection (Lee et al., 2013). Also, White *et al* used a Lm-ova protection assay with the adoptive transfer of T_{VM} cells isolated from antigen-specific (OT-I) or antigen-irrelevant (gBT-1) donors into recipient mice without IL-15-dependent T_{VM}, and found T_{VM} are capable of mediating potent immunological protection against a bacterial challenge even in the absence of their cognate antigen, of which bystander protection is maintained by IL-15 in C57BL/6 mice (White et al., 2016). In addition to IL-15 mediated mechanisms, Renkema *et al.* found that IL-4 can also promote type 1 immune response and IFN-γ in T_{VM}, and regulates the $CD8^+$ T cell response to acute viral infection (LCMV Armstrong) (Renkema et al., 2016). Interestingly, $CCR2^+$ T_{VM} were found to rapidly infiltrate the lungs early after primary IAV infection with a high dose, which was dependent on CXCR3. Recruited T_{VM} to the lung promoted early viral control by producing IFN-γ and granzyme B, and contributed to the pool of resident memory T cells (Hou et al., 2021). In the thymus, Baez *et al.* reported that thymic IL-4 and IL-15 expression triggered by Th1-associated *Trypanosoma cruzi* infection induced an adequate niche for development of innate $CD8^+$ T cells as early as the double positive (DP) stage in WT mice (Baez et al., 2019). Hence, all these findings support that T_{VM} have a potent capacity to mediate immune control against pathogens. In addition, an important study from the host laboratory showed that during helminth exposure, IL-4 drives the expansion and activation of IFN-γ producing T_{VM}, which was directly associated with the control of a subsequent viral infection in the lung (Rolot et al., 2018). Similarly, T_{VM} expanded by helminth infection were shown to confer protection against subsequent bacterial infection dependent on IFN-γ (Lin et al., 2018). Thus, these studies provided a new prospect where type 1 and type 2 immune axes converge to drive the activation of immune protection through antigen-inexperienced $CD8^+$ T cell populations.

Besides their function in response to viral or bacterial infections, T_{VM} also display enhanced responses against tumors. In order to define the contribution of T_{VM} cells in the immune response to

human and murine cancers, Miller *et al.* used a mouse model of prostate adenocarcinoma and studied the co-transfer of congenic polyclonal T_N and T_{VM} cells. Of interest, they found that the donor T_{VM} constituted a substantial fraction of the tumor-infiltrating $CD8^+$ T cells and the majority expressed high densities of the inhibitory receptor PD-1, which may functionally impact antitumor immunity or directly be impacted by anti-PD-1 or anti-PD-L1 checkpoint blockade antibodies (Miller et al., 2020). The role of T_{VM} in cancer immunotherapy was also addressed in another study using different tumor models (Wang et al., 2021). Here, chemotherapy-treated cancer cells directly activated T_{VM} in a MHC-I-independent manner requiring cell–cell contact and activation of the PI3K pathway, which preferentially promoted T_{VM} tumor-infiltrating and potent granzyme B-dependent cytotoxicity (Wang et al., 2021). Whereas IL-4 or IL-15 were shown to promote T_{VM} maintenance and function, their role during chemotherapy-treated tumor cell-mediated activation of T_{VM} remains under investigation. In addition, a recent study found systemic expression of IL-12 and IL-18, which could activate $CD8^+$ T cells in a bystander manner, and an enrichment of T_{VM} displaying antitumor characteristics was found in a melanoma (B16) and pancreatic ductal adenocarcinoma (KPC) tumor models (Savid-Frontera et al., 2022). In that context, the absence of IFN-γ abolished the high antitumor capacity. Thus, these studies provide significantly new information for antitumor immunotherapy by focusing on T_{VM}.

In addition to their role during infection or in tumor development, characteristic differences of T_{VM} were observed during aging. It has long been assumed that memory T cells accumulate with aging as a result of lifelong foreign antigen exposure (Nikolich-Žugich, 2008). However, similarly to conventional memory $CD8^+$ T cells, the proportion of T_{VM} were shown to also increase with aging (Rudd et al., 2011b). Chensue *et al.* analyzed the contribution of T_{VM} to aging-related accumulation of memory $CD8^+$ T cells and found that aging-related accumulation of T_{CM} was due to lifelong accumulation of T_{VM} rather than conventional memory $CD8^+$ T cells, which was independent of $CD4^+$ T cells, CCR5 and CXCR3 (Chensue et al., 2013). Interestingly, T_{VM} were reported to lose proliferative capacity in response to TCR signals, but not to IL-15 (Quinn et al., 2018). Aged T_{VM} exhibited a profile consistent with senescence, but not exhaustion, similar to aged naive $CD8^+$ T cells (Quinn et al., 2018). To further clarify the altered longevity and functionality of T_{VM} with age, the metabolism of T_{VM} responses was also investigated (Quinn et al., 2020). An elevated spare respiratory capacity (SRC, a higher mitochondrial energy reserve) was found to be a feature of T_{VM}, but not conventional memory $CD8^+$ T cells, SRC increased with age in both subsets, driven by physiological IL-15, and it could also be induced by IAV infection (Quinn et al., 2020). Interestingly, high SRC is not necessarily associated with enhanced $CD8^+$ T cell function, even in aged individuals, but associated with anti-apoptotic Bcl-2 expression to reflect the survival capacity in $CD8^+$ T cells (Quinn et al., 2020).

Asymmetric cell division (ACD) is an important mechanism to generate T-cell diversity and maintain a memory T-cell pool (Borsa et al., 2019). Borsa *et al.* found that ageing, known to negatively impact T

cell functionality, also impaired ACD in murine $CD8^+$ T cells, and that this phenotype can be rescued by transient mTOR inhibition. However, T_{VM} retained their ability to divide asymmetrically while accumulating with age (Borsa et al., 2021). These findings further demonstrated that T_{VM} with intrinsically high ACD rates contribute to the establishment and maintenance of a memory T cell pool and the authors proposed that ACD might be a compensatory mechanism to sustain efficient T cell response upon ageing. These observations supported previous work showing that conventionally defined T_{CM} are predominantly T_{VM} in the aged mice (Quinn et al., 2020), and could explain why T_{VM} dominate the response of memory $CD8^+$ T cells from aged naive mice to primary IAV infection by effectively producing granzyme B and mediating viral clearance (Lanzer et al., 2018). Thus, differences exist between young and aged T_{VM}. In fact, there is a stable gene expression program in unconventional memory $CD8^+$ T cells that is regulated by the age and genetic background, while the most notable variation is that T_{VM} express higher levels of CD122, IL-18R and activating NKG2D receptor when compared to T_{IM} (Moudra et al., 2021).

The existence of unconventional memory $CD8^+$ T cells provides new horizons in T cell-mediated immunity. The phenomenon of type 2 cytokines driving their expansion establishes an axis between type 1 and type 2 immunity in $CD8^+$ T cells. However, more studies are still required to complete the understanding of their origin, even though some studies have that demonstrated T_{IM}/T_{VM} are converted from naive $CD8^+$ T cells. Notably, it could be difficult to prove by transferring naive $CD8^+$ T cells into recipient host producing T_{IM} /T_{VM}, since donor and recipient host might have potentially different self-antigens and cannot induce a similar process *via* self-antigen recognition during differentiation and development of these cells. Indeed, more and more studies have identified these antigen-inexperienced memory $CD8^+$ T cells in several conditions, and many names have been used to describe and define them, like ‘memory-like’, ‘memory-phenotype’, ‘virtual memory’, ‘innate memory’, ‘antigen-inexperienced memory-like T cells’, and even ‘bystander’. Such discrepancy in terminology renders the task difficult to precisely define them. An interesting study performed by Akane *et al.* rather defined $CD8^+CD122^+CD49d^{low}$ T cells as “regulatory $CD8^+$ T cells”, and showed that these cells could kill activated T cells via Fas/FasL-mediated cytotoxicity to maintain T-cell homeostasis (Akane et al., 2016), while $Foxp3^+$ regulatory $CD4^+$ T cells limited expansion of T_{VM} *via* restraining IL-15 trans-presentation by DCs to maintain stable T_{VM} population (Da Costa et al., 2019). As T_{VM} are both defined as self-tolerant and self-reactive which could be seen as contradictory, such particular cell population could represent regulatory cells involved in the maintenance of immune homeostasis by expressing regulatory receptors and cytokines, which in turn could also regulate their own development. Thus, it is important to phenotype T_{IM}/T_{VM} in more details. For instance, the study by Hou *et al.* provided important information on the origin of lung T_{RM}, which seem to originate from $CCR2^-$ T_{VM} (Hou et al., 2021). Although it remains challenging to determine from which tissue and which cell precursors T_{IM}/T_{VM}

originate, as well as their fate, a better understanding of these key aspects of T_{VM} biology and regulation could help us better understand how T_{VM} function.

Objectives

Objectives

Virtual memory CD8$^+$ T cells (T_{VM}) are foreign antigen-inexperienced memory-like CD8$^+$ T cells, of which expansion can be driven by IL-4 and IL-15. Recent studies have demonstrated that IL-4-dominated immune responses like it occurs during helminth infections can expand the population of T_{VM} in the periphery and that these cells contribute to the control of bystander viral infections. However, the exact definition of IL-4-induced T_{VM} gene expression program and how these cells are regulated while expanding remains unclear. A previous study in our group has used *Il4ra*$^{-/-}$ mice to demonstrate that IL-4 signaling is essential for T_{VM} expansion. However, such germ-line knockout mice do not allow to study the role of IL-4 signaling specifically in CD8$^+$ T lymphocytes in the peripheral lymphoid tissues.

The main objective of this thesis was to investigate the gene expression program of IL-4-dependent T_{VM} during helminth infection to study how these cells are regulated. To achieve this global objective, we followed different goals based on our findings.

First, we aimed to develop a mouse model to interrogate the effect of IL-4 signaling in peripheral CD8$^+$ T lymphocytes specifically. To achieve this, we generated a *E8i*^{Cre}*Il4ra*$^{lox/lox}$ (IL-4Rα$^{\Delta CD8}$) mouse model, which we characterized as partial knockout for the expression of IL-4Rα specifically in peripheral single-positive CD8$^+$ T cells.

Second, we sought to define IL-4-dependent T_{VM} expansion during helminth infection at the transcriptomic levels. There, single-cell RNA sequencing approaches were performed on CD8$^+$ T cells isolated from the spleen or mesenteric lymph nodes isolated from WT and IL-4Rα$^{\Delta CD8}$ mice by artificially inducing an IL-4 dominated environment by IL-4 complex (recombinant mouse IL-4 and anti-IL-4 antibody) injection or after infection with the parasite *H. polygyrus*.

Third, based on the observation that IL-4-restricted gene signature of expanding T_{VM} after helminth infection included the strong upregulation of CD22, we further developed experimental approaches including RNA-seq, TCR-seq, as well as bone-marrow mixed chimeras of WT and *Cd22*$^{-/-}$ or *Cd22*^{Y2,5,6F} knock-in mice to study the potential role of CD22$^+$ T_{VM}.

CD22

Sialic acid binding immunoglobulin like lectin (**Siglec**) 2, named **CD22**, is a transmembrane receptor restricted on B lymphocytes (Torres et al., 1992), which carries immunoreceptor tyrosine-based inhibitory motifs (ITIMs) within their cytoplasmic tail and recruit the tyrosine phosphatase SHP-1 that inhibits cell signaling (Blasioli et al., 1999; Doody et al., 1995; Otipoby et al., 2001). Studies using CD22 knockout mice have shown that the lack of CD22 leads to a pre-activated B cell phenotype with a higher calcium mobilization. Thus, CD22 as a negative regulator involved in the BCR signaling program, and mainly functions on conventional B cells (also called B2 cells) (Nitschke et al., 1997; O'Keefe et al., 1996; Otipoby et al., 1996; Sato et al., 1997). CD22 binds to α2,6-linked sialic acids present on glycans of membrane proteins (Engel et al., 1995; Powell et al., 1993), in *cis* (on the same cell surface) and in *trans* (on other cells) (Collins et al., 2004; Crocker et al., 2007).

A number of studies have focused on the signaling pathways of CD22 in the past years. Six tyrosine residues have been identified in the cytoplasmic domain of CD22, and different binding partners involved in downstream signaling have also been identified, like Lyn, growth-factor receptor-bound protein-2 (Grb2), protein tyrosine phosphatase 1C, SHP-1, Syk and phospholipase C-γ1 (Doody et al., 1995; Law et al., 1996; Otipoby et al., 2001; Smith et al., 1998). Recent studies have used two different knockin mice, $Cd22^{R103E}$ (of which CD22 cannot bind α2,6-linked sialic acids) and $Cd22^{Y2,5,6F}$ (of which downstream signaling via CD22 phosphorylation is impaired), to dissect CD22 ligand binding and cytoplasmic signaling function. Using these mutants, it has been demonstrated that both ligand binding and phosphorylation of the ITIM domain of CD22 are crucial to inhibit calcium signaling in B cells (Muller et al., 2013).

To regulate BCR signaling, CD22 inhibits the calcium responses after BCR stimulation via interacting with the plasma membrane calcium pump ATPase PMCA and potentiate its activity by depleting Ca^{2+} from the cytoplasm (Chen et al., 2004). Interestingly, it has been shown that CD22 mediates regulation of calcium signaling through two different signaling pathways via two associated signaling proteins (SHP-1 and Grb-2), binding to distinct phosphorylated tyrosines of its intracellular signaling domain (Chen et al., 2016; Poe et al., 2000; Yohannan et al., 1999). In addition, it has been demonstrated that CD22 interacts to CD45, a highly glycosylated protein in *cis* (Hermiston et al., 2009; Van Der Merwe et al., 1996), at the steady state. Such CD22-CD45 interaction limits the association of CD22 with the BCR in resting B cells (Coughlin et al., 2015). CD22 can also homo-dimerize, also resulting in a sequestration on the membrane to avoid interaction with BCR (Figure 13).

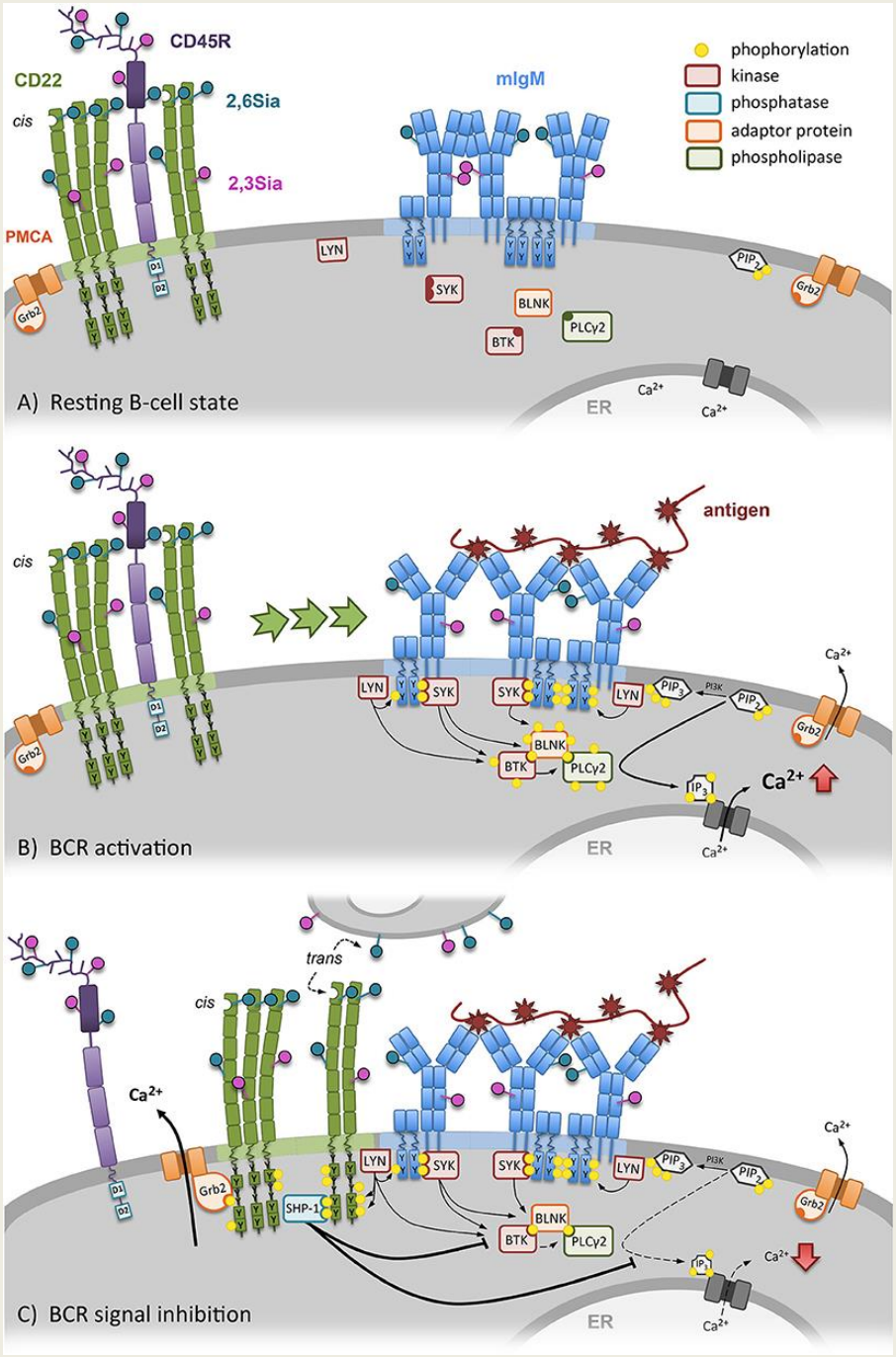
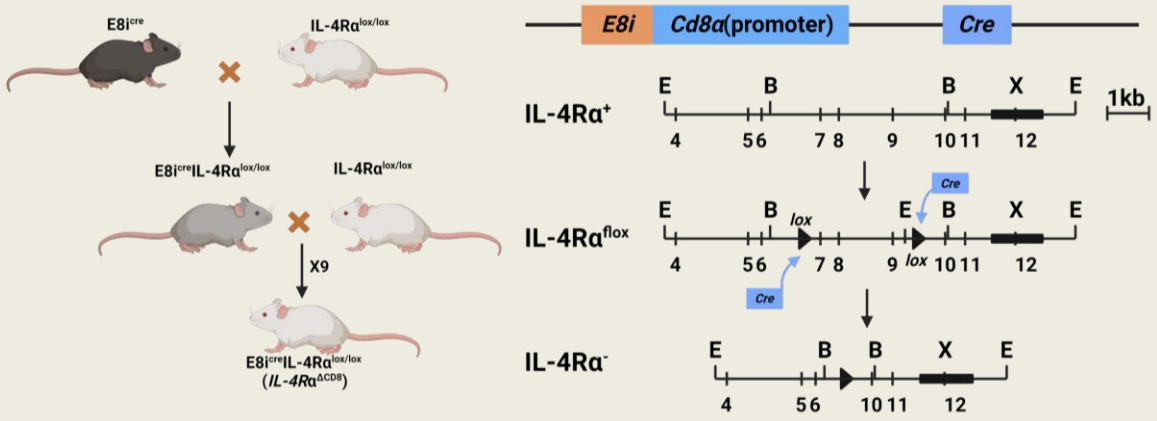
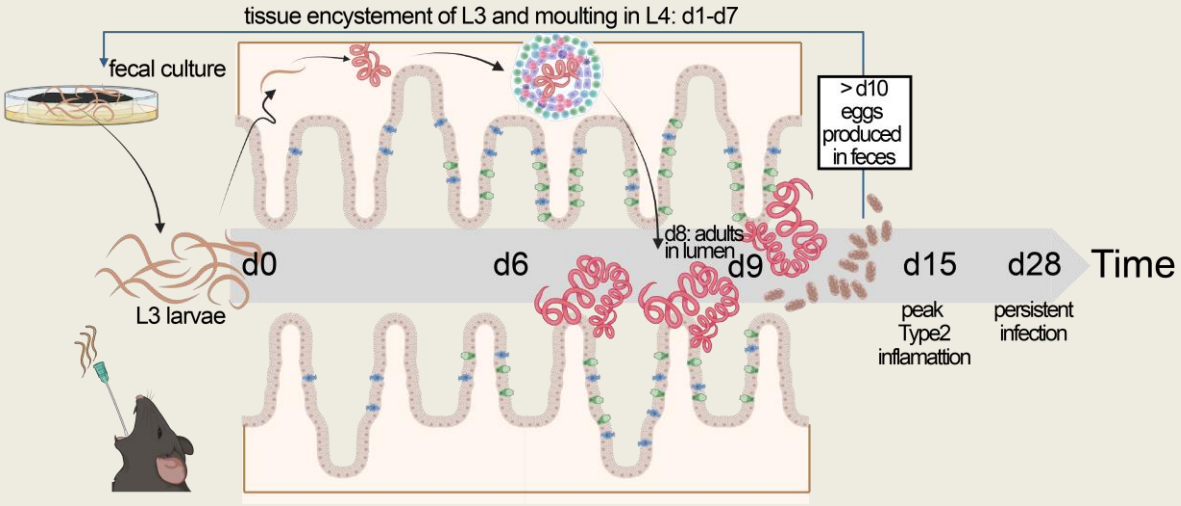


Figure 13. CD22 dependent regulation of the BCR signal. **(A)** In resting B cells the conformation of the BCR is closed and CD22 is forming homo-oligomers (*cis*-interaction) distinct from the BCR. **(B)** Specific antigen binding induces conformational opening of the BCR, followed by activation and phosphorylation of ITAMs of the Igα/Igβ-complex. Increased Syk recruitment and activation of the BTK-BLNK-PLCγ2-complex leads to more Ca^{2+}-release out of the endoplasmatic reticulum. Additionally, CD22 clusters are recruited to the BCR. **(C)** After BCR activation, CD22 recruitment inhibits the BCR signaling. Additionally, CD22 can also be recruited to the BCR by binding to its ligands on other cells (*trans*-interaction). Due to proximity of CD22 and BCR, ITIMs of CD22 get phosphorylated by LYN. SHP-1 binds to CD22 and inhibits further Ca^{2+} release. In addition, through the formed CD22-Grb2-PMCA complex, Ca^{2+} is transported out of the cell into the extracellular space by PMCA. From (Meyer et al., 2018).

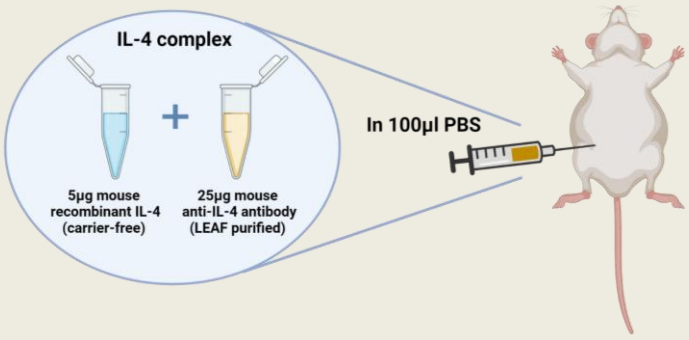
Main experimental models



The *E8i*^{Cre}*Il4ra*$^{lox/lox}$ mouse were generated by backcrossing for 9 generations E8i^{Cre} mice in *Il4ra*$^{lox/lox}$ BALB/c mice. *E8i*^{Cre} transgene is expressed transiently and the specifically in single-positive CD8 T cells once egressing the thymus. Cre-mediated deletion removes exons 7, 8 and 9 of the *Il4ra* locus.



The lifecycle and infection of *H. polygyrus* in mice (see details in Materials and methods).



Mice received 2 times intraperitoneal injections of IL-4c: 5 µg of recombinant IL-4 (carrier-free) and 25 µg of anti-IL-4 antibody (LEAF purified) per mouse at d0 and d2.

Experimental section

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IL-4 induces CD22 expression to restrain the effector program of self-reactive virtual memory T cells

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IL-4 induces CD22 expression to restrain the effector program of self-reactive virtual memory T cells

Manuscript in revision

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Abstract

Parasitic helminths induce the production of interleukin (IL)-4 which causes the expansion of virtual memory $CD8^+$ T cells (T_{VM}), a cell subset contributing to the control of viral coinfection. The mechanisms regulating IL-4-dependent T_{VM} activation and expansion during worm infection remain ill defined. We used single-cell RNA sequencing of $CD8^+$ T cells to investigate IL-4-dependent T_{VM} responses. Gene signature analysis of $CD8^+$ T cells identified a cell cluster marked by CD22, a canonical regulator of B cell activation, as a selective surface marker of IL-4-induced T_{VM} cells. $CD22^+$ T_{VM} were enriched for IFN-γ and granzyme A and retained a diverse TCR repertoire, while enriched in self-reactive CDR3 sequences. Impaired CD22 signaling enhanced the effector program of $CD8^+$ T cells in response to IL-4, indicating that CD22 modulates T_{VM} responses during helminth infection. Thus, helminth-induced IL-4 drives the expansion and activation of self-reactive T_{VM} in the periphery that is counter-inhibited by CD22.

Introduction

Helminths are widespread parasitic worms responsible for the infection of about a quarter of the human population (*1*). In high burden infections, helminths can induce debilitating and chronic disease with a strong impact on health and welfare (*2, 3*). However, most helminth infections are of low burden, with a limited number of parasites persisting in their host for several months and even years. Worm persistence can be explained by a finely regulated balance between immune control and evasion to tolerate the parasites, while avoiding critical harm (*4*, *5*). Helminths generally induce a robust and protective type 2 immunity, which is characterized by the production of type 2 cytokines including interleukin (IL)-4, IL-5, and IL-13. Type 2 cytokines are produced by and activate key immune cells such as group 2 innate lymphoid cells (ILC2s), eosinophils, mast cells, macrophages and $CD4^+$ T helper 2 (Th2) cells, which coordinate immune protection (*5*). In addition to these effector mechanisms, helminth infection also induces the expansion of a specific population of memory-phenotype $CD8^+$ T cells in the secondary lymphoid organs that are referred to as virtual memory T cells (T_{VM}) (*6–9*).

T_{VM} have many characteristics in common with true memory $CD8^+$ T cells (T_{TM}), which arise in the periphery in response to a foreign antigen and are characterized by their ability to develop fast and effective responses after repeated encounters with the same antigen (*10*). Likewise, T_{VM} can also rapidly respond in the early phase of infection with pathogens (*11–14*). However, in contrast to T_{TM}, T_{VM} have the particularity to differentiate and develop in absence of foreign antigen (*13*, *15*, *16*). In naive conditions, T_{VM} are $CD8^+$ αβ T cells that are found in substantial proportions in conventionally housed mice, as well as in germ-free (GF) mice (*17*). While T_{VM} exhibit a $CD44^{hi}CD122^+CXCR3^{hi}$ memory phenotype, they differ from T_{TM} since they downregulate the α4 integrin (*Itga4*, CD49d), which is normally upregulated in response to TCR activation (*18*). T_{VM} have been described in human, but there is still a significant lack of specific markers to distinguish them from T_{TM}, in both human and mice (*16*, *19–21*). The developmental origin of the memory phenotype of T_{VM} has been recently explained by the recognition of self-ligands during maturation in the thymus, driving the upregulation of the transcription factor eomesodermin (EOMES) in T cell precursors (*17*, *22*). In addition to self-specificity, T_{VM} differentiation in the thymus and expansion in the periphery also requires signals from IL-4 and/or IL-15, depending on the mouse strain (*8*, *23–26*). Invariant natural killer T cells produce IL-4 in BALB/c mice (*24*, *27*), driving memory-phenotype T cell differentiation in the thymus. In addition, IL-4 signaling is also required in the periphery to maintain T_{VM} in this strain (*6*, *25*). In contrast, C57BL/6 mice seem to mainly rely on IL-15 for T_{VM} differentiation and maintenance at steady-state (*8*, *21*, *26*, *28*). Efforts have been made to decipher the function of T_{VM} in homeostasis as well as in response to infection or tumor development (*6*, *7*, *17*, *29*, *30*), but the mechanisms regulating helminth-induced T_{VM} expansion, activation and maintenance are yet to be fully uncovered. T_{VM} significantly expand in the periphery during helminth infection, but the role of IL-4 and IL-15 in such peripheral expansion appears

controversial and/or redundant, irrespective of the mouse strain (*6, 7, 9, 29*). Nevertheless, expansion of T_{VM} after helminth infection can result in enhanced $CD8^+$ T cell-mediated control against bystander viral coinfection (*6*), which was also observed in bacterial coinfection (*7*). Thus, the increased numbers of T_{VM} in helminth infection can drive protection against concurrent intracellular pathogens. However, the mechanisms regulating T_{VM} response to IL-4 during helminth infection remain incompletely defined.

Here, we addressed this important question by studying IL-4 signaling and consequences in the circulating $CD8^+$ T cell population during helminth infection. We employed single-cell RNA sequencing (scRNA-seq) to investigate transcriptomic gene signatures and interrogate cellular heterogeneity to identify unique features of IL-4-induced T_{VM}. Our findings identified a specialized program of differentiation instructed by IL-4 in T_{VM}, including the unanticipated upregulation of CD22, a canonical inhibitory receptor of B lymphocytes. CD22 was upregulated in IL-4-induced self-reactive T_{VM} specifically and the absence of CD22 signaling resulted in an enhanced T_{VM} response to IL-4 during helminth infection.

Results

Required IL-4Rα expression on peripheral CD8$^+$ T cells for T_{VM} maintenance. In BALB/c mice, IL-4 is required for the differentiation of unconventional memory T cell in both the thymus and the periphery (*6*, *27*). To study IL-4-mediated T_{VM} expansion in the periphery, a BALB/c mouse model was generated in which peripheral CD8$^+$ T lymphocytes have impaired expression of the IL-4 receptor α chain (IL-4Rα). Specifically, *E8i^{Cre}* C57BL/6 mice were backcrossed for 9 generations with *Il4ra$^{lox/lox}$* BALB/c mice (WT) to generate IL-4Rα$^{\Delta CD8}$ BALB/c mice. In *E8i^{Cre}* mice, Cre expression is driven through the control of an E8i-CD8α enhancer/promoter construct (*31*). The enhancer E8i is activated in maturating single positive CD8$^+$ T lymphocytes (SP8) before exiting the thymus to the periphery. In naive conditions, single positive CD4$^+$ (SP4) and SP8 expressed similar levels of IL-4Rα (CD124) in both WT and IL-4Rα$^{\Delta CD8}$ mice (**Fig. 1a**). E8i enhancer has been reported to be activated in CD24$^-$ SP8 cells (*32*), and we observed that a small subset of CD24$^-$ SP8 cells started to lose CD124 expression in IL-4Rα$^{\Delta CD8}$ mice suggesting an efficient deletion of the *Il4ra* locus in mature CD8$^+$ T cells exiting the thymus (**Fig. 1b**). As expected, splenic CD3$^+$CD8$^+$ T cells expressed significantly lower levels of CD124 (**Fig. 1c**), whereas CD3$^+$CD8$^-$ T cells and other cell types retain high CD124 expression in IL-4Rα$^{\Delta CD8}$ mice (**Fig. 1c**, **Fig. S1a**). T_{VM} were gated as CD44^{hi}CXCR3$^+$CD49d^{low}CD3$^+$CD8$^+$ T cells (**Fig. S1b**), and IL-4Rα$^{\Delta CD8}$ mice had significantly less T_{VM} when compared to WT littermate controls (**Fig. 1d**). These results support previous observations regarding the IL-4Rα requirement for peripheral T_{VM} differentiation (*6*, *25*) and further demonstrate that IL-4 significantly contributes to T_{VM} maintenance in the periphery in naive conditions. However, a population of CXCR3$^+$ T_{VM} retained the expression of CD124 in IL-4Rα$^{\Delta CD8}$ mice (**Fig. 1e**), suggesting incomplete deletion of *Il4ra* in IL-4Rα$^{\Delta CD8}$. Such incomplete deletion could explain the higher proportions of T_{VM} found in the remaining CD124$^+$ cells in IL-4Rα$^{\Delta CD8}$ mice (**Fig. 1f and Fig. S1c-d**). Thus, peripheral IL-4 signaling is required for T_{VM} maintenance, and IL-4Rα$^{\Delta CD8}$ mice are partial knockout in which the remaining T_{VM} population mainly arises from IL-4Rα-expressing CD8$^+$ T cells, likely escaping genetic deletion driven by the *E8i^{Cre}* transgene.

T_{VM} expansion is reduced in IL-4Rα$^{\Delta CD8}$ mice after IL-4c treatment and helminth exposure. During helminth infection, IL-4 can expand T_{VM} (*6*, *7*). To study the effect of IL-4 signaling on peripheral CD8$^+$ T cells, IL-4Rα$^{\Delta CD8}$ and WT littermate control mice were treated with recombinant IL-4 complexed with a monoclonal antibody to IL-4 (IL-4c), which extends the bioactive half-life of the cytokine and artificially induces an IL-4 dominant environment (**Fig. 1g-i**). We found that although maturating thymic CD24$^-$ SP8 started to lose CD124 and IL-4c upregulated expression of the transcription factor eomesodermin (EOMES) in SP8, there were no significant difference in CD24$^-$ SP8 proportions or EOMES expression between WT and IL-4Rα$^{\Delta CD8}$ mice (**Fig. 1g**). In the spleen, IL-4c induced the expansion of T_{VM} and upregulated the expression of EOMES in WT mice, while T_{VM}

expansion and EOMES expression levels were reduced in IL-4Rα$^{\Delta CD8}$ mice (**Fig. 1h**), confirming significant deletion of the *Il4ra* locus. However, IL-4c treatment increased the proportions of CD124^{+} CD8^{+} T cells in IL-4Rα$^{\Delta CD8}$ mice (**Fig. 1i**), likely through selection of cells retaining IL-4Rα expression. When focusing on CXCR3^{+} T_{VM}, we found that although significantly reduced compared to WT controls, a significant proportion of CXCR3^{+} cells was still present in IL-4Rα$^{\Delta CD8}$ animals. However, the majority of CXCR3^{+} T_{VM} cells retained surface expression of CD124, confirming that IL-4Rα$^{\Delta CD8}$ mice are partial knockout mice for *Il4ra* locus (**Fig. S1e**). We further explored T_{VM} expansion in IL-4Rα$^{\Delta CD8}$ mice exposed to helminths using injection of *S. mansoni* eggs or infected with the gastro-intestinal nematode *Heligmosomoides polygyrus*. A significant reduction of T_{VM} expansion was observed in IL-4Rα$^{\Delta CD8}$ mice (**Fig. 1j**). Importantly, expansion of T_{VM} during helminth infection required IL-4Rα expression by CD8^{+} T cells, since the majority of T_{VM} in IL-4Rα$^{\Delta CD8}$ mice were found in the CD124^{+} compartment but significantly lower in CD124^{-} T cells (**Fig. 1k**). However, *H. polygyrus* infection was not affected in IL-4Rα$^{\Delta CD8}$ mice as attested by similar worm burden at day 15 after infection (**Fig. 1l**). Finally, IL-4c treatment resulted in a significant early control of the lung replication of a luciferase-expressing viral strain of murid gammaherpesvirus 4 (MuHV-4-Luc) by day 8 after infection (**Fig 1m**), supporting our previous observations (*6*). However, luminescent signals revealing replication levels of MuHV-4-Luc in infected IL-4Rα$^{\Delta CD8}$ mice remained significantly high, further confirming a role of IL-4-dependent T_{VM} in controlling viral replication in the lung. Together, these data demonstrate that during helminth infection IL-4 directly induces T_{VM} expansion in peripheral CD8^{+} T cells, impaired in IL-4Rα$^{\Delta CD8}$ mice.

Single-cell transcriptomic analysis reveals peripheral T_{VM} signature. To investigate the transcriptomic program of CD8^{+} T cell responses to IL-4 during helminth infection, single-cell RNA-seq (scRNA-seq) was performed on CD8^{+} T cells enriched from the spleen of IL-4Rα$^{\Delta CD8}$ and littermate control WT mice after IL-4c treatment or *H. polygyrus* infection (**Fig. S2a**). Doublets and contaminating non-CD8^{+} T cells were excluded (exclusion of cells expressing *Cd19, Cd4, Ncr1, Itgax, Tcrg-C1, Tcrg-C3, Tcrg-C4, Trdc*) and a total of 33,553 cells were analysed in which ~30-50,000 reads and ~2,000 genes per cell were detected. Six datasets were obtained based on the treatment (PBS, IL-4c and *H. polygyrus*) and genotype (IL-4Rα$^{\Delta CD8}$ and littermate WT control). Clustering on the Uniform Manifold Approximation and Projection (UMAP) representation identified 17 distinct clusters (**Fig. 2a, Fig. S2b-c**). Hash tagging allowed separation of cells from individual mice in each dataset to visualize the proportional dispersion of each cluster for each individual mouse (**Fig. S2d**). Based on gene signatures and increased proportions upon IL-4 response, cluster 6 was identified as the cluster in which T_{VM} would be most likely found as memory genes were upregulated and *Itga4* downregulated (**Fig. S2d-f**). To better define the T_{VM} population, SingleR analysis was performed on the same merged datasets to identify naive, memory and effector T cells based on signature genes (**Fig. 2b-c**) (*33*). Cells having a signature of memory or effector T cells were then extracted from the main object and re-analysed with Seurat for

clustering (**Fig. 2d-e, Fig. S3a-b**). In total 15 clusters were identified, and differential gene expression was performed to furtherly characterize the new clusters. We could identify Cluster 14 as terminally effector T cells (*Zeb2, Klrg1, Cx3cr1, Bhlhe40, Gzmb*), while Cluster 1 contained cells upregulating *Gzmm*. Cluster 3 and 4 upregulated genes associated with true memory (*Itga4, Gzmk, Ccl5, Cxcr3)*. Cluster 5 contained cells upregulating *Cd226, Lef1, Ccr9* and *Foxp1*. While cluster 6 upregulated *Ccr7, Il7r* and *Tcf7* and likely includes central memory T cells, the exhaustion gene *Tox* and *Lag3* were differentially upregulated in Cluster 8. Signature genes reminiscent of mucosal associated invariant T cells and/or γδ T cells such as *Cd160, Klra7* and *Klrc1*, as well as *Trg-C1, Trg-C1* were found in Cluster 9. In addition, *Cd69, Cd83, Icam1* grouped potential tissue resident T cells in Cluster 10, and interferon stimulated genes (*Isg15, Ifit1, Ifit3, Oasl1, Usp18*) were up in Cluster 11. Cluster 12 contained cells in active division potentially representing stem-like memory T cells upregulating *Mki67, Birk5, Ccnb2, Ccna2*, and *Cdk1*. Finally, cluster 2 had a gene signature related to T_{VM}, characterized by the reduced expression of *Itga4* combined with the upregulation of memory/effector genes like *Eomes, Ctla2a, Ccr2, Ccr5, Il2rb* and *Gzma* (**Fig. 2d-f**). Confirming that cluster 2 represents T_{VM}, the proportion of cells in cluster 2 was increased upon IL-4c or *H. polygyrus* infection in littermate WT control mice, while less abundant in IL-4Rα$^{\Delta CD8}$ mice (**Fig. S3c-d**).

IL-4-induced T_{VM} upregulate signature genes including *Cd22* during helminth infection. When looking at the effects of treatment and genotype, we observed that IL-4c and *H. polygyrus* infection induced an enrichment of cells having a gene signature of memory T cells with upregulation of *Eomes*, *Cxcr3* and *Il4ra* and reduced *Itga4* (**Fig. 3a-b**). Although there was no significant difference between IL-4Rα$^{\Delta CD8}$ and littermate WT controls, likely due to the partial deletion of *Il4ra* in IL-4Rα$^{\Delta CD8}$, we observed that IL-4c treatment or *H. polygyrus* infection had a more limited effect on the expansion of memory T cells and signature gene expression levels in these mice. Trajectory pathway analysis using RNA velocity (*34*) showed main trajectories of differentiation from cluster 0 to clusters 1 and 2, then to cluster 4, 3 and 14 irrespective of the treatment (**Fig. S4a**). These data suggest that T_{VM} cluster 2 originates from memory cluster 0 and that the main pathway of T_{VM} differentiation are conserved upon IL-4 activation. Interestingly, gene set enrichment analyses of individual clusters for regulation of transcription identified *Atf6* and *Cebpd* among the top enriched transcription program in response to IL-4c and *H. polygyrus*, suggesting increased cellular stress in cluster 2 (**Fig. S4b**) (*35–38*). Then, focusing on the differential gene expression in cluster 2 in response to both IL-4c or *H. polygyrus* infection, we observed a significant upregulation of *Gzma, Gzmm, Ctla2a, Xcl1, Bcl11b, Fyn, Ccr2, Ccr5* and *Cd22*, whereas *S1pr1, Cd55, Ly6c1, Ly6c2 and Itgb7* were downregulated (**Fig. 3c-d**). Interestingly, most of the regulated genes were also similarly regulated in data obtained by RNA sequencing on sorted T_{VM} from mice exposed to *S. mansoni* eggs (**Fig. S4c**) (*6*), CD5$^+$ or CD5$^-$ T_{VM} (*39*), and CCR2$^+$ T_{VM} (*14*), further supporting that cluster 2 are T_{VM}. Moreover, differential gene expression analysis in cluster 2 upon IL-4c treatment and at day 15 after *H. polygyrus* infection confirmed the significant increased

expression of *Gzma, Gzmm, Ccr5, Xcl1* and *Cd22* (**Fig. 3e**). At day 15 after *H. polygyrus* infection, although frequencies of T_{VM} increased in the spleen (**Fig. 4a**), a most severe expansion of T_{VM} was found in the mesenteric LN (mesLN) (**Fig. 4b**), where a significant IL-4Rα-dependent upregulation of EOMES in $CD8^+$ T cells could be observed (**Fig. 4c**). To further determine the IL-4Rα-dependent T_{VM} gene signature in the mesLN after *H. polygyrus* infection, we took advantage of the partial deletion of the *Il4ra* locus in IL-4R$\alpha^{\Delta CD8}$ mice. $CD124^+$ and $CD124^-$ $CD8^+$ T cells were FACS-sorted from the mesLN at day 15 after *H. polygyrus* infection of IL-4R$\alpha^{\Delta CD8}$ mice to tag each cell population before re-pooling them and further proceed to scRNA-seq (**Fig. 4d, Fig. S4d**). A total of 6,808 cells were validated with ~60% $CD124^+$ and ~40% $CD124^-$ cells. In $CD124^+$ cells specifically, Seurat analysis identified clusters 1 and 5 as IL-4-induced T_{VM} based on the upregulation of *Eomes, Il2rb*, while reduced expression of *Itga4*. However, cells composing cluster 5 displayed additional signature genes of activation such as *Gzma, Ccr2, Ccr5,* and *Cd22* (**Fig. 4e-f, Fig. S4d**), as observed in the identified IL-4-dependent cluster 2 of $CD8^+$ T cells from the spleen of IL-4c-treated mice (**Fig. 3d-e)**. Interestingly, none of the IL-4-dependent clusters upregulated *Gzmb* or exhaustion markers like *Pdcd1*. Thus, these results specifically identified the nature of transcriptional changes in IL-4-induced T_{VM} during helminth infection.

CD22 expression is restricted to IL-4-induced T_{VM} cells. CD22 expression is largely restricted to B cells (*40, 41*). While CD22 has recently been shown to regulate other cell types such as microglia (*42*), the role of CD22 in T lymphocytes remains poorly defined (*43*, *44*). *Cd22* RNA expression was mainly restricted to the T_{VM} clusters in the spleen of mice treated with IL-4c or infected with *H. polygyrus* (**Fig. 3c-d and Fig. 5a**), and in the mesLN of *H. polygyrus* infected mice (**Fig. 4e-g**). However, scRNA-seq barely detected *Cd22* expression in naive T cells (**Fig. 3d**). This observation was confirmed by immunostaining for surface CD22 where a significant proportion of $CD22^+$ cells could be detected by flow cytometry on $CD8^+$ T lymphocytes after IL-4c treatment and *H. polygyrus* infection (**Fig. 5b**). We could confirm the specific expression of CD22 by $CD8^+$ T cells by ImageStream (**Fig. 5c**), excluding any artefact due to potential cell doublets. Flow cytometry analysis of $CD22^+CD8^+$ T cells confirmed that IL-4-induced $CD22^+CD8^+$ T cells mainly express T_{VM} markers (**Fig. 5d**), further suggesting that CD22 expression is induced in T_{VM} through IL-4 signaling. Indeed, the expansion of $CD22^+$ T_{VM} by IL-4c and *H. polygyrus* infection was significantly reduced in IL-4R$\alpha^{\Delta CD8}$ mice (**Fig. 5e-f**). Following concatenation of flow data from multicolor staining of $CD8^+$ T cells, FlowSOM unbiased clustering analysis was performed. These analyses identified cluster C0 as being T_{VM} cells expressing high levels of CD44, CXCR3 and CD22 but lower levels of CD49d (**Fig. S5a-b**). Importantly, cluster C0 also expressed high levels of CD124, suggesting that IL-4 signaling in T_{VM} drives CD22 expression, which was further confirmed as CD22 was only expressed by the Cre recombinase-escaping $CD124^+$ T cells in IL-4R$\alpha^{\Delta CD8}$ mice, whereas $CD22^+$ cells could not be detected in $CD124^-$ T cells (**Fig. 5g, Fig. S5c**). In addition, expansion of $CD22^+$ T cells was not observed in $CD4^+$ T cells after helminth infection (**Fig. S5d**). Interestingly, $CD22^+$ T_{VM} strikingly expanded in the mesenteric LN (mesLN) at day 15 after

worm infection and were restricted to CD124$^+$ CD8$^+$ T cells (**Fig. 5h**)**,** and upregulated EOMES (**Fig. S5e**). Although IL-4 was shown to be required for T_{VM} maintenance in BALB/c mice, we could also observe a strong expansion of CD22$^+$ T_{VM} after IL-4c treatment or *H. polygyrus* infection of C57BL/6 mice (**Fig. S5f**). Moreover, CD22$^+$ T_{VM} was observed in the spleen, the mesLN but also in the inguinal LN and bone marrow after *H. polygyrus* infection (**Fig. S5f-h**), suggesting a systemic effect of IL-4 during worm infection. However, whereas T_{VM} expansion was maintained after a single injection of IL-4c, surface CD22 was transient (**Fig. S5i**). Importantly, IL-4c treatment could not induce CD22 expression on thymic SP8 cells (**Fig. S5j**), or on virus-specific effector/memory T cells at 30 days after MuHV-4 infection (**Fig. S5k**). Finally, we could not detect any significant expression of CD22 on CD8$^+$ T cells from IL-4c treated or *H. polygyrus*-infected BALB/c or C57BL/6 *Il4ra*$^{-/-}$, or C57BL/6 *Stat6*$^{-/-}$ mice (**Fig. 5i**). Similarly, CD22 expression was not increased on *Il4ra*$^{-/-}$ CD8$^+$ T cells from WT:*Il4ra*$^{-/-}$ mixed bone marrow chimeras treated with IL-4c or infected with *H. polygyrus* (**Fig. 5j**). These data collectively demonstrate that CD22 is specifically upregulated by IL-4 in expanding T_{VM}.

CD22$^+$ T_{VM} induced by IL-4 display an activated phenotype. T_{VM} expressing CD22 at their surface in response to IL-4 could have a specific functional program. Thus, an in-depth transcriptomic comparative analysis by RNA-seq was performed on FACS-sorted CD44^{low} T_{NAIVE}, CD22$^+$, and CD22$^-$ CD44^{hi}CD49^{low} T_{VM} populations from PBS- or IL-4c-treated mice. Principal component analysis (PCA) revealed clustering of each analyzed cell population, suggesting that the surface expression of CD22 in T_{VM} is associated with phenotypic changes (**Fig. S6a**). Among genes upregulated in CD22$^+$ T_{VM}, we observed upregulation of *Cd22*, as well as genes related to effector functions and activation such as *Gzma, Ifng, Eomes, Il2rb, Mki67, Ctla2a, Slc16a2, Gzmm, Xcl1, Ccl4, Cxcr3, Ccr2* or *Ccr5* (**Fig. 6a-b)**. Interestingly, we observed significant gene set enrichment in CD22$^+$ T_{VM} of T cell signaling, cell cycle, NK mediated killing, and IL-2/STAT5 signaling (**Fig. 6c**), as well as MAPK and BCR signaling (**Fig. S6b**). Moreover, *Cd22* was the only upregulated siglec gene (**Fig. S6c**), whereas genes related to T cell exhaustion such as *Pdcd1*, *Lag3*, *Havcr2* (TIM3), *Ctla4* or *Tox* were not significantly upregulated, apart from *Tigit* (**Fig. S6d**). In comparison to CD22$^-$ T_{VM}, CD22$^+$ T_{VM} significantly upregulated inhibitory receptors such as, *Entpd1* (CD39), *Cd160, Klra3* (Ly49c), *Klra5* (Ly49e), *Klra7* (Ly49g), *Klrc1* and *Klrd1* (NKG2A/CD94) and downregulated *Klrg1* and *Itga4* (CD49d) (**Fig. 6d, Fig. S6e**). Interestingly, similar gene signatures with upregulation of *Cd22* together with its known interacting factors *Ptpn6*, *Grb2* and *Lyn*-related *Fyn* as well as upregulation of *Entpd1* or *Klra3* could be identified in the T_{VM} cluster in the mesLN during *H. polygyrus* infection (**Fig. S6f**), suggesting that a common regulation program occurs in T_{VM} in both spleen and mesLN in response to IL-4 and during *H. polygyrus* infection. Importantly, the observed upregulation of EOMES, GZMA, CD39, CCR2, and CD160 were further confirmed by flow cytometry after IL-4c in the spleen (**Fig. 6e**) or at 2 weeks post-infection with *H. polygyrus* (**Fig. S7a**). Moreover, CD22$^+$ T_{VM} were shown to be the main population of CD8$^+$ T cells producing IFN-γ upon IL-4 stimulation using an IFN-γ reporter mouse model (**Fig. 6f-g**). During *H.*

polygyrus infection, IL-4 signaling via STAT6 activation was further demonstrated to specifically drive the expansion of peripheral T_{VM} upregulating CD22, CD39 and GZMA (**Fig. S7b-d**). Whereas T_{VM} exist in germ-free (GF) mice (*17*), microbiota might contribute to CD22 upregulation. However, expansion of T_{VM} co-expressing CD22, CD39 and GZMA also occurred in GF mice infected with axenic *H. polygyrus*, demonstrating that microbiota is not critical for $CD22^+$ T_{VM} expansion (**Fig. S7e**). Moreover, an increase in the frequency of $GATA3^+$ Th2 cells in the mesLN of mice infected with *H. polygyrus* under GF conditions was associated with an increase in CD39 and GZMA expression by $CD22^+$ T_{VM} (**Fig. S7f**). In addition, absence of IFN-I signaling was shown to be dispensable for the induction of CD22, CD39 and GZMA in T_{VM} during *H. polygyrus* infection (**Fig. S7g**). Taken together, these data suggest that $CD22^+$ T_{VM} display a phenotype of activation when compared to $CD22^-$ T_{VM}, and that the magnitude of *in vivo* IL-4 signals is a determinant of $CD22^+$ T_{VM} activation.

$CD22^+$ T_{VM} cells possess a diverse TCR repertoire enriched in self-reactive signatures. T_{VM} mainly arise from a pool of $CD8^+$ thymocytes that display reactivity towards endogenous self-ligands (*17, 22*). Thus, we hypothesized that the IL-4 driven expression of the inhibitory molecule CD22 on peripheral T_{VM} cells could be restricted to self-reactive cells. In line with this, we observed an increased expression in $CD22^+$ T_{VM} of *Cd5* which is associated to self-antigen recognition (*45*, *46*) (**Fig. 6d**). Additionally, analysis of a public RNA-seq dataset revealed that $CD5^+$ T_{VM} also upregulate *Cd22* (*39*) (**Fig. 7a**), which was similarly found to be upregulated in $CCR2^+$ T_{VM} (*14*). To investigate whether self-reactive T_{VM} could be preferentially expanded upon IL-4 signaling and whether CD22 could mark highly self-reactive TCRs, TCR sequencing was performed on $CD44^{low}$ T_{NAIVE}, $CD22^+$ T_{VM}, and $CD22^-$ T_{VM} populations from PBS- or IL-4c-treated mice (**Fig. S8a**). Both $CD22^-$ and $CD22^+$ T_{VM} possessed a highly diverse TCR repertoire, indicating a polyclonal expansion (**Fig. 7b-c, Fig. S8b**). To verify whether this polyclonal expansion was stochastic or whether self-reactive TCRs were favored, CDR3α and CDR3β amino acid composition was further analyzed for several key physicochemical features described to influence self-antigen recognition (*47–49*). CDR3 sequences from IL-4-induced T_{VM}, especially the $CD22^+$ subset, displayed significantly enriched hydrophobic doublets in positions 6 and 7 (**Fig. 7d-e**), were enriched for CDR3 strength and volume, and showed reduced CDR3 polarity (**Fig. 7f-g and Fig. S8c-h**). Cumulative contribution of these and additional CDR3 features were assessed by principal component analysis and revealed a significant separation of $CD22^+$ T_{VM} from other groups (**Fig. 7h and Fig. S8i**). Supporting the hypothesis that CD22 expression is restricted to self-reactive TCR populations, IL-4c treatment induced strong T_{VM} expansion and CD22 expression in OT-I TCRtg cells, which are reported to exhibit relatively high levels of autoreactivity (**Fig. 7i**) (*22*, *50*, *51*). Conversely, T_{VM} expansion and CD22 expression were significantly reduced in the non-autoreactive CSP TCRtg mice (*52*) after IL-4c treatment (**Fig. 7i**). Collectively, these data demonstrate that IL-4 drives the expression of CD22 at the surface of expanding self-reactive T_{VM} cells.

The lack of CD22 leads to increased activation of T_{VM} during helminth infection. The expression of the inhibitory receptor CD22 on the most self-reactive T_{VM} might contribute to counter self-peptide activation. Thus, we studied T_{VM} expansion in CD22-deficient mice (*Cd22*$^{-/-}$) (*53*) after IL-4c administration and during helminth infection. As expected, CD22^{+} T_{VM} could not be found in *Cd22*$^{-/-}$ mice (**Fig. 8a**). Interestingly, *Cd22*$^{-/-}$ mice had significantly more T_{VM} at steady-state and their numbers were significantly increased after IL-4c when compared to WT mice (**Fig. 8b**). Whereas IL-4c administration in *Cd22*$^{-/-}$ mice resulted in similarly increased expression of EOMES in T_{VM}, suggesting the initial T_{VM} activation by IL-4 is not altered by the absence of CD22. However, *Cd22*$^{-/-}$ T_{VM} showed an enhanced response to IL-4 driven expansion by producing significantly more CD39 and GZMA (**Fig. 8c and Fig. S9a-b**). After *H. polygyrus* infection, CD22^{+} T_{VM} strongly expanded in spleen and mesLN of WT mice, but the expansion of T_{VM} was surprisingly not affected in *Cd22*$^{-/-}$ mice (**Fig. 8d-e**) and no difference in parasite burden was observed (**Fig. 8f**). Importantly, Th2 responses including IL-4 production was significantly impaired in *Cd22*$^{-/-}$ mice **(Fig. S9c-d**), likely explaining the reduced activation of T_{VM} in *Cd22*$^{-/-}$ mice infected with *H. polygyrus*. Since CD22 is strongly expressed in B lymphocytes to regulate B cell responses, the absence of CD22 expression on B cells in *Cd22*$^{-/-}$ mice could indirectly interfere with T_{VM} responses. Thus, mixed bone marrow WT:*Cd22*$^{-/-}$ chimeras were generated to investigate in a competitive setting how a cell-intrinsic lack of CD22 could impact T_{VM} responses to IL-4c or helminth infection (**Fig. 8g**). Strikingly, CXCR3^{+}CD49d^{low} T_{VM} expansion upon IL-4c treatment or *H. polygyrus* infection was significantly increased in the *Cd22*$^{-/-}$ CD8^{+} T cell compartment (**Fig. 8g-h**). WT and *Cd22*$^{-/-}$ T_{VM} expressed similar levels of EOMES but the levels of expression of GZMA and CD39 were significantly increased after both IL-4c treatment or *H. polygyrus* infection in *Cd22*$^{-/-}$ T_{VM} (**Fig. 8g-h and Fig. S9e**). Interestingly, when comparing the levels of CD22, CD39 and GZMA expression in T_{VM} of chimeric mice, the highest CD22 expression were observed in mice displaying higher CD39 and GZMA expression as well, suggesting the association of these 3 markers in IL-4-induced T_{VM} (**Fig. S9f**). These data indicate that CD22 regulates the magnitude of the response to IL-4 activation and T_{VM} expansion. If so, we reasoned that mutation of the cytoplasmic ITIM motifs in CD22 should mimic the absence of CD22. To address this, we used transgenic *Cd22*^{Y2,5,6F} mice in which the ITIM signaling domains were mutated to prevent CD22 downstream signaling (*54*) (**Fig. 8i**). Mixed bone marrow WT:*Cd22*^{Y2,5,6F} chimeras were generated to investigate IL-4-dependent T_{VM} expansion. Again, whereas EOMES expression was not affected in T_{VM} by the absence of CD22 signaling, IL-4 activation of *Cd22*^{Y2,5,6F} CD8^{+} T cells resulted in a significant increased CD49d^{low} T_{VM} expansion, expressing higher levels of CD39 and GZMA (**Fig. 8j-k**). Next, we further examined the role of CD22 in regulating TCR and/or bystander activation upon *ex vivo* stimulation of IL-4-expanded T_{VM}. We observed that CD22 deficiency resulted in a significant increase of IFN-γ production upon restimulation *ex vivo* (**Fig. 9a-b**). Moreover, impaired CD22 signaling in T_{VM} resulted in an altered

"effector-like" T_{VM} response, with increased KLRG1, CD39, and GZMA and increased $CD44^{+}CD62L^{low}$ effector T cells, as well as increased CD107a detection during stimulation (**Fig. 9c-d**). To further investigate how the absence of $CD22^{+}$ T_{VM} would affect the $CD8^{+}$ T cell response to viral respiratory infection, WT:*Cd22*$^{-/-}$ chimeras were infected with strain PR8 of influenza virus (IAV) after IL-4c treatment (**Fig. 9e**). At day 6 after IAV infection, we observed a significant infiltration of the airway with $CD8^{+}$ T cells (**Fig. 9f**), displaying a similar chimerism as observed in peripheral blood before IAV infection (**Fig. 9g**). However, significantly higher proportions of $GZMA^{+}$, $GZMB^{+}$ and $CD39^{+}$ T cells were observed in the *Cd22*$^{-/-}$ T cell compartment after IAV infection (**Fig. 9h**), together with an increased ability to produce IFN-γ upon restimulation (**Fig. 9i**). Thus, these data demonstrate that IL-4 induces the expansion of $CD22^{+}$ T_{VM} resulting in a counter-inhibition of T_{VM} expansion, and a reduction of the degree of T_{VM} activation after bystander stimulation.

Discussion

Overall, our results reveal the unexpected finding that CD22 is specifically expressed by IL-4-driven self-specific T_{VM} to control their expansion and activation during helminth infection. Specifically, we have demonstrated that IL-4 signaling during *H. polygyrus* infection in peripheral $CD8^+$ T cells drives the engagement of a specific gene signature program of activation, together with CD22 and other counter-inhibitory receptors that are not related to T cell exhaustion.

We found that IL-4R$\alpha^{\Delta CD8}$ mice, although partially knockout for *Il4ra*, had significantly reduced T_{VM} population in the spleen, whereas CD124 expression was not affected in the thymus. We could demonstrate the selective expression of the Cre recombinase in mature $CD8^+$ T cells exiting the thymus in IL-4R$\alpha^{\Delta CD8}$ mice and the requirement of IL-4 signaling in $CD8^+$ T cells to drive the expansion and functional changes in peripheral T_{VM}. Indeed, T_{VM} expansion and upregulation of signature markers of IL-4-induced T_{VM} like CD22, CD39, and GZMA were specifically found in $CD124^+$ T_{VM} after *H. polygyrus* infection. Moreover, we could not observe the upregulation of these markers in *Il4ra*$^{-/-}$ or in *Stat6*$^{-/-}$ mice, irrespective of the strain background. These findings clearly highlight the requirement of IL-4 signaling in driving a specific program in T_{VM} upon helminth infection. These findings are important as IL-15 signaling has also been shown to expand T_{VM} upon *H. polygyrus* infection (*9*). As both cytokines signal through the common gamma chain (γc), they might trigger overlapping intracellular activation pathways in T_{VM} (*55*).

The heterogeneity of the gene expression program in splenic $CD8^+$ T cells in response to IL-4c and *H. polygyrus* infection was revealed by scRNA-seq. Given the remarkable expansion of T_{VM} during helminth infection, our study enhanced the description of this subset, thereby adding to our understanding of memory/effector $CD8^+$ T cell diversity previously reported using scRNA-seq approaches in other settings (*56*, *57*). Further, gene signature analyses highlighted that IL-4-induced T_{VM} differ from classical T_{TM} or exhausted T cells and are rather characterized by a unique combination of *Eomes*, *Il2rb*, *Ctla2a*, *Gzma*, *Gzmm*, *Ccr2*, *Ccr5*, and *Itm2a* expression. While confirming previous transcriptomic characterizations of T_{VM} (*6*, *14*, *21*), our scRNA-seq data suggests similar trajectories of differentiation followed by IL-4 or *H. polygyrus*-induced T_{VM}, and further provides unique information about the expanding T_{VM} subset. Although we also observed changes in the expression of these signature genes in IL-4R$\alpha^{\Delta CD8}$, the magnitude of their differential expression was overall reduced when compared to WT littermate controls in the spleen, likely because IL-4R$\alpha^{\Delta CD8}$ are partially knockout and a remaining population of $CD124^+CD8^+$ T cells exist. Indeed, when single cell transcriptomes were studied in sorted $CD124^+$ or $CD124^-$ $CD8^+$ T cells from the mesLN of *H. polygyrus* infected mice, IL-4-induced T_{VM} signature was only found in IL-4Rα-competent cells. When computing the significant differentially expressed genes in both IL-4 and *H. polygyrus*-induced T_{VM}, effector genes *Gzma*, *Gzmm*,

Ctla2a, *Ccr2* and *Ccr5* were upregulated, but we also unexpectedly observed the upregulation of *Cd22*. CD22 is a canonical inhibitory receptor of B lymphocytes which contributes to control autoimmune disease (*40*), and its expression on other cell types has only been scarcely reported (*42–44*, *58*). CD22 is upregulated by microglia in aging brains, where it inhibits phagocytosis (*42*), and one report identified functional CD22 in primary T cells but with a very low surface expression level (*43*). Interestingly, CD22 was the only sialic acid-binding immunoglobulin-type lectin (siglec) gene upregulated in T cells upon IL-4 signaling and we demonstrated that CD22 is expressed at the surface of IL-4-induced T_{VM} specifically, whereas IL-4 did not upregulate CD22 in virus-specific effector/memory T cells, thymocytes or $CD4^+$ T cells. At the cell surface of B lymphocytes, CD22 creates homo-oligomers and also binds to CD45 *in cis* via interactions with α2,6-linked sialic acids (*41*, *59*). However, the B cell receptor (BCR) does not interact with CD22 via sialic acids and both BCR and CD22 are rather found in separate membrane domains (*41*). In mouse models where CD22 cannot bind sialic acid (*54*), less CD22 homo-oligomers are formed, resulting in increased association with the BCR. We have not investigated to which ligand(s) T_{VM} CD22 binds, but we did observe that the $CD22^+$ T_{VM} had a distinct functional phenotype and expressed a diverse TCR repertoire enriched in features of self-reactivity when compared to naive T cells and from $CD22^-$ T_{VM} in response to IL-4. Previous reports showed that IL-4-responding antigen-specific T_{TM} had an altered effector phenotype with reduced NKG2D or CCL5 expression (*60*), but our results highlighted that IL-4 production in response to helminth infection drives an enrichment of genes involved in TCR signaling, cytotoxicity, cell cycle, and the STAT-5 activation pathway. TCR activation is supported by the enriched features of CDR3 sequences associated with self-reactivity, similar to that observed in regulatory T cells (Tregs) (*17*, *22*, *61*). EOMES has been involved in driving self-reactive T_{VM} in the thymus (*17*). Thus, it is possible that IL-4 induces an effector/memory program in self-specific T_{VM} via EOMES activation which is then counter-regulated by CD22 expression. This possibility is also supported by the observed upregulation of the proto-oncogene Src tyrosine protease *Fyn*, related to the oncogene *Lyn* which is required for CD22 inhibitory activity in B lymphocytes as well as increased expression of the adaptor protein-encoding gene *Grb2* and *Ptpn6* (encoding phosphatase SHP-1), both interacting with the immunoreceptor tyrosine-based inhibitory motif (ITIM) of CD22 (*62*). However, CD22 might not be the only inhibitory strategy to counter-regulate IL-4-responding T_{VM}, since increased expression of CD39, CD160, or inhibitory Ly49e/g was also observed in $CD22^+$ T_{VM}. Interestingly, $CD8^+$ T cells expressing inhibitory Ly49 or related killer cell immunoglobulin-like (KIR) receptors in mouse or human, respectively, have been reported to ensure immunoregulation and control immunopathology (*63*, *64*). CD39 was even more strongly expressed in *Cd22*$^{-/-}$, suggesting the absence of CD22 might be at least partially compensated by upregulation of alternative regulatory receptors. Interestingly, naive germline *Cd22*$^{-/-}$ mice had higher numbers of T_{VM} in steady-state and *Cd22*$^{-/-}$ mice had similar worm control than WT mice. However, *Cd22*$^{-/-}$ mice responded to helminth infection with significantly reduced Th2 responses while expanding similar levels of T_{VM}, which suggests CD22 on T cells controls IL-4-driven T_{VM} expansion and activation. Confirming

this hypothesis, *Cd22*$^{-/-}$ or *Cd22*^{Y2,5,6F} T_{VM} in mixed-bone marrow chimeras strongly responded to IL-4 with an increased expansion of T_{VM} producing higher levels of CD39 and GZMA, reporting an increased activation phenotype. When *Cd22*$^{-/-}$ or *Cd22*^{Y2,5,6F} T_{VM} were subjected to TCR-triggering stimulation or bystander IL-12/IL-18 treatment, T_{VM} responded by producing higher levels of IFN-γ as well as increased expression of activation markers like KLRG1, CD39 with an enrichment of CD44^{hi}CD62L^{low} effector T cells. In addition, IAV infection of mixed-bone marrow chimeras resulted in a stronger activation and cytotoxic potential of *Cd22*$^{-/-}$ CD8^{+} T cells in the airway. These data further support the hypothesis that CD22 is directly regulating T cell activation, through yet to uncover mechanisms. Thus, our findings identify CD22 as an inhibitory receptor contributing to peripheral tolerance of self-reactive T_{VM} expansion in response to IL-4 during worm infection. It now remains to be determined how such IL-4-activation of self-reactive T_{VM} and induction of CD22 is a transient phenomenon or if it leaves an imprint to train CD22^{+} T_{VM} in the long term.

Materials and methods

Mice. The experiments, maintenance and care of mice and rats complied with the guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (CETS n° 123). The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Liège, Belgium (Permit nos. 2001 and 2371). All efforts were made to minimize suffering. Female BALB/cAnNCrl and C57BL/6NCrl wild-type mice, 6–8 weeks old, were purchased from Charles River. *E8i*^{Cre} genitor mice were obtained from Prof. A. Thiel (C57BL/6-Tg(Cd8a-cre)1Itan/J, Charité Berlin, Germany). BALB/c CD45.1^{+} genitor mice were generously provided by Prof. U. Eriksson (Center for Molecular Cardiology, University of Zurich). CSP-TCR transgenic Thy1.1 mice were obtained from Dr R. Amino (Institut Pasteur, Paris, France). OT-I TCR transgenic *Rag*$^{-/-}$ mice were obtained from Prof. S. Goriely, Université Libre de Bruxelles, Belgium). Thy1.2.1 (Thy1.1^{+}Thy1.2^{+}) BALB/c mice were obtained by crossing Thy1.1 BALB/c with WT BALB/c. *Il4ra*$^{-/-}$ BALB/c and *Il4ra*$^{lox/lox}$ BALB/c mice were initially obtained from Prof. F. Brombacher (University of Cape Town, South Africa). CD45.1.2 (CD45.1^{+}CD45.2^{+}) C57BL/6 mice were obtained by crossing CD45.1 with CD45.2 C57BL/6 mice. *Cd22*$^{-/-}$ (C57BL/6-*Cd22*^{tm1Lam} /J) mice and *Cd22*^{Y2,5,6F} knockin bone-marrow were obtained from colonies in Erlangen, Germany. Female BALB:B mice (C.B10-*H2*^{b}/LilMcdJ), that are BALB/c congenic for the C57BL/10-derived H-2^{b} region, were obtained from Dr O. Denis (Sciensano, Belgium). GREAT IFN-γ reporter mice (B6.129S4-*Ifng*$^{tm3.1Lky}$/J) have an IRES-eYFP reporter cassette inserted between the translational stop codon and 3' UTR/polyA tail of the *Ifng* gene and were obtained from Jax laboratories. *Ifnar1*$^{-/-}$ C57BL/6 mice were obtained from Kai Dallmeier (Rega Institute, KUleuven). WT C57BL/6 and *Stat6*$^{-/-}$ mice obtained from The Jackson Laboratory and germ-free obtained from the McGill Centre for Microbiome Research were handled with accordance with the McGill University Health Centre Research Institute Animal Resource Division with approved animal use permit no. 7977. Six- to eight-week-old female littermates were randomly assigned to experimental groups. During experiments, 4 to 5 mice were cohoused per cage, food and water was provided ad libitum. All the animals were bred and/or housed at the University of Liège, GIGA-ULiège and Department of Infectious Diseases or at the McGill University Health Centre Research Institute.

Reconstitution of germ-free mice. To colonize GF mice with specific-pathogen-free microbiota (exGF), fresh fecal pellets from C57BL/6 WT mice were homogenized in PBS to a concentration of 62.5 g mL^{-1}. The fecal slurry was centrifuged at 700 × g for 5 min to remove large debris. The supernatant was collected and diluted by ten-fold in PBS before being administered to 6-8 week old GF mice (150 µL per mouse by gavage). exGF were used 3-4 weeks after microbial reconstitution.

Parasites. The lifecycle of *H. polygyrus* was maintained in male C57BL/6 mice as described (*65*). Briefly, 6-8-week-old male C57BL/6 mice were infected with 300 *H. polygyrus* L3 larvae in 200 µL of distilled water by oral gavage. After 14 days post infection, the mice were placed on a grid and fecal pellets collected after several hours. Then, the feces were mixed with granulated charcoal at a ratio of at least a 1:1, to achieve a consistency just damp enough with distilled water to adhere to filter paper. A thin layer was smeared on the center of dampened filter paper in a petri dish placed in a humid box in the dark. The larvae form a ring around the edge of the filter paper and were collected from day 7 onwards for at least three occasions before the paper was discarded. The larvae were collected in distilled water in a 15 mL tube and washed in cold dH_2O and centrifuged at 150 x *g* for 5 min a total of ten times. The purified *H. polygyrus* L3 larvae were stored in distilled water at 4 °C for up to 6 months. For experiments, L3 larvae were washed in distilled water 3 times before administration of 200 × L3 larvae in 200 µL distilled water by gavage. For infection of GF and exGF mice, the *H. polygyrus* lifecycle was maintained in axenic conditions as described (*66*). *S. mansoni*-exposed Swiss-Webster mice were provided by the Schistosome Research Reagent Resource Center for distribution by BEI Resources, NIAID, NIH: *Schistosoma mansoni*, Strain NMRI exposed *Biomphalaria glabrata*, Strain NMRI (NR-21962), *S. mansoni*, Strain NMRI exposed Swiss-Webster mice (NR-21963). *S. mansoni* cercariae were collected from *S. mansoni* exposed *B. glabrata* and used for natural infection. *S. mansoni* eggs used for egg immunization, were collected from *S. mansoni*-exposed Swiss-Webster mouse liver and stored in PBS at −80 °C, as previously described with minor modifications(*6*). Treatment with *S. mansoni* eggs consisted of an intraperitoneal immunization on day 0 (5000 eggs per mouse) followed by one intravenous injection of 5000 eggs on day 14.

Viruses. The strain MHV-68 of Murid gammaherpesvirus-4 (MuHV-4) expressing luciferase under the control of the M3 promoter (MuHV-4-Luc) was propagated, semi-purified and titrated in BHK-21 cells, as described (*6*). Influenza H1N1 strain A/Puerto Rico/8/34 (PR8) was propagated and isolated from Madin–Darby canine kidney (MDCK) cells and titrated with standard plaque assay in MDCK cells. In brief, MDCK cells were cultivated in Dulbecco's modified Eagle's medium (DMEM; Life Technologies), supplemented with 2 mM glutamine, 100 U penicillin, 100 mg streptomycin, and 5% heat-inactivated fetal calf serum (FCS) at 37°C in an atmosphere of 5% CO_2. For PR8 growth, TPCK (L-1-tosylamido-2-phenylethyl chloromethyl ketone)-treated trypsin (2 mg/mL) was added to culture medium. Mice were infected intratracheally (i.t.) under gas anesthesia (isoflurane) with 50 µL sterile PBS containing 10^4 PFU of MuHV-4 or 25 PFU of IAV.

IL-4 complex treatment. Mice received 2 intraperitoneal injections of IL-4c: 5 µg of recombinant IL-4 (BioLegend, carrier-free) and 25 µg of anti-IL-4 antibody (BioLegend, clone 11B11, LEAF purified) per mouse at day 0 and day 2.

Cell preparation. Spleen, thymus and lymph nodes were harvested, cut in small pieces using scissors and filtered through a 100 µm cell strainer with a sterile syringe plunger. Erythrocytes were lysed in red cell lysis solution (155 mM NH_4Cl, 0.12 mM EDTA, 10 mM $KHCO_3$), and leukocytes suspended in PBS and filtered through a 40 µm cell strainer. Airways were flushed twice with 1 mL of ice cold PBS, and cells were harvested by centrifugation of the BAL fluid. After section of the vena cava, lungs were perfused with 5 mL of ice-cold PBS through the right ventricle. Lungs were dissociated with the gentleMACS dissociator (Miltenyi Biotec) in C-tube (Miltenyi Biotec), incubated in HBSS (Gibco), 5% FCS, 1 mg mL^{-1} of collagenase D (Roche) and 0.1 mg mL^{-1} of DNase I (Roche) for 30 min at 37°C under agitation and further dissociated with the gentleMACS dissociator. The resulting suspension was washed in cold PBS/2mM EDTA and filtered on a 100µm cell strainer (Falcon). Cells were counted in a Neubauer cytometer chamber in 0.4% buffered trypan blue dye for exclusion of dead cells.

Antibodies and flow cytometry. Incubations were performed in FACS buffer (PBS containing 0.1% BSA and 0.05% NaN3) at 4°C. Cells were first incubated with anti-mouse CD16/32 antibody (clone 93, 1 µg mL^{-1}, isotype Rat IgG2a, λ, BioLegend) before fluorochrome-conjugated antibodies against surface antigens were added and incubated during 20 min at 4 °C. Various panels were used including antibodies to CD3ε (145-2C11, 0.4 µg mL^{-1}, APC-Cy7, Armenian Hamster IgG1, κ), CD183 (CXCR3-173, 2 µg mL^{-1}, BV421 or PE, Armenian Hamster IgG1, κ), CD49d (R1-2, 1 µg mL^{-1}, BV650 or BV786, CDF IgG2b, κ), CD124 (mIL4R-M1, 1 µg mL^{-1}, PE or BV421, Lewis IgG2a, κ), CD195 (C34-3448, 2 µg mL^{-1}, BV711, Rat IgG2c, κ), CD62L (MEL-14, 1 µg mL^{-1}, FITC or BV785, CDF IgG2a, κ), CD4 (GK1.5, 2 µg mL^{-1}, BUV395, Rat IgG2b, κ), CD44 (IM7, 0.4 µg mL^{-1}, BV786, Rat IgG2b, κ) all from BD Biosciences, antibody to CD3ε (145-2C11 or 17A2, 0.4 µg mL^{-1}, V450 or APC, Armenian Hamster IgG or Rat IgG2b, κ), KLRG1 (2F1, BV786, Syrian Hamster IgG2, κ), TCRβ chain (H57-597, 0.8 µg mL^{-1}, BV711, Armenian Hamster IgG), CD8α (53-6.7, 1 µg mL^{-1}, FITC or APC or BV785 or APC/Fire 750 or BUV615, Rat IgG2a, κ; 2 µg mL^{-1}, BV605, Rat IgGa, κ), CD44 (IM7, 0.4 µg mL^{-1}, PE-Cy7, Rat IgG2b, κ), CD183 (CXCR3-173, 2 µg mL^{-1}, FITC or APC, Armenian Hamster IgG), CD124 (I015F8, 1 µg mL^{-1}, APC, Lewis IgG2a, κ), CD39 (Duha59, 2 µg mL^{-1}, APC or PE-Cy7, Rat IgG2a, κ), CD4 (GK1.5, 0.4 µg mL^{-1}, FITC or APC, Rat IgG2b, κ), CD24 (M1/69, 2 µg mL^{-1}, APC/Fire 750, Rat IgG2b, κ), CD45.1 (A20, 2 µg mL^{-1}, BV421 or APC-Cy7 or APC or Alexa Fluor 700, Mouse (A.SW) IgG2a, κ), CD45.2 (104, 1 µg mL^{-1}, BV510 or PE-Cy7 or Alexa Fluor 700, Mouse (SJL) IgG2a, κ), CD90.1 (OX-7, 2,5 µg mL^{-1}, APC or Alexa Fluor 700, Mouse IgG1, κ), CD90.2 (53-2.1, 1 µg mL^{-1}, BV421, Rat IgG2a, κ), CD122 (TM-β1, APC-Cy7, 1 µg mL^{-1}, Rat IgG2b, κ), CD192 (SA203G11, 2 µg mL^{-1}, BV650, Rat IgG2b, κ), CD160 (7H1, 2 µg mL^{-1}, PerCP-Cy5.5, Rat IgG2a, κ), TCR Vβ8.1, 8.2 (MR5-2, 2 µg mL^{-1}, PE, Mouse IgG2a, κ) from BioLegend, CD45.2 (104, 2 µg mL^{-1}, eFluor450, Mouse IgG2a, κ), TCRβ chain (H57-597, 2 µg mL^{-1}, APC-eF780, Armenian Hamster IgG), CD49d (R1-2, 2 µg mL^{-1}, PerCP710 or PE, Rat IgG2b, κ), CD4 (RM4-5, 2 µg mL^{-1}, AF700, Rat IgG2a,

κ), CD44 (IM7, 2 µg mL^{-1}, PE, Rat IgG2b, κ) from ThermoFisher and antibodies to CD22 (Cy34.1, 3 µg ml^{-1}, PE or biotinylated, isotype mouse IgG1κ) from Miltenyi Biotec. Biotinylated antibodies were detected using Qdot 625-conjugated streptavidin (5 nM, ThermoFisher). Dead cells were stained using Zombie Aqua Fixable Viability Kit (1000x dilution, Biolegend) or Fixable Viability Dye (1000× dilution, eFluor780 or eFluor 506, eBioscience). For detection of intracellular IFN-γ (XMG1.2, BV711 (2 µg mL^{-1}, Biolegend) or PE (2 µg mL^{-1}, Biolegend), or AF488 (5 µg mL^{-1}, ThermoFisher) or APC (2 µg mL^{-1}, ThermoFisher), Rat IgG1, κ), GZMA (3G8.5, 2 µg mL^{-1}, PE (BioLegend) or APC ThermoFisher, Mouse IgG2b, κ), or GZMB (QA16102, 2 µg mL^{-1}, PE, Mouse IgG1 κ, BioLegend), cells were fixed in paraformaldehyde 2% in PBS on ice for 20 min, before permeabilized and stained in FACS buffer containing 0.1% saponin for 30 min at 4°C. In experiments in which intranuclear staining for transcription factors was needed, cells were fixed and permeabilized using Foxp3/Transcription factor staining buffer set (eBioscience) following manufacturer's instruction and incubated 30 min at 4°C with antibody against EOMES (Dan11mag, 2 µg mL^{-1}, PE or PE-eFluor610, Rat IgG2a, κ, eBioscience, or X4-83, 2 µg mL^{-1}, Alexa Fluor 488, Mouse IgG1, κ, BD Biosciences) or GATA3 (TWAJ, 5 µL per test, AF488 or PerCP710, Rat IgG2b, κ, ThermoFisher), diluted in permeabilization buffer. Samples were analyzed on a Fortessa X-20 or FACSymphony A5SE flow cytometer (BD Biosciences). Flow cytometry acquisitions were analyzed using FlowJo 10.8 or 10.9. In some analyses, samples were concatenated and further analyzed using the built-in plugins UMAP and FlowSOM. In some experiments, samples were analyzed using an ImageStream X Mk II (Amnis, VIB Flow Core Facility, UGhent). For tetramer stainings, cells were incubated with BV421-conjugated tetramers H-$2D^b$-$ORF6^{487-495}$ (AGPHNDMEI, 90 nM) or H-$2K^b$-$ORF61^{521-531}$ (TSINFVKI, 45 nM) (NIH Tetramer Core Facility) for 30min at RT before further staining.

***Ex vivo* restimulation and cytokine production.** Cytokine production upon restimulation was assessed by intracellular cytokine staining (ICCS) and flow cytometry. For ICCS, cells were cultured at 37°C, 5% CO_2 in IMDM complemented with 2 mM β-mercaptoethanol, 100 U mL^{-1} penicillin, 100 mg mL^{-1} streptomycin and 10% fetal calf serum for 4h in presence of brefeldin A (10 µg mL^{-1}, Sigma-Adlrich), monensin (2 µM, eBioscience). Unbiased restimulation was performed using phorbol 12-myristate 13-acetate (PMA, 20 ng mL^{-1}, Sigma-Adlrich) and ionomycin (1 µg mL^{-1}, Sigma-Aldrich). Innate restimulation was performed using IL-12 (5 ng mL^{-1}, BioLegend) and IL-18 (10 ng mL^{-1}, BioLegend) for 16h and brefeldin A was added for the last 3h of incubation. In some experiments, anti-CD107a (1D4B, 0.5 µg mL^{-1}, PerCP-Cy5.5, Rat IgG2a, κ) was added to the stimulation media. TCR-specific restimulation was done using anti-CD3 (145-2C11, 1 µg mL^{-1}, purified, BioLegend) and anti-CD28 (37.51, 1 µg mL^{-1}, purified, BioLegend) mixed with brefeldin A and monensin for 4h. Following surface and viability stainings, cells were fixed in 2% paraformaldehyde overnight and washed with Permeabilization Buffer (eBioscience) before being incubated with antibodies against IFN-

γ (clone XMG1.2, 2 µg mL^{-1}, BV711 or PE, Rat IgG1, κ, BioLegend) in Permeabilization Buffer for 20 min at 4°C.

Cell isolation for single-cell RNA-seq assay. Single-cell suspension were obtained from harvested spleen or mesLN before enrichment of $CD8^+$ T lymphocytes using the MojoSort Mouse CD8 T Cell Isolation Kit (Biolegend) and LS columns (Miltenyi Biotec). For spleen samples, enriched $CD8^+$ T cells isolated from mice of the same genotype and having received the same treatment were individually stained with 0.5 µg per 1.5 million cells of TotalSeq anti-mouse hashtag antibodies (A0301 to A0305, Biolegend) and incubated at 4°C during 20 min before pooling (n=4 to 5). Pools were then filtered through a 40 µm cell strainer and the concentration adjusted to 700 to 1000 cells μL^{-1} before single cell capture using the Chromium controller (10x Genomics). For mesLN samples, enriched $CD8^+$ T cells from IL-4RαΔ^{CD8} mice were pooled and further sorted to high purity based on the expression of CD124. Then, $CD124^+$ or $CD124^-$ were stained with 20 µL per 1 million cells of Sample Tag 1 or Sample Tag 2 from the Mouse Immune Single-Cell Multiplexing Kit (BD Biosiences) and pooled before filtered through a 40 µm cell strainer, before being subjected to single cell capture and quality control using the BD Rhapsody Express and BD Rhapsody Scanner (BD Biosiences).

Mixed bone-marrow chimeric mice models. Mixed bone-marrow chimeric mice were produced by treating $Thy1.1^+Thy1.2^+$ BALB/c mice (Thy1.2.1) or $CD45.1^+CD45.2^+$ C57BL/6 mice (CD45.1.2) with intraperitoneal injection of busulfan (25 µg g^{-1} of mouse body weight per day for 3 consecutive days, Cayman Chemical) for myeloablation, as previously reported (*67*). Then, mice were reconstituted by intravenous injection of 2×10^6 bone marrow cells 24h after the last busulfan injection. Bone marrow cells were obtained from femurs and tibias of donor $CD45.1^+$ WT and $CD45.2^+$ *Il4ra*$^{-/-}$ mice, or of donor $CD45.1^+$ WT and $CD45.2^+$ *Cd22*$^{-/-}$ or *Cd22*^{Y2,5,6F} mice and mixed at a 1:1 ratio before intravenous injection in 100 µL sterile PBS into Thy1.2.1 mice or CD45.1.2 mice, respectively. Mice were left untreated for 6 weeks to allow complete reconstitution and chimerism of circulating $CD8^+$ T cells was then confirmed by flow cytometry, before used in experiments.

Single-cell RNA library preparation. Spleen samples were processed using the 10x genomics Chromium Single Cell 3' v3 chemistry as per the manufacturer's recommendations (10x Genomics). Samples obtained from mesLN were processed using the BD Rhapsody Whole Transcriptome Analysis cDNA synthesis and amplification kit as per the manufacturer's recommendations (BD Biosiences). Barcoded RNA was collected and processed following the manufacturer's recommendations. After quantification, equal molar concentrations of each library were pooled and sequenced using the NovaSeq S4 300 cycles XP workflow (GIGA ULiege, Belgium).

Single-cell RNA-seq analysis. *Raw data processing.* For 10x Genomics platform, the sequencing run was processed using Cell Ranger (v3.0.2). For BD Rhapsody platform, sequencing run was processed using SevenBridges Genomics. First using mkfastq function to produce fastq files and then the count function to perform alignment, filtering, barcode counting, and UMI counting. The merged data were transferred to the R statistical environment for analysis using the package Seurat (v3.1.5) (*68*). *Doublet cell filtering.* Cell Hashing/HTO libraries were processed using CITE-seq Count python package v1.4.3 (https://github.com/Hoohm/CITE-seq-Count) and used in Seurat to remove identified Doublet cells. Sample Tag demultiplexing for BD Rhapsody platform was performed using the build-in function in SevenBridges Genomics. *Data cleaning.* We additionally applied two filtering steps as we selected cells with a total number of molecules detected within a range between 2000 and 10,000 and a percentage of reads that map to the mitochondrial genome lower than 10%. *Samples integration & Transcriptome-based clustering.* We used Seurat SCTransform workflow for Multiple Dataset Integration in order to merge all the datasets and proceed with joint analysis. We used FindIntegrationAnchors and IntegrateData functions based on SCT normalization. We then performed PC analysis with RunPCA algorithm producing 50 PCs and we applied FindNeighbors and FindClusters functions from Seurat with two chosen parameters (dims = 1:50; resolution = 0.5) to identify clusters. *Data visualization.* Data were then dimensionally reduced for visualization using RunUMAP function and using (dims = 1:50). UMAP figures were obtained using DimPlot function with UMAP reduction. Other plot types were generated using implemented Seurat functions, custom R scripts or combinations of both. *SingleR for cluster annotation and selection.* SingleR package was performed for unbiased annotation and was performed against the build-in ImmGen database in SingleR package. Clusters showing expression of CD8a and CD3e were selected, and other clusters were removed. Cells presenting T8.memory or T8.effector profiles were selected for additional analysis. New clustering of this subset of cells was performed using same methodology as previously described. *Single-cell differential gene expression analysis.* Cluster specific markers were obtained by the FindAllMarkers function of Seurat with default parameters. Pairwise single-cell differential gene expression analysis was performed between condition inside clusters using the FindMarkers function with default parameters. *Gene set enrichment analysis*. TF-gene interactions were extracted from mouse DoRothEA database (*69*), while filtering for high-confidence interactions (score A, B, or C). TF activities were then inferred from differential gene expression results using fgsea (*70*), with signed logarithm of p-value used as the metric to rank the genes. Method details are available here https://github.com/BlanQwall/VM_T.

RNA sequencing. Single-cell suspension were obtained from harvested spleens before enrichment of $CD8^+$ T lymphocytes using the MojoSort Mouse CD8 T Cell Isolation Kit (Biolegend) and LS columns (Miltenyi Biotec). Then, around 100,000 cells were sorted in IMDM 50% FCS using a FACS Aria IIIu (BD biosciences). Total RNA was purified using the RNAeasy Micro Kit (Qiagen) and eluted in 14µL of water. RNA integrity was further verified using a 2100 Agilent Bioanalyzer and RIN

> 9 were used for further analyses. Total RNA was then used as input for the SMART-seq HT cDNA synthesis kit (Takara). Libraries were quality checked on an Agilent Bioanalyzer, pooled at an equimolar ratio and sequenced on a Novaseq S4 V1.5 with 300 cycles XP workfow. Reads were mapped to the mouse reference genome (mm10) using STAR (version 3.4.0). Subsequently the analysis was performed with R Bioconductor packages Rsamtools (version 1.18.3) and GenomicAlignments (version 1.2.2) were used to count the reads by exons, and gene count datasets were then analyzed to determine DE genes (DEGs) using DESeq2 (version 1.16.1). A gene was determined to be a DEG by passing FDR < 0.01 and log 2-fold change ≥ ±1.

TCR sequencing. Single-cell suspensions were obtained from harvested spleens before enrichment of $CD8^+$ T lymphocytes using the MojoSort Mouse CD8 T Cell Isolation Kit (Biolegend) and LS columns (Miltenyi Biotec). Then, 200,000 cells were sorted in IMDM 50% FCS using a FACS Aria IIIu (BD biosciences). RNA extraction was carried out using the RNeasy Micro Kit (Qiagen, 217084). High-throughput sequencing was performed as previously described with template-switch anchored RT-PCR but using oligodT during the cDNA generation and the following mouse-specific Cα and Cβ primers for the PCR amplification: TRAC '5-*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG*GTCCTGAGACCGAGGATCTTT and TRBC '5-*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG*GGGTAGCCTTTTG-TTTGTTTG (adapters in italic).

CDR3 sequence analysis. CDR3 sequences were extracted from raw sequencing fastq files after aligning the reads to reference V, D and J genes from 'TRA' or 'TRB' loci of GenBank database using the MiXCR software (version 3.0.12) (*71*). CDR3 sequences were further analyzed using VDJtools software version 1.2.1 (*72*). CDR3 extraction was also performed on raw fastq files derived from RNA-seq experiments to increase the robustness of the data in all analyzed groups, this analysis was performed following the standard pipeline for CDR3 extraction from RNA-seq data described in MiXCR documentation. In all cases out of frame sequences were excluded from the analysis, as well as non-functional TRA and TRB segments using IMGT (the international ImMunoGeneTics information system®) annotation. Cumulative gene segment plots were generated using the output from CalcSegmentUsage function. Tree maps were generated using the Treemap Package (version 2.4.3) on RStudio. Diversity read-outs (Normalized Shannon-Wiener, Inverse Simpson, Chao1 and efronThisted indexes) were obtained from the re-sampled file generated using CalcDiversityStats function. Cysteine usage was determined following previously described indications (*73*). Hydrophobic doublets in CDR3α and CDR3β sequences were determined by calculating the percentage of sequences using any of the 175 amino acid doublets previously identified as promoters of self-reactivity (*47*). Physicochemical properties were computed on the 5 central amino acids from the CDR3 sequences using CalcCdrAaStats function from VDJtools software. Principal component analysis of CDR3α and CDR3β repertoire was

performed using R package FactoMineR (version 2.7) and using the variables described in **Fig. S8i** which were scaled to similar range using Z-score normalization prior to PCA analysis. PCA results were visualized using factoextra (version 1.0.7) R package.

Statistical analysis. Statistical evaluation of different groups was performed either by analysis of variance (ANOVA) followed by the Dunnett or Sidak multiple-comparison test or by non-parametric Mann-Whitney test, as indicated. A p-value < 0.05 was considered significant. Statistical analyses were performed using Prism v8 and v9 (GraphPad, La Jolla, CA).

Data repository. Sequencing data access number GSE228564.

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Figures

Figure 1

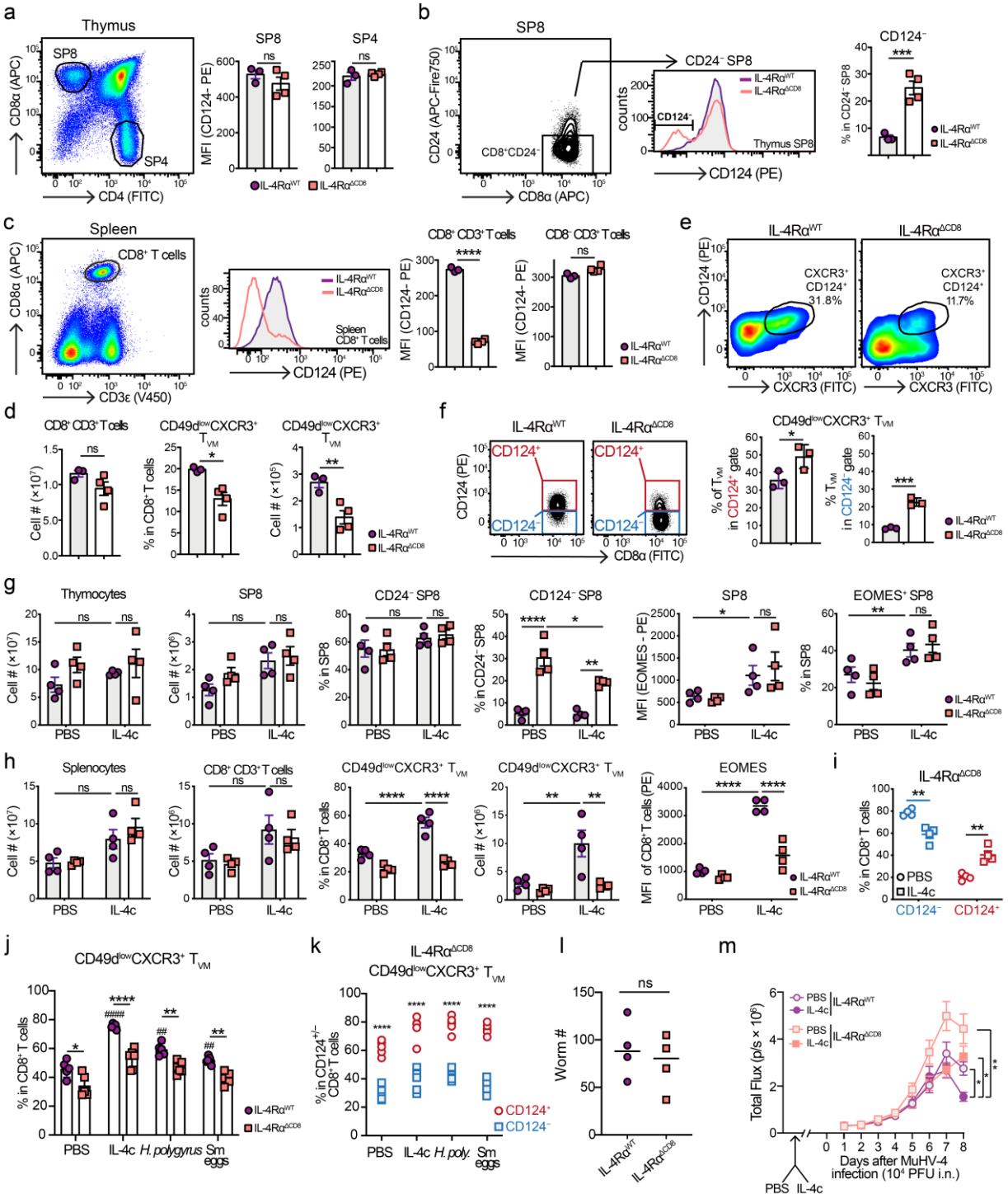


Figure 1. Impaired peripheral T_{VM} expansion in IL-4Rα$^{\Delta CD8}$ mice during helminth infection.
(**a**) Representative pseudocolour flow plot of TCRβ$^+$ thymocytes. Median fluorescence intensities (MFI) of CD124 in single positive CD8$^+$ (SP8) and CD4$^+$ thymocytes (SP4).
(**b**) Representative contour flow plot of SP8 thymocytes and representative histogram of CD24$^-$ SP8 thymocytes. Summary data of CD124$^-$ cell percentages in CD24$^-$ SP8 are shown, as determined by flow cytometry.
(**c**) Representative pseudocolour flow plot of spleen live lymphocytes and representative histogram of CD3$^+$CD8$^+$ T splenocytes. Median fluorescence intensities (MFI) of CD124 of the indicated population.
(**d**) Summary data of CD8$^+$CD3$^+$ T cell numbers and CD44^{hi}CXCR3$^+$ CD49d^{low} CD8$^+$ T cell numbers and percentages, as determined by flow cytometry.
(**e**) Representative pseudocolour flow plot of splenic CD8$^+$ T lymphocytes. Numbers indicate percent of events in each gate.
(**f**) Representative contour flow plot of splenic CD8$^+$ T lymphocytes and summary data of CD49d^{low}CXCR3$^+$ T_{VM} percentages in CD124$^+$ and CD124$^-$ CD8$^+$ T cells, as gated by flow cytometry.
(**g**-**h**) IL-4c was injected to IL-4Rα^{WT} or IL-4Rα$^{\Delta CD8}$ mice at d0 and d2 before analysis at d4. Data show summary plots of cell numbers, percentages, and median fluorescence intensities (MFI) of EOMES of the indicated populations in the thymus (**g**) or the spleen (**h**).
(**i**) Summary plots of CD124$^+$ and CD124$^-$ CD8$^+$ T cells percentages in naive or IL-4c-treated IL-4Rα$^{\Delta CD8}$ mice.
(**j**) Summary data of CD49d^{low}CXCR3$^+$ T_{VM} percentages in the spleen of IL-4Rα^{WT} or IL-4Rα$^{\Delta CD8}$ mice after treatment with IL-4c, at day 15 after *H. polygyrus* infection, and at d22 after administration of *S. mansoni* (Sm) eggs.
(**k**) Summary data of CD49d^{low}CXCR3$^+$ T_{VM} percentages in CD124$^+$ and CD124$^-$ CD8$^+$ T cells in IL-4Rα$^{\Delta CD8}$ mice, based on the results in (j).
(**l**) *H. polygyrus* adult worm burden in the gut at day 15 after infection in the indicated mouse group.
(**m**) IL-4c was injected to IL-4Rα^{WT} or IL-4Rα$^{\Delta CD8}$ mice at d0 and d2 before MuHV-4-Luc infection intranasally (i.n.) at d4. Combined dorsal and ventral measurements by live imaging of thoracic light emission following D-luciferin injection are shown. p s^{-1} = photons per second.
Statistical significance calculated using Mann-Whitney test (**a-f, l**) or two-way analysis of variance (ANOVA) and Sidak's multiple comparison-test (**g-k, m**) (*P < 0.05, *P < 0.01, ***P < 0.001, ****P < 0.0001; $^{\#\#}P$ < 0.01, $^{\#\#\#\#}P$ < 0.0001). Data are representative of 2 to 3 independent experiments with 3-5 mice per group. Mean ± s.e.m., each symbol represents one individual mouse.

Figure 2

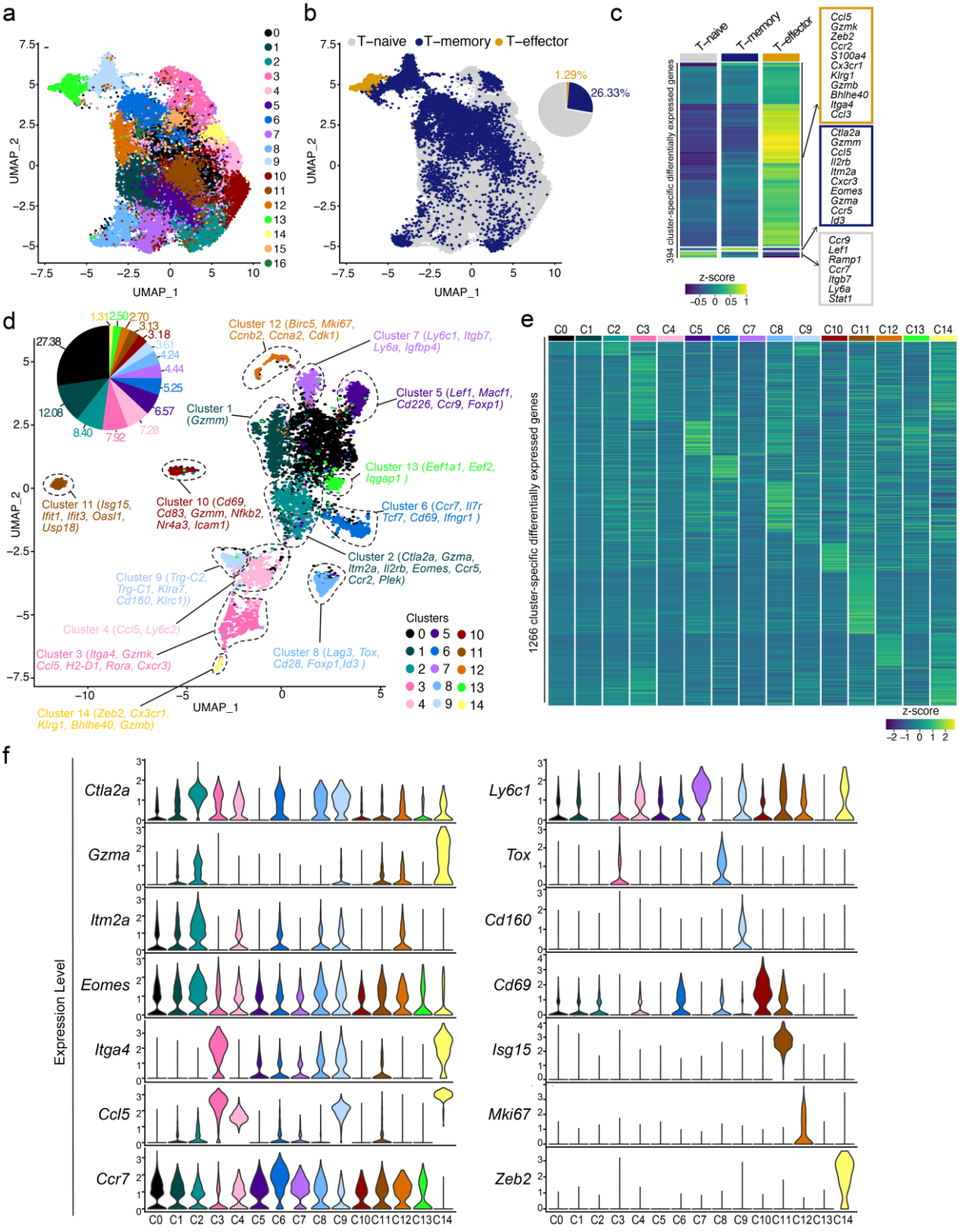

a
b
c
d
e
f
T−naive
T−memory
T−effector
1.29%
26.33%
UMAP_1
UMAP_2
394 cluster-specific differentially expressed genes
z-score
Ccl5 Gzmk Zeb2 Ccr2 S100a4 Cx3cr1 Klrg1 Gzmb Bhlhe40 Itga4 Ccl3
Ctla2a Gzmm Ccl5 Il2rb Itm2a Cxcr3 Eomes Gzma Ccr5 Id3
Ccr9 Lef1 Ramp1 Ccr7 Itgb7 Ly6a Stat1
Cluster 1 (Gzmm)
Cluster 2 (Ctla2a, Gzma, Itm2a, Il2rb, Eomes, Ccr5, Ccr2, Plek)
Cluster 3 (Itga4, Gzmk, Ccl5, H2-D1, Rora, Cxcr3)
Cluster 4 (Ccl5, Ly6c2)
Cluster 5 (Lef1, Macf1, Cd226, Ccr9, Foxp1)
Cluster 6 (Ccr7, Il7r Tcf7, Cd69, Ifngr1)
Cluster 7 (Ly6c1, Itgb7, Ly6a, Igfbp4)
Cluster 8 (Lag3, Tox, Cd28, Foxp1,Id3)
Cluster 9 (Trg-C2, Trg-C1, Klra7, Cd160, Klrc1))
Cluster 10 (Cd69, Cd83, Gzmm, Nfkb2, Nr4a3, Icam1)
Cluster 11 (Isg15, Ifit1, Ifit3, Oasl1, Usp18)
Cluster 12 (Birc5, Mki67, Ccnb2, Ccna2, Cdk1)
Cluster 13 (Eef1a1, Eef2, Iqgap1)
Cluster 14 (Zeb2, Cx3cr1, Klrg1, Bhlhe40, Gzmb)
Clusters
1266 cluster-specific differentially expressed genes
Expression Level
Ctla2a
Gzma
Itm2a
Eomes
Itga4
Ccl5
Ccr7
Ly6c1
Tox
Cd160
Cd69
Isg15
Mki67
Zeb2

Figure 2. Single-cell RNA sequencing reveals T_{VM} cluster.
(**a**) UMAP visualization of unsupervised Seurat clustering analysis of combined 33,553 single $CD8^+$ T cell transcriptomes of IL-4Rα^{WT} or IL-4R$\alpha^{\Delta CD8}$ mice (n=29) based on the experimental design in Fig. S2a.
(**b**) Supervised SingleR clustering analysis to identify naive, memory and effector T cell clusters. The pie chart shows the cell number proportion of each cluster.
(**c**) Heat map of row-wise *z*-score-normalized expression for 394 cluster-specific differentially expressed genes used to establish the SingleR clustering in (b). Rows are ordered by hierarchical clustering. Genes for biologically relevant example genes for each cluster are shown.
(**d**) UMAP visualization of unsupervised Seurat clustering analysis of combined 8,198 single memory and effector $CD8^+$ T cell transcriptomes of IL-4Rα^{WT} or IL-4R$\alpha^{\Delta CD8}$ mice (n=29), based on SingleR clustering in (b). Cluster labels indicate selected differentially expressed genes.
(**e**) Heat map of row-wise *z*-score-normalized expression for 1266 cluster-specific differentially expressed genes used to establish the Seurat clustering analysis in (d). Columns represent the average expression for each cluster.
(**f**) Violin plots show normalized expression in each cluster of 14 cluster-specific signature genes.

Figure 3

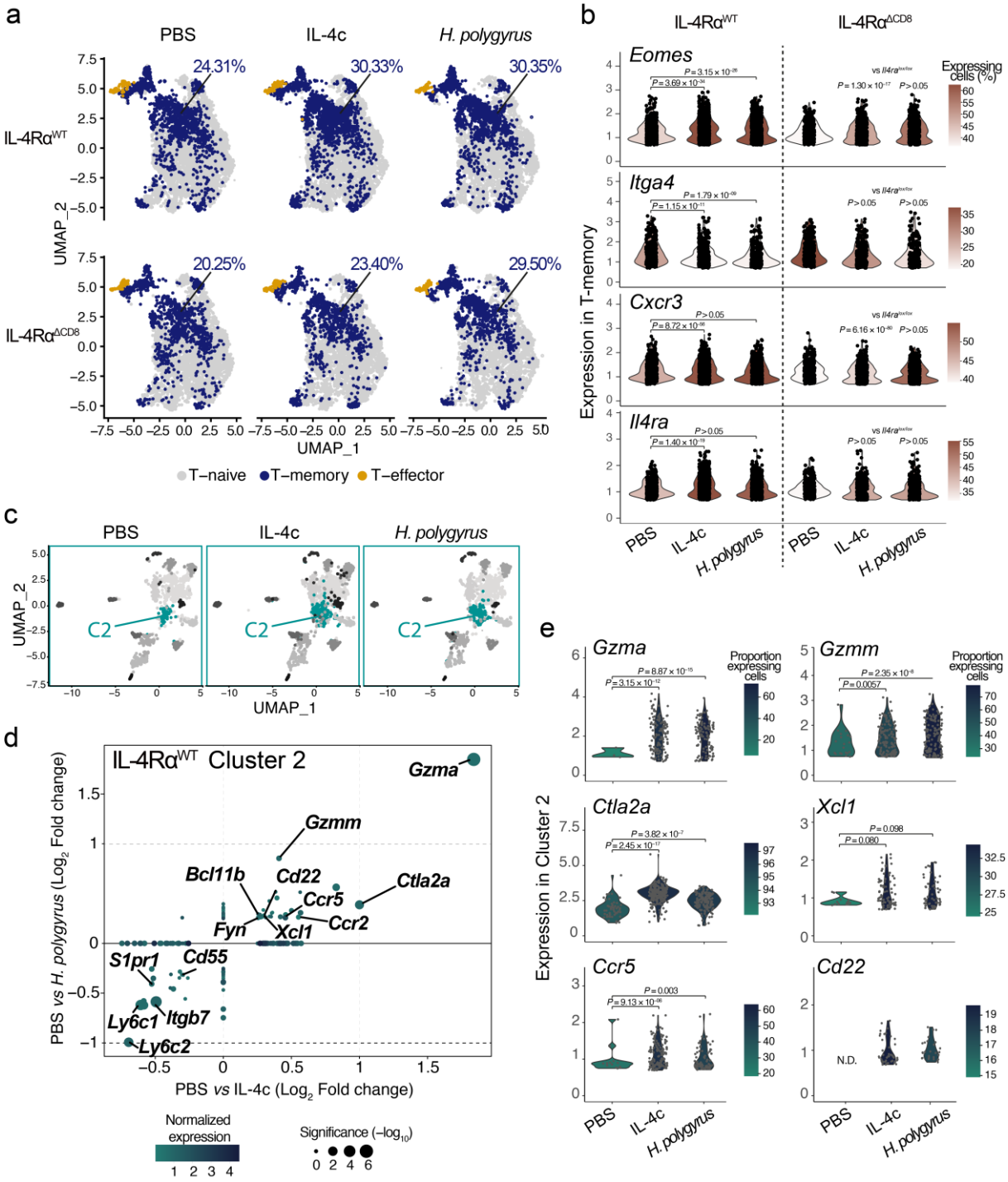

a
PBS
IL-4c
H. polygyrus
24.31%
30.33%
30.35%
20.25%
23.40%
29.50%
IL-4Rα^WT
IL-4Rα^ΔCD8
UMAP_2
UMAP_1
T−naive
T−memory
T−effector
b
IL-4Rα^WT
IL-4Rα^ΔCD8
Eomes
Itga4
Cxcr3
Il4ra
Expressing cells (%)
Expression in T-memory
PBS
IL-4c
H. polygyrus
c
PBS
IL-4c
H. polygyrus
C2
UMAP_2
UMAP_1
d
IL-4Rα^WT Cluster 2
Gzma
Gzmm
Bcl11b
Cd22
Ctla2a
Ccr5
Ccr2
Fyn
Xcl1
S1pr1
Cd55
Ly6c1
Itgb7
Ly6c2
PBS vs H. polygyrus (Log2 Fold change)
PBS vs IL-4c (Log2 Fold change)
Normalized expression
Significance (−log10)
e
Gzma
Gzmm
Ctla2a
Xcl1
Ccr5
Cd22
N.D.
Proportion expressing cells
Expression in Cluster 2
PBS
IL-4c
H. polygyrus

Figure 3. Single-cell transcriptomic analysis of spleen T_{VM} upon IL-4 response.
(**a**) Split UMAP visualization of supervised SingleR clustering analysis of combined $CD8^+$ T cell transcriptomes of IL-4Rα^{WT} and IL-4R$\alpha^{\Delta CD8}$ mice, following treatment with PBS, IL-4c, or *H. polygyrus* infection. Percent of cells in cluster T-memory is indicated for each dataset.
(**b**) Violin plots show normalized expression in the T-memory cluster of selected signature genes. Each dot represents a single cell included in the T-memory cluster and expressing the given gene. The color scale represents the percentage of cells within each cluster expressing the given gene, excluding cells with no expression.
(**c**) Split UMAP visualization of T-memory and T-effector SingleR clustering, highlighting cluster 2 (C2) as identified in Fig. 2d.
(**d**) Scatter plot shows the $\log_2$ fold change of selected biologically relevant genes between IL-4c vs. PBS (x-axis) and *H. polygyrus* vs. PBS (y-axis) mice among cells in cluster 2 from memory/effector SingleR analysis in Fig. 2d. Data are from IL-4Rα^{WT} mice. Each dot represents one gene. The color scale indicates normalized expression. The size of the dot indicates statistical significance.
(**e**) Violin plots show normalized expression in cluster 2 of six selected signature genes. Each dot represents a single cell included in cluster 2 and expressing the given gene. The color scale represents the percentage of cells within each cluster expressing the given gene, excluding cells with no expression.

Figure 4

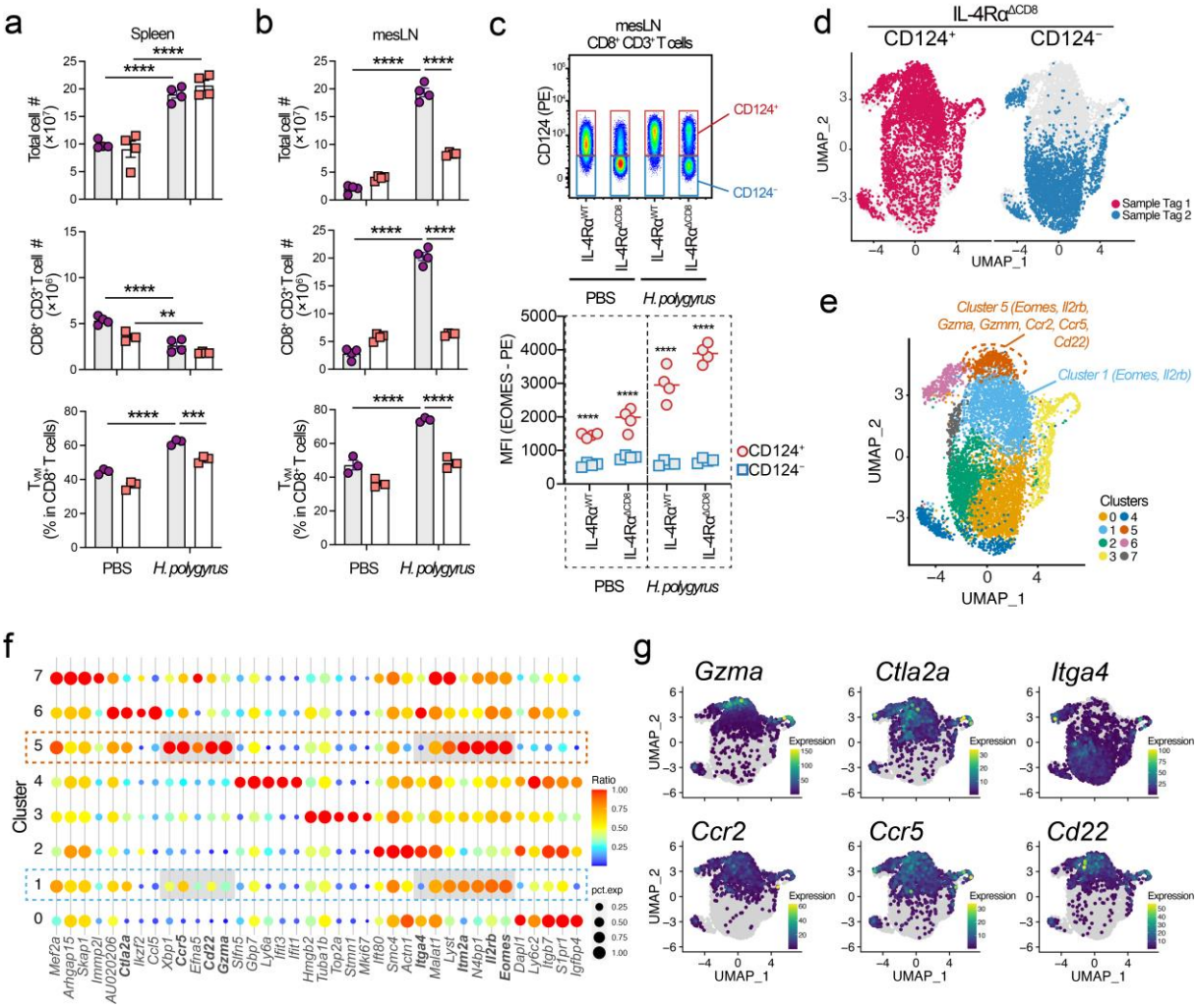

a
Spleen
Total cell # (×10⁷)
CD8⁺ CD3⁺ T cell # (×10⁶)
TVM (% in CD8⁺ T cells)
PBS
H.polygyrus
b
mesLN
c
mesLN
CD8⁺ CD3⁺ T cells
CD124 (PE)
CD124⁺
CD124⁻
IL-4Rα^WT
IL-4Rα^ΔCD8
MFI (EOMES - PE)
d
IL-4Rα^ΔCD8
CD124⁺
CD124⁻
UMAP_2
UMAP_1
Sample Tag 1
Sample Tag 2
e
Cluster 5 (Eomes, Il2rb, Gzma, Gzmm, Ccr2, Ccr5, Cd22)
Cluster 1 (Eomes, Il2rb)
Clusters
f
Cluster
Ratio
pct.exp
Mef2a
Arhgap15
Skap1
Immp2l
AU020206
Ctla2a
Ikzf2
Ccl5
Xbp1
Ccr5
Efna5
Cd22
Gzma
Slfn5
Gbp7
Ly6a
Ifit3
Ifit1
Hmgb2
Tuba1b
Top2a
Stmn1
Mki67
Ifit80
Smc4
Actn1
Itga4
Malat1
Lyst
Itm2a
N4bp1
Il2rb
Eomes
Dapl1
Ly6c2
Itgb7
S1pr1
Igfbp4
g
Gzma
Ctla2a
Itga4
Ccr2
Ccr5
Cd22
Expression
UMAP_2
UMAP_1

Figure 4. Gene expression signature of T_{VM} in the mesLN after helminth infection.

(**a**-**b**) Summary data of total leukocytes, $CD8^+$ T cell numbers and T_{VM} percentages in the spleen (**a**) and mesLN (**b**) at day 15 after *H. polygyrus* infection.

(**c**) Representative contour plots of CD124 expression of concatenated samples, based on the experimental condition. Summary data of median fluorescence intensities (MFI) of EOMES expression in $CD124^+$ or $CD124^-$ cells are shown.

Statistical significance calculated using two-way analysis of variance (ANOVA) and Sidak's multiple comparison-test (***P* < 0.01, ****P* < 0.001, *****P* < 0.0001). Data are representative of three independent experiments with 3-4 mice per group. Mean ± s.e.m., each symbol represents one individual mouse.

(**d**) Split UMAP visualization of combined 6,808 single $CD8^+$ T cell transcriptomes obtained by scRNA-seq analysis of FACsorted then pooled sample tagged $CD124^+$ and $CD124^-$ $CD8^+$ T cells from mesLN of IL-4R$\alpha^{\Delta CD8}$ mice at day 15 after *H. polygyrus* infection (n=3).

(**e**) UMAP visualization of Seurat clustering analysis. Cluster 5 labels indicate selected differentially expressed genes.

(**f**) Dot plot representation of the top 5 upregulated gene expression in each cluster, based on (f).

(**g**) FeaturePlot representation of selected gene expression in single $CD8^+$ T cells, based on (f).

Figure 5

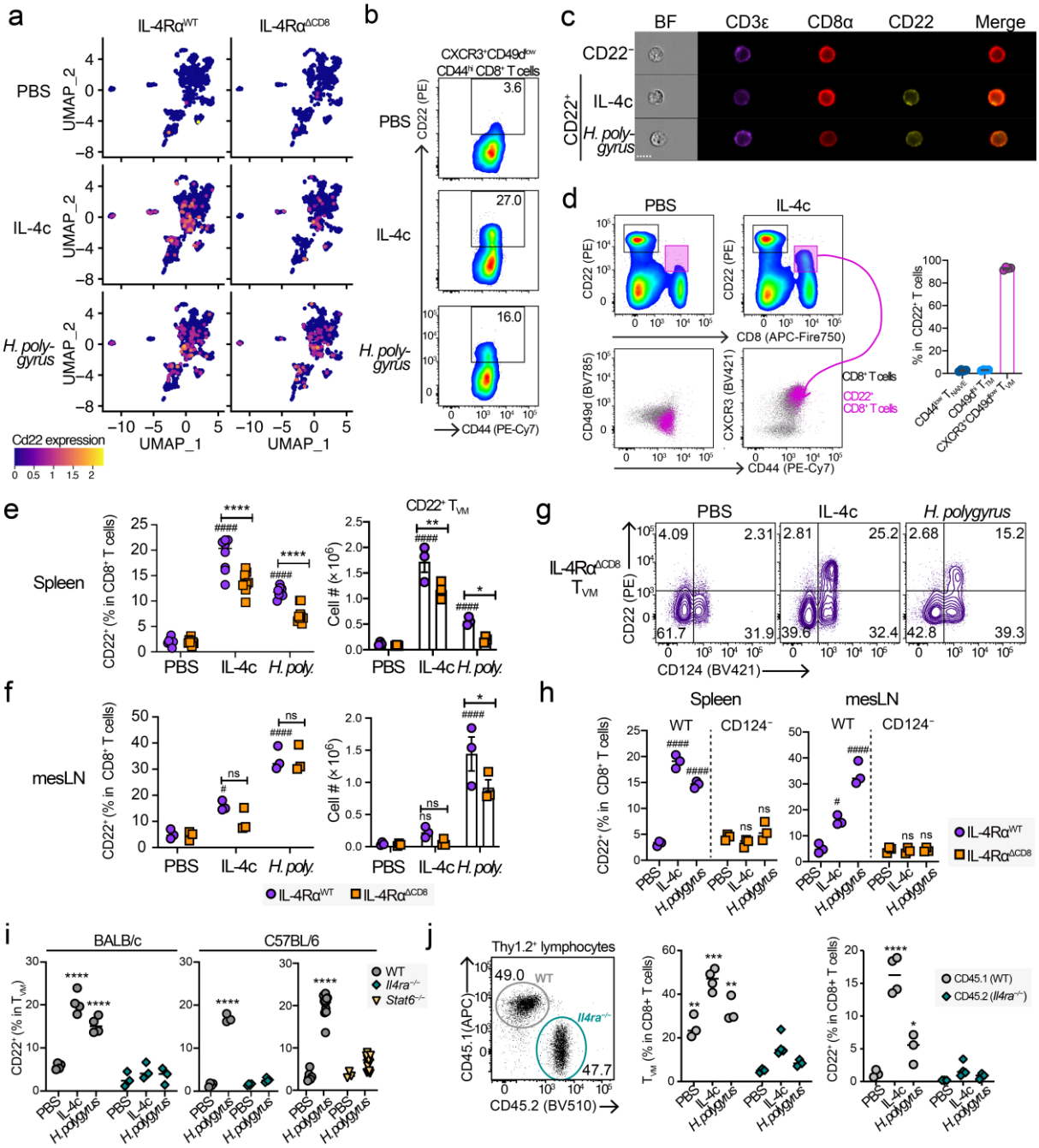

a
IL-4Rα^WT
IL-4Rα^ΔCD8
PBS
IL-4c
H. poly-gyrus
UMAP_2
UMAP_1
Cd22 expression
b
CXCR3+CD49dlow CD44hi CD8+ T cells
CD22 (PE)
CD44 (PE-Cy7)
3.6
27.0
16.0
c
BF
CD3ε
CD8α
CD22
Merge
CD22−
CD22+
IL-4c
H. poly-gyrus
d
PBS
IL-4c
CD22 (PE)
CD8 (APC-Fire750)
CD49d (BV785)
CXCR3 (BV421)
CD44 (PE-Cy7)
CD8+ T cells
CD22+ CD8+ T cells
% in CD22+ T cells
e
Spleen
CD22+ (% in CD8+ T cells)
CD22+ TVM
Cell # (×10^6)
PBS
IL-4c
H.poly.
f
mesLN
IL-4RαWT
IL-4RαΔCD8
g
PBS
IL-4c
H. polygyrus
IL-4Rα^ΔCD8 TVM
CD22 (PE)
CD124 (BV421)
4.09
2.31
61.7
31.9
2.81
25.2
39.6
32.4
2.68
15.2
42.8
39.3
h
Spleen
mesLN
WT
CD124−
CD22+ (% in CD8+ T cells)
i
BALB/c
C57BL/6
CD22+ (% in TVM)
WT
Il4ra−/−
Stat6−/−
j
Thy1.2+ lymphocytes
CD45.1(APC)
CD45.2 (BV510)
49.0
47.7
TVM (% in CD8+ T cells)
CD45.1 (WT)
CD45.2 (Il4ra−/−)

Figure 5. Specific CD22 expression in IL-4-induced T_{VM}.
(**a**) Feature plot of *Cd22* expression in memory/effector single $CD8^+$ T cell transcriptomes, based on scRNA-seq analysis in Fig. 2d.
(**b**) Representative pseudocolour flow plot of CD22 surface staining on gated T_{VM} from the spleen of WT BALB/c mice at d4 after treatment with IL-4c or at day 15 after infection with *H. polygyrus*. Numbers indicate percent of events in each gate.
(**c**) Imaging cytometry of single splenic $CD8^+$ T cells co-stained for CD3ε, CD8α and CD22 (ImageStream X Mk II; ×60, scale bar, 7 μm)
(**d**) Representative flow cytometry analysis of spleen cells from PBS or IL-4c-treated WT BALB/c mice and stained with anti-CD22. Back-gating of $CD22^+CD8^+$ T cells is shown for CD49d, CD44 and CXCR3 expression. Percentages of $CD22^+$ cells in $CD44^{low}$ T_{NAIVE}, $CD49d^{hi}$ T_{TM}, and T_{VM} are shown. Data are representative of three independent experiments with 4 mice per group (mean ± s.e.m.).
(**e**-**f**) Summary plots of $CD22^+CD8^+$ T cell counts and percentages in the spleen (**e**) and mesLN (**f**) of IL-4Rα^{WT} or IL-4Rα$^{\Delta CD8}$ mice at day 4 after PBS or IL-4c treatment, or at day 15 after *H. polygyrus* infection. Statistical significance calculated using two-way analysis of variance (ANOVA) and Sidak's multiple comparison-test (***P* < 0.01, ****P* < 0.001, *****P* < 0.0001; comparing IL-4Rα^{WT} and IL-4Rα$^{\Delta CD8}$, and $^{\#\#\#\#}P$ < 0.0001, comparing treated group with WT PBS-treated group). Data are representative of three independent experiments with 3-4 mice per group (mean ± s.e.m.).
(**g**) Representative contour flow plot of CD22 and CD124 co-expression of spleen T_{VM} from IL-4Rα$^{\Delta CD8}$ mice at day 4 after PBS or IL-4c treatment, or at day 15 after *H. polygyrus* infection. Numbers indicate percent of events in each quadrant.
(**h**) Percentages of $CD22^+$ T_{VM} in the spleen and mesLN based on the expression of IL-4Rα at day 4 after PBS or IL-4c treatment, or at day 15 after *H. polygyrus* infection. In IL-4Rα$^{\Delta CD8}$ mice, percentages of $CD22^+$ T_{VM} were reported to $CD124^-$ $CD8^+$ T cells. Data are representative of three independent experiments with 3-4 mice per group (mean ± s.e.m.).
(**i**) Percentages of $CD22^+$ T_{VM} in the spleen of WT and *Il4ra*$^{-/-}$ BALB/c mice, and WT, *Il4ra*$^{-/-}$ and *Stat6*$^{-/-}$ C57BL/6 mice at day 4 after PBS or IL-4c treatment, or at day 15 after *H. polygyrus* infection. Statistical significance calculated using two-way analysis of variance (ANOVA) and Sidak's multiple comparison-test (***P* < 0.01, ****P* < 0.001, *****P* < 0.0001). Data are representative or show pooled data of three independent experiments with 3-4 mice per group (mean ± s.e.m.).
(**j**) Percentages of $CD45.1^+Thy1.2^+$ WT and $CD45.2^+Thy1.2^+$ *Il4ra*$^{-/-}$ cells in the spleen of bone-marrow mixed chimeras, in recipient $CD45.2^+Thy1.1^+$ BALB/c mice and percentages of $CD22^+$ T_{VM} in each $CD45.1^+$ and $CD45.2^+$ compartment of transferred $Thy1.2^+$ cells, at day 4 after PBS or IL-4c treatment, or at day 15 after *H. polygyrus* infection. Statistical significance calculated using two-way analysis of variance (ANOVA) and Sidak's multiple comparison-test (***P* < 0.01, ****P* < 0.001, *****P* < 0.0001). Data are representative of two independent experiments with 3-4 mice per group (mean ± s.e.m.).

Figure 6

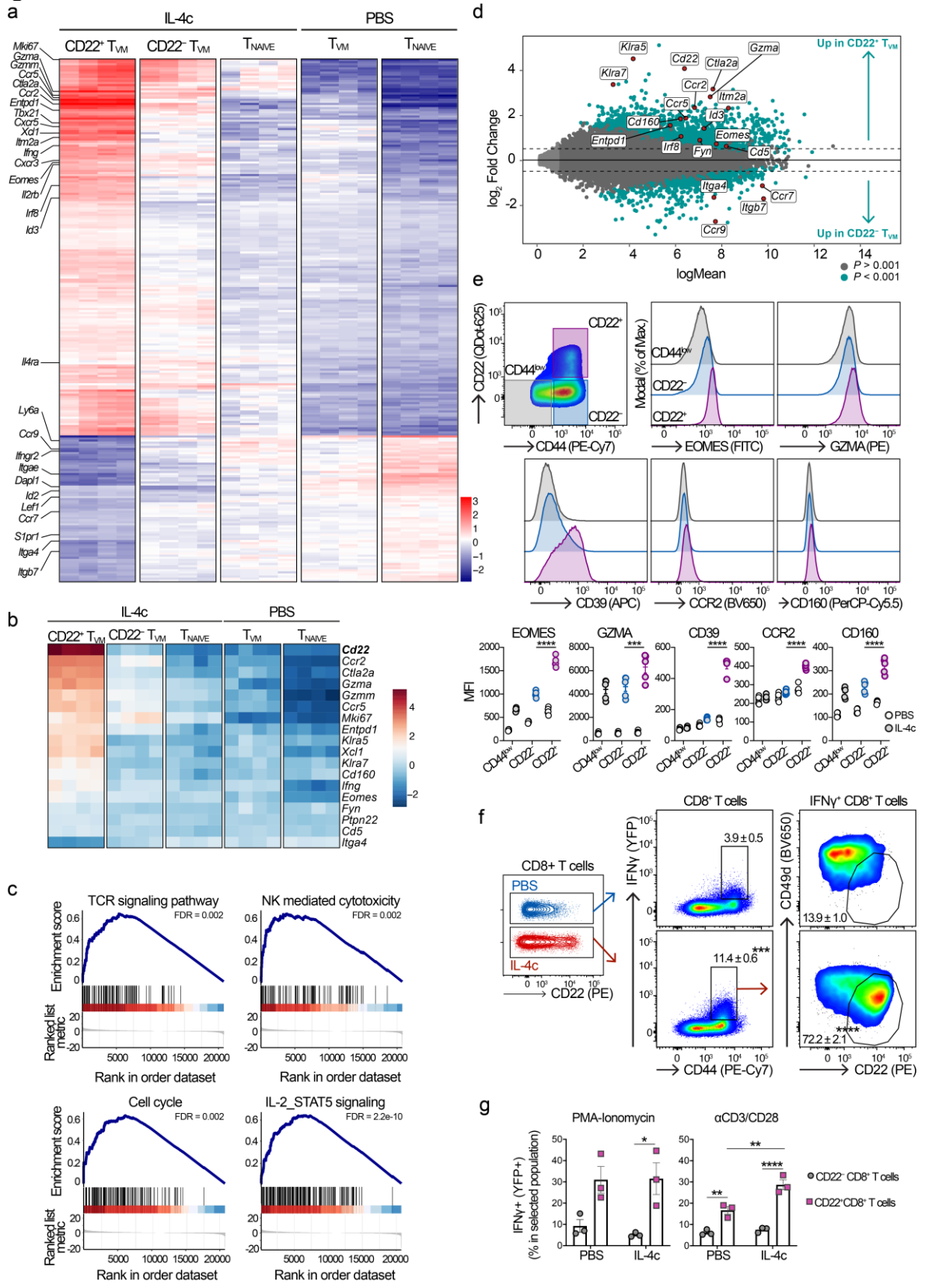

a
IL-4c
PBS
CD22+ TVM
CD22− TVM
TNAIVE
TVM
TNAIVE
Mki67
Gzma
Gzmm
Ccr5
Ctla2a
Ccr2
Entpd1
Tbx21
Cxcr5
Xcl1
Itm2a
Ifng
Cxcr3
Eomes
Il2rb
Irf8
Id3
Il4ra
Ly6a
Ccr9
Ifngr2
Itgae
Dapl1
Id2
Lef1
Ccr7
S1pr1
Itga4
Itgb7
b
Cd22
Ccr2
Ctla2a
Gzma
Gzmm
Ccr5
Mki67
Entpd1
Klra5
Xcl1
Klra7
Cd160
Ifng
Eomes
Fyn
Ptpn22
Cd5
Itga4
c
TCR signaling pathway
FDR = 0.002
NK mediated cytotoxicity
FDR = 0.002
Cell cycle
FDR = 0.002
IL-2_STAT5 signaling
FDR = 2.2e-10
Enrichment score
Ranked list metric
Rank in order dataset
d
Klra5
Cd22
Gzma
Ctla2a
Klra7
Ccr2
Itm2a
Ccr5
Id3
Cd160
Eomes
Entpd1
Irf8
Fyn
Cd5
Itga4
Ccr7
Itgb7
Ccr9
Up in CD22+ TVM
Up in CD22− TVM
log2 Fold Change
logMean
P > 0.001
P < 0.001
e
CD22 (QDot625)
CD44 (PE-Cy7)
Modal (% of Max.)
EOMES (FITC)
GZMA (PE)
CD39 (APC)
CCR2 (BV650)
CD160 (PerCP-Cy5.5)
MFI
EOMES
GZMA
CD39
CCR2
CD160
PBS
IL-4c
f
CD8+ T cells
IFNγ+ CD8+ T cells
PBS
IL-4c
IFNγ (YFP)
CD22 (PE)
CD49d (BV650)
CD44 (PE-Cy7)
3.9 ± 0.5
13.9 ± 1.0
11.4 ± 0.6
72.2 ± 2.1
g
PMA-Ionomycin
αCD3/CD28
IFNγ+ (YFP+) (% in selected population)
CD22− CD8+ T cells
CD22+CD8+ T cells
PBS
IL-4c

Figure 6. $CD22^+$ T_{VM} are transcriptionally distinct from $CD22^-$ T_{VM} and naive T cells.
(**a-d**) WT BALB/c mice were treated with PBS or IL-4c before spleen harvest and purification of $CD44^{low}$ T_{NAIVE} and $CD22^-$ and $CD22^+$ $CD44^{high}CD49d^{low}$ T_{VM} for RNA-seq analysis.
(**a**) Unsupervised hierarchical clustering of the top 250 differentially expressed genes. Specific biologically relevant example genes are listed on the left. Heatmap color scale indicates *z*-score.
(**b**) Heatmap of biologically relevant example gene expression. Color scale indicates normalized mean expression.
(**c**) GSEA enrichment score plots for the indicated KEGG or HALLMARK gene sets between $CD22^-$ and $CD22^+$ T_{VM} in IL-4c treated mice.
(**d**) MA plot of differentially expressed genes between $CD22^-$ and $CD22^+$ T_{VM} in IL-4c treated mice. Each dot represents one gene. Manually curated genes are indicated.
(**e**) Representative flow cytometry analysis of concatenated samples and summary data of the MFI of selected markers in spleen $CD44^{low}$ T_{NAIVE}, $CD22^-$ T_{VM} and $CD22^+$ T_{VM} of IL-4c-treated WT BALB/c mice. Data are representative of three independent experiments with 4-5 mice per group.
(**f**) Representative flow cytometry analysis of concatenated samples revealing IFN-γ expression by eYFP detection in Great reporter C57BL/6 mice (n=3). Numbers in gates indicate mean percent of events (± s.e.m.). Data are representative of two independent experiments.
(**g**) Summary data of IFN-γ^+ ($eYFP^+$) as in (**f**) upon restimulation with PMA and ionomycin or anti-CD3/CD28. Data are representative of two independent experiments.
Statistical significance calculated using two-way analysis of variance (ANOVA) and Sidak's multiple comparison-test (***P* < 0.01, ****P* < 0.001, *****P* < 0.0001).

Figure 7

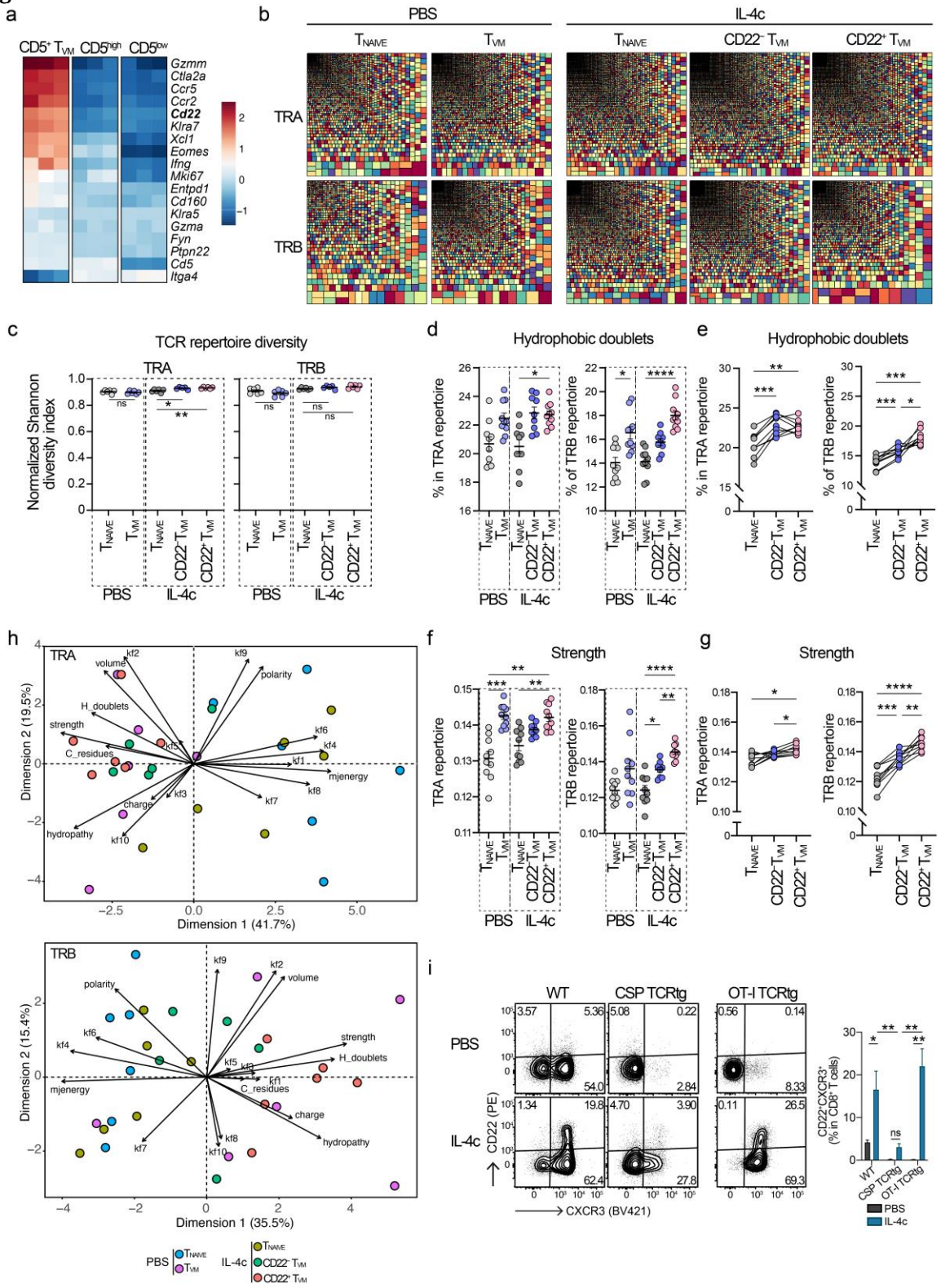

a
CD5+ TVM
CD5high
CD5low
Gzmm
Ctla2a
Ccr5
Ccr2
Cd22
Klra7
Xcl1
Eomes
Ifng
Mki67
Entpd1
Cd160
Klra5
Gzma
Fyn
Ptpn22
Cd5
Itga4
b
PBS
IL-4c
TNAIVE
TVM
CD22- TVM
CD22+ TVM
TRA
TRB
c
TCR repertoire diversity
Normalized Shannon diversity index
d
Hydrophobic doublets
% in TRA repertoire
% of TRB repertoire
e
f
Strength
TRA repertoire
TRB repertoire
g
h
Dimension 1 (41.7%)
Dimension 2 (19.5%)
Dimension 1 (35.5%)
Dimension 2 (15.4%)
volume
polarity
H_doublets
strength
C_residues
mjenergy
charge
hydropathy
i
WT
CSP TCRtg
OT-I TCRtg
CD22 (PE)
CXCR3 (BV421)
CD22+CXCR3+ (% in CD8+ T cells)

Figure 7. The TCR repertoire of IL-4-induced CD22$^+$ T_{VM} is diverse but enriched in self-reactive CDR3 sequences.

(**a**) Heatmap of biologically relevant example gene expression from publicly accessible RNA-seq dataset from naive CD5$^+$ and CD5$^-$ T_{VM}[21]. Color scale indicates normalized mean expression.

(**b-h**) CD44^{low} T_{NAIVE}, CD22$^-$ CD44^{high}CD49d^{low} T_{VM} and CD22$^+$ CD44^{high}CD49d^{low} T_{VM} were purified by FACS from the spleen of PBS or IL-4c-treated WT BALB/c mice. CDR3α and CDR3β sequences were extracted from RNA-seq and TCR-seq.

(**b**) Representative tree maps showing CDR3 clonotype usage for the different T cell subsets in PBS- or IL-4c-treated mice; each rectangle represents one CDR3 clonotype and its size corresponds to its relative frequency in the repertoire (rectangle colors are chosen randomly and do not match between plots). Data show CDR3α and CDR3β sequences obtained by TCR-seq.

(**c**) Summary plots of normalized Shannon diversity index of TRA and TRB repertoires for the different T cell subsets in PBS- or IL-4c-treated mice. The normalized index ranges from 0 (no diversity) to 1 (maximal diversity). Each symbol represents an individual mouse. Data show CDR3α and CDR3β sequences obtained by TCR-seq (n=6 in each T cell subset from both conditions (PBS, IL-4c)). Mean ± s.e.m. is indicated. Statistical significance calculated using one-way analysis of variance (ANOVA) and Holm-Sidak's multiple comparison test (ns, not significant, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$).

(**d-e**) Summary plots of hydrophobic doublets in positions 6 and 7 of CDR3α and CDR3β of the different T cell subsets in PBS- or IL-4c-treated mice (**d**) or of paired samples in IL-4c-treated mice (**e**). Each symbol represents an individual mouse, with CDR3 sequences obtained by TCR-seq and RNA-seq (n=10).

(**f-g**) Summary plots of CDR3 strength based on the presence of bulky amino acid side chains for CDR3α and CDR3β in the different T cell subsets in PBS- or IL-4c-treated mice (**f**) or of paired samples in IL-4c-treated mice (**g**). Each symbol represents an individual mouse, with CDR3 sequences obtained by TCR-seq and RNA-seq (n=10). Summary data show mean ± s.e.m. (d-g). Statistical significance calculated using one-way analysis of variance (ANOVA) and Tukey's multiple comparison test (d, f) or one-way analysis of variance (ANOVA) for repeated measures and Dunnett's multiple comparison test (e, g), (ns, not significant, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$).

(**h**) Cumulative contribution of 18 CDR3-derived parameters (see Fig. S8i) in principal component analysis for the repertoires of the different T cell subsets in PBS- or IL-4c-treated mice. Plot shows two-dimensional principal component analysis projections of 5,000 functional read sequences of CDR3α and CDR3β that were randomly selected for each of the different samples.

(**i**) Representative flow cytometry analysis and summary data of CD22$^+$ T_{VM} percentages in WT, CSP- or OT-I TCR transgenic mice treated with PBS or IL-4c. Mean ± s.e.m. is indicated (n=4). Statistical significance calculated using two-way analysis of variance (ANOVA) and Sidak multiple comparison test, (ns, not significant, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$).

Figure 8

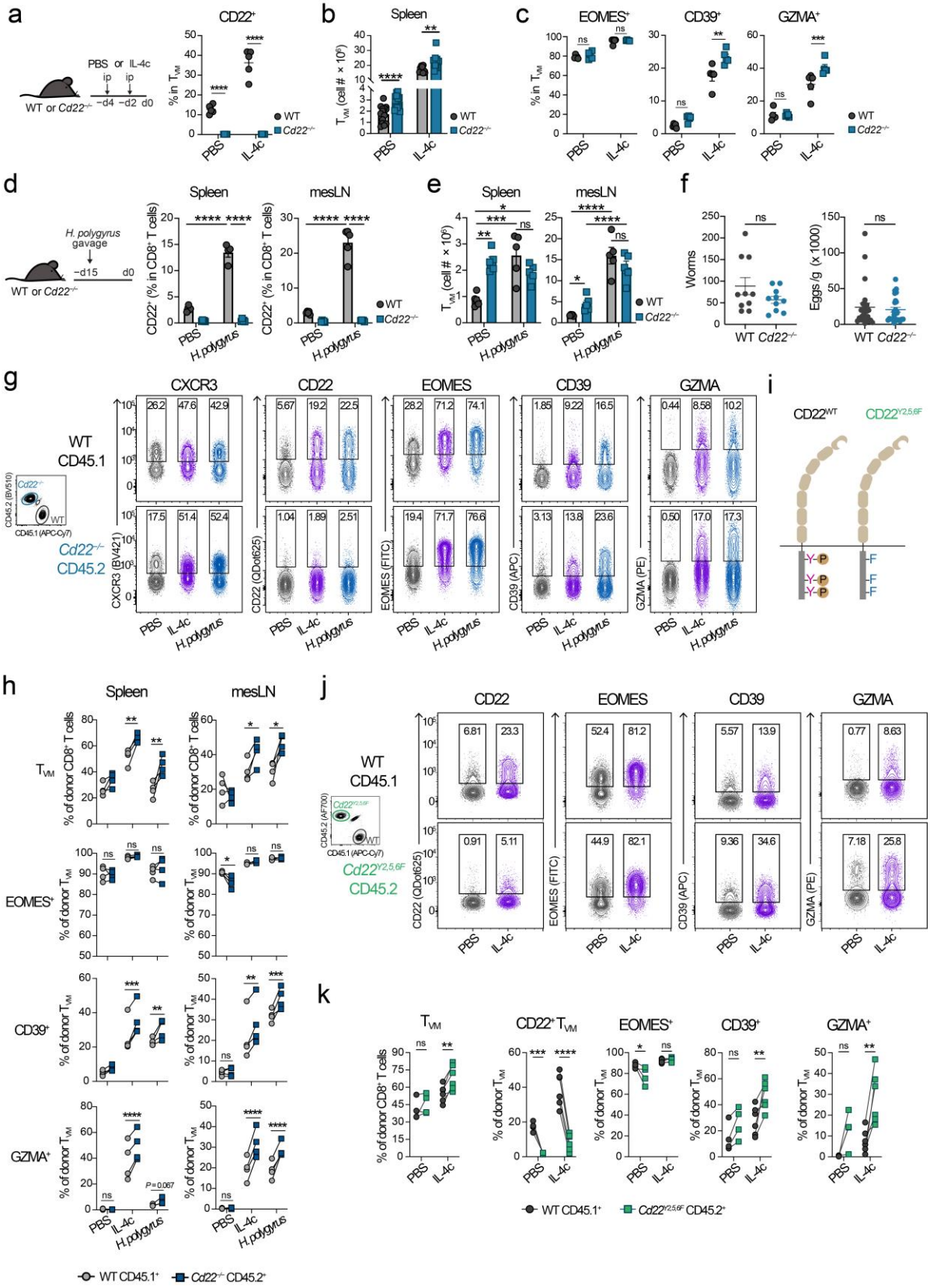

a
PBS or IL-4c
ip ip
−d4 −d2 d0
WT or Cd22−/−
CD22+
% in TVM
WT
Cd22−/−
b
Spleen
TVM (cell # × 10⁶)
c
EOMES+
CD39+
GZMA+
d
H. polygyrus gavage
−d15 d0
Spleen
mesLN
CD22+ (% in CD8+ T cells)
H.polygyrus
e
TVM (cell # × 10⁶)
f
Worms
Eggs /g (x1000)
g
CXCR3
CD22
EOMES
CD39
GZMA
WT CD45.1
Cd22−/− CD45.2
CD45.2 (BV510)
CD45.1 (APC-Cy7)
CXCR3 (BV421)
CD22 (QDot625)
EOMES (FITC)
CD39 (APC)
GZMA (PE)
PBS
IL-4c
H.polygyrus
i
CD22WT
CD22Y2,5,6F
Y-P
F
h
Spleen
mesLN
TVM
EOMES+
CD39+
GZMA+
% of donor CD8+ T cells
% of donor TVM
P=0.067
WT CD45.1+
Cd22−/− CD45.2+
j
CD22
EOMES
CD39
GZMA
WT CD45.1
Cd22Y2,5,6F CD45.2
k
TVM
CD22+ TVM
EOMES+
CD39+
GZMA+
WT CD45.1+
Cd22Y2,5,6F CD45.2+

Figure 8. The absence of CD22 renders T_{VM} more reactive to IL-4 during helminth infection.
(**a**) WT or *Cd22*$^{-/-}$ mice treatment with IL-4c. Summary data of CD22^{+} CD8^{+} T cell percentages. Bar indicates mean ± s.e.m.
(**b-c**) Summary data of T_{VM} numbers (b) and EOMES^{+}, CD39^{+} or GZMA^{+} T_{VM} cells in the spleen (c). Bar indicates mean ± s.e.m.
(**d-f**) WT or *Cd22*$^{-/-}$ mice were infected with *H. polygyrus* (200 × L3) by gavage and samples analyzed at day 15 after infection. (**d**) Experimental design and summary data of CD22^{+} CD8^{+} T cell percentages. (**e**) Summary data of T_{VM} numbers in the indicated tissue. (**f**) *H. polygyrus* adult worm burden in the gut of WT and *Cd22*$^{-/-}$ mice at day 15 after infection, and fecal egg counts between day 10 and day 14 after infection. Bar indicates mean ± s.e.m..
(**g**) Contour flow plots of CD8^{+} T cells from mesLN of mixed bone marrow WT:*Cd22*$^{-/-}$ chimeric mice. Plots show concatenated samples of 4 mice per group, separated by experimental condition. Numbers indicate percent of events in each gate for each experimental condition (PBS, IL-4c or *H. polygyrus* at day 15 after infection).
(**h**) Summary data of the percentage of T_{VM} in donor CD45.1^{+} or CD45.2^{+} CD8^{+} T cell compartment, and percentage of EOMES^{+}, CD39^{+} and GZMA^{+} cells in T_{VM} from the spleen and mesLN of mixed bone marrow WT: *Cd22*$^{-/-}$ chimeric mice after treatment with IL-4c or infection with *H. polygyrus* (day 15). Lines connect data from one individual chimeric mouse.
(**i**) Schematic representation of WT CD22 (CD22^{WT}) and CD22^{Y2,5,6F} knock-in.
(**j**) Contour flow plots of CD8^{+} T cells from the spleen of mixed bone marrow WT:*Cd22*^{Y2,5,6F} chimeric mice. Plots show concatenated samples of 4 to 6 mice per group, separated by experimental condition. Numbers indicate percent of events in each gate for each experimental condition (PBS or IL-4c).
(**k**) Summary data of the percentage of T_{VM} in donor CD45.1^{+} or CD45.2^{+} CD8^{+} T cell compartment, and percentage of EOMES^{+}, CD39^{+} and GZMA^{+} cells in T_{VM} from the spleen and mesLN of mixed bone marrow WT: *Cd22*^{Y2,5,6F} chimeric mice after treatment with IL-4c. Lines connect data from one individual chimeric mouse.
Statistical significance calculated using two-way analysis of variance (ANOVA) and Sidak's multiple comparison test, (ns, not significant, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$). Each symbol represents an individual mouse. Data are representative of 2 to 3 independent experiments.

Figure 9

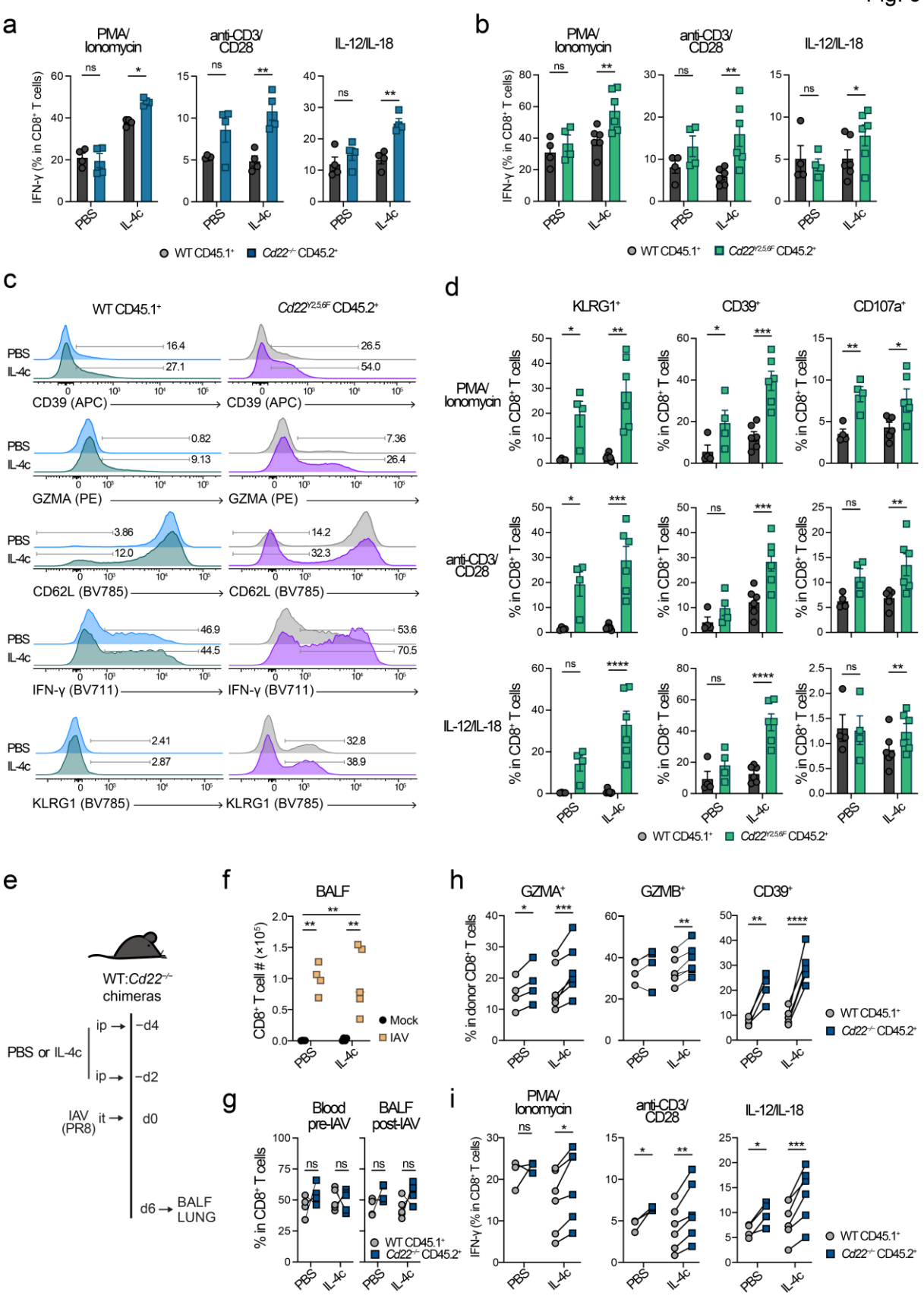


Fig. 9
a
PMA/ Ionomycin
anti-CD3/ CD28
IL-12/IL-18
IFN-γ (% in CD8+ T cells)
ns
*
**
PBS
IL-4c
WT CD45.1+
Cd22-/- CD45.2+
b
PMA/ Ionomycin
anti-CD3/ CD28
IL-12/IL-18
IFN-γ (% in CD8+ T cells)
WT CD45.1+
Cd22Y2,5,6F CD45.2+
c
WT CD45.1+
Cd22Y2,5,6F CD45.2+
PBS
IL-4c
16.4
27.1
26.5
54.0
CD39 (APC)
0.82
9.13
7.36
26.4
GZMA (PE)
3.86
12.0
14.2
32.3
CD62L (BV785)
46.9
44.5
53.6
70.5
IFN-γ (BV711)
2.41
2.87
32.8
38.9
KLRG1 (BV785)
d
KLRG1+
CD39+
CD107a+
PMA/ Ionomycin
anti-CD3/ CD28
IL-12/IL-18
% in CD8+ T cells
****
WT CD45.1+
Cd22Y2,5,6F CD45.2+
e
WT:Cd22-/- chimeras
PBS or IL-4c
ip
−d4
−d2
IAV (PR8)
it
d0
d6
BALF LUNG
f
BALF
CD8+ T cell # (×10^5)
Mock
IAV
g
Blood pre-IAV
BALF post-IAV
% in CD8+ T cells
WT CD45.1+
Cd22-/- CD45.2+
h
GZMA+
GZMB+
CD39+
% in donor CD8+ T cells
WT CD45.1+
Cd22-/- CD45.2+
i
PMA/ Ionomycin
anti-CD3/ CD28
IL-12/IL-18
IFN-γ (% in CD8+ T cells)
WT CD45.1+
Cd22-/- CD45.2+

Figure 9. CD22 controls the effector program of activation of T_{VM} upon stimulation.
Mixed bone-marrow WT:*Cd22*$^{-/-}$ (**a**) and WT:*Cd22*^{Y2,5,6F} (**b-d**) chimeric mice were treated with PBS or IL-4c and spleen cells subjected to PMA-ionomycin, anti-CD3ε/CD28 or IL-12 and IL-18 stimulation *ex vivo*.
(**a**) Summary data of the percentage of IFN-γ$^+$ cells in CD8$^+$ T cells following the indicated *ex vivo* stimulation in WT:*Cd22*$^{-/-}$ chimeric mice.
(**b**) Summary data of the percentage of IFN-γ$^+$ cells in CD8$^+$ T cells following the indicated *ex vivo* stimulation in WT:*Cd22*^{Y2,5,6F} chimeric mice.
(**c**) Half-off-set histograms adjusted to mode of concatenated samples (n=4 to 6) showing the fluorescence intensities of the indicated markers upon analysis by flow cytometry on spleen cells from WT:*Cd22*^{Y2,5,6F} chimeric mice. Data for IFN-γ and KLRG1 are from spleen cells restimulated with PMA and ionomycin. Bars indicate positive population set on isotype control. Numbers indicate percent of positive population.
(**d**) Summary data of the percentage of KLRG1$^+$, CD39$^+$ and CD107a$^+$ cells in CD8$^+$ T cells following the indicated *ex vivo* stimulation in WT:*Cd22*^{Y2,5,6F} chimeric mice.
(**e-i**) Mixed bone-marrow WT:*Cd22*$^{-/-}$ chimeric mice were treated with PBS or IL-4c before IAV infection intratracheally (25 PFU) and BALF and lung cells harvested at day 6 after IAV infection.
(**f**) Absolute numbers of CD8$^+$ T cells collected from the airway (BALF) of mock-infected mice or mice at day 6 after IAV infection.
(**g**) CD8$^+$ T cells chimerism in peripheral blood before infection and in the BALF at day 6 after IAV infection.
(**h**) Summary data of the percentage of GZMA$^+$, GZMB$^+$ and CD39$^+$ cells in CD8$^+$ T cells in the BALF at day 6 after IAV infection.
(**i**) Summary data of the percentage of IFN-γ$^+$ cells in CD8$^+$ T cells following the indicated *ex vivo* stimulation of lung cells at day 6 after IAV infection.
Statistical significance calculated using two-way analysis of variance (ANOVA) and Sidak's multiple comparison test (ns, not significant, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$). Each symbol represents an individual mouse. Data are representative of 2 independent experiments.

Supplementary Figures

Figure S1

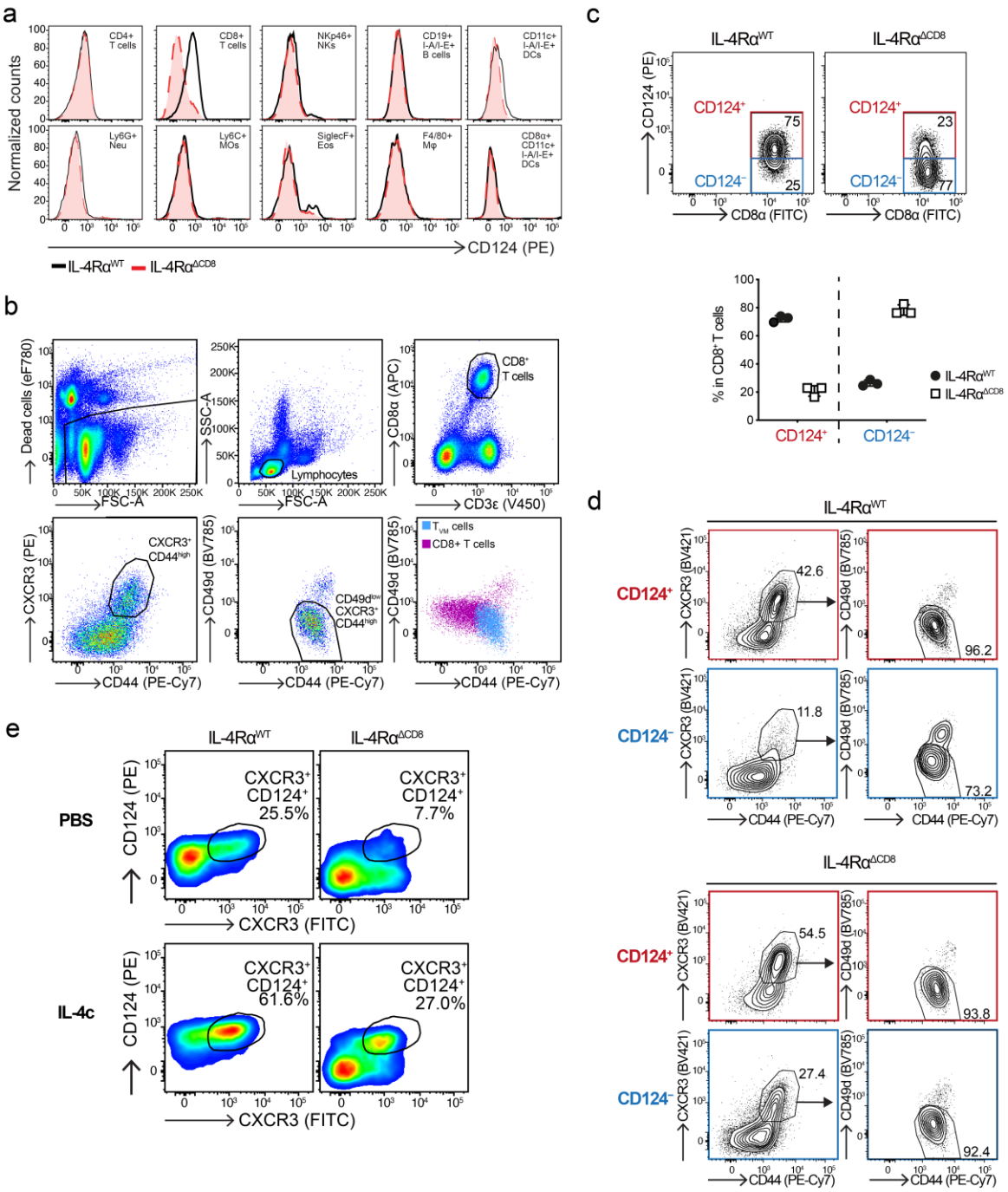


Figure S1. Characterization of IL-4Rα$^{\Delta CD8}$ mice.

(**a**) Representative histograms of CD124 expression in the indicated population from the spleen of IL-4Rα^{WT} or IL-4Rα$^{\Delta CD8}$ naive mice. (**b**) Gating strategy for CD49d^{low}CXCR3^{+} T_{VM} detection. (**c**) Representative contour flow plot of spleen CD8^{+} T lymphocytes. Numbers indicate percent of events in each gate. Percentages of CD124^{+} and CD124^{-} CD8^{+} T cells in IL-4Rα^{WT} and IL-4Rα$^{\Delta CD8}$ mice. (**d**) Representative contour plots depicting the gating strategy of CD124^{+} and CD124^{-} CD49d^{low}CXCR3^{+} T_{VM} in the spleen of IL-4Rα^{WT} or IL-4Rα$^{\Delta CD8}$ mice. (**e**) Representative pseudocolour flow plot of spleen CD8^{+} T lymphocytes. Numbers indicate events percent in each gate. Statistical significance calculated using Mann-Whitney test (**f**) or two-way analysis of variance (ANOVA) and Sidak's multiple comparison-test (**$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$). Data are representative of two independent experiments with 4 mice per group. Mean ± s.e.m., each symbol represents one individual mouse.

Figure S2

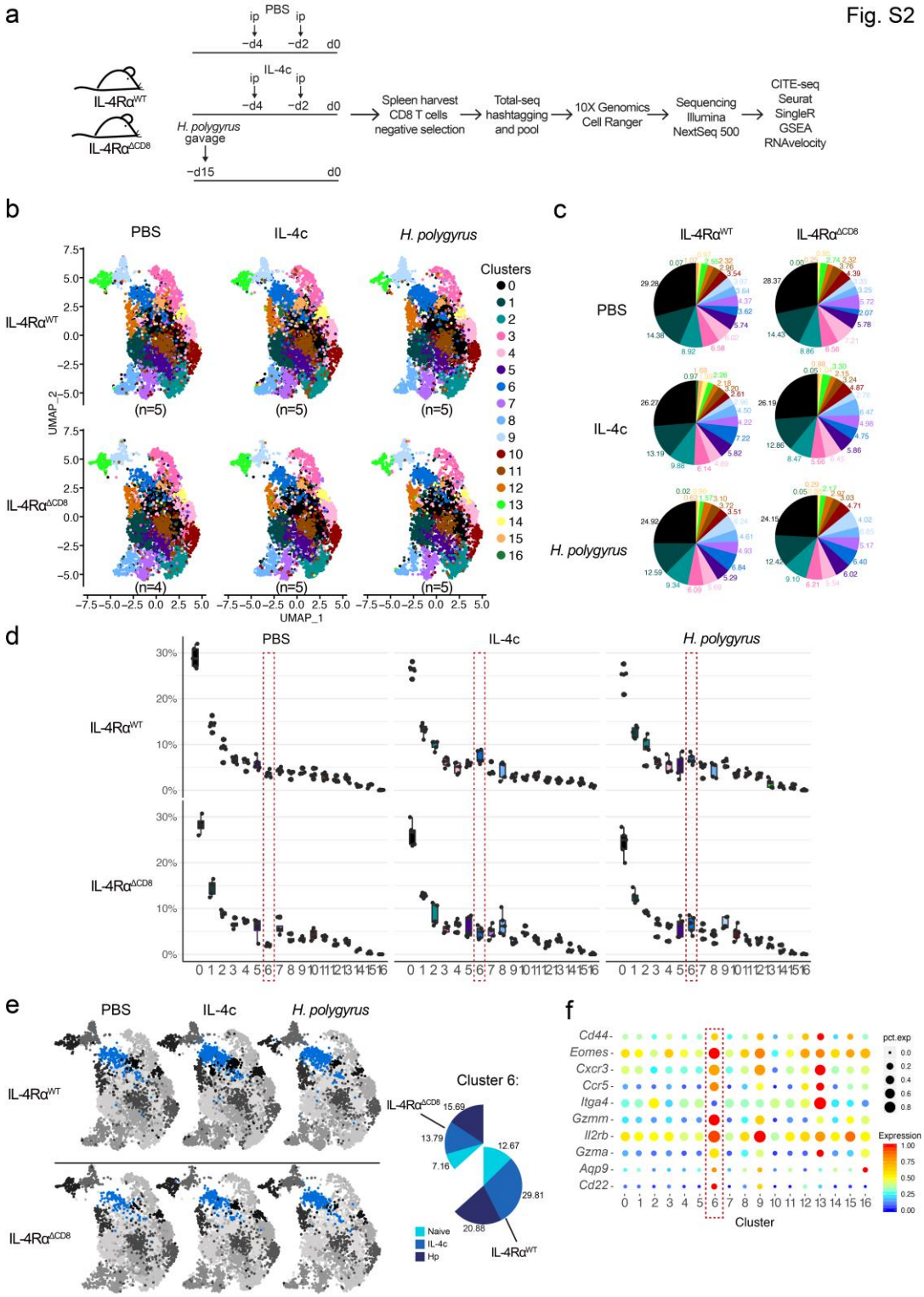


Fig. S2
a
PBS
IL-4c
H. polygyrus
gavage
IL-4Rα^WT
IL-4Rα^ΔCD8
Spleen harvest
CD8 T cells
negative selection
Total-seq
hashtagging
and pool
10X Genomics
Cell Ranger
Sequencing
Illumina
NextSeq 500
CITE-seq
Seurat
SingleR
GSEA
RNAvelocity
b
Clusters
UMAP_1
UMAP_2
c
d
e
Cluster 6:
Naive
IL-4c
Hp
f
pct.exp
Expression
Cluster

Figure S2. Single-cell RNA sequencing of spleen CD8$^+$ T cells upon IL-4 response.
(**a**) Experimental design of treatment with IL-4c or infection with *H. polygyrus* before CD8$^+$ T cell enrichment, hash-tagging, single cell capture, library preparation, and sequencing. The main packages used to analyze scRNA-seq data are listed.
(**b**) Split UMAP visualization of Seurat clustering analysis of combined single CD8$^+$ T cell transcriptomes of IL-4Rα^{WT} and IL-4Rα$^{\Delta CD8}$ mice, following treatment with PBS, IL-4c, or *H. polygyrus* infection. (n = 5, except IL-4Rα$^{\Delta CD8}$-PBS (n=4)).
(**c**) Pie charts of cell number proportions of each cluster in each dataset.
(**d**) Box plot distribution of the frequency of each cluster based on the hashtagging of each individual mouse sample included in each dataset.
(**e**) Split UMAP visualization of Seurat clustering analysis as in (b), highlighting cluster 6. The split pie chart shows the cell number proportion of cluster 6 in each dataset.
(**f**) Dot plot showing the average expression levels and percentage of expression of selected genes in each cluster obtained in Fig. 2a.

Figure S3

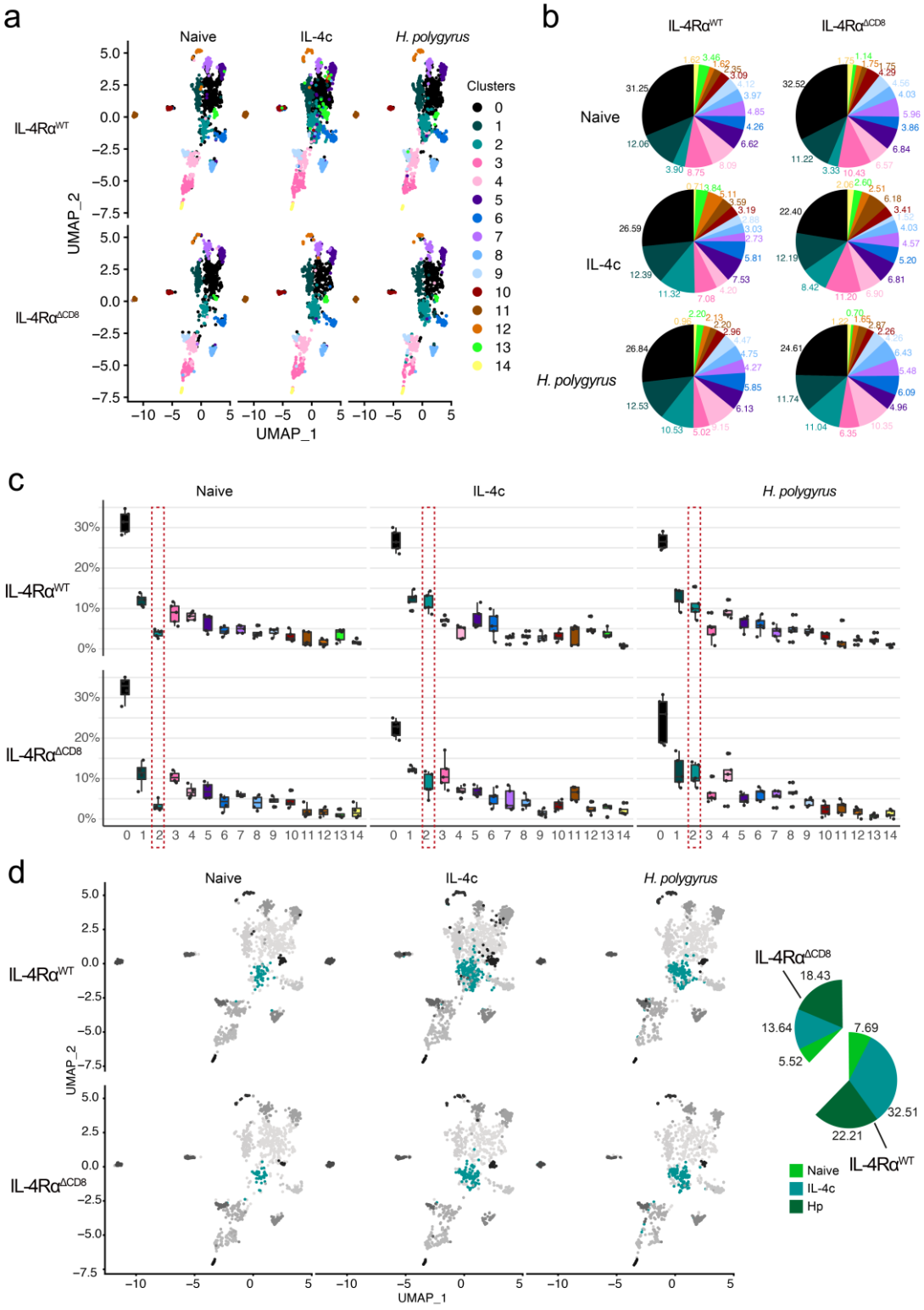

a
Naive
IL-4c
H. polygyrus
IL-4Rα^WT
IL-4Rα^ΔCD8
UMAP_2
UMAP_1
Clusters
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
b
IL-4Rα^WT
IL-4Rα^ΔCD8
Naive
IL-4c
H. polygyrus
c
Naive
IL-4c
H. polygyrus
IL-4Rα^WT
IL-4Rα^ΔCD8
d
Naive
IL-4c
H. polygyrus
IL-4Rα^WT
IL-4Rα^ΔCD8
UMAP_2
UMAP_1
IL-4Rα^ΔCD8
18.43
13.64
7.69
5.52
32.51
22.21
IL-4Rα^WT
Naive
IL-4c
Hp

Figure S3. Single-cell RNA sequencing of spleen memory/effector CD8$^+$ T cells upon IL-4 response.
(**a**) Split UMAP visualization of Seurat clustering analysis of combined single memory/effector CD8$^+$ T cell transcriptomes of IL-4Rα^{WT} and IL-4Rα$^{\Delta CD8}$ mice as in Fig. 2d and following treatment with PBS, IL-4c, or *H. polygyrus* infection. (n = 5, except IL-4Rα$^{\Delta CD8}$-PBS (n=4)).
(**b**) Pie charts of cell number proportions of each cluster in each dataset.
(**c**) Box plot distribution of the frequency of each cluster based on the hashtagging of each individual mouse sample included in each dataset.
(**d**) Split UMAP visualization of Seurat clustering analysis as in (a), highlighting cluster 2. The split pie chart shows the cell number proportion of cluster 2 in each dataset.

Figure S4

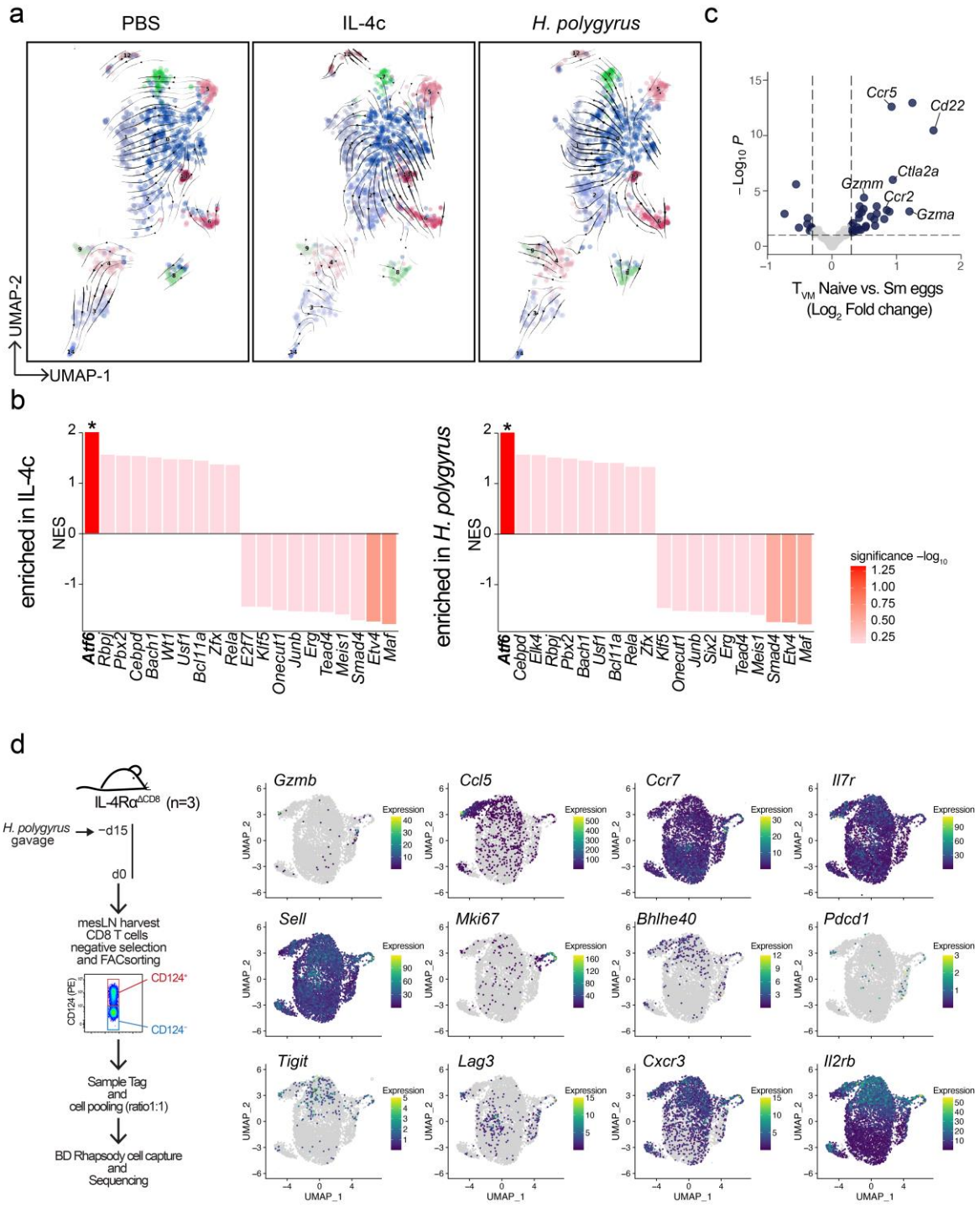

a
PBS
IL-4c
H. polygyrus
UMAP-2
UMAP-1
c
Ccr5
Cd22
Ctla2a
Gzmm
Ccr2
Gzma
– Log10 P
TVM Naive vs. Sm eggs
(Log2 Fold change)
b
enriched in IL-4c
enriched in H. polygyrus
NES
Atf6 Rbpj Pbx2 Cebpd Bach1 Wt1 Usf1 Bcl11a Zfx Rela E2f7 Klf5 Onecut1 Junb Erg Tead4 Meis1 Smad4 Etv4 Maf
Atf6 Cebpd Elk4 Rbpj Pbx2 Bach1 Usf1 Bcl11a Rela Zfx Klf5 Onecut1 Junb Six2 Erg Tead4 Meis1 Smad4 Etv4 Maf
significance –log10
1.25
1.00
0.75
0.50
0.25
d
IL-4Rα ΔCD8 (n=3)
H. polygyrus gavage
–d15
d0
mesLN harvest
CD8 T cells
negative selection
and FACsorting
CD124 (PE)
CD124+
CD124–
Sample Tag
and
cell pooling (ratio1:1)
BD Rhapsody cell capture
and
Sequencing
Gzmb
Ccl5
Ccr7
Il7r
Sell
Mki67
Bhlhe40
Pdcd1
Tigit
Lag3
Cxcr3
Il2rb
Expression
UMAP_1
UMAP_2

Figure S4. Transcription regulation of memory/effector CD8$^+$ T cells upon IL-4 and *H. polygyrus* infection
(**a**) Split UMAP visualization of RNAVelocity trajectory analysis of memory/effector CD8$^+$ T cell transcriptomes of IL-4Rα^{WT} mice.
(**b**) GSEA of the indicated transcription factor in the T_{VM} cluster 2. Top 10 gene set enrichment scores (NES) in scRNA-seq as in Fig. 2.
(**c**) Volcano plot of differentially expressed (DE) genes (P<0.1) showing DE genes in blue from bulk RNAseq data of sorted T_{VM} from PBS- or Sm-treated BALB/c WT mice, obtained in a previously published study[6].
(**d**) Experimental design before CD8$^+$ T cell enrichment, FACsortingand sequencing. UMAP visualizations show FeaturePlots of selected gene expression in single CD8$^+$ T cells from the mesLN of IL-4Rα$^{\Delta CD8}$ mice at day 15 after *H. polygyrus* infection, based on Fig. 3f.

Figure S5

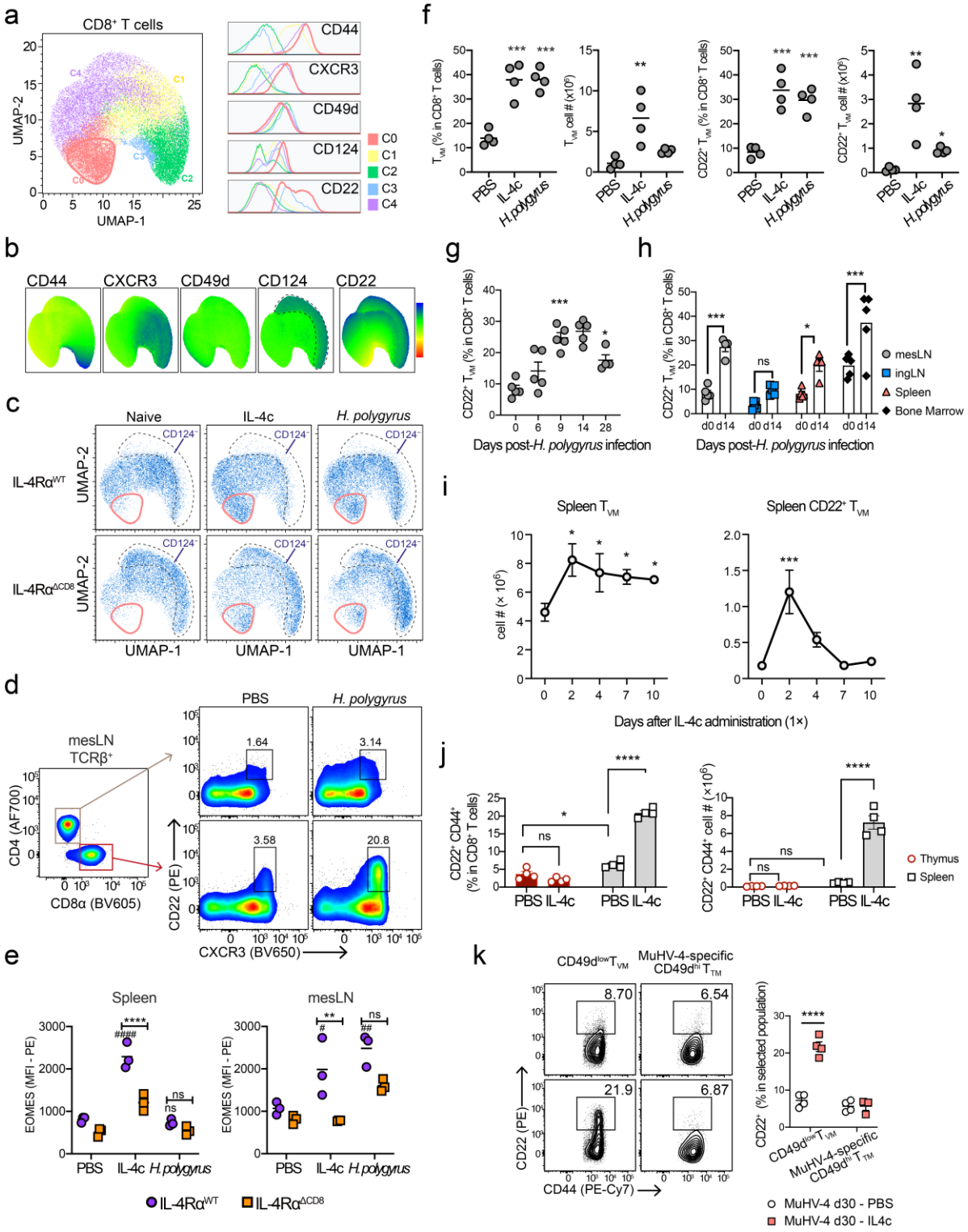

a
CD8⁺ T cells
UMAP-2
UMAP-1
C0
C1
C2
C3
C4
CD44
CXCR3
CD49d
CD124
CD22
b
c
Naive
IL-4c
H. polygyrus
IL-4Rα^WT
IL-4Rα^ΔCD8
CD124⁻
d
PBS
H. polygyrus
mesLN
TCRβ⁺
CD4 (AF700)
CD8α (BV605)
CD22 (PE)
CXCR3 (BV650)
1.64
3.14
3.58
20.8
e
Spleen
mesLN
EOMES (MFI - PE)
PBS
IL-4c
H.polygyrus
IL-4Rα^WT
IL-4Rα^ΔCD8
f
T_VM (% in CD8⁺ T cells)
T_VM cell # (x10⁶)
CD22⁺ T_VM (% in CD8⁺ T cells)
CD22⁺ T_VM cell # (x10⁶)
g
CD22⁺ T_VM (% in CD8⁺ T cells)
Days post-H. polygyrus infection
h
mesLN
ingLN
Spleen
Bone Marrow
d0 d14
i
Spleen T_VM
Spleen CD22⁺ T_VM
cell # (× 10⁶)
Days after IL-4c administration (1×)
j
CD22⁺ CD44⁺ (% in CD8⁺ T cells)
CD22⁺ CD44⁺ cell # (×10⁶)
PBS IL-4c
Thymus
Spleen
k
CD49d^lowT_VM
MuHV-4-specific CD49d^hi T_TM
8.70
6.54
21.9
6.87
CD22 (PE)
CD44 (PE-Cy7)
CD22⁺ (% in selected population)
MuHV-4 d30 - PBS
MuHV-4 d30 - IL4c

Figure S5. Dynamics of CD22 expression in IL-4-induced T_{VM} during helminth infection.
(**a**) UMAP visualization of concatenated spleen $CD8^+$ T cells analyzed by flow cytometry. FlowSOM clustering analysis was applied based on the fluorescence signals of CD3, CD8, CD44, CXCR3, CD49d, CD124, and CD22. Histograms show the expression levels of the given marker in each cluster.
(**b**) UMAP visualization of concatenated spleen $CD8^+$ T cells analyzed by flow cytometry. Color scale indicates relative expression for each marker.
(**c**) Split UMAP visualization of concatenated spleen $CD8^+$ T cells analyzed by flow cytometry. IL-4Rα negative ($CD124^-$) population is indicated by the dashed area. The area surrounded in red highlights $CD22^+$ events (cluster 0).
(**d**) Representative flow cytometry analysis of concatenated samples (n=5) for CD22 expression in $CD4^+$ and $CD8^+$ T cells in the mesLN of WT C57BL/6 mice at day 15 after *H. polygyrus* infection. Numbers indicate percent of events in each gate.
(**e**) Analysis EOMES expression in $CD22^+CD8^+$ T cells in spleen and mesLN of IL-4Rα^{WT} and IL-4R$\alpha^{\Delta CD8}$ BALB/c mice at day 4 after PBS or IL-4c treatment, or at day 15 after *H. polygyrus* infection. Symbols indicate results from each individual mouse.
(**f**) Summary data of T_{VM} and $CD22^+$ T_{VM} percentages and numbers in the spleen of C57BL/6 WT mice at day 15 after *H. polygyrus* infection.
(**g**) $CD22^+$ T_{VM} percentages in the spleen of *H. polygyrus*-infected WT C57BL/6 mice at different time points post-infection.
(**h**) $CD22^+$ T_{VM} percentages in the mesLN, spleen, inguinal LN, and bone marrow of WT C57BL/6 mice at day 15 after *H. polygyrus* infection.
(**i**) Persistence of T_{VM} and $CD22^+$ T_{VM} over time after one i.p. injection of IL-4c and day 0.
(**j**) Percentages and absolute numbers of spleen and thymic $CD22^+$ T cells after IL-4c treatment (2× at 2 days interval, analysis at day 4).
(**k**) WT BALB:B mice were infected with 10^4 PFU of MuHV-4-Luc intranasally, and treated with IL-4c (2× at 2 days interval) at day 30 after infection. Data show representative flow cytometry analysis and summary data of CD22 expression in spleen T_{VM} or in $D^bORF6^{487\text{-}495}$ and $K^bORF61^{524\text{-}531}$ MuHV-4-specific $CD8^+$ T cells. Numbers in gates indicate percentage of tetramer positive events in $CD8^+$ T cells. Scatter plots show mean ± s.e.m. are shown.
Statistical significance calculated using two-way analysis of variance (ANOVA) and Sidak's multiple comparison-test (**P < 0.01, ***P < 0.001, ****P < 0.0001). Data are representative of three independent experiments with 3-5 mice per group (mean ± s.e.m.).

Figure S6

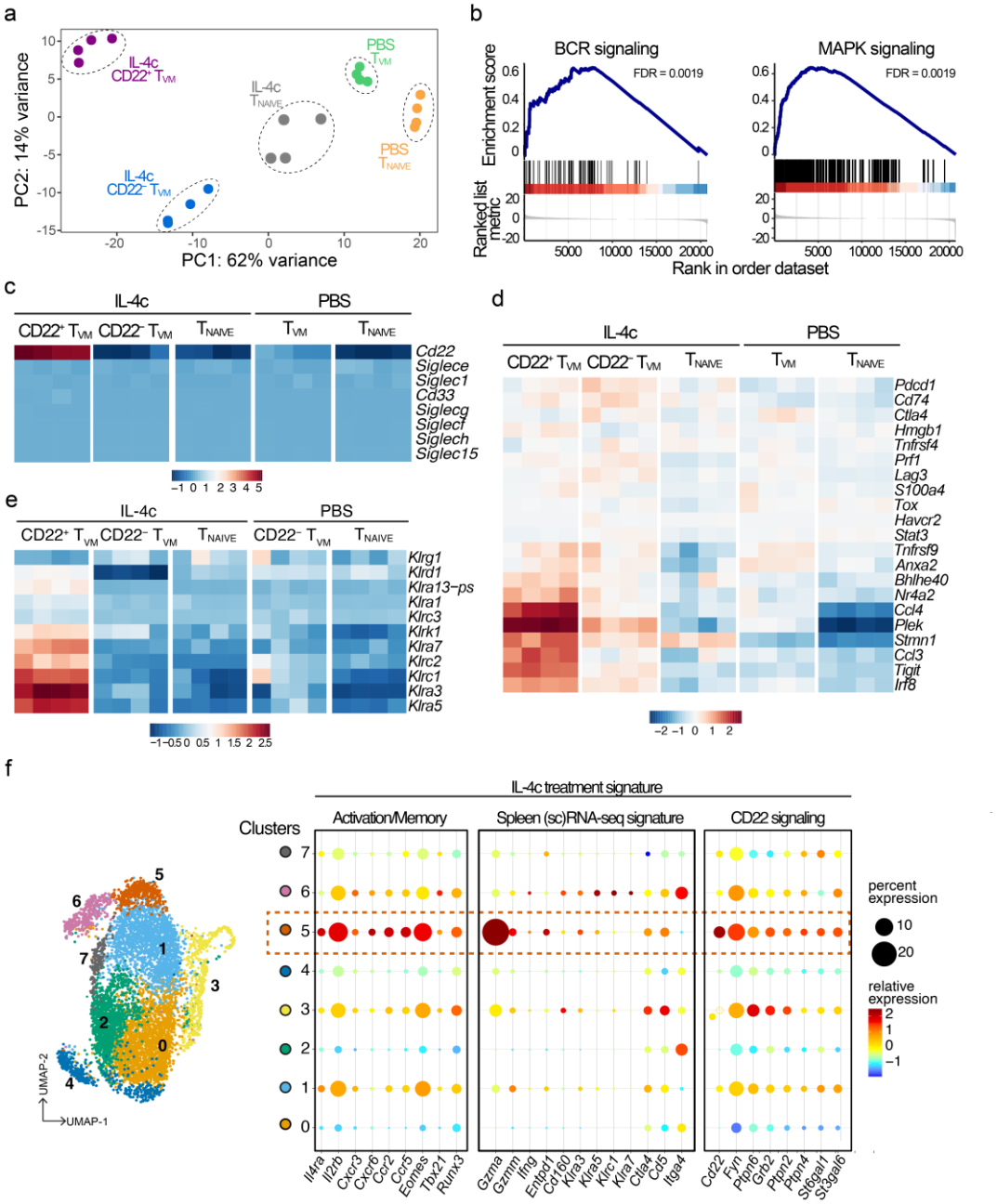

a
PC2: 14% variance
PC1: 62% variance
IL-4c CD22+ TVM
IL-4c CD22- TVM
IL-4c TNAIVE
PBS TVM
PBS TNAIVE
b
BCR signaling
MAPK signaling
FDR = 0.0019
Enrichment score
Ranked list metric
Rank in order dataset
c
IL-4c
PBS
CD22+ TVM
CD22- TVM
TNAIVE
TVM
Cd22
Siglece
Siglec1
Cd33
Siglecg
Siglecf
Siglech
Siglec15
d
Pdcd1
Cd74
Ctla4
Hmgb1
Tnfrsf4
Prf1
Lag3
S100a4
Tox
Havcr2
Stat3
Tnfrsf9
Anxa2
Bhlhe40
Nr4a2
Ccl4
Plek
Stmn1
Ccl3
Tigit
Irf8
e
Klrg1
Klrd1
Klra13−ps
Klra1
Klrc3
Klrk1
Klra7
Klrc2
Klrc1
Klra3
Klra5
f
IL-4c treatment signature
Clusters
Activation/Memory
Spleen (sc)RNA-seq signature
CD22 signaling
percent expression
relative expression
UMAP-1
UMAP-2
Il4ra
Il2rb
Cxcr3
Cxcr6
Ccr2
Ccr5
Eomes
Tbx21
Runx3
Gzma
Gzmm
Ifng
Entpd1
Cd160
Klra3
Klra5
Klrc1
Klra7
Ctla4
Cd5
Itga4
Cd22
Fyn
Ptpn6
Grb2
Ptpn2
Ptpn4
St6gal1
St3gal6

Figure S6. Unconventional activation of CD22$^+$ T_{VM} by IL-4.
(**a**) Principal component analysis of the RNA-seq analysis on sorted CD44^{low} T_{NAIVE} and CD22$^-$ and CD22$^+$ CD44^{high}CD49d^{low} T_{VM} upon PBS or IL-4c treatment.
(**b**) GSEA enrichment score plots for the indicated KEGG gene sets between CD22$^-$ and CD22$^+$ T_{VM} in IL-4c treated mice.
(**c**) Heatmap of Siglec-encoding gene expression in *Mus musculus*. Color scale indicates normalized mean expression.
(**d**) Heatmap of curated T cell exhaustion gene expression. Color scale indicates normalized mean expression.
(**e**) Heatmap of gene expression of killer cell lectin-like receptor subfamily members (*Klr*). Color scale indicates normalized mean expression.
(**f**) Dot plot showing the average expression levels and percentage of expression of selected genes in each cluster from scRNA-seq experiment performed on mesLN CD124$^+$ and CD124$^-$ CD8$^+$ T cells at day 15 after *H. polygyrus* infection, as depicted in Fig. 4 and Fig. S4. Selected genes are indicated by blocks based on signature genes identified in bulk RNA-seq on splenic CD22$^+$ T_{VM} after IL-4c treatment, and genes involved in CD22 signaling in B lymphocytes.

Figure S7

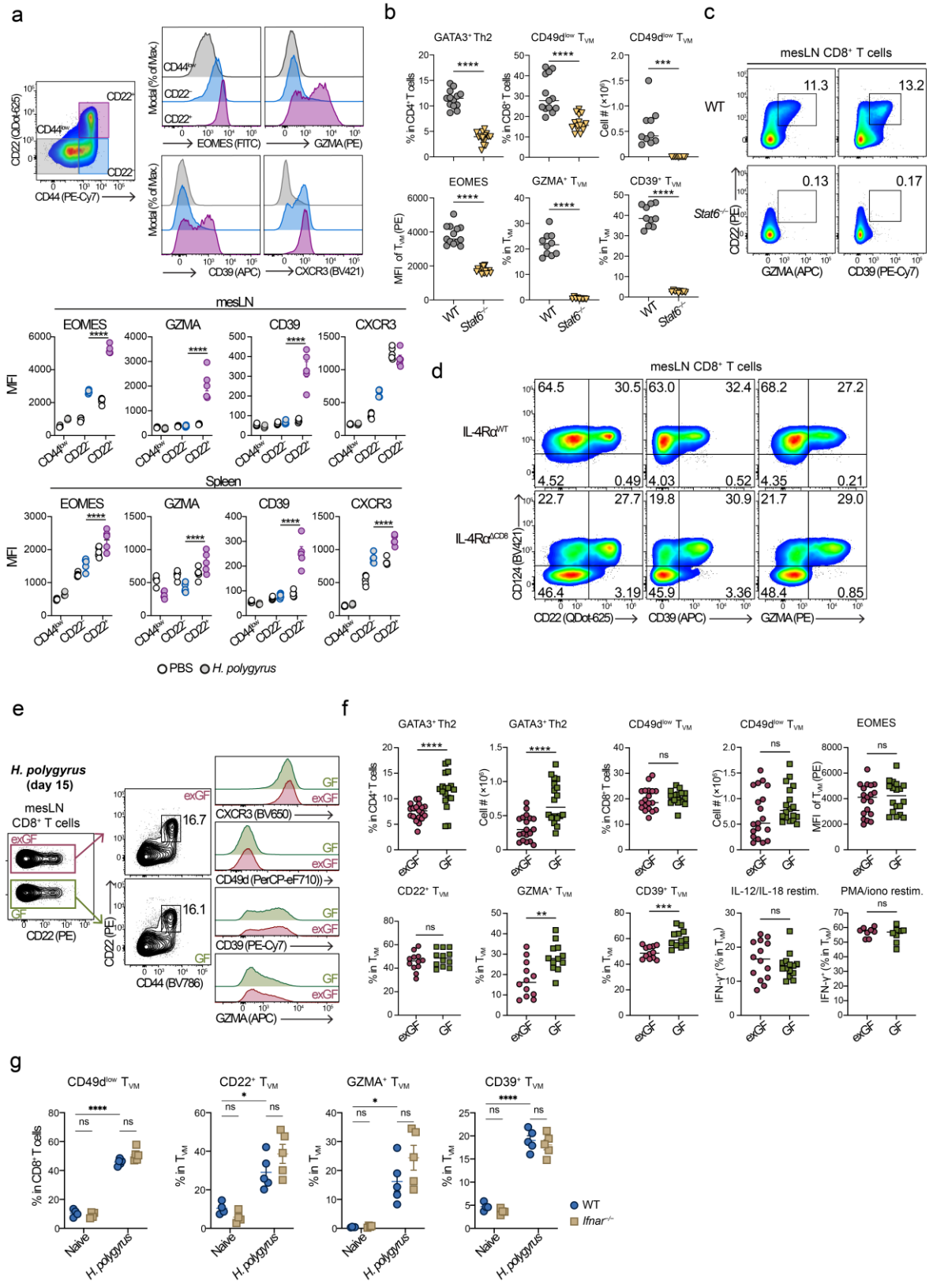
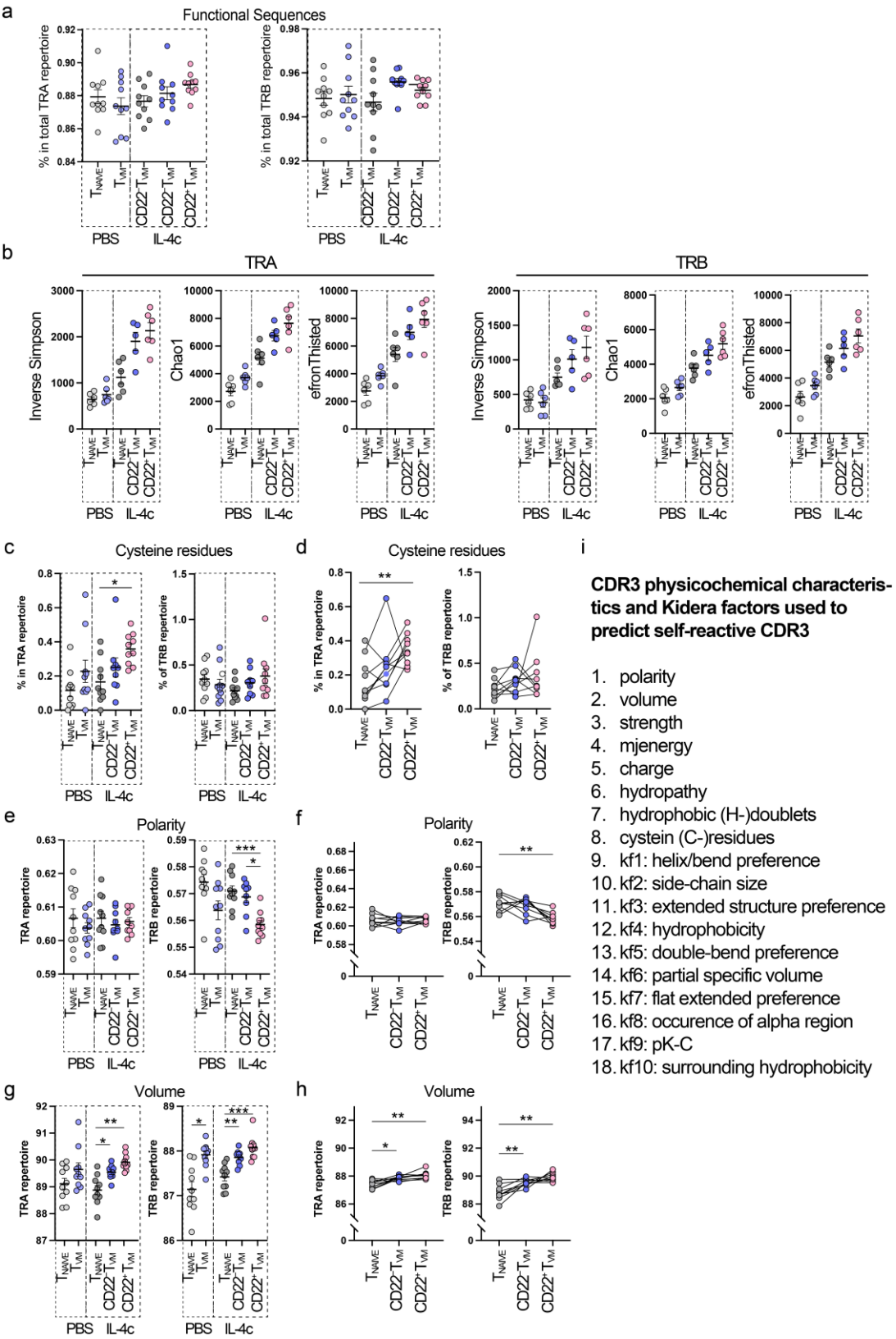

a
b
c
d
e
f
g
mesLN
Spleen
PBS
H. polygyrus
mesLN CD8⁺ T cells
GATA3⁺ Th2
CD49d^low T_VM
EOMES
GZMA⁺ T_VM
CD39⁺ T_VM
WT
Stat6⁻/⁻
IL-4Rα^WT
IL-4Rα^ΔCD8
H. polygyrus (day 15)
exGF
GF
CD22⁺ T_VM
IL-12/IL-18 restim.
PMA/iono restim.
Ifnar⁻/⁻
Naive

Figure S7. Activation of CD22$^+$ T_{VM} is IL-4Rα/STAT6-dependent but microbiota independent.
(**a**) Representative flow cytometry analysis of concatenated samples and summary data of the MFI of selected markers in mesLN CD44^{low} T_{NAIVE}, CD22$^-$ T_{VM} and CD22$^+$ T_{VM} of WT BALB/c mice at day 15 after infection with *H. polygyrus* (n=4-5).
(**b**) Summary data of GATA3$^+$ Th2 and T_{VM} responses in mesLN of C57BL/6 WT and *Stat6*$^{-/-}$ mice at day 15 after infection with *H. polygyrus* (pooled data from 3 independent experiments, n=4-5).
(**c**) Representative flow cytometry analysis of concatenated samples for the co-expression of CD22 and GZMA and CD39 in mesLN of C57BL/6 WT and *Stat6*$^{-/-}$ mice at day 15 after infection with *H. polygyrus* (n=5).
(**d**) Representative pseudocolour flow plots of concatenated CD8$^+$ T cells from mesLN of IL-4Rα^{WT} and IL-4Rα$^{\Delta CD8}$ BALB/c mice at day 15 after *H. polygyrus* infection. Numbers indicate percent of events in each quadrant (n=5).
(**e**) Representative flow cytometry analysis of concatenated samples revealing CD22 expression by T_{VM}, and coexpression of the indicated markers as shown by the histograms in mesLN of exGF and GF C57BL/6 mice at day 15 after infection with axenic *H. polygyrus* (n=5).
(**f**) Summary data of GATA3$^+$ Th2 and T_{VM} responses in mesLN of exGF and GF C57BL/6 mice at day 15 after infection with axenic *H. polygyrus* (pooled data from 3 independent experiments, n=4-5).
(**g**) Summary data of T_{VM} responses in mesLN of of C57BL/6 WT and *Ifnar*$^{-/-}$ mice at day 15 after infection with *H. polygyrus* (n=4-5).
Statistical significance calculated using two-way analysis of variance (ANOVA) and Sidak's multiple comparison-test (ns, not significant, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$). Data are representative of three independent experiments (mean ± s.e.m.).

Figure S8



i

CDR3 physicochemical characteristics and Kidera factors used to predict self-reactive CDR3

1. polarity
2. volume
3. strength
4. mjenergy
5. charge
6. hydropathy
7. hydrophobic (H-)doublets
8. cystein (C-)residues
9. kf1: helix/bend preference
10. kf2: side-chain size
11. kf3: extended structure preference
12. kf4: hydrophobicity
13. kf5: double-bend preference
14. kf6: partial specific volume
15. kf7: flat extended preference
16. kf8: occurence of alpha region
17. kf9: pK-C
18. kf10: surrounding hydrophobicity

Figure S8. Diverse TCR repertoire in IL-4-induced CD22^{+} T$_{VM}$ is enriched in self-reactive CDR3 sequences.
CD44^{low} T$_{NAIVE}$, CD22^{-} CD44^{high}CD49d^{low} T$_{VM}$ and CD22^{+} CD44^{high}CD49d^{low} T$_{VM}$ were purified by FACS from the spleen of PBS or IL-4c-treated WT BALB/c mice. CDR3α and CDR3β sequences were extracted from RNA-seq and TCR-seq.
(**a**) Summary plots of functional CDR3 sequences retrieved from the RNA-seq and TCR-seq experiments. Each symbol represents an individual mouse (n=10).
(**b**) Summary plots of inverse Simpson, Chao1 and efronThisted diversity indexes of TRA and TRB for the different T cell subsets in PBS- or IL-4c-treated mice. Each symbol represents an individual mouse. Data show CDR3α and CDR3β sequences obtained by TCR-seq. Mean ± s.e.m. is indicated (n=6).
(**c-d**) Summary plots of enrichment of cysteine residues of the central amino acids in CDR3α and CDR3β repertoire of the different T cell subsets in PBS- or IL-4c-treated mice (**d**) or of paired samples in IL-4c-treated mice (**f**).
(**e-f**) Summary plots of CDR3 polarity based on the presence of bulky amino acid side chains of CDR3α and CDR3β repertoire of the different T cell subsets in PBS- or IL-4c-treated mice (**e**) or of paired samples in IL-4c-treated mice (**f**).
(**g-h**) Summary plots of CDR3 volume based on the presence of bulky amino acid side chains of CDR3α and CDR3β of the different T cell subsets in PBS- or IL-4c-treated mice (**g**) or of paired samples in IL-4c-treated mice (**h**).
(**i**) CDR3 physicochemical characteristics used in Fig. 7h.
Statistical significance calculated using one-way analysis of variance (ANOVA) and Holm-Sidak's multiple comparison test. When repeat measurements were analyzed (**d,f,h**), one-way ANOVA for repeated measurements and Dunnett's multiple comparison test was applied. (ns, not significant, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$).

Figure S9

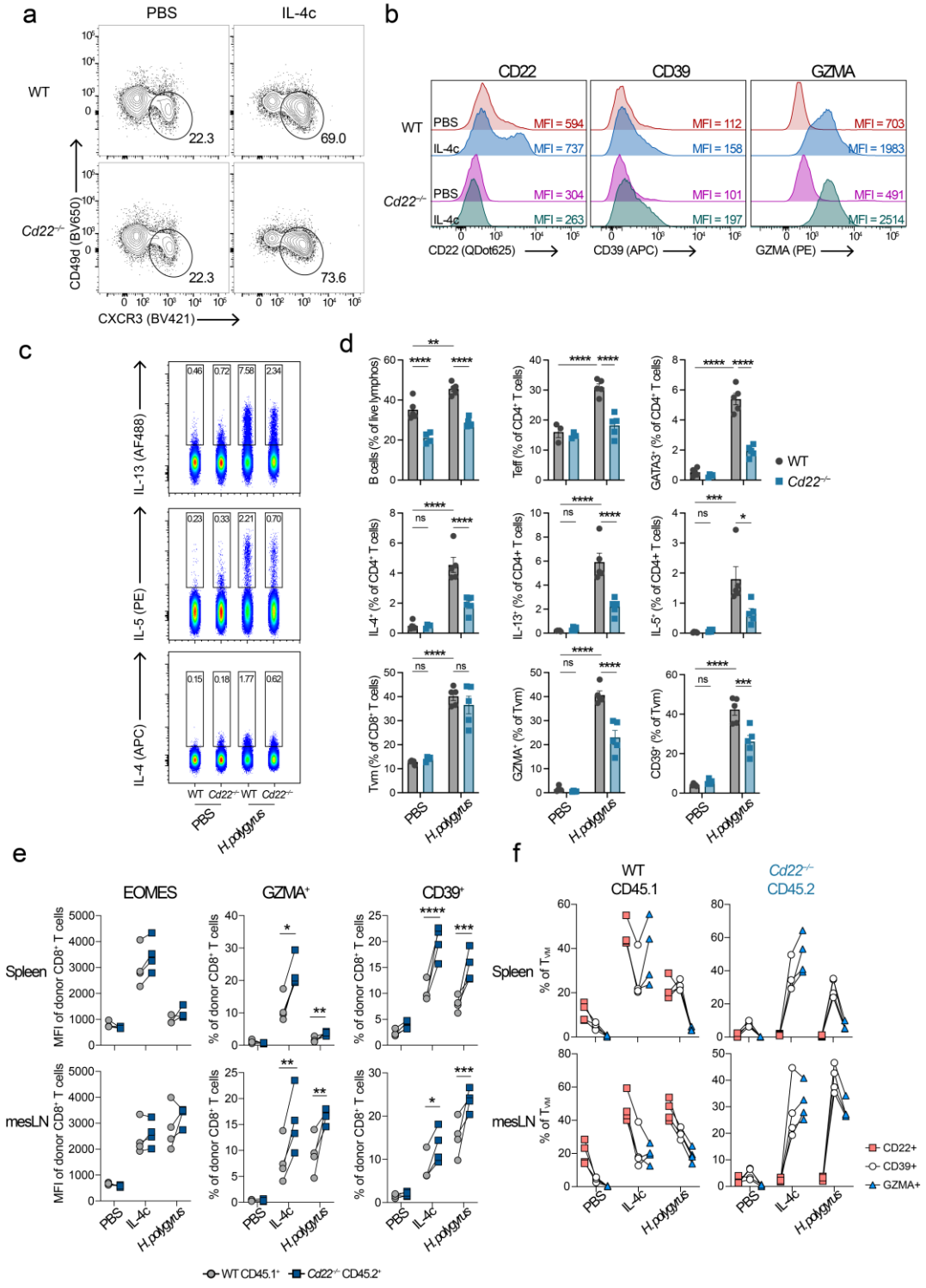

a
PBS
IL-4c
WT
Cd22-/-
CD49d (BV650)
CXCR3 (BV421)
22.3
69.0
22.3
73.6
b
CD22
CD39
GZMA
WT
Cd22-/-
PBS
IL-4c
MFI = 594
MFI = 737
MFI = 112
MFI = 158
MFI = 703
MFI = 1983
MFI = 304
MFI = 263
MFI = 101
MFI = 197
MFI = 491
MFI = 2514
CD22 (QDot625)
CD39 (APC)
GZMA (PE)
c
IL-13 (AF488)
IL-5 (PE)
IL-4 (APC)
0.46
0.72
7.58
2.34
0.23
0.33
2.21
0.70
0.15
0.18
1.77
0.62
WT
Cd22-/-
PBS
H.polygyrus
d
B cells (% of live lymphos)
Teff (% of CD4+ T cells)
GATA3+ (% of CD4+ T cells)
IL-4+ (% of CD4+ T cells)
IL-13+ (% of CD4+ T cells)
IL-5+ (% of CD4+ T cells)
Tvm (% of CD8+ T cells)
GZMA+ (% of Tvm)
CD39+ (% of Tvm)
WT
Cd22-/-
PBS
H.polygyrus
e
EOMES
GZMA+
CD39+
Spleen
mesLN
MFI of donor CD8+ T cells
% of donor CD8+ T cells
PBS
IL-4c
H.polygyrus
WT CD45.1+
Cd22-/- CD45.2+
f
WT
CD45.1
Cd22-/-
CD45.2
Spleen
mesLN
% of TVM
PBS
IL-4c
H.polygyrus
CD22+
CD39+
GZMA+

Figure S9. T_{VM} response in absence of CD22.
(**a**) Contour plots of concatenated samples (n=5) of spleen samples from WT or *Cd22*$^{-/-}$ mice treated with PBS of IL-4c. Numbers indicate the percent of CD49d^{low}CXCR3^{+} T_{VM} cells in each gate.
(**b**) Half-off-set histograms adjusted to mode of concatenated samples (n=5) showing the fluorescence intensities of the indicated markers upon analysis by flow cytometry on spleen cells from WT or *Cd22*$^{-/-}$ mice treated with PBS of IL-4c. Numbers indicate median fluorescence intensities (MFI).
(**c-d**) WT or *Cd22*$^{-/-}$ mice were infected with *H. polygyrus* (200 × L3) by gavage. At day 15 after infection, mesLN were harvested and Th2 and T_{VM} responses analyzed by flow cytometry. Pseudocolour plots of concatenated samples (n=5) of CD4^{+} T cells show percentage of IL-13^{+}, IL-5^{+} and IL-4^{+} CD4^{+} T cells for each experimental condition (PBS or *H. polygyrus*) and for each mouse genotype (c). Summary data of the percentages of the indicated populations in the mesLN at 15 days after *H. polygyrus* infection (d).
(**e**) Summary data of EOMES median fluorescence intensity, percentage of GZMA^{+} and CD39^{+} cells in CD8^{+} T cells from the spleen and mesLN of mixed bone marrow WT: *Cd22*$^{-/-}$ chimeric mice after treatment with IL-4c or infection with *H. polygyrus*.
(**f**) Summary data obtained from the spleen and mesLN of individual mixed bone marrow WT: *Cd22*$^{-/-}$ chimeric mice showing the co-expression of CD22, CD39 and GZMA.
Statistical significance calculated using two-way analysis of variance (ANOVA) and Sidak's multiple comparison test (ns, not significant, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$). Each symbol represents an individual mouse. Data are representative of 2 independent experiments.

Discussion - Perspectives

Discussion

In this thesis, we investigated how IL-4 dependent T_{VM} expansion is defined in term of gene expression signature during helminth infection, and how such expansion is regulated in response to IL-4. Unexpectedly, we discovered that CD22, an inhibitory receptor normally expressed on B cells, is specifically expressed by IL-4-driven expanding self-reactive T_{VM}. We also identified that $CD22^+$ T_{VM} express an inhibitory expression program including CD22 in response to IL-4, which likely controls their expansion and activation during helminth infection and bystander concurrent activation. Overall, we have demonstrated that IL-4 signaling during *H. polygyrus* infection in peripheral $CD8^+$ T cells drives the engagement of a specific gene signature program of activation, together with CD22 and other counter-inhibitory receptors that are not related to T cell exhaustion.

Immune control of persistent helminth infections mainly requires the development of effective $CD4^+$ Th2 cells, and experiments depleting $CD8^+$ T lymphocytes during helminth infections did not result in major effects on parasite burden. As a consequence, the role of $CD8^+$ T cells in the control of helminths has long been disregarded. Interestingly, an early study found that *S. mansoni* infection lead to the development of a strong type 1-like antigen-specific $CD8^+$ T cell response, by which production of IFN-γ and IL-10 are dependent on help from $CD4^+$ cells, but can be substituted by IL-2 or IL-4 (Pedras-Vasconcelos and Pearce, 1996). These findings demonstrated that $CD8^+$ T cells could represent an important modulator in establishment of Th2 response since they persist in a strongly type 2 environment and utilize IL-4 as helper. In addition, *H. polygyrus* could induce a cell population defined as regulatory $CD8^+$ T cells in the intestine, which modulated the induction of colitis and T cell proliferation in a mouse model independently of IL-10 but requiring MHC-I interaction (Metwali et al., 2006). These rare studies revealed that helminth infections can significantly modify $CD8^+$ T cells and their potential functions. More recently, the role of helminth-induced IL-4 on $CD8^+$ T cell responses was highlighted, and the key impact of helminth infection on the expansion of T_{VM} has been demonstrated as well as their potential role in controlling during bystander viral or bacterial co-infection (Lin et al., 2018; Rolot et al., 2018). Thus, although $CD8^+$ T cells do not readily control parasite infection, their responses to helminth infection and associated cytokines produced during the parasite infection have been shown to significantly alter their phenotype and functions, possibly in the long term.

Some studies have investigated the role of IL-4 on the development of T_{VM} by using $Il4^{-/-}$ or $Il4ra^{-/-}$ mice. However, these models do not allow to clearly investigate $CD8^+$ T cell-specific IL-4 signaling. Hence, we generated an $E8i^{Cre}Il4ra^{lox/lox}$ (IL-4Rα$^{\Delta CD8}$) mouse model, in which the Cre recombinase is specifically expressed in mature single-positive $CD8^+$ T cells exiting the thymus, and upregulating the enhancer E8i. In these mice, $CD8^+$ T cells are therefore expected to be deficient for the IL-4Rα (CD124)

expression in the periphery and would then be useful to study the effect of IL-4 signaling in these cells during helminth infection. Interestingly, although the impaired expression of CD124 was specific to $CD8^+$ T cells, a fraction of $CD8^+$ T cells retained the expression of the receptor at the protein level. Thus, IL-4R$\alpha^{\Delta CD8}$ model did not show a complete deletion of *Il4ra* in mature $CD8^+$ T cells, as similarly observed in *Il4ra*$^{lox/lox}$*Lyz2*^{cre} even *Il4ra*$^{-/lox}$*Lyz2*^{cre} mouse model (Vannella et al., 2014). Nonetheless, such partial impaired expression did not significantly impact our findings as IL-4R$\alpha^{\Delta CD8}$ mice had significantly reduced T_{VM} population in the spleen in the steady-state and after IL-4c treatment or helminth infection. Also, IL-4R$\alpha^{\Delta CD8}$ showed increased MuHV-4-Luc replication loads in the lung when compared to IL-4c-treated littermate controls, supporting our previous results showing that the IL-4-induced expansion of T_{VM} is associated with enhanced control of MuHV-4. In addition, the specific deletion in peripheral T lymphocytes was confirmed as CD124 expression was not affected in the thymus, demonstrating the selective expression of the Cre in mature $CD8^+$ T cells exiting the thymus and the requirement of IL-4 signaling in $CD8^+$ T cells to drive the expansion and functional changes in peripheral T_{VM}. Although it seems that IL-4c treatment cannot induce an increased cell number of SP8 or mature $CD24^-$ SP8 in the thymus, it did induce the upregulation of EOMES in SP8. However, reduced expression of CD124 on $CD8^+$ T cells once in the periphery impaired its response to IL-4 signaling, triggering a downregulation of EOMES and resulted into reduced peripheral expansion of T_{VM} after IL-4c treatment, which supported that IL-4 signaling could direct $CD8^+$ T_{VM} fate when TCR signals are limiting (Carty et al., 2014). Although the preference of IL-4 and IL-15 for T_{VM} expansion in different mouse strains can variate, we found that IL-4 signaling is required for the expansion of T_{VM} in both BALB/c and C57BL/6 mice, which is consistent with the results obtained by Lin *et al*. (Lin et al., 2018).

Helminths induce a complex immune response upon infection, including high levels of IL-4 after enteric colonization, together with other cytokines also involved in the persistence of the parasite. Here a scRNA-seq approach was first used to better reveal the heterogeneity of the gene expression program in splenic $CD8^+$ T cells in response to IL-4c and *H. polygyrus* infection. Interestingly, the use of CD124 as surface marker could reveal by flow cytometry analysis that IL-4R$\alpha^{\Delta CD8}$ mice could be considered as natural chimeric mouse model, in which both *Il4ra*$^{+/+}$ ($CD124^+$) and *Il4ra*$^{-/-}$ ($CD124^-$) $CD8^+$ T cells coexist. Thus, IL-4R$\alpha^{\Delta CD8}$ mice are suitable for studying how IL-4 signaling drives T_{VM} expansion in the same environment. However, scRNA-seq approach using 10X Genomics did not enable sufficient depth of sequencing to identify the populations of $CD8^+$ T cells having escaped Cre recombination. Indeed, Cre delated loxed exons 7 to 9 of the *Il4ra* locus, which means *Il4ra* RNA is still produced after Cre-mediated deletion. Despite attempts to identify cells expressing reads matching the exons 7-9 of the *Il4ra* locus, these analyses identified too few cells to be further processed in downstream analyses (data not shown). Nonetheless, our study provided in-depth description of $CD8^+$ T cells responding to IL-4 (IL-4c treatment) and to *H. polygyrus* infection. Our findings further added important information to the

understanding of memory/effector CD8$^+$ T cell diversity previously reported using scRNA-seq approaches in other settings (Galletti et al., 2020; Sekine et al., 2020); and efficiently distinguished T_{VM} from other T cell subsets, notably by using CD49d (*Itga4*) as a marker gene being downregulated. Moreover, gene signature analyses highlighted that T_{VM} differ from classical T_{TM} or exhausted T cells and are rather characterized by a unique combination of signature genes including *Eomes*, *Il2rb*, *Ctla2a*, *Gzma*, *Gzmm*, *Ccr2*, *Ccr5*, and *Itm2a* expression. In addition, an enrichment of the transcription factor responses mediated by *Atf6* and *Cebpd* was observed in IL-4-dependent T_{VM}. Such transcription factors are involved in endoplasmic reticulum (ER) stress (Cao et al., 2019; Gade et al., 2012; Haze et al., 1999; Walter and Ron, 2011). Thus, whereas IL-4 seems to induce increased cellular stress, some potential factors likely regulate the response to maintain the balance of the intracellular environment and cell survival. Additional investigations should clarify how such mechanisms are involved in T_{VM} biology. While confirming previous transcriptomic characterizations of T_{VM} (Hou et al., 2021; Rolot et al., 2018; White et al., 2016), our scRNA-seq data suggests similar trajectories of differentiation by IL-4 or *H. polygyrus*-induced T_{VM} using Velocity analyses, and further provides unique information about the expanding T_{VM} subset. Although we also observed changes in the expression of these signature genes in IL-4Rα$^{\Delta CD8}$, the magnitude of their differential expression was overall reduced when compared to WT littermate controls in the spleen, likely because IL-4Rα$^{\Delta CD8}$ are partially knockout and a remaining population of CD124$^+$CD8$^+$ T cells exist. Importantly, IL-4Rα chain is shared by both IL-4 and IL-13 receptors through hetero-dimerization with the common γ chain (*Il2rg*) or the IL-13 receptor α chain 1 (*Il13ra1*). However, IL-13Rα1 is not referenced to be expressed on T lymphocytes, and we did not find any expression of *Il13ra1* in our sequencing datasets. Thus, we are confident that the maintenance of T_{VM} is supported uniquely by IL-4. During *H. polygyrus* infection, we observed a stronger expansion of T_{VM} in the mesLN compared to the spleen, which could likely be explained by the strong local type 2 immune response in the mesenteric and enteric tissues compared to the spleen. Considering the mesenteric and enteric tissues as the main tissues exposed to continuous stimulation from worms, we decided to further investigate the mesLN to perform single cell transcriptomics analysis on sorted CD124$^+$ or CD124$^-$ CD8$^+$ T cells at two weeks after *H. polygyrus* infection. Strikingly, IL-4-induced T_{VM} signature was only found in CD124$^+$ (and thus IL-4Rα-competent) cells, whereas CD124$^-$ CD8$^+$ T cells did not display the main signature genes of T_{VM}. Also interestingly, clusters 1 and 5 were both only observed in CD124$^+$ and shared T_{VM} signature genes as observed in the spleen upon IL-4c treatment such as *Eomes*, *Il2rb*, *Itm2a* and downregulation of *Itga4*. However, cluster 5 show additional marker genes including *Gzma*, *Ccr5* and *Cd22*, suggesting these two populations might develop in response to IL-4 in the T_{VM} population.

When computing differentially expressed genes in both IL-4 and *H. polygyrus*-induced T_{VM}, genes encoding effector molecules like *Gzma*, *Gzmm*, *Ctla2a*, *Ccr2* and *Ccr5* were upregulated, but we also

unexpectedly observed the upregulation of *Cd22*. CD22 is a sialic acid-binding immunoglobulin-like lectin (siglec-2), a canonical inhibitory receptor of B lymphocytes. CD22 contributes to the regulation of B cell responses therefore controlling autoimmune diseases (Müller and Nitschke, 2014), and its expression on other cell types has only been scarcely reported (Hudson et al., 2019; Pluvinage et al., 2019; Sathish et al., 2004; Wen et al., 2012). CD22 is upregulated by microglial cells in aging brains, where it inhibits phagocytosis (Pluvinage et al., 2019), and one report identified functional CD22 in primary T cells but with a very low surface expression level (Sathish et al., 2004). Although RNA-seq data often reveals signature genes for which expression at the protein level is difficult or not possible to demonstrate, we however could obtain clear evidence by flow cytometry with exclusion of $CD19^+$ B lymphocytes, as well as using ImageStream analyses that CD22 is expressed at significant levels in $CD8^+$ T lymphocytes. More specifically, we further demonstrated that $CD22^+$ T_{VM} expansion was directly dependent on IL-4 as they were not expanding in *Il4ra*$^{-/-}$ and *Stat6*$^{-/-}$ mice after *H. polygyrus* infection. Thus, CD22 expression on T_{VM} was significantly increased, but the level of expression remains lower when compared to B lymphocytes, which could explain why this has never been reported before. Interestingly, CD22 was the only sialic acid-binding immunoglobulin-like lectin (siglec) gene upregulated in T cells upon IL-4 signaling. Also, we used *Cd22*$^{-/-}$ mice as controls to demonstrate that the CD22 staining is specific and not an artefact, which is important to overrule any risk of false positivity. Whereas CD22 could be upregulated in T_{VM} by IL-4, this cytokine was unable to upregulate CD22 in virus-specific effector/memory T cells, thymocytes or $CD4^+$ T cells. Thus, our detailed characterization clearly demonstrated that CD22 can be expressed in $CD8^+$ T cells, and that the expansion of $CD22^+$ T_{VM} in directly dependent on IL-4 stimulation. Whereas CD22 is directly upregulated at the gene level by IL-4 remains to be investigated. Preliminary *ex vivo* experiments on sorted naïve $CD8^+$ T cells or splenocytes incubated with various concentrations of rIL-4 failed to induce detectable levels of CD22 at the surface of the cells (data not shown), suggesting that IL-4 might rather driving the expansion of T_{VM} *in vivo* which in turn results in an indirect upregulation of CD22.

Our findings revealed that not all T_{VM} upregulated expression of CD22 in response IL-4. The phenotypic changes between $CD22^+$ T_{VM}, $CD22^-$ T_{VM}, and T_N were revealed at the transcriptomic level through bulk RNA-seq analysis of sorted populations. Genes like *Gzma, Ifng, Eomes, Il2rb, Mki67, Ctla2a, Slc16a2, Gzmm, Xcl1, Ccl4, Cxcr3, Ccr2* or *Ccr5* were significantly upregulated in $CD22^+$ T_{VM}, whereas $CD22^-$ T_{VM} also upregulated a few of these activation genes when compared to T_N or T cells from naïve mice. We could imagine that $CD22^+$ T_{VM} and $CD22^-$ T_{VM} would represent either a continuum of differentiation representing 'semi-differentiated' $CD22^-$ T_{VM} and 'fully differentiated' $CD22^+$ T_{VM}. Another explanation would be that both $CD22^+$ and $CD22^-$ T_{VM} respond to IL-4 similarly, but a subset of T_{VM} would upregulate CD22 in response to other stimuli independently of direct IL-4 signaling. Further characterization revealed significant gene set enrichment in $CD22^+$ T_{VM} of T cell signaling, NK

mediated killing and IL-2/STAT5 signaling, suggesting CD22$^+$ T_{VM} might have enforced activation and cytotoxicity. However, we did not observe any upregulation of other cytotoxic granules such as *Gzmb* or *Prf1* although IFN-γ was preferentially expressed in CD22$^+$ T_{VM}. IL-4 was previously shown to induce IFN-γ production in T_{VM} upon restimulation, further explaining why expanded T_{VM} could quickly respond to viral infection. While investigating the phenotype of CD22$^+$ T_{VM}, we found that IL-4-induced T_{VM} expansion was associated with the upregulation of additional signature genes like CD39 (*Entpd1*) and GZMA. Importantly, both CD39 and GZMA could be efficiently detected by flow cytometry and were specifically found in CD124$^+$ CD22$^+$ T_{VM} after *H. polygyrus* infection or IL-4c treatment. Similar to what we observed regarding CD22 expression, no upregulation of either CD39 or GZMA could be observed in *Il4ra*$^{-/-}$ or in *Stat6*$^{-/-}$ mice, irrespective of the BALB/c or C57BL/6 strain background. These findings clearly highlight that IL-4/STAT6 signaling is essential for driving a specific program in T_{VM} upon helminth infection. These findings are important, since expansion of T_{VM} in BALB/c and C57BL/6 strains have been shown to rely on different cytokines (Tripathi et al., 2016). While IL-15 signaling has also been shown to expand T_{VM} upon *H. polygyrus* infection (Hussain et al., 2023), both cytokines signal through the common gamma chain (γc) and might trigger overlapping intracellular activation pathways in T_{VM} (Jameson et al., 2015). However, our findings place IL-4/STAT6-dependent signaling as being essential in helminth-induced upregulation of CD22, CD39 and GZMA upregulation by T_{VM}. CD39 is an ectonucleotidase that modulates the immune response by hydrolyzing ATP to ADP in the extracellular space. CD39$^+$ CD8$^+$ T cells could express excessive IFN-γ via ROS signaling after anti-CD3 and/or CD28 stimulation. These cells then initiate purinergic signaling by generating extracellular adenosine, which binds to the adenosine A2A receptor leading to inhibition of JNK and NFκB signaling and decrease IFN-γ production by surrounding CD39$^-$ T cells and other immune cells (Bai et al., 2015). Thus, CD39 expressed by expanding CD22$^+$ T_{VM} in response to IL-4 could modulate the immune environment during helminth infection. Experiments using *Cd39*$^{-/-}$ mice or administration of the CD39 inhibitor POM-1 (sodium polyoxotungstate) could be developed to investigate the role of CD39 during helminth infection (Fang et al., 2022; Lima et al., 2018).

In addition to CD39, a strong upregulation of GZMA was also observed to be restricted to IL-4-induced CD22$^+$ T_{VM}. Interestingly, a previous study found that *in vitro* activation of naive CD8$^+$ T cells, in the presence of IL-4, enhanced STAT6-dependent GZMA expression and was associated with GATA3 binding and enrichment of transcriptionally permissive histone post-translational modifications (PTMs) across the *Gzma* gene locus (Nguyen et al., 2016). We found that helminth-induced IL-4 increased GATA3$^+$ Th2 population in CD4$^+$ T cells, and strong STAT6-dependent GZMA expression in CD22$^+$ T_{VM}, especially in mesLNs during *H. polygyrus* infection, likely by continuous potent IL-4 stimulation. However, no GZMA upregulation was observed in CD4$^+$ T cells. Like other granzymes, GZMA is a serine protease which has been shown to have more functions than simply being cytotoxic

(Lieberman, 2010; van Daalen et al., 2020). Indeed, GZMA can be pro-inflammatory while not directly cytotoxic, and rather act as an inflammatory modulator (Metkar et al., 2008). This could support the results obtained by Lin *et al* that T_{VM} expanded by helminth infection can protect against bacterial infection (Lin et al., 2018). Seen the high quantities of GZMA detected in $CD22^+$ T_{VM}, it would be of high interest to further investigate the role of GZMA as well as GZMM which was also detected by scRNA-seq in these cells and how they contribute to the yet underappreciated functions of IL-4-induced T_{VM}. There, the newly generated *Gzma*-knockin mice would be of interest to study the role of this serine protease during helminth infection and in the functions of T_{VM} (Rawle et al., 2022).

Importantly, we further demonstrated that the intestinal microbiota does not contribute to the expansion of T_{VM} during helminth infection, and does not affect the expression of marker genes in $CD22^+$ T_{VM}. In germ-free mice, *H. polygyrus*-induced type 2 immune response was increased and resulted in a robust expansion of $CD22^+$ T_{VM} conserving the expression of CD39 and GZMA. Thus, although *H. polygyrus* infection in WT mice has been reported to alter the microbiota composition in the intestine, it did not affect the upregulation of CD22, GZMA, CD39, or proliferation of $GATA3^+$ Th2 cells. Interestingly as well, $Ifnar^{-/-}$ mice also expanded in the mesLN upon *H. polygyrus* infection, suggesting that although type I IFN signaling is required to maintain T_{VM} pool in naive conditions, the IL-4-driven expansion of T_{VM} does not require type I IFN response (Martinet et al., 2015). In addition to CD39 and GZMA, we observed the upregulation of inhibitory receptors like, *Cd160, Klra3* (Ly49c), *Klra5* (Ly49e), *Klra7* (Ly49g), *Klrc1 and Klrd1 (NKG2A/CD94)* and downregulated *Klrg1* indicating that IL-4-induced development of $CD22^+$ T_{VM} is regulated by several endogenous modulators, or could regulate other immune responses.

At the cell surface of B lymphocytes, CD22 creates homo-oligomers and also binds to CD45 *in cis* via interactions with α2,6-linked sialic acids (Akatsu et al., 2022; Meyer et al., 2018). However, the B cell receptor (BCR) does not interact with CD22 via sialic acids and both BCR and CD22 are rather found in separate membrane domains (Meyer et al., 2018). In $Cd22^{-/-}$ mice, B cell activation is not controlled and leads to hyperreactivity. However, in mouse models where CD22 cannot bind sialic acids ($Cd22^{R130E}$ knockin mice) (Muller et al., 2013), less CD22 homo-oligomers are formed, resulting in increased association with the BCR, which then rather results in over-inhibition of the BCR signaling. In our findings, we observed that the $CD22^+$ T_{VM} have a distinct functional phenotype and express a diverse TCR repertoire enriched in features of self-reactivity when compared to naive T cells and from $CD22^-$ T_{VM} in response to IL-4. Thus, it is possible that CD22 counter regulate signals coming from TCR activation by self-peptide presentation, although this mechanism would be difficult to demonstrate. Previous reports showed that IL-4-responding antigen-specific T_{TM} had an altered effector phenotype with reduced NKG2D or CCL5 expression (Ventre et al., 2012), but our results showed increased

NKG2D and CCL5 expression in CD22$^+$ T_{VM} (data not shown); and highlighted that IL-4 production in response to helminth infection drives an enrichment of genes involved in TCR signaling, cytotoxicity, cell cycle, and the STAT-5 activation pathway. TCR activation is supported by the enriched features of CDR3 sequences associated with self-reactivity, similar to that observed in Tregs (Drobek et al., 2018; Logunova et al., 2020; Miller et al., 2020). Increased expression in CD22$^+$ T_{VM} of *Cd5*, which is associated to self-antigen recognition (Azzam et al., 1998; Tarakhovsky et al., 1995), supports the fact that CD22$^+$ T_{VM} are self-reactive. Considering the more complexed and diversity of TCRβ repertoire, because of the additional diversity (D) regions in the TRB locus, CDR3 sequencing revealed comparable TCR repertoire diversity but more self-reactive in CD22$^+$ T_{VM}. It would be interesting to use a strategy developed in Miller *et al.* that uses transgenic mice for one specific Vβ chain in order to fix the TRB diversity and only study TRA diversity. Such strategy would also enable the use of retrogenic mice expressing given TCRα CDR3 sequences in adoptive transfer experiments. However, our TCR-seq analyses support previous observations and convincingly show that CD22$^+$ T_{VM} are enriched in T cells displaying TCR having self-reactivity features. Mechanistically, EOMES has been involved in driving self-reactive T_{VM} in the thymus (Miller et al., 2020). Thus, it is possible that IL-4 induces an effector/memory program in self-specific T_{VM} via EOMES activation, which is then counter-regulated by CD22 expression. This possibility is also supported by the observed upregulation of the proto-oncogene Src tyrosine protease *Fyn* in our RNA sequencing data. Fyn is expressed in T cells and is directly related to the B cell oncogene Lyn which is required for CD22 inhibitory activity in B lymphocytes as well as increased expression of the adaptor protein-encoding gene *Grb2* which interacts with the immunoreceptor tyrosine-based inhibitory motif (ITIM) of CD22 to modulate calcium flux (Smith et al., 1998). When B cells are activated by recognizing antigens, CD22 is recruited to the BCR vicinity and its ITIMs become phosphorylated leading to recruitment of phosphatases such as SHP-1, SHP-2 and SHIP-1, which could inhibit B cell activation (Aujla et al., 2019). On the contrary, we found IL-4 unregulated expression of CD22 on T_{VM}. However, we did not find upregulation of SHP-1 (*Ptpn6*), SHP-2 (*Ptpn11*) and SHIP-1(*Inpp5d*), but upregulated *Ptpn2* and *Ptpn22*, in CD22$^+$ T_{VM} compared with that in CD22$^-$ T_{VM}. It suggested that CD22 signaling in T cells might not be exactly the same as observed in B lymphocytes. Besides CD22 as regulator of T_{VM} responses, other inhibitory receptors could also be involved such as CD39, CD160, or inhibitory Ly49e/g. Interestingly, CD8$^+$ T cells expressing inhibitory Ly49 or related killer cell immunoglobulin-like (KIR) receptors in mouse or human, respectively, have been reported to ensure immunoregulation and control immunopathology (Kim et al., 2011; Li et al., 2022). Thus, we could describe CD22$^+$ T_{VM} as potential regulatory-like CD8$^+$ T cells in terms of functionality although they do not express Foxp3, as T_{VM} can maintain immune homeostasis by killing activated T cells, as previously reported (Akane et al., 2016).

Whereas IL-4 induces expansion of CD22$^+$ T_{VM}, it was reported to reduce CD22 expression on B cells (Rudge et al., 2002). In any case, CD22 most likely acts as an inhibitory receptor in T cells although the exact mechanisms remain to be uncovered. Indeed, *Cd22*$^{-/-}$ mice have T_{VM} numbers in the spleen significantly higher than WT controls, suggesting CD22 regulates their development. Moreover, IL-4c treatment induced an enhanced expansion of T_{VM} in absence of expression of CD22 globally. However, *H. polygyrus* infection unexpectedly did not result in increased T_{VM} expansion in *Cd22*$^{-/-}$ mice. Although this result might seem contradictory, the significant reduced GATA3$^+$ Th2 response in the mesLN of *H. polygyrus* infected *Cd22*$^{-/-}$ mice, together with impaired type 2 cytokine production (IL-4, 5 and 13) likely explains such reduced effects, as well as the reduced CD39 and GZMA expression in *Cd22*$^{-/-}$ mice. Thus, the absence of CD22 on B lymphocytes likely impairs Th2 response establishment and should be further explored. Indirectly, these results in germline *Cd22*$^{-/-}$ mice during *H. polygyrus* infection strengthen the association between CD22, CD39 and GZMA with IL-4-induced expansion of T_{VM}. Using two complementary models of bone-marrow mixed chimeric mice in which WT immune cells can develop in parallel to *Cd22*$^{-/-}$ or *Cd22*^{Y2,5,6F} cells, we were able to decipher the cell-intrinsic role of CD22 in the regulation of T_{VM}. Indeed, in response to IL-4c or *H. polygyrus* infection, both *Cd22*$^{-/-}$ and *Cd22*^{Y2,5,6F} compartment showed increased T_{VM} expansion as well as increased CD39 and GZMA expressing cells in the T_{VM} compartment, when compared to the WT cell compartment. Thus, it appears that CD22 regulates the expansion of T_{VM} after IL-4 activation. Interestingly, CD22 seems to act downstream of an IL-4/EOMES axis, as no difference in EOMES expression was observed in WT and *Cd22*$^{-/-}$ or WT and *Cd22*^{Y2,5,6F} compartment of the bone marrow chimeras. Thus, while IL-4 signaling activates T_{VM} expansion by driving an EOMES-dependent program of memory differentiation though yet to uncover mechanisms, CD22 expression is likely independent of EOMES. Supporting this hypothesis, EOMES-transgenic mice did not upregulate *Cd22* expression (Istaces et al., 2019), whereas BALB/c EOMES$^+$ innate memory T cells in the thymus had increased *Cd22* expression in the same study. However, we could not detect CD22 expression in thymic SP8 in our study, either in naïve or in IL-4c treated mice. Importantly, IL-4-induced expansion of T_{VM} in the bone marrow mixed chimeras resulted in enhanced responses of CD22-deficient CD8$^+$ T cells upon *ex vivo* restimulation with PMA/ionomycin, anti-CD3/CD8 or IL-12/18. Such TCR-targeting or bystander cytokine activation resulted in increased IFN-γ production by *Cd22*$^{-/-}$ or *Cd22*^{Y2,5,6F} CD8$^+$ T cells. Moreover, such increased activation was also associated with increased proportions of CD8$^+$ T cells displaying a phenotype of effector T cells with increased expression of CD39, KLRG1 and CD107a (LAMP-1). Thus, these data suggest that CD22 expression on CD8$^+$ T cells reduces the degree of T_{VM} activation during helminth infection. CD22 would then contribute as a mechanism of regulation of peripheral tolerance of self-reactive T_{VM} expansion in response to IL-4 during worm infection.

As discussed above, common γ chain cytokines, like IL-4 and IL-15, can induce the expansion of T_{VM}. However, whether it remains possible that IL-15 could drive CD22 expression, our data demonstrate via several approaches that IL-4 signaling through the IL-4Rα in $CD8^+$ T cells is essential for $CD22^+$ T_{VM} expansion and activation. Nonetheless, we expect that IL-15 expression is retained during *H. polygyrus* infection of IL-4R$\alpha^{\Delta CD8}$ mice. However, we did not observe T_{VM} expansion in the $CD124^-$ cells in these mice. Thus, our results indirectly demonstrate that IL-15 cannot induce expansion of CD22 in T_{VM} in these settings; or it is also possible that IL-4R$\alpha^{\Delta CD8}$ mice have a defect in the expression of effective IL-15 receptor, which we did not verify. Interestingly, C57BL/6 mice express low levels of CD22 on $CD8^+$ T cells at the steady state, while CD22 expression is barely detectable in BALB/c mice. This observation is worth paying attention as IL-15 might be required for the generation and maintenance of $CD22^+$ T_{VM} in naive C57BL/6 mice. Indeed, $CD22^+$ T_{VM} in naive WT, *Il4ra*$^{-/-}$ or *Stat6*$^{-/-}$ mice remain at very similar low but detectable levels in C57BL/6 mice, even after *H. polygyrus* infection. Also, our results suggest that the baseline expression of IL-4 by thymic $PLZF^+$ cells in BALB/c mice cannot induce expression and upregulation of CD22 on SP8, whatever these cells are mature or not. Based on these observations, the origin of $CD22^+$ $CD8^+$ T cells might be different and dependent on the strain background. In BALB/c mice, our group has suggested that helminth-induced expansion of T_{VM} does not result from proliferation of existing T_{VM} and recruitment of thymus T cells (Rolot et al., 2018); but likely from the conversion of naive cells. However, if this would be the case, it becomes difficult to explain that IL-4-induced T_{VM} are enriched in self-reactive features like in naive T_{VM}, whereas $CD22^-$ T_{VM} do not. Thus, given the self-reactivity in T_{VM}, and the fact that highly self-reactive clones preferentially differentiate into antigen-inexperienced memory-like cells (Paprckova et al., 2022), the preferred hypothesis is that naive $CD8^+$ T cells with high binding affinity for self-antigens could convert into T_{VM} and be expanded by IL-4 in the periphery; which fits with the description of T_{VM} having similar self-tolerance as T_N and defined as 'semi-naive' or 'semi-differentiated'. Supporting the expansion from existing T_{VM} during helminth infection, helminth-induced expansion of T_{VM} has been recently shown to be primarily driven by proliferation of existing T_{VM} and not by *de novo* generation from T_N (Hussain et al., 2023). We observed that helminth-induced $CD22^+$ T_{VM} was also found in inguinal LN and bone marrow, which suggested a recirculation in the periphery of these cells, similar to T_{CM}. Hence, expanded $CD22^+$ T_{VM} in C57BL/6 mice after IL-4c treatment likely results from direct proliferation of existing $CD22^+$ T_{VM}. To further investigate the origin of $CD22^+$ T_{VM}, pre- or pro- $CD22^+$ T_{VM} could be investigated using fate-mapping or timestamping systems as in Smith *et al.* (Smith et al., 2018). It is possible that the fate decision of cells with the potential to express CD22 could be governed by developmental origin, and expression and upregulation of CD22 mark and regulate IL-4-responding activation. Using transfers of TCR transgenic mice such as OT-I T_N and $CD22^-$ T_{VM} to naive recipients and detecting which compartment express CD22 after IL-4c or IL-15c administration would at least demonstrate which subsets of self-reactive $CD8^+$ T cells could become $CD22^+$ T_{VM}. In addition, as we

found that CD22 expression is transient, and that *Il4ra* expression is slightly downregulated in $CD22^+$ T_{VM} compared to $CD22^-$ T_{VM} (data not shown), it is possible that the more activated $CD22^+$ T_{VM} reduce their sensitivity to IL-4 and IL-4 signaling might not be required for the maintenance of $CD22^+$ T_{VM}.

In B cells, CD22 is a recycling endocytic receptor distributed between the cell surface and endosomal compartments shared with Toll-like receptors (TLRs), resulting in a strong constitutive suppression of TLR signaling (Duan and Paulson, 2020; O'Reilly et al., 2011). Thus, although RNA-seq data clearly demonstrate the upregulation of *Cd22* gene expression, once upregulated CD22 surface expression is lost after a few days post-IL-4c treatment whereas proportions of T_{VM} remain high. To determine whether CD22 expression in T_{VM} is really downregulated overtime or simply recycled in endosomes, intracellular detection of CD22 is required.

Interestingly, the expression of CD22 on T_{VM} in naïve C57BL/6 mice was recently reported at steady state at the RNA level (Hou et al., 2021; White et al., 2016), and it was also mentioned to be detected in stem-like $CD8^+$ T cells but no role was associated to CD22 (Hudson et al., 2019). Given that $CCR2^+$ T_{VM} could quickly infiltrate the lung against IAV infection (Hou et al., 2021), we have observed that $CD22^+$ T_{VM} cells upregulate *Ccr2* RNA and they might also infiltrate the lung upon viral challenge. CXCR5, related to cell stemness, was also found upregulated in $CCR2^+$ or $CD22^+$ T_{VM} (Hou et al., 2021) (data not shown), which further supports that T_{VM} have a high capacity of asymmetric division, and likely better respond to cognate antigens. Using transfers of $CD22^+$ and $CD22^-$ T_{VM} to naive recipients followed by viral infection could address this hypothesis. Indeed, as $Cd22^{-/-}$ have increased T_{VM} numbers and restimulation results in enhanced IFN-γ expression and increased proportion of effector T cells, $Cd22^{-/-}$ T_{VM} could be more responsive to viral infection, which could be verified by co-transfers of WT and $Cd22^{-/-}$ T_{VM} to WT or $Rag^{-/-}$ recipients followed by IAV infection and detecting which compartment is the majority in long term for protection. Considering CCR2 expression is beneficial for recruitment, defining the kinetics of cell recruitment and the quality of the response to the viral infection in the lung is important to address this question.

In recent studies, T_{VM} were found to accumulate during ageing with a unique proliferation and metabolic profile, as well as increased ability of asymmetric cell division which correlated with memory potential (Borsa et al., 2021). It is also established that $CD8^+$ T cell populations previously defined as T_{CM} cells, from young, aged or infected mice, may have been predominantly composed of T_{VM} cells, and display a high heterogeneity in the quality of their responses (Quinn et al., 2020). Our scRNA-seq data illustrated that IL-4 can enlarge the heterogeneity of $CD8^+$ T cells and induce T_{VM} into an activated phenotype. However, not all T_{VM} could express/upregulate CD22. CD22 expression on T_{VM} might counter-inhibit the further development of $CD22^+$ T_{VM}, and $CD22^-$ T_{VM} might represent the

compartment with capability of asymmetric division. Indeed, we found the upregulation of *Itgae* (CD103) and downregulation of *Ccr2* in $CD22^{-}$ T_{VM}, while $CCR2^{-}$ T_{VM} prefer to develop into resident memory $CD8^{+}$ T cell after viral infection (Hou et al., 2021). Therefore, it is possible that $CD22^{-}$ T_{VM} would have higher capacity of asymmetric division and contribute to bystander immune response more effectively, whereas $CD22^{+}$ T_{VM} would have reached a more advanced level of differentiation potentially due to their self-reactive features. Moreover, high activation observed in $CD22^{+}$ T_{VM} would result in a short-lived fate during immune response, while $CD22^{-}$ T_{VM} would be developing as long-lived memory $CD8^{+}$ T cells and keep high heterogeneity and self-tolerance to respond to cognate antigens. CD22 has been shown to regulate Ca^{2+} signaling in B cells via binding to sialic acids in α2,6 linkages *in cis* or *in trans* (Muller et al., 2013). We identified an enriched *Atf6* dependent transcription program in IL-4-responding T_{VM}, suggesting that IL-4 induces endoplasmic reticulum (ER) stress in T_{VM} which could change Ca^{2+} homeostasis. Thus, upregulation of CD22 might promote Ca^{2+} release from ER and induce immune signaling, while Bcl2 could negatively regulate Ca^{2+} signaling to maintain cell survival (Lam et al., 1994; Rodriguez et al., 2011). This hypothesis could explain why CD22 expression on T_{VM} was transient, and the relative downregulation of *Bcl2*, an anti-apoptotic gene, in $CD22^{+}$ T_{VM} (data not shown) also supporting that $CD22^{+}$ T_{VM} could have a short-lived fate. Thus, CD22 expression in T_{VM} might indirectly distinguish the more self-reactive subset, whereas the more self-tolerant subset do not express CD22, explaining why T_{VM} display both contradictory characteristics.

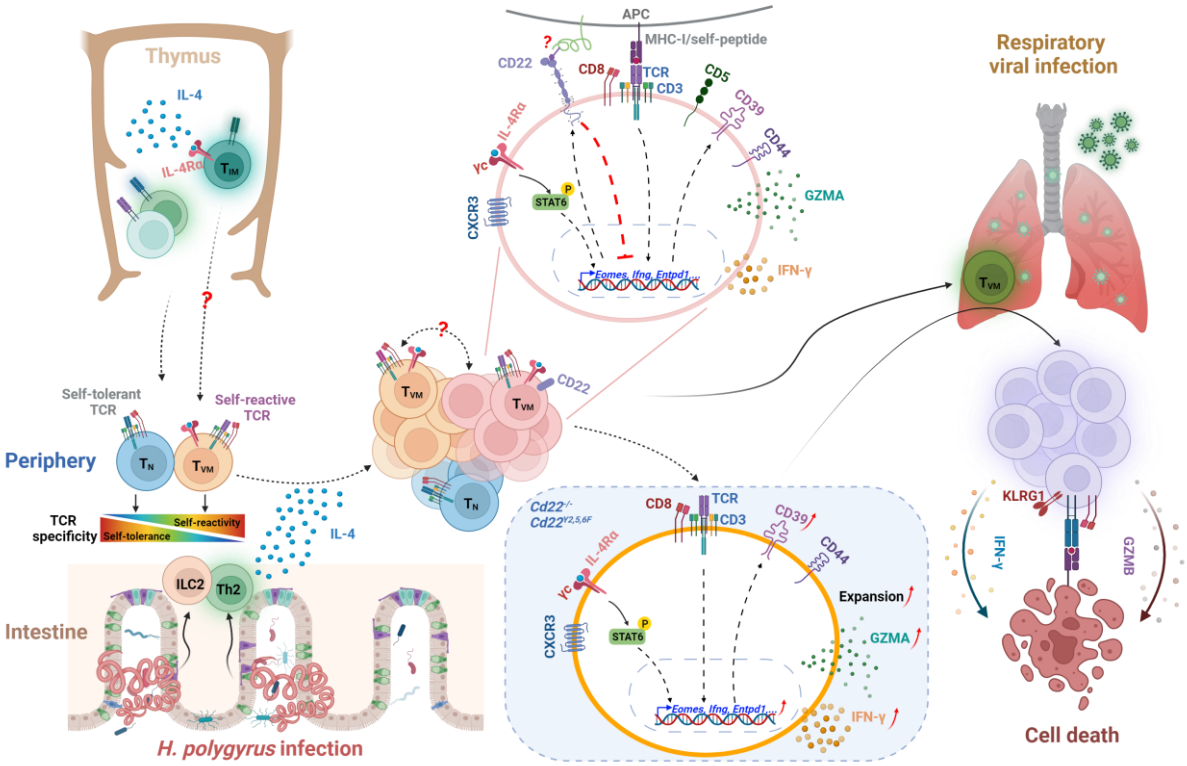
Based on the proposed hypothesis, IL-4 would activate self-reactive T_{VM} which would result in their expansion and upregulation of CD22. Thus, if TCR signaling and self-peptide recognition is involved in such mechanism, blocking MHC-I signaling followed by IL-4c and/or *H. polygyrus* infection could reduce the levels of expansion and/or CD22 expression. This experiment could be performed *in vitro* and *in vivo*. Similarly, given upregulation of the phosphatases *Ptpn2* and *Ptpn22* in $CD22^{+}$ T_{VM}, some inhibitors (Ho et al., 2021) could be administrated to investigate how downstream genes could be involved in IL-4-induced activation of T_{VM}. To further understand how CD22 functions in T_{VM}, existing CD22-knockin mouse model could be used. These models consist in $Cd22^{R130E}$ and $Cd22^{Y2,5,6F}$ mice respectively impaired for sialic acid binding domain or intracellular phospho-signaling. Our data show that in WT: $Cd22^{Y2,5,6F}$ bone-marrow chimeras, absence of phospho-signaling in T_{VM} resulted in a very similar phenotype as observed in WT: $Cd22^{-/-}$ chimeric mice. Indeed, the $Cd22^{Y2,5,6F}$ compartment showed a significant increased expansion of $CD49d^{low}$ T_{VM} together with increased CD39 and GZMA expression by T_{VM} upon IL-4c treatment, as well as increased KLGR1 and IFN-γ expression upon restimulation. In IL-4c treated WT: $Cd22^{-/-}$ chimeric mice followed by IAV infection, more $Cd22^{-/-}GZMB^{+}$ $CD8^{+}$ T cells in BALF further suggested a stronger activation and cytotoxic potential of $CD8^{+}$ T cells with absense of *Cd22*. It remains to address the role of sialic acid binding, using bone-marrow WT: $Cd22^{R130E}$ chimera mice; but it is likely that mutating the sialic acid binding domain will

result in an alteration of the T_{VM} responses. In addition, to clearly address the mechanism of regulation mediated by CD22 on T_{VM}, an *E8i*^{cre} *Cd22*$^{lox/lox}$ mouse model would be ideally developed. Unfortunately, there is no *Cd22*$^{lox/lox}$ mice as CD22 has always been regarded as being specific to B lymphocytes. An alternative to address the specific role of CD22 in $CD8^+$ T cells would be to generate mixed bone-marrow chimeras in *Rag*$^{-/-}$ mice using bone-marrow from *Cd8a*$^{-/-}$ (or alternatively from *Cd3e*$^{-/-}$ mice as we have shown that CD4 T cells do not express CD22) mixed with the bone marrow of *Cd22*$^{-/-}$ or *Cd22*^{Y2,5,6F}. Control mice would consist in a similar setting but using WT instead of CD22-deficient bone marrow. These mice could then be used in helminth infection experiments as well as in co-infection experiments using for example viruses like IAV or MuHV-4.

On another aspect, there has been reports of CD19 escape when using CD19 chimeric antigen receptor T-cell (CAR-T) therapy against refractory/relapsed (R/R) B-cell hematological malignancies. Alternatives to target and kill malignant B cells are proposing to use CD22 CAR-T Cocktail Therapy for refractory B cell lymphoma, which are safe and effective (Huang et al., 2022). Although the existence of $CD22^+$ T_{VM} in human remain to be clarified, a better understanding of the biological consequences of $CD22^+$ expression on T_{VM} is important to distinguish the epitope specificity between T and B lymphocyte, avoid off-target effects of CD22 CAR-T cells on $CD22^+$ T_{VM}, or utilize the T_{VM} for therapy based on its cytotoxicity.

Interestingly, CD22 is not the only B-cell inhibitory receptor found to be also expressed T lymphocytes. Inhibitory fragment crystallizable (Fc) receptor FcγRIIB (CD32) was identified on a subset of effector memory $CD8^+$ T cells following activation and multiple rounds of division (Morris et al., 2020). FcγRIIB was shown to function intrinsically in $CD8^+$ T cells to limit T cell responses without requirement of IgG. However, T cell FcγRIIB is targeted by fibrinogen-like 2 (Fgl2), an anti-inflammatory cytokine produced by $Foxp3^+$ Treg, and leading to apoptosis (Morris et al., 2020). Thus, FcγRIIB acts as a regulator of effector T cell responses. It is also interesting that FcγRIIB is not detectable on naive T cells, similar as what we identified CD22. However, $FcγRIIB^+$ OT-I T cells were depleted following the use of a negative selection $CD8^+$ MACS enrichment kit which explains that its expression was not identified before on T cells. However, $CD22^+$ T_{VM} are not depleted by negative selection using standard commercialized kits. In addition to B cell-restricted receptors, CD14, a component of the LPS receptor that is usually expressed on myeloid cells, was also identified to be expressed on a population of tissue-resident memory $CD8^+$ T cells in human liver. Interestingly, $CD14^+$ $CD8^+$ T cells mark these myeloid-instructed $CD8^+$ T cells, which could be reduced by antibiotics or other drugs that decrease the amount of gut bacterial LPS in the portal circulation (Pallett et al., 2023).

In conclusion, we have in this thesis clarified the transcriptomic changes of IL-4-induced T_{VM}, which provided more details to better define IL-4 dependent T_{VM} cells and understand how IL-4 regulates the peripheral expansion and phenotype presentation of T_{VM} (Summary Figure). IL-4-responding T_{VM} showed distinct transcriptomic profile from T_{EM}/T_{CM} and specifically expressed CD22, an inhibitor receptor of B lymphocytes. This unique finding offered us a new horizon to investigate how common γ chain cytokines regulate the development of T_{VM} and understand the relationship between Th1 and Th2 immune responses. In addition, the expression of CD39, Ly49e and some other inhibitory receptors on $CD22^+$ T_{VM} illustrated their potential roles in regulating the immune response during helminth infection and coinfections with viruses. Based on our findings, we propose a model in which IL-4-dependent expansion of T_{VM} results in a strong activation of self-reactive T cells which upregulate an inhibitory program, which includes CD22, to counter-regulate such activation. In absence of CD22 signaling in T_{VM}, T_{VM} acquire a phenotype of effector-like T cells while losing features of their virtual memory phenotype. As such, CD22 is a key regulator of the T_{VM} responses, active during type 2 inflammatory processes, dependent on IL-4-induced expansion and activation, and distant from T cell exhaustion.



Summary Figure. T_{VM} are memory-phenotype T cells which are derived from self-reactive $CD8^+$ T cells in the thymus, via an EOMES-dependent program. During helminth infection, IL-4 is produced in the periphery and induces the expansion of T_{VM}, which requires STAT6 and leads to upregulation of CD22, present at the cell surface of IL-4-dependent T_{VM}. $CD22^+$ T_{VM} are enriched in self-reactive TCR features and display a specific activation/regulatory program including upregulation of CD39 and GZMA, independently of microbiota contribution. Strikingly, the absence of *Cd22* signaling in T_{VM} can enhance the activation and cytotoxic potential of $CD8^+$ T cells during respiratory viral infection, suggesting that CD22 contains the activation and effector program of self-reactive T_{VM} during peripheral expansion. CD22 could therefore represent an important contributor of the peripheral tolerance to regulate T_{VM} activation and expansion during helminth infection and resulting type 2 immunity.

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