THE UNIVERSITY OF CHICAGO

CELL DEATH AND TYPE 1 INTERFERON COLLABORATIVELY PROMOTE VIRUS-INDUCED TYPE I IMMUNITY TO DIETARY ANTIGENS

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This thesis is dedicated to my mother Marie-Chantal Kouame
I can do all things through Christ who strengthens me – Philippians 4: 13

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ABBREVIATIONS

BATF3: Basic Leucine Zipper ATF-like transcription factor 3

BCL6: B-cell lymphoma 6

Bst2: Bone marrow stromal cell antigen 2

CCR2: CC motif chemokine receptor 2

CCR7: CC motif chemokine receptor 7

CD103: Cluster of differentiation 103

CD11b: Cluster of differentiation 11B

CD40: Cluster of differentiation 11B

cDC1: Conventional Dendritic cells 1

cDC2: Conventional Dendritic cells 2

CeD: Celiac Disease

Clec9a: C-type Lectin domain containing 9A

DCs: Dendritic cells

FOXP3: Forkhead box P3

HLA: Human Leukocyte Antigen

IECs: Intestinal epithelial cells

IFN : Interferon

IFNAR : Interferon alpha beta receptor

IL-12 : Interleukin 12

ILFs :Isolated lymphoid follicles

IFNg : Interferon gamma – Type II interferon

IRF4 : Interferon regulatory factor 4

IRF8 : Interferon regulatory factor 8

IRF9 : Interferon regulatory factor 9

ISGs : Interferon stimulating genes

ISVPs: Infectious subvirions particles

JAM-A : Junctional adhesion molecule A

LCs : Langerhans cells

LCMV: Lymphocytic choriomeningitis virus

LP : lamina propria

MHC: Major Histocompatibility complex

MLKL: MLKL mixed lineage kinase domain like pseudo-kinase

mLNs : Mesenteric lymph nodes

MyD88: Myeloid differentiation primary response 88

NOX2: NADPH oxidase 2

PAMPs: Pathogen-associated molecular patterns

pDCs : plasmacytoid dendritic cells

PFU : plaque forming units

PIC: Polyinosinic:polycytidylic

PPs: Peyer's patches

pSTAT1: phosphorylated signal transducer and activator of transcription

pTreg : Peripheral regulatory T cells

SLE : systemic lupus erythematosus

STAT1: signal transducer and activator of transcription

SYK : Spleen tyrosine kinase

T1L : Type-1 Lang

T3D - Type-3 Dearing

T3D-RV : Type-3 Dearing reassortant

T-bet : T-box expressed in T cells

Th1 : T helper 1

Th17 : T helper 17

TLR3 : Toll-like receptor 3

TLR7: Toll-like receptor 7

TLR9: Toll-like receptor 9

TNFa - tumor necrosis alpha

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ABSTRACT

Celiac Disease (CD) is a lifelong immune-mediated enteropathy in which susceptible individuals develop an inflammatory T-helper-1 (Th1) immune response against dietary gluten leading to intestinal tissue damage and production of autoantibodies [1–3]. Environmental factors have been proposed to play a role in the development of CD as only 1% of the genetically predisposed individuals develop the disease [2]. Although epidemiological studies have highlighted a role of viral infections in the development of CD, mechanistic understanding by which viruses can trigger CD is unknown. Previous studies have shown that viruses from the same family that can induce protective immunity exhibit different immunopathology in the context of the immune response to food antigens. Specifically, acute infection with reovirus strains T1L and T3D-RV induced protective immunity but only T1L disrupted the immune response to dietary antigens as seen in CD [4]. However, how T1L infection but not T3D-RV induced Th1 immunity against dietary antigens remains to be investigated. Here we show that differences between T1L and T3D-RV M1 and M2 genes determined the induction of Th1 immunity against dietary antigens. Furthermore, only T1L infection led to type I IFN signaling in dietary antigen-specific T cells in the mLNs and necroptosis in the epithelium. Through the expression of Clec9a, migratory cDC1s sensed necroptotic cells, which was necessary for the secretion of Th1-inducing cytokine IL-12, and uptook viral particles which drove induction of type I IFN. Collectively, our data demonstrated for the first time the coupling of cDC1-specific Clec9A sensing to Th1 immunity and established that several checkpoints are needed to drive Th1 immunity and prevent the tolerogenic response against dietary antigens. Overall, we propose that early events

during viral infection could determine the ability of a virus to trigger immunopathology like CD later on in life.

CHAPTER 1: INTRODUCTION

1.1 Immune response to food antigens

The immune system in the intestine is challenged by a wide array of foreign antigenic materials such as protein from our diet, microbiota, and pathogens [5–7]. Inducing the appropriate response to diverse triggers is essential to establishing homeostasis and preventing diseases [8]. The immune system can do so by inducing protective responses against harmful invaders like pathogenic bacteria and viruses and promoting non-inflammatory responses to harmless triggers from food and resident microbiota [7,8]. Although many studies have been done to describe protective immune responses against pathogens and to understand the interactions between the microbiota and the immune system [9–11] not much is known about the immune response toward food antigens.

The intestine can be divided into the small intestine and the colon and the small intestine is further divided into the duodenum, jejunum, and ileum. Most of the food absorption occurs in the small intestine along a decreasing gradient from the duodenum to the ileum which correlates with decreasing frequency of peripheral regulatory T cells (pTregs) [12,13]. The presence of these pTreg cells is independent of the microbiota and solely dependent on proteins from the diet in mice [14]. However, how dietary antigens induce pTreg cells is not fully understood.

To understand how the immune system responds to food antigens, immunologists have developed an oral tolerance assay that describes systemic immunity from orally ingested antigens and adoptive T cells transfer as models to understand the immune response to food [5]. With food-related immunopathology on the rise [15], it is more than

ever important to uncover mechanisms underlying food tolerance to inform therapeutic strategies. In this section, we review some of the mechanisms and factors that have been described using these methods.

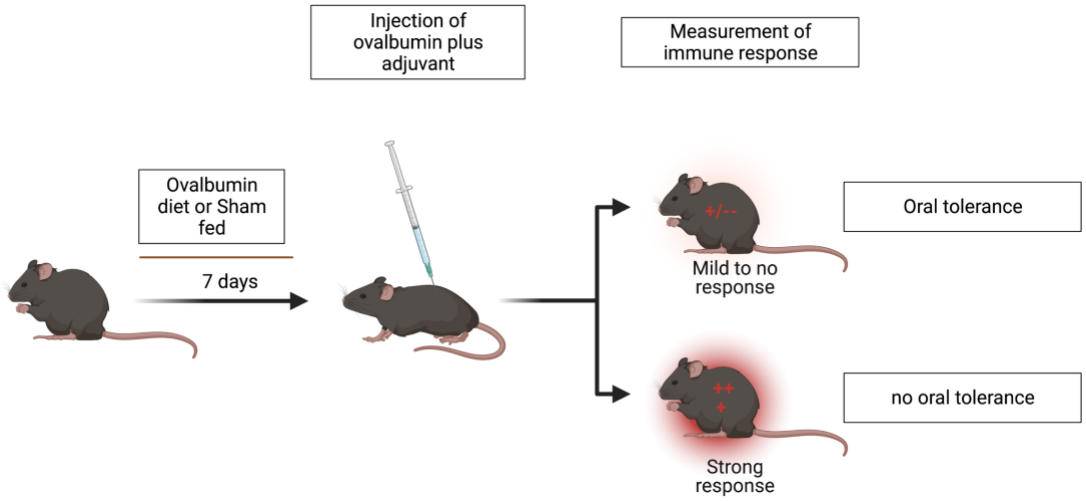


Figure 1: Delayed type hypersensitivity assay

1.1.1 T cell response to dietary antigens

T cell development in the thymus leads to a diverse T cell repertoire that recognizes a wide array of antigens, including self and food-derived antigens. To prevent autoimmune responses, self-reactive T cells are purged through negative selection and central tolerance [16,17]. However, both mechanisms are based on TCR cognate interactions, which do not include food-derived antigen-specific TCR as food antigens are not present in the thymus (Mowat et al., 2012). Therefore, peripheral tolerance is needed to regulate dietary antigen-specific T cells and prevent disease.

It was once believed that the immune system responds to dietary antigens through passive mechanisms such as T cell deletion and anergy. In both scenarios, T cells were unable to mount a response. However, new insights came about since the discovery of regulatory T cells. The immune system is now believed to actively respond to dietary antigens through Treg differentiation in the mesenteric lymph nodes (mLNs). In humans, mutations in FOXP3, the master transcription factor for Treg, are associated with a break in peripheral tolerance and inflammatory responses against food [18]. Mice lacking *Foxp3* failed to mount tolerance to food antigens [19] and adoptive T cell transfer showed Treg differentiation upon food antigens feeding (Esterhazy et al., 2016). Once pTreg cells are primed in the mLNs, they migrate to the lamina propria (LP) where they are maintained [19]. In addition, retinoic acid and TGF-β which promoted pTreg differentiation are highly abundant in the gut. Altogether, these studies highlighted a vital role for pTreg differentiation in peripheral tolerance to dietary antigens.

1.1.2 Gut microenvironment influences immune response to food

Despite the acidic nature of the stomach and the high concentration of enzymes that can degrade proteins in the upper gastrointestinal tract, food components such as gluten are resistant to complete degradation [20]. This leads to the presence of immunogenic, incompletely digested food proteins in the intestinal lumen. Studies in mice have shown that food antigens can be detected as soon as 1hr after oral administration in the epithelium and the LP [21]. Several mechanisms exist to create the appropriate gut microenvironment to support effective tolerance induction to innocuous antigens and protective immunity to pathogens. One of such mechanisms is gut compartmentalization, whereby immunological sites in the gut have specialized functions.

The immune system in the gut can be divided into two sites: inductive and effector sites. On one hand, the inductive sites where adaptive cells are primed are composed of the mesenteric lymph nodes (mLNs), Peyer's patches (PPs), and isolated lymphoid follicles (ILFs) [8]. On another hand, the effector sites are composed of the epithelium and the lamina propria (LP) [8,22]. Here we review the contribution of each site to the immune response to dietary antigens.

1.1.2.1 PPs and ILFs

PPs and ILFs are thought to be instrumental in the mounting of protective immunity against microbiota or pathogens, potentially due to the presence of M cells. Pathogens and antigens can be transcytosed through M cells allowing antigens in the lumen to

reach the gut. Furthermore, studies in mice have shown that PPs and ILFs are not required for induction and maintenance of the homeostatic response against dietary antigens [5,22]. Still, antigen uptake at these sites could amplify the homeostatic response [21]. In conclusion, PPs and ILFs are immune sites that promote protective immune responses but are dispensable in response to dietary antigens.

1.1.2.2 mLNs at the center of immune response to food

Unlike PPs and ILFs, mLNs are the site of immune tolerance to dietary antigens. Studies by [23] have demonstrated that immune response against oral antigens cannot be induced in the absence of mLNs, suggesting the requirement for mLNs in inducing oral tolerance. The nature of the antigens plays a vital role in determining where the antigen will end up. Soluble antigens like undigested food proteins in the lumen are more likely to be taken up by dendritic cells (DCs) and presented to the massive pool of naïve T cells in the mLNs [22]. Low molecular weight particles and large molecules will preferentially diffuse through the epithelium and cross the epithelium barrier through transcytosis in the PPs, respectively [8].

Another level of compartmentalization in the mLNs regulates immune responses. Each gut draining lymph node is functionally distinct from the others, with the duodenal lymph nodes specializing in oral tolerance with the highest potential for pTreg induction to food antigens (Esterházy & Mucida, 2019).

1.1.2.3 LP and the epithelium

The effector sites are composed of the epithelium and the LP. While the epithelium, a single barrier of intestinal epithelial cells, keeps the microbiota and pathogens at bay, the lamina propria maintain fully differentiated and memory immune cells primed in the mLNs. Although the epithelium plays an essential role in the immune responses to the microbiota, its contribution to the response to food antigens is focused on DCs conditioning and instruction of the appropriate immune response. The LP, however, in the context of food antigens, is a crucial site for maintaining a homeostatic response to dietary antigens. More specifically, in mice, it was shown that secretion of IL-10 by CX3CR1+ macrophages in the LP was required to maintain the non-inflammatory response to orally administered antigens [19].

1.1.3 Local conditioning in the mLNs

Transforming growth factor-β and retinoic acid have been shown to promote a unique environment in the gut that favors tolerogenic responses [8].

Transforming growth factor-β is a potent cytokine that is abundant in the gut and promotes differentiation of pTregs [25]. There are several isoforms of this protein with the TGF-β1 isoform as well as its receptor predominantly expressed in immune cells [26]. The gut is an environment rich in TGF-β1 due to the presence of the microbiome as germ-free mice showed severe impairment of levels of TGF-β1 [27], and oral feeding as an increased level of TGF-β1 was observed in tolerized mice [28]. TGF-β1 is first secreted in its inactive form and needs to undergo conformational changes and/or cleavage to be activated [26]. This activation can be mediated by several molecules such as integrins

αvβ6 and αvβ8 which are expressed by intestinal epithelial cells and CD103+ dendritic cells respectively [29] which are key players in the induction of pTregs. *In vitro* studies have shown that TGF-β1alone is sufficient to induce *Foxp3* in murine and human CD4+ T cells and dysregulation of TGF-β1 signaling has been associated with inflammatory disorders and autoimmune lesions [30–32]. Altogether, these findings demonstrate that TGF-β1 promotes pTregs differentiation in the gut. It is important to note that TGF-β1 can also promote the differentiation of CD4+ T-helper 17 in the presence of IL-6 highlighting its pleiotropic role in gut immune responses [33].

Retinoic acid (RA) which is a diet-obtained vitamin A metabolite plays an important in instructing pTreg differentiation in collaboration with TGF-β1 [8]. CD103+ DCs and intestinal epithelial cells can metabolize vitamin A precursors into RA through upregulation of retinaldehyde dehydrogenase(*Raldh*) and retinol dehydrogenase 7 (*Rdh7*) [34,35]. This leads to a local production of retinoic acid that can drive T cells differentiation into pTregs. However, in the presence of IL-15, RA has been shown to promote CD4+ T-helper 1 T cell and drive inflammatory immunity to dietary antigens [36].

1.1.4 Dendritic cells at the center of the immune response against food antigens

Dendritic cells (DCs) integrate signals from the microenvironment and induce the necessary T-cell response to neutralize threats [37,38]. DCs were first described by [39], and it was not until the 1990s that there were recognized as a heterogeneous group of cells [40,41]. Today, DCs can be classified into four groups based on their ontogeny [38,42]. There are plasmacytoid DCs (pDCs), monocyte-derived DCs (moDCs), Langerhans cells (LCs), and conventional DCs (cDCs) (Murphy et al., 2012).

cDCs can be further divided into two lineages termed cDC1 and cDC2 based on the expression of specific transcription factors and surface markers. While transcription factors IRF8 and BATF3 control differentiation of cDC1[37] Notch and IRF4 on the other hand control differentiation of cDC2 [43]. They are present across all tissues and lymph nodes in humans and mice [44]. Each lineage can be further divided into migratory and lymph node resident cells based on their initial seeding location and characterized by the expression of CD103 [45]. Resident DCs reside in the lymph nodes and do not migrate out while migratory DCs reside in the tissue and migrate to the lymph nodes via the lymphatics [42].

cDC1 can be distinguished from cDC2 on the surface by the expression of XCR1 and lack of expression of CD11b and Sirp1a [37]. In the mLNs, cDC1s display the most tolerogenic profile with high expression of genes involved in retinoic acid metabolism, TGFβ, and gut homing [46]. Due to their tolerogenic profile, it was hypothesized that they are more predisposed to induce pTreg cells and oral tolerance than their migratory counterpart in the context of food antigens [46,47]. This hypothesis was tested by [46] using mice lacking IRF8 in the cDC compartment. Interestingly, they observed only a reduction in the induction of *Foxp3*, the master transcription factor for Treg, suggesting that migratory cDC2 can compensate for the induction of pTreg. It was proposed that there is a division of labor in cDCs' ability to induce pTreg, with cDC1 having the highest potential [48]. However, an oral tolerance assay demonstrated that cDC1s alone were not required for establishing tolerance to orally fed antigens [46]. This suggests that reduction of pTreg alone is insufficient to break oral tolerance, and perhaps other CD4 T helper cells that recognize the antigen are needed.

Interestingly, cDC1 exhibit the highest potential for inflammatory responses due to higher expression of inflammatory genes such as IL-15 and IL-12 compared to cDC2s and resident DC subsets [46,48]. In the context of infection, cDC1s were shown to secrete IL-12 required for cross-priming of CD8 T cells and clearance of the pathogens. IL-12 alone was able to rescue infected toxoplasma gondii-infected batf3 knockout mice in the context of *Toxoplasma Gondii* infection [49]. In addition, cDC1s express unique receptors such as Clec9a (DNGR-1) that allow antigens from necrotic cells to be loaded on MHC-I for cross-presentation [50]. Clec9a is a C-type lectin receptor that recognizes F-actin on necrotic cells and signals through SYK to redirect the cargo to either the cytosol through the cytosolic pathway or to the endosome via the vacuolar pathway [50,51]. Interestingly, antigen presentation from dying cells can induce tolerance [47] therefore suggesting that additional signals might be needed to activate dendritic cells. Reis e Sousa et al demonstrated that TLR3 signaling in the context of viral infection can induce the expression of costimulatory molecules and the production of inflammatory cytokines leading to an increase in the cross-priming response [52].

Overall in the gut, various DCs populations are present in the Peyer's patches, mLNs, LP, and ILFs and they exhibit functional differences. For instance, small intestine LP DCs could induce oral tolerance in a DTH assay while Peyer's patches DCs could not [53]. Furthermore, the DC compartment in the LP is dominated by CX3CR1+ and CD103+ DCs which are instrumental in driving and maintaining the T cell response against dietary antigens [8,54]. CX3CR1+ DCs in particular have been shown to uptake dietary antigens from the lumen and transfer antigens to CD103+ DCs [21]. In addition, previous work highlighted their requirement in the maintenance of dietary antigens-specific

pTregs and establishment of oral tolerance demonstrated using adoptive T cell transfer and DTH assay respectively [19]. However, using a targeted mouse model to specifically delete precursors monocytes cells that give rise to CX3CR1+ DCs, Esterhazy et al demonstrated a significant reduction in pTregs cell induction but with no impact on oral tolerance using DTH assay [46]. Furthermore, unlike other phagocytes, DCs have various mechanisms that enable them to preserve the antigenic cargo that they uptake. They express different types of proteases at low concentrations which results in reduced efficiency in protein degradation. Expression of NOX2 by DCs contributes to preventing phagosome and early endosome acidification through stabilization of pH above neutrality [55].

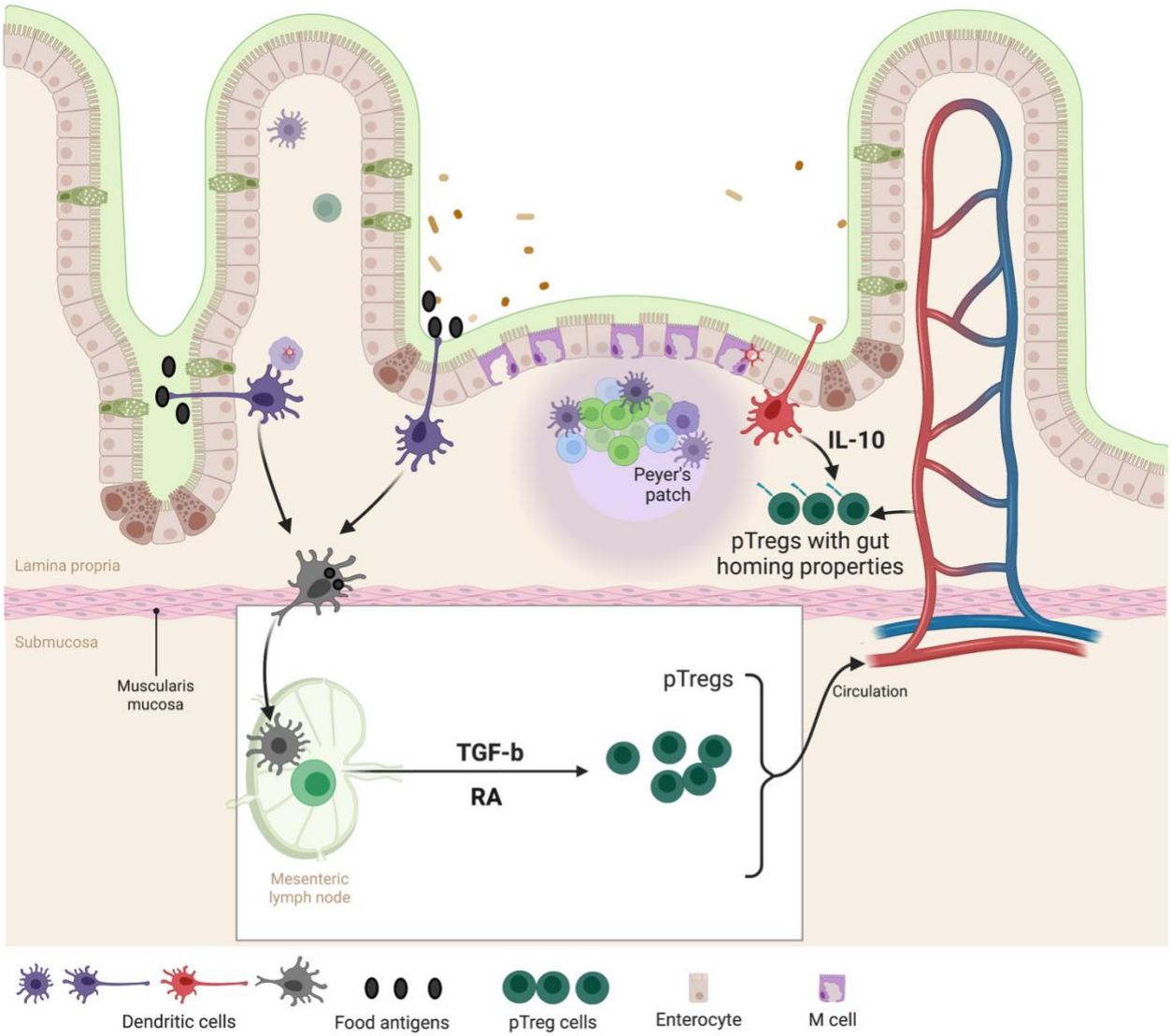


Figure 2 : Induction of pTreg cells in the gut

Adapted from Pabst et al, 2012. Following dietary antigens feeding, CX3CR1+ DCs in the lamina propria can uptake dietary antigens and transfer them to CD103+ DCs and migrate to the mLNs. Alternatively, CD103+ DCs could themselves uptake dietary antigens and migrate to the mLNs. Once in the mLNs, CD103+ DCs drive the differentiation of naïve T cells into pTreg cells and the expression of gut homing markers in the presence of TGF-β and RA. pTreg cells will then join circulation and home the LP where they will be maintained by IL-10 secretion from CX3CR1 DCs.

1.2 Celiac Disease : immune dysregulation to dietary antigens

Celiac Disease (CD) is a T-cell-mediated autoimmune-like disorder characterized by the induction of a CD4+ T helper 1 response against dietary gluten, the presence of autoantibodies, and the killing of intestinal epithelial cells leading to villous atrophy by Intraepithelial lymphocytes [56]. CD affects approximately 1% of the US population with a higher incidence in children than in adults [57]. Like other autoimmune diseases, CeD has genetic associations with Human Leukocyte Antigen (HLA) [58] with all CeD patients carrying either HLA DQ2 or DQ8 molecules. Notably, HLA testing is used in the clinic as part of the diagnosis of CD. Other genetic associations are related to IL-15, B and T cell immunity and genes associated with type I interferon (IFN) signaling [59].

Loss of tolerance to dietary gluten is due to induction of a Th1 immune response against gluten, instead of pTreg cells, which is the main driver of CD pathology. Gluten is a type of protein found in wheat, barley, and rye. Gluten proteins contain many proline residues, making them resistant to enzymatic degradation in the stomach and enabling the formation of the immunogenic peptide to reach the intestine. Upon removal of gluten from the diet, CD symptoms disappear and reappear once gluten is reintroduced [56]. Gluten-specific Th1 cells are present in the gut tissue of CD and preferentially recognize deamidated gluten peptides [3]. Notably, deamidated gluten peptides have a stronger affinity for HLA-DQ2 and DQ8, which may explain their strong association with the disease. Why CD patients develop this pathogenic response to gluten is not fully understood. Though gluten is immunogenic, the presentation of gluten peptides alone cannot explain the loss of tolerance. In addition, genetics alone cannot

explain the disease as only a fraction of HLA DQ2, and DQ8 positive individuals develop the disease suggesting a role for environmental factors in CD development. Enteric viral infections have been associated with CD incidence and have been proposed to trigger the inflammatory T cell response to dietary gluten [60]. An epidemiological study in which genetically predisposed children were followed from birth for CD development showed that frequent rotavirus infections predicted a higher risk of CD (Stene et al., 2006). Furthermore, results from a rotavirus efficacy and safety trial suggested that the risk of CD was decreased in vaccinated children [61]. Although another vaccine trial did not show any impact on CeD incidence [62]. However, a potential pitfall of the latter study was a relatively short follow-up period and was done at an age when CeD incidence is low [60].

Our lab was the first to demonstrate experimentally that acute infection with reovirus T1L and murine norovirus CW3 can disrupt the immune response to dietary antigens, as seen in CD [4,63]. Furthermore, we showed that infection with these viruses could activate innate immunity and promote

Th1 immune response to dietary gluten. However, the viral determinants and the integration of cellular and molecular events leading to Th1 immune response against dietary antigens are not known.

1.3 Reovirus

1.3.1 Features of mammalian reovirus (physical description/genes with table, replication, and tropism/strain)

Mammalian orthoreovirus, called Reovirus, stands for Respiratory Enteric Orphan virus and was first isolated from the stools of children experiencing gastrointestinal illness by Albert Sabin in 1950. In humans, infections are common during early childhood, and seroprevalence remains intact throughout life. Studies with reovirus were the first to discover and identify the role of methylated cap structure at the 5' end needed to recruit ribosomes to mRNA. Reoviruses are non-enveloped double-stranded RNA viruses that elicit subclinical symptoms.

Reovirus virions are formed by two concentric protein shells: outer capsid and inner core. The RNA genome of the reovirus is enclosed in the inner capsid and surrounded by the outer capsid. The reovirus genome is composed of ten segments. The genes are classified based on their molecular weight and grouped into three classes: large, medium, and small. In total, reovirus has three Large (L), three medium (M), and four small (S) genes. Eight of the twelve encoded proteins are structural components of virions (λ1, λ2, λ3, μ1, μ2, σ1, σ2, and σ3), and four are nonstructural (μNS, μNSC, σ1s, and σNS) (Figure 4) (Dermody et al., 2013).

In vitro, during replication, the structural protein encoded by the S1 gene on the surface of the virion binds to receptors such as Junctional adhesion molecule (JAM-A) or sialic acid on host cells through a process called receptor-mediated endocytosis. The presence of host cathepsins in the endolysosome enables the uncoating of the virions to form infectious subvirions particles (ISVPs). ISVPs are characterized by the absence of outer capsid protein sigma three encoded by gene S3 and proteolytic cleavage of another capsid protein mu1 encoded by reovirus M2 gene. Cleaved mu1 on ISVPs enables exit from the endolysosome and access to the cytoplasm, where viral mRNA

can be synthesized within viral factories. In vivo, and more precisely in the intestine, it was shown that reovirus could infect the gut through transcytosis across M cells in the Peyer's patches. However, it is not known whether infection in vivo is receptor-mediated. Due to the presence of endosomal cathepsins and acidic pH in the gut lumen, reovirus virions are modified into infectious subvirions particles (ISVPs). The infectivity of reovirus at the ISVPs stage is increased for certain strains such as T1L but decreased in others like T3D, thereby rendering T3D unable to replicate in the gut. However, a modified version of T3D named T3D-RV which has T1L outer capsid protein-encoding genes S1 and L2 genes can infect the gut (Dermody et al., 2013) [65].

1.3.2 Strain determinants of reovirus pathogenesis

Reoviruses can be divided into four serotypes based on their antigenic properties and hemagglutination profile of the S1 gene. There are Type-1 Lang (T1L), Type-3 Dearing (T3D), Type-2 Jones (T2J), and Type-4 Ndelle (T4N) [66].

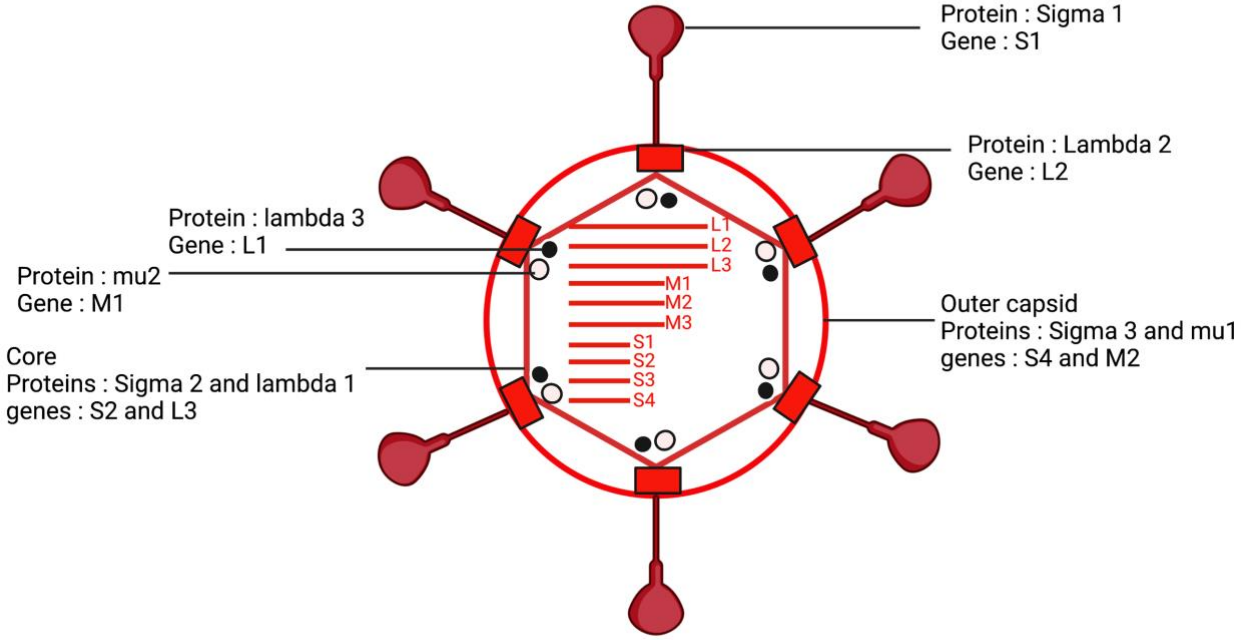
Route of entry, primary replication, viral spread, and secondary replication were all associated with differences between viral genes across strains. These differences between the strains are associated with unique pathology. For instance, several interesting biological features are associated with reovirus M genes. Strain differences in the M1 gene encoding mu2 protein segregated with differences in the IFN response between T1L and T3D strains. On the one hand, the T1L M1 gene inhibited IFN through sequestration of IRF9 in the nucleus, while M1 from the T3D-RV strain did not prevent IFN induction [67]. The ability of T1L M1 to repress type I IFN signaling was linked to a

signal amino acid polymorphism [68]. Interestingly, the opposite phenotype is observed in vivo, with T1L inducing more type I IFN than T3D [4].

Furthermore, differences in apoptosis induction segregate with the reovirus M2 gene [69]. The T3D M2 gene is required and sufficient to induce apoptosis, while the T1L M2 gene is necessary to block apoptosis in L929 cell infection models. In vivo, the T3D M2 gene was associated with severe myocarditis, while T1L M2 was not. Integrating the data in vitro and in vivo suggested that the ability of T3D to induce myocarditis was linked to its M2 gene enabling apoptosis in the heart in mice, therefore, suggesting that the M2 gene is the determinant of virally induced myocarditis (Boehme et al. 2022). In addition, the ability of T3D to cause cell death has been leveraged for several years as a therapeutic solution for cancer (Dermody et al., 2013).

Reovirus is considered a non-pathogenic virus as it does not cause clinical symptoms. However, depending on the strains, it can disrupt the immune response to food with high anti-reovirus antibody titers in active CeD (Bouziat et al, 2016). More specifically, T1L infection but not T3D-RV induces Th1 immunity against dietary antigens although both viruses induce strong protective immunity.

In this thesis, we set out to understand how reovirus T1L but not T3D-RV induces the loss of tolerance to dietary antigens. Specifically, we investigated the mechanisms underlying T1L M genes' interactions with host factors driving Th1 immunity. Overall, our study provides a novel mechanism by which viruses promote autoimmunity, and uncovering these interactions will give us a better understanding of key factors that enable only certain viruses to induce autoimmunity.



non-structural proteins : muNS, sigma NS and Sigma1S
genes: M3 and S1

Figure 3:Reovirus virion

CHAPTER 2: METHODS

2.1 Mice

All knockout and transgenic mice used in these studies are on a C57BL/6J background are between 6-8 weeks old. C57BL/6J (WT), CCR7-KO (B6.129P2(C)-*Ccr7*^{tm1Rfor}/J), BATF3-KO (B6.129S(C)-*Batf3*^{tm1Kmm}/J), IFNAR$^{-/-}$ (B6.129S2-Ifnar1^{tm1Agt}/Mmjax), Clec9a-/- (B6(Cg)-*Clec9a*$^{tm1.1Crs}$/J) , IL-12KO (B6.129S1-*Il12b*^{tm1Jm}/J) and p40-IRES-eYFP (C.129S4(B6)-IL12b$^{tm1.1LKy}$/J), STAT1$^{-/-}$ (B6.129S(Cg)-*Stat1*^{tm1Dlv}/J) and TLR7-/- (B6.129S1-Tlr7^{tm1Flv}/J) mice were purchased from The Jackson Laboratory and housed in our facility. RAG$^{-/-}$ OT-II$^{+/-}$ CD45.1$^{+/+}$, RAG$^{+/+}$ OT-II$^{+/-}$ CD45.2 and MLKL$^{-/-}$ mice were provided by Dr. Peter Savage Dr. Dr. Daria Esterhazy and Dr. Seungmin Hwang respectively and bred in our mouse facility. IFNAR$^{-/-}$ or STAT$^{-/-}$ mice were crossed to RAG$^{+/+}$ OT-II$^{+/-}$ CD45.2 to produce IFNAR$^{-/-}$ RAG$^{+/+}$ OT-II$^{+/-}$ CD45.2 mice and STAT1$^{-/-}$ RAG$^{+/+}$ OT-II$^{+/-}$ CD45.2. Vilin-creERT2 (B6.Cg-Tg(Vil1-cre/ERT2)23Syr/J) were purchased from The Jackson Laboratory and crossed with Ai9 (B6.Cg-*Gt(ROSA)26Sor*$^{tm9(CAG-tdTomato)Hze}$/J) provided by Dr. Daria Esterhazy to generate Vilin-creERT2-RFP mice. All mice were housed at the University of Chicago and maintained under SPF conditions. For all experiments using knockout mice, WT mice were either co-housed for 15 days or littermate controls were used.

2.2 Adoptive T cell transfer

CD4+ T cells were isolated and purified from the spleen and inguinal lymph nodes of RAG$^{-/-}$ OT-II$^{+/-}$ CD45.1$^{+/+}$ or RAG$^{+/+}$ OT-II$^{+/-}$ CD45.2$^{+/+}$, STAT1-/- RAG$^{+/+}$ OT-II$^{+/-}$

CD45.2$^{+/+}$ and IFNAR$^{-/-}$ RAG$^{+/+}$ OT-II$^{+/-}$ CD45.2$^{+/+}$ mice by negative selection using CD4+ T cell isolation kit (Miltenyi). Cell purity was evaluated by flow cytometry using these markers/fluorophores combination CD45-BUV395, CD45.1-BV421, Vα2-PE, Vβ5-FITC, CD44-PEcy7, and CD62L-PEtexared. In general, 1x10^5 to 3x10^5 cells were transferred by retro-orbital injection in mice. One day post-transfer, mice were perorally infected with indicated viruses at 10^8 PFU and put on an OVA-containing diet (Harlan Envigo TD 130362 10mg/kg) for the indicated duration of the experiments.

2.3 Mice Infection and treatment

Mice were perorally inoculated using a 22-gauge needle (Cadence Science) with indicated viruses at 10^8 PFU diluted in phosphate-buffered saline (PBS) and sham PBS infected for control. Two-, Three- or six days post-infection, mice were euthanized and mesenteric lymph nodes and/or lamina propria and colonic content were harvested.

For anti-IFNAR treatment, mice received blocking antibody (MAR1-5A3) intraperitoneally three times at 100 μg/day one day before infection, on the day of infection, and one-day post-infection.

For Poly(I: C) treatment, mice received peritoneally 100 μg or 200 μg every day until the duration of the experiment.

For Clec9A and anti-IL12p75 treatment, mice received peritoneally 100 μg of blocking antibody every day starting on the day of infection till the end of the experiment.

For NK1.1 treatment, mice received peritoneally 400 μg one day before and one day after infection.

Antibiotics treatment: cocktails preparation and treatment plan were adapted methods from [70]. In brief, 6 weeks old C57BL/6J (WT) received 200ul mixture antibiotics cocktail of kanamycin at 4mg/ml; gentamicin at 3.5 mg/ml, colistin at 8500U/ml, metronidazole at 2.15 mg/ml, and vancomycin at 4.5mg/ml (Sigma) only once. One week later, mice were put on antibiotics through their drinking water at 20ml antibiotics per 100 ml of water for two weeks. Fresh fecal samples from antibiotics-treated and control mice were obtained and processed for 16s sequencing.

2.4 Virus preparation and reassortants generation

Viruses were prepared following protocol from [69] by Dermody lab. Spinner-adapted murine L cells were maintained in either suspension or monolayer cultures in Joklik's modified Eagle's minimal essential medium (SMEM; Lonza) supplemented to contain 5% fetal bovine serum (FBS; Gibco), 2 mM l-glutamine, 100 U/ml of penicillin, 100 µg/ml of streptomycin (Gibco), and 25 ng/ml of amphotericin B (Sigma). Baby hamster kidney (BHK) cells that constitutively express the bacteriophage T7 RNA polymerase (BHK-T7 cells) were maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM; Gibco) supplemented to contain 5% FBS, 2 mM l-glutamine, 1 mg/ml of Geneticin (Gibco), and nonessential amino acids (Sigma).

Recombinant reoviruses were engineered using a plasmid-based reverse genetics [71]. Recombinant strain T1L is a stock recovered by plasmid-based rescue from cloned T1L cDNAs [72]. The engineered reassortant virus strain, T3D-RV, was recovered following transfection of BHK-T7 cells with plasmid constructs encoding the S1 and L2 gene segments of strain T1L and the remaining eight gene segments of strain T3D. T1L ×

T3D-RV reassortant viruses were isolated using cloned T1L or T3D cDNAs. After 3 to 5 days of incubation, cells were frozen and thawed three times, and virus was isolated by plaque purification using monolayers of L cells [73]. Reovirus virions were purified from second- or third-passage L-cell lysate stocks [74]. Viral particles were extracted from infected cell lysates using Vertrel XF (DuPont), layered onto 1.2- to 1.4-g/cm^3 CsCl gradients, and centrifuged at 62,000 × *g* for 16 h. Bands corresponding to virions (1.36 g/cm^3) were collected and dialyzed in virion storage buffer (150 mM NaCl, 15 mM $MgCl_2$, and 10 mM Tris-HCl [pH 7.4]) [75]. Viral titer was determined by plaque assay using L cells [73]. Purified viral particles were electrophoresed in SDS-polyacrylamide gels, which were stained with ethidium bromide to visualize viral gene segments.

2.5 Cell isolation

Mesenteric lymph nodes: Small intestine mesenteric lymph nodes were isolated and treated in collagenase VIII (Sigma) in RPMI media at 1mg/ml for 35 minutes at 37 degrees Celsius. The reaction was stopped using EDTA at 5 mM for 5 minutes. Samples were processed by mechanical disruption through a 100 μm cell strainer. Cells were used for staining and/or lysed for RNA extraction. For cDC11c purification, after EDTA incubation cells, cD11c+ were MACS enriched by positive selection and processed for RNA extraction.

Lamina propria: the whole small intestine was isolated and Peyer's patches were removed. The intestine was then opened longitudinally and washed with cold PBS. The tissue was then cut into smaller pieces and shaken vigorously in 1% FBS RPMI media supplemented with 2 mM EDTA and 1.5 mM $MgCl_2$ for 20 min twice. Tissues were

filtered through a 100 μm cell strainer and then digested again with 20% FBS RPMI supplemented with 100 U/ml of collagenase VIII for 40 min. Digests were centrifuged at 1600 RPM and resuspended in 40% Percoll and spun at 3000 rpm for 12 min. Pellets were resuspended into 10% FBS PBS and used for downstream processes.

2.6 Cell sorting

For plaque assay in cD11c+ cells: mice were inoculated perorally with 10^8 PFU of purified reovirus diluted in phosphate-buffered saline (PBS) using a 22-gauge round-tipped needle (Cadence Science). At 2 days post-infection mLNs were removed, treated with collagenase VIII (Sigma) at 37ºC for 35 minutes, quenched with 0.5M EDTA, pH 8.0 (Corning), and mechanically disrupted by passage through a 100-μm cell strainer. Cells were stained with CD45.2 (104), Aqua LIVE/DEAD® Fixable Aqua Dead Cell Stain Kits (Biolegend), CD19 (1D3), CD11c (N418), and CD11b (M1/70). Cells were sorted through ARIA III Sorter and twice freeze-thawed and viral titer was assessed by plaque assay.

For sequencing: mice were inoculated perorally with 10^8 PFU of purified reovirus diluted in phosphate-buffered saline (PBS) using a 22-gauge round-tipped needle (Cadence Science). At 2 days post-infection mLNs were removed, treated with collagenase VIII (Sigma) at 37ºC for 35 minutes, quenched with 0.5M EDTA, pH 8.0 (Corning), and mechanically disrupted by passage through a 100-μm cell strainer. Cells were stained with CD45-APCcy7, CD11b-PEcy7, CD11c-BV421, CD8a-BUV395, F4/80-FITC, CD103-APC, and MHCII-BV711. Migratory cDC1 (CD103+ CD11b- CD8a+ and

CD103+ CD11b- CD8-) and migratory cDC2 (CD103+ CD11b+) were sorted through Symphony 6 cell sorter into RLT+ buffer and stored at -80C.

2.7 Antibodies and flow cytometry

The following fluorophore-conjugated antibodies were used : p-STAT1 (Tyr701), Clec9A(7H11), NK1.1(PK136), T-bet (4B10), Foxp3 (FJK-16s), MHCII (M5/114.15.2), CD11b (M1/70), IL-12p40 (C17.8), CD62L (MEL-14), CD25 (PC61.5), Rat IgG1, Rat IgG2a, IFNγ (XMG1.2), CD103 (M290), CD45 (30-F11), and Fc BlockTM (2.4G2). TCD4 (GK1.5), TCRb (H57-597), CD45.1 (A20), CD11c (N418), CD8a (53-6.7), CD86 (GL-1), CD44 (IM7), and F4/80 (BM8). Aqua LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Life Technologies). Cells were permeabilized with the Foxp3 fixation/permeabilization kit for transcription factor (eBioscience) or Cytofix/Cytoperm (BD Biosciences) for cytokine staining. Flow cytometry was performed with a 9-color BD FACSCanto (BD Biosciences) or 42-color Cytek Aurora and data were analyzed using FlowJo software (Treestar).

2.8 RT-PCR

Whole small intestine mesenteric lymph nodes were isolated, digested using collagenase D (Sigma Aldrich), processed into single-cell suspension, and lyse into RLT buffer containing 1% Mercaptoethanol. RNA extraction was done using the RNeasy Mini Kit (Qiagen) or RNeasy Micro Kit (Qiagen). Samples were normalized at 400 ng RNA/ sample for cDNA synthesis. cDNA synthesis was performed using SuperScript^{TM} III First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions.

Master mixes were prepared with 5 μl of Power SYBR Green (Thermo Fisher Scientific), 0.25 μl at 10 μM forward and reverse primer mix, and water up to 8 μl. Samples were multi-channeled in duplicates into 384 well plates. Plates were run on a Roche LightCycler 480. Delta Ct was calculated by subtracting the average of housekeeping gene CT value from the average of our gene of interest. Relative expression was calculated using the equation: $2^{-\Delta CT}\ x\ 1000$

Table 1: Primers

Species	Gene	Forward Primer	Reverse Primer
Murine	Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Virus	S4	CGCTTTTGAAGGTCGTGTATCA	CTGGCTGTGCTGAGATTGTTTT
Murine	Isg15	GGTGTCCGTGACTAACTCCAT	TGGAAAGGGTAAGACCGTCCT
Murine	Mx1	GACCATAGGGGTCTTGACCAA	AGACTTGCTCTTTCTGAAAAGCC
Murine	Il27	CTCTGCTTCCTCGCTACCAC	GGGGCAGCTTCTTTTCTTCT

2.9 Modified SMARTseq RNA sequencing library preparation and analysis

Full-length cDNA and sequencing libraries were generated using a modified version of the single-cell Smart-seq2 protocol [76]Briefly 100 to 30,000 cells CD103+ CD11b- CD8a+, CD103+ CD11b- CD8a- and CD103+ CD11b+ dendritic cells were sorted in lysis buffer. RNA was extracted (Qiagen MicroKit). Reverse transcription was performed after hybridization of oligo-dT to poly-A RNA. The resulting cDNA was amplified, purified using an AMPure XP Kit (Beckman Coulter), and quality controlled using a Bioanalyzer High Sensitivity DNA Kit (Agilent). A total of 100 to 150 pg of cDNA from each sample was tagmented, ligated with adapters (Nextera XT), and amplified. and quality controlled using a Bioanalyzer High Sensitivity DNA Kit (Agilent). Libraries were then pooled in equimolar amounts and sequenced on two lanes in duplicate using a HiSeq4000 system (Illumina).

Raw reads were subjected to quality control checks, aligned using STAR (v2.6.1d, GRCm38, Gencode vM25), and summarized with featureCounts (subread v1.5.3). Batch correction was performed using ComBATseq [77] and differential expression analysis was performed using DESeq2. The linear models were fit using coefficients based on the immune analysis and outcomes of in vivo infections (ie. ~Th1 induction, ~Cell Death, ~Type 1 interferon) and resulting gene lists were used for gene set enrichment and ontology analysis.

2.1 Statistical analysis

Data were first analyzed for normal distribution using D'Agostino and Pearson omnibus normality tests. Normally distributed data were analyzed using unpaired two-tailed

Student's *t*-test for single comparisons, and one-way ANOVA for multiple comparisons. ANOVA analysis was followed by a Tukey's post-hoc test. Not normally distributed data was analyzed using unpaired two-tailed Mann-Whitney *U*-test for single comparisons. The statistical test used and *P* values are indicated in each figure legend. P values of < 0.05 were considered to be statistically significant. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001. ns = non significant

CHAPTER 3 : CELL DEATH AND TYPE I INTERFERON COOPERATIVELY DRIVE VIRUS-MEDIATED TYPE I IMMUNITY TO DIETARY ANTIGENS

One sentence summary

Integration of necroptotic cells sensing and secretion of type I IFN by cDC1s drives virus-induced Type I immunity to dietary antigens.

Results

A high viral load is not sufficient to disrupt homeostatic T cell response to dietary antigens

Infection with T1L and T3D-RV reovirus strains, which differ by eight genes, induced similar antiviral responses. However, only T1L infection induced loss of tolerance to dietary antigens as demonstrated by induction of Th1 immunity and blockade of pTregs against dietary antigens [4]. This finding implies that properties unique to T1L underlie the loss of tolerance to dietary antigens. In vitro, T1L and T3D-RV are capable to trigger an anti-viral response suggesting that both viruses can inherently activate the immune system [4]. However, despite the fact that T3D-RV which is a reassortant virus with a T3D background with T1L S1 and L2 genes necessary for replication in the gut, it replicated slightly less efficiently than T1L [69]. We tested whether increasing the viral dose of T3D-RV to a similar level as T1L is sufficient to disrupt the homeostatic T cell response to dietary antigens. Wild-type (WT) mice were infected with increasing doses of T3D-RV and compared to the standard dose of T1L. At a high dose of T3D-RV, we observed a

similar viral burden as the T1L standard dose (Figure 5. A, B). To determine the impact on the immune response to dietary antigens, we use an adoptive T cell strategy by transferring naïve OT-II T cells to track the dietary ovalbumin-specific T cell response (Figure 4). Even though at a high dose where T3D-RV replicated similarly to T1L, it was still unable to drive Th1 immunity and block pTregs against dietary antigens (Figure 5. C, D, E and F). We concluded that differences in viral load between T1L and T3D-RV cannot explain why only T1L can disrupt the immune response to dietary antigens. These results indicate that unique differences between T1L and T3D-RV genes underlie the capacity to induce loss of tolerance to dietary antigens.

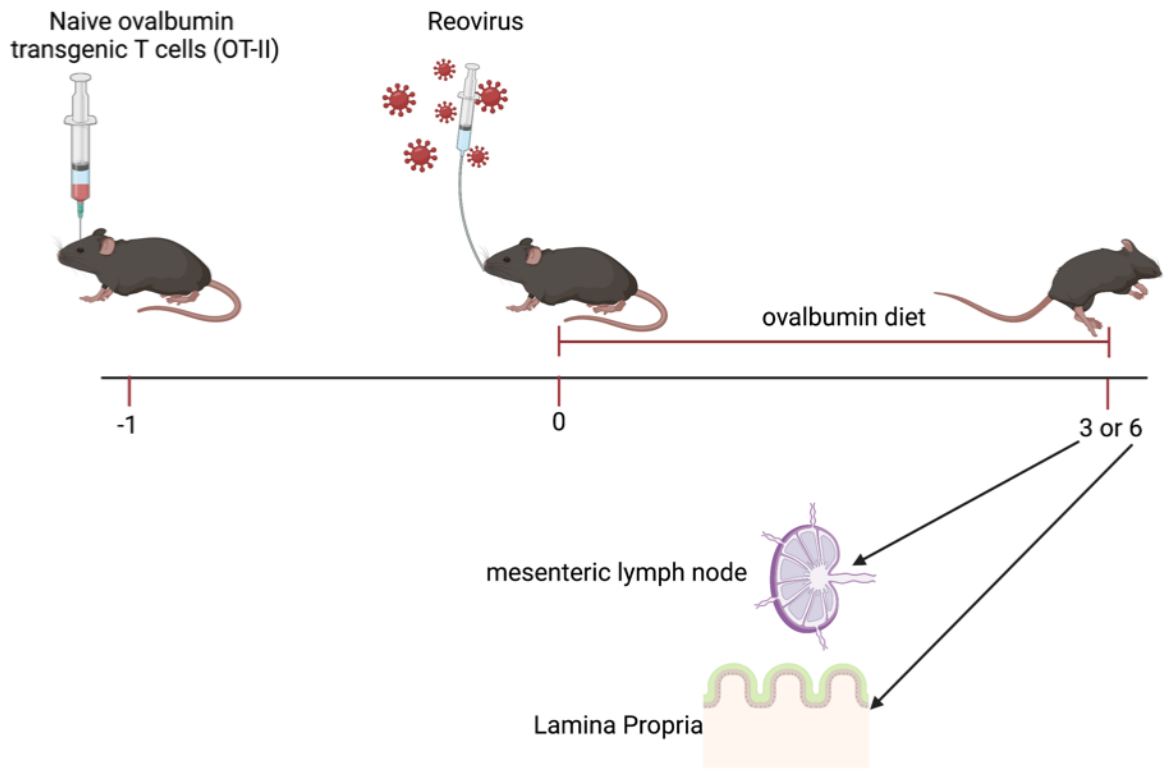


Figure 4: ***Experimental setup to track dietary antigen-specific T cell response during reovirus infection***

Naïve ovalbumin-specific transgenic T cells were transferred in wild-type C57BL6 (WT) or mutant mice a day before infection. On the next day, mice were infected with reovirus T1L or reassortants at 10^8 PFU or indicated PFU and put on ovalbumin containing diet. Small intestine mesenteric lymph nodes (mLNs) and lamina propria (LP) were harvested at 3- and 6-days post-infection to assess priming and effector response, respectively.

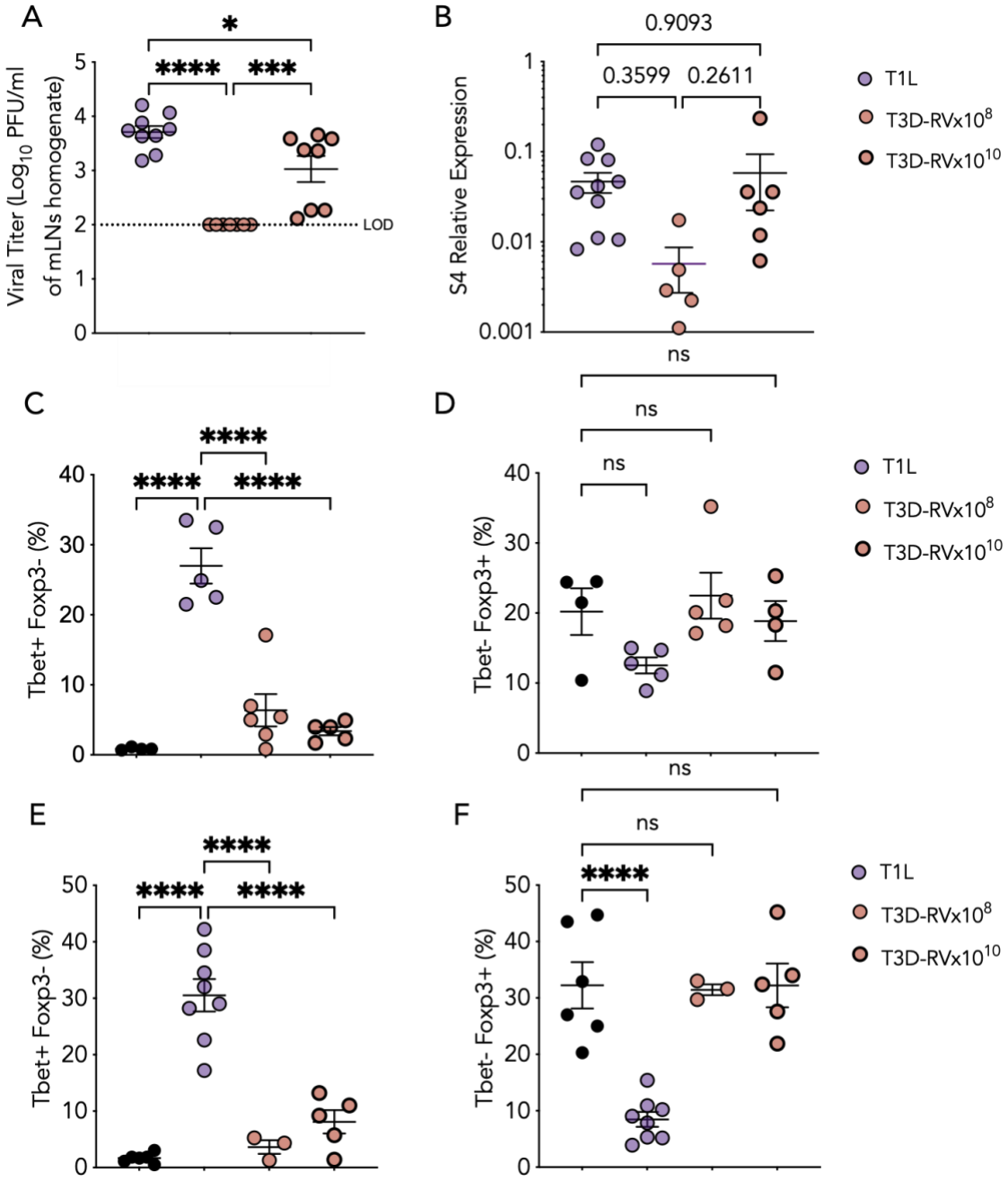


Figure 5 : T1L M genes determine loss of tolerance to dietary antigens
(A,B,C,D,E,F) OTII$^+$ CD45.1$^+$ CD4$^+$ T cells were transferred into WT C57BL/6 CD45.2$^+$ mice. One day post-transfer, mice were inoculated with T1L 10^8 PFU, 10^8 PFU of T3D-RV, and T3D-RV 10^{10} PFU and put on an ovalbumin-containing diet for three days and six days. (A) Titers of T1L and T3D-RV were quantified in the mLNs. (B) Relative expression of viral S4 genome copies in the mLNs was evaluated by RT-PCR (C,D,E,F) the frequency of T-bet+ Foxp3- and T-bet- Foxp3+ expressing OT-II cells in the mLNs

(C,D) and LP (E,F) was assessed by flow cytometry. Graphs depict three experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; one-way analysis of variance (ANOVA)/Tukey's multiple comparison.

T1L M genes are the viral determinants for inflammatory responses against dietary antigens

The genome of reoviruses consists of 10 double-stranded RNA segments classified into three groups based on their size 4 small (S), 3 medium (M), and 3 large (L) segments [78]. Several studies have identified polymorphisms, especially in M genes, between T1L and T3D-RV that underlie differences in the activation of innate immunity (Zurney et al, 2018, Ooms et al, 2010; Sarkar et al., 2010). Therefore, we investigated whether differences in M genes could explain why T1L and not T3D-RV can induce Th1 immunity against dietary antigens. To do so, we generated M genes loss-of-function reassortant P (Reast P), which contains T3D-RV M1, M2, and M3 genes in a T1L background (Figure 6 A). We knew that Reast P replicates similarly in vitro as T1L and T3D-RV [69], so we first wanted to check whether it can replicate in vivo. We infected mice with Reast P, T1L, and T3D-RV and assessed viral burden. We observed a slight decrease in viral load by transcripts in the mLNs of Reast P infected mice (Figure 6 B), similar to T3D-RV. Using an in vivo T cell conversion assay (Figure 4), we assessed the requirement of M genes to differentiate dietary antigen-specific T cells into Th1 and blockade of pTreg induction. We adoptively transferred naïve ovalbumin-specific OT-II T cells into wild-type (WT) mice, infected them with T1L, T3D-RV, and Reast P, and put the mice on an ovalbumin-containing diet. Our results showed that infection with Reast P did not induce differentiation of Th1 T cells as defined by the failure to express T-bet (T-bet+ Foxp3-) in transferred OT-II T cells in the mLNs, similar to T3D-RV (Figure 6. C, D). However, there was a partial effect on pTregs blockade, with Reast P infected group having an intermediate phenotype in pTreg blocking ability (Figure 6 C, D). In our

previous study, we showed that Th1 immunity and pTreg blockade were regulated by distinct mechanisms, which may explain why T1L M genes seem to play a significant role in Th1 but only partially affect pTreg blockade.

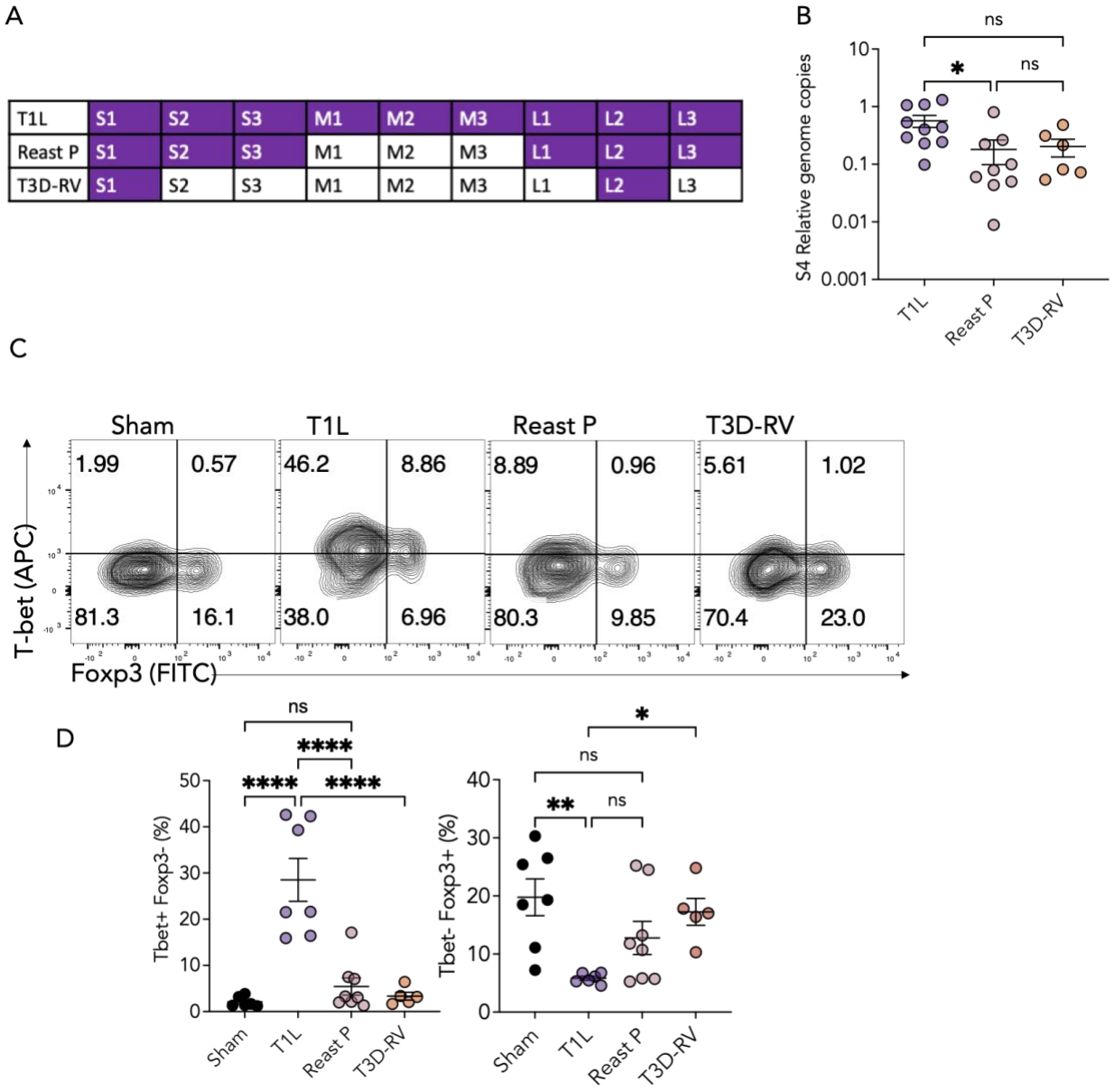


Figure 6: Reovirus T1L M genes are required to induce Th1 immunity but not pTreg against dietary antigens

(A) gene segments of parental viruses T1L and T3D-RV and reassortant P (Reast P). (B, C, and D) OTII^{+} CD45.1^{+} CD4^{+} T cells were transferred into WT C57BL/6 CD45.2^{+} mice. One day post-transfer, mice were inoculated with T1L 10^{8} PFU, 10^{8} PFU of Reast P, and T3D-RV 10^{8} PFU for 3three days (B, C) and six days (D). Relative expression of viral S4

genome copies in the mLNs was evaluated by RT-PCR (B) and representative flow cytometry plots, and the frequency of T-bet+ Foxp3- and T-bet- Foxp3+ expressing OT-II cells in the mLNs (C) and LP (D) was assessed by flow cytometry. Graphs depict three experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; one-way analysis of variance (ANOVA)/Tukey's multiple comparison.

T1L M1 and M2 genes are viral determinants of Th1 immunity against dietary antigens

To determine the role of individual M genes in driving Th1 immunity, we generated single M gene reassortants. Individual M1 reassortant: Reast T, T3D M1 in a T1L background; individual M2 reassortant: Reast U, T3D M2 in a T1L background and Individual M3 reassortant: Reast V, T3D M3 in a T1L background (Figure 7 A, E, I). We observed similar relative viral S4 genome copies between T1L, Reast U, and Reast V two days post-infection in the mLNs (Figure 7. F, J). However, in Reast T-infected mice, we observe significantly less relative viral S4 genome copies similar to Reast P compared to T1L suggesting that the T1L M1 gene is necessary for efficient replication in the mLNs.

Reast T and Reast V failed to drive Th1 immunity, while Reast V induced a similar frequency of Th1 cells to T1L (Figure 7 C, D, G, H, K, and L). Despite differences in their viral burden, Reast T and Reast U failed to induce Th1 immunity (Figure 7 B, D, F, and H), reinforcing that high viral load alone cannot explain the induction of Th1 against dietary antigens. Taken together, our results suggest that T1L M1 and M2 genes are required and sufficient to induce dietary antigen-specific Th1 response.

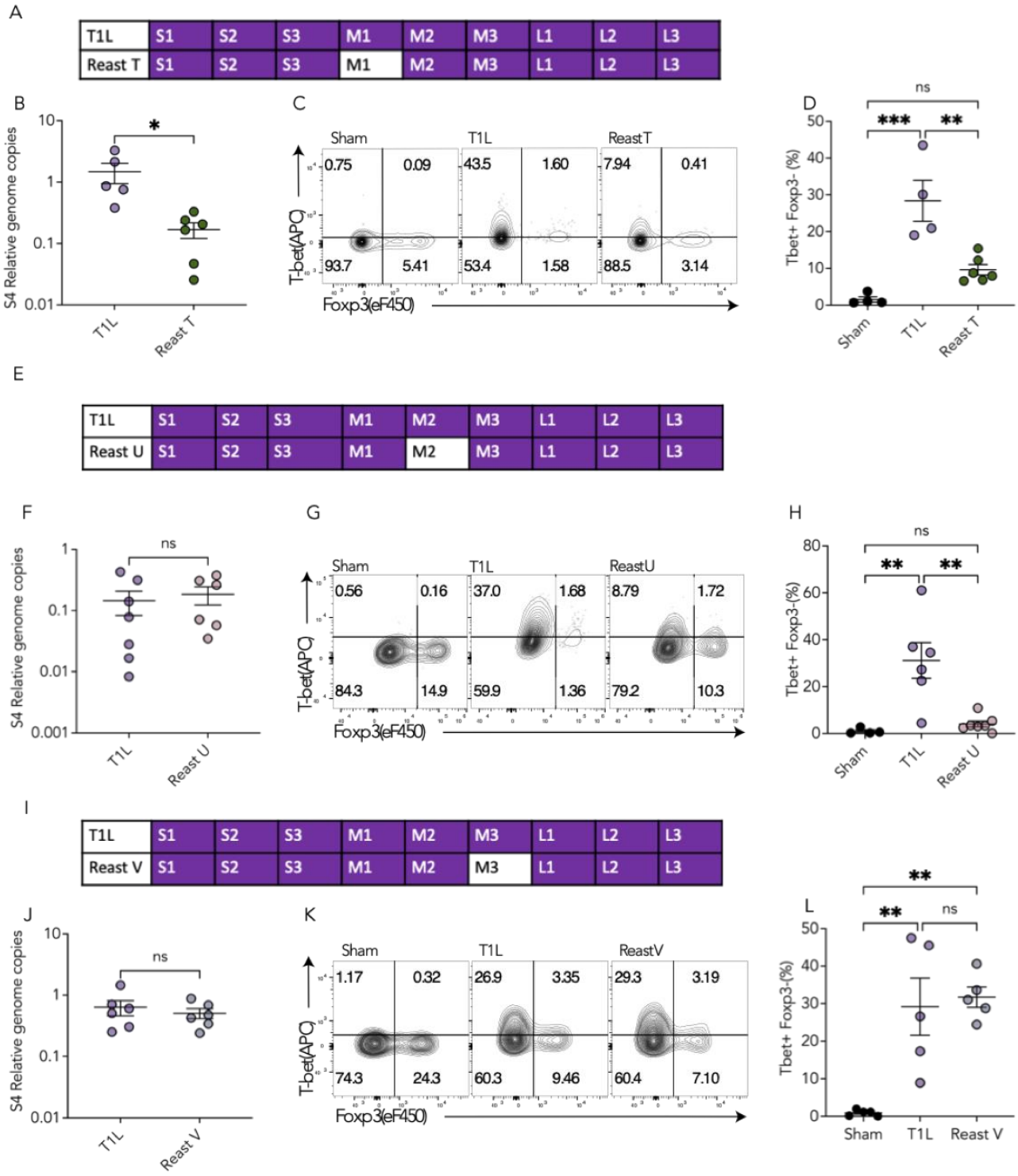

A
T1L S1 S2 S3 M1 M2 M3 L1 L2 L3
Reast T S1 S2 S3 M1 M2 M3 L1 L2 L3
B
S4 Relative genome copies
T1L
Reast T
C
Sham
T1L
ReastT
0.75
0.09
43.5
1.60
7.94
0.41
93.7
5.41
53.4
1.58
88.5
3.14
T-bet(APC)
Foxp3(eF450)
D
Tbet+ Foxp3- (%)
ns
***
**
Sham
T1L
Reast T
E
T1L S1 S2 S3 M1 M2 M3 L1 L2 L3
Reast U S1 S2 S3 M1 M2 M3 L1 L2 L3
F
S4 Relative genome copies
ns
T1L
Reast U
G
Sham
T1L
ReastU
0.56
0.16
37.0
1.68
8.79
1.72
84.3
14.9
59.9
1.36
79.2
10.3
T-bet(APC)
Foxp3(eF450)
H
Tbet+ Foxp3-(%)
ns
**
**
Sham
T1L
Reast U
I
T1L S1 S2 S3 M1 M2 M3 L1 L2 L3
Reast V S1 S2 S3 M1 M2 M3 L1 L2 L3
J
S4 Relative genome copies
ns
T1L
Reast V
K
Sham
T1L
ReastV
1.17
0.32
26.9
3.35
29.3
3.19
74.3
24.3
60.3
9.46
60.4
7.10
T-bet(APC)
Foxp3(eF450)
L
Tbet+ Foxp3-(%)
**
**
ns
Sham
T1L
Reast V

Figure 7: Reovirus T1L M1 and M2 genes are required to induce Th1 immunity and pTreg blockade against dietary antigens

(A) gene segments of parental virus T1L and reassortant T (Reast T). (B, C, D) $OTII^+$ $CD45.1^+$ $CD4^+$ T cells were transferred into WT C57BL/6 $CD45.2^+$ mice. One day post-transfer, mice were inoculated with PBS Sham, T1L 10^8 PFU, and 10^8 PFU of Reast T and put on an ovalbumin-containing diet for two (B) and three (C, D) days. Relative expression of viral S4 genome copies in the mLNs was evaluated by RT-PCR (B). Representative flow cytometry plots and the frequency of T-bet+ Foxp3- expressing OT-II cells in the mLNs (C, D) were assessed by flow cytometry. (E) gene segments of parental virus T1L and reassortant U (Reast U). (F, G, H) $OTII^+$ $CD45.1^+$ $CD4^+$ T cells were transferred into WT C57BL/6 $CD45.2^+$ mice. One day post-transfer, mice were inoculated with PBS Sham, T1L 10^8 PFU, and 10^8 PFU of Reast U and put on an ovalbumin-containing diet for two (F) and three days(G, H). Relative expression of viral S4 genome copies in the mLNs was evaluated by RT-PCR (F). Representative flow cytometry plots and the frequency of T-bet+ Foxp3- expressing OT-II cells in the mLNs (G, H) were assessed by flow cytometry. (I) gene segments of parental virus T1L and reassortant V (Reast V). (J, K, L) $OTII^+$ $CD45.1^+$ $CD4^+$ T cells were transferred into WT C57BL/6 $CD45.2^+$ mice. One day post-transfer, mice were inoculated with PBS Sham, T1L 10^8 PFU, and 10^8 PFU of Reast V and put on an ovalbumin-containing diet for two (J) and three (K, L) days. Relative expression of viral S4 genome copies in the mLNs was evaluated by RT-PCR (J). Representative flow cytometry plots and the frequency of T-bet+ Foxp3- expressing OT-II cells in the mLNs (K, L) were assessed by flow cytometry. Graphs depict two experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; one-way analysis of variance (ANOVA)/Tukey's multiple comparison.

Type I IFN is required for induction of Th1 immunity and pTregs blockade

We were particularly interested in the T1L M1 gene because it has been associated with the regulation of type I IFN signaling in vitro [67,68]. It was shown that T3D infection promotes type I IFN signaling while T1L infection repressed it. The ability of T1L to repress type I IFN signaling was due to the sequestration of IRF9 by its M1 gene [67]. However, in the gut, infection with T1L induces a higher type I IFN signature than T3D-RV [4] highlighting differences in the host-viral interactions between the in vitro and in vivo systems. Nonetheless, it is unknown whether T1L M1 is responsible for the induction of type I IFN signaling in vivo. Knowing that type I IFN could play a role in Th1 priming through pSTAT1 [79], we hypothesized that the T1L M1 gene is necessary for the differentiation of naïve OT-II T cells into Th1 due to its ability to activate the type I IFN pathway. To test that, we first infected mice with Reast T and assessed the induction of *Mx1* as a proxy for type I IFN stimulating gene (ISGs). Infection with Reast T resulted in a significant decrease in *Mx1* (Figure 8 A). To confirm that defect in type I IFN signaling was the reason why Reast T failed to induce T-bet, we investigated whether pSTAT1 was upregulated in T cells in the absence of T1L M1. As predicted, pSTAT1 was decreased in OT-II which correlated with the decrease in T-bet. (Figure 8 B,C Figure 4 C,D). To directly address the role of type I IFN, we assessed the differentiation of naïve T cells into Th1 T cells in T1L infected mice treated with anti-type I IFN receptor blocking antibody (anti-IFNAR). Treatment with the anti-IFNAR antibody was successful at blocking type I IFN which correlated with an increase in viral load (Figure 8 D, E). This result is in line with the literature suggesting that interferons are required to control viral replication [80]. Although we previously showed that Th1 immunity against dietary

antigens was not impaired in T1L-infected type I IFN receptor-deficient (IFNAR-KO) mice [4], here we showed that Th1 was impaired when type I IFN signaling was blocked on host cells and transferred T cells (Figure 8 F, G). Taken together, we hypothesized that type I IFN is required at the level of T cells. To test that, we generated OT-II T cells that lacked the type I IFN receptor and transferred them into WT mice. IFNAR-KO OTIIs did not express pSTAT1 and T-bet (Figure 9 A, B, C, and D) upon T1L infection. Our results demonstrated that the requirement of Type I IFN signaling in Th1 immunity is T cell-intrinsic.

Of note, we observed induction of Foxp3 in OT-II T cells in T1L-infected anti-IFNAR treated mice. As poly IC alone was sufficient to block pTregs [4], taken together our data suggests that type I IFN signaling in host cells and transferred T cells is required for pTreg blockade suggesting a different mechanism from Th1 induction. Overall, our data demonstrated that the T1L M1 gene is necessary for type I IFN and the signaling of type I IFN is T-cell intrinsic for Th1 immunity against dietary antigens.

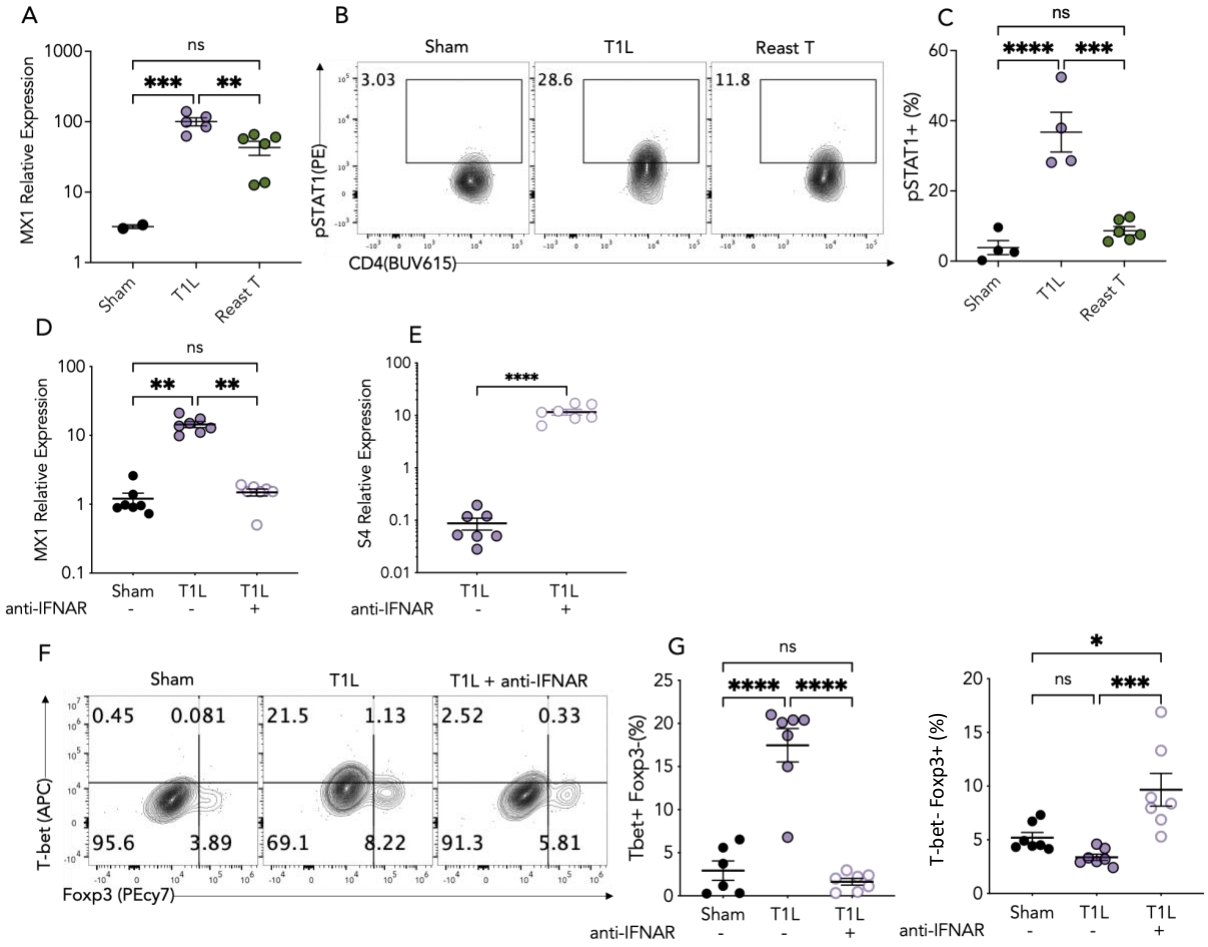


Figure 8: Type I IFN regulate Th1 immunity and pTregs immunity

(A) WT mice were infected with Sham, T1L and Reast T. Relative expression of MX1 was assessed by RT-PCR in the mLNs 2 days post-infection. (B, C) OT-II$^+$ CD45.1$^+$ CD4$^+$ T cells were transferred into WT C57BL/6 CD45.2$^+$ mice. One day post-transfer, mice were inoculated with PBS Sham, T1L 10^8 PFU, and 10^8 PFU of Reast T and put on an ovalbumin-containing diet for 3 days. Representative flow cytometry plots and the frequency of pSTAT1 expressing OT-II cells in the mLNs. (D to G) OTII$^+$ CD45.1$^+$ CD4$^+$ T cells were transferred into WT C57BL/6 CD45.2$^+$ mice. One day post-transfer, mice were inoculated perorally with 10^8 PFU of T1L or PBS and fed an OVA-containing diet for either 3 days. Mice received 100 μg of anti-IFNAR every day starting from the day of infection till the end of the experiment. (D, E) Relative expression of *MX1* and viral S4 genome copies was evaluated with RT-PCR (F, G) Representative flow cytometry plots and frequency of T-bet+ Foxp3- and T-bet- Foxp3+ expressing OT-II cells in the mLNs were assessed. The graph depicts two (A, B, C) and three (D, E,F) experiments.
*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; one-way analysis of variance (ANOVA)/Tukey's multiple comparison.

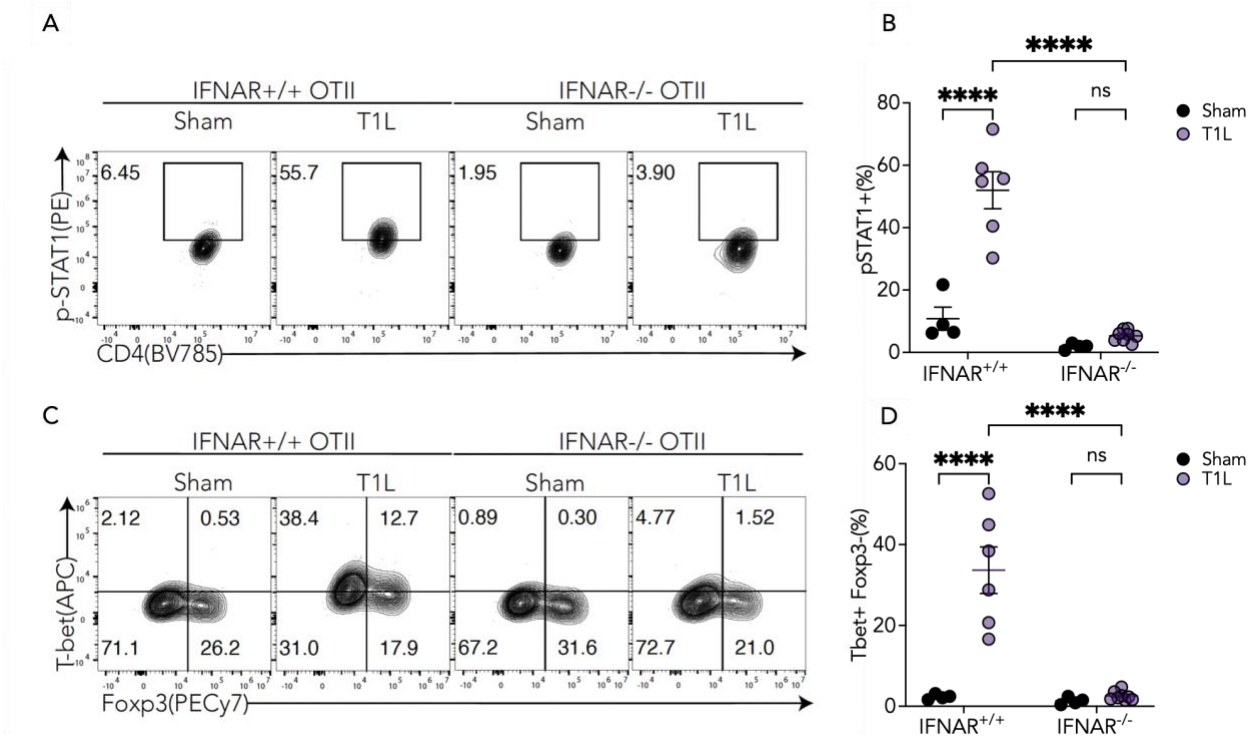


Figure 9 : T cell-intrinsic type I IFN signaling drives Th1 immunity to dietary antigens

(A to D) WT OTII$^+$ CD45.2$^+$ CD4$^+$ T cells or IFNAR-/- OTII$^+$ CD45.2$^+$ CD4$^+$ T cells were transferred into WT C57BL/6 CD45.1$^+$ mice. One day post-transfer, mice were inoculated perorally with 10^8 PFU of T1L or PBS and fed an OVA-containing diet for either 3 days. Representative flow cytometry plots and frequency (A,B) pSTAT1 and (C,D)T-bet+ Foxp3- expressing OT-II cells in the mLNs was assessed. Graphs depict two experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; one-way analysis of variance (ANOVA)/Tukey's multiple comparison.

Type I IFN alone is not sufficient to induce Th1 immunity

We next investigated whether the M2 reassortant mutant had a similar defect in type I IFN signaling which may explain its failure to induce Th1 immunity. Interestingly, Reast U did not interfere with the type I IFN response (Figure 10 A), suggesting that type I IFN signaling alone is not sufficient to induce Th1 immunity. To directly test that, we treated mice with an increasing dose of Poly I:C (PIC). Interestingly, PIC alone was sufficient to induce in a dose dependent manner type I IFN signaling in migratory DCs via upregulation of pSTAT1 (Figure 10 B). In addition to that, PIC alone was sufficient in a dose dependent manner to activate migratory DCs which was evaluated by the expression of CD86 (Figure 10 C). Although PIC was sufficient to induce DCs maturation, it failed to induce T-bet in transferred OT-II T cells (Figure 10 D,E). In sum, our data showed that type I IFN is required but not sufficient to induce loss of tolerance to dietary antigens suggesting the contribution of another pathway.

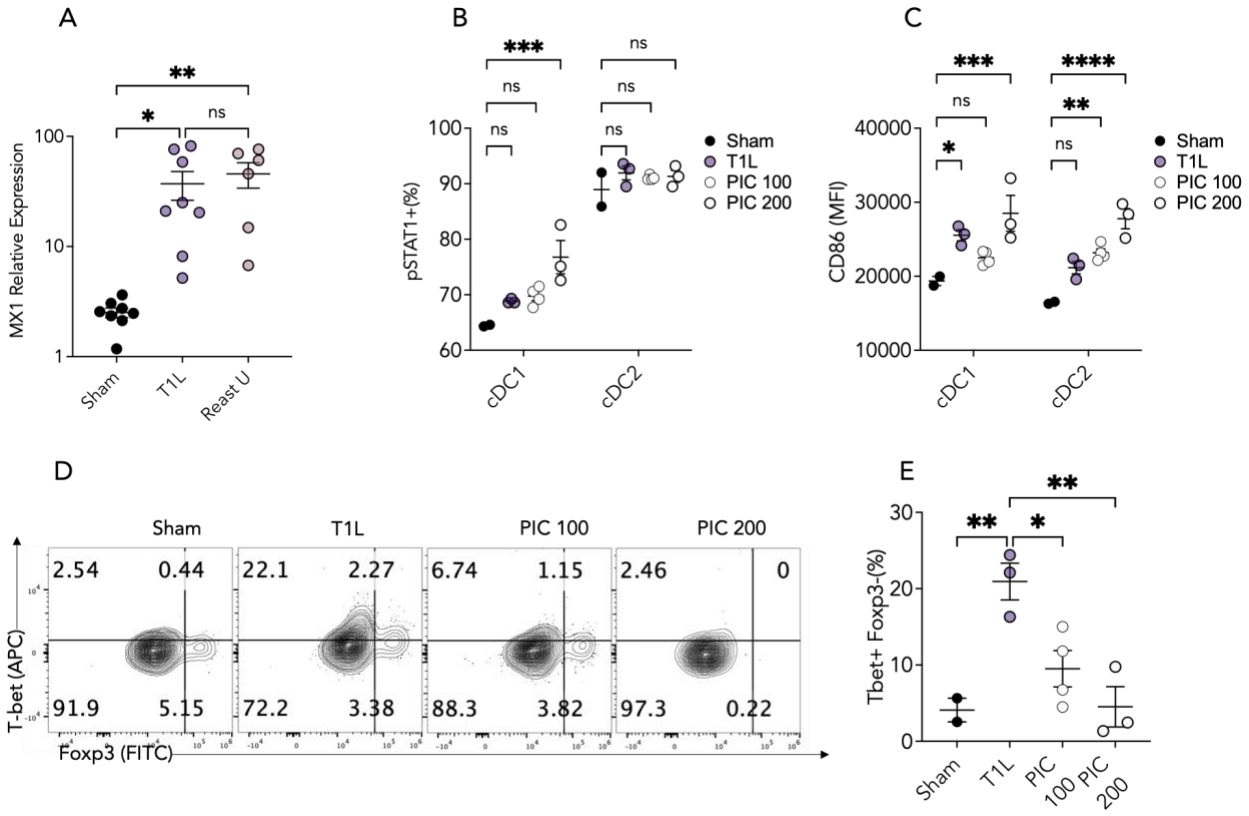


Figure 10: Type I IFN is not sufficient to induce Th1 immunity

(A) WT mice were perorally inoculated with Sham, T1L, and Reast U at 10^8 PFU. mLNs were harvested 2 days later and *MX1* was quantified by RT-PCR. The graph depicts three independent experiments. (B to E) WT OTII$^+$ CD45.1$^+$ CD4$^+$ T cells were transferred into WT C57BL/6 CD45.2$^+$ mice. One day post-transfer, mice were inoculated perorally with 10^8 PFU of T1L or 100μg of poly IC (PIC 100) and 200μg of Poly IC (PIC 200) and fed an OVA-containing diet for either 3 days. The expression of pSTAT1 (B) and CD86 (C) was evaluated in migratory DCs. (D, E) Representative flow cytometry plots and frequency T-bet+ Foxp3- expressing OT-II cells in the mLNs was assessed. Graphs depict one representative experiment. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; one-way analysis of variance (ANOVA)/Tukey's multiple comparison.

Necroptosis is the second pathway required for Th1 induction.

As Reast U failed to induce Th1 immunity independently of type I IFN and this gene along with M1 has been associated with cell death, we explored the contribution of cell death in Th1 induction. Reovirus infections have been associated with the induction of different types of cell death. For instance, in certain conditions in vitro, T3D-RV induced apoptosis while T1L blocked apoptosis [69,81]. The ability to modulate cell death was linked to the M2 gene with a minimal contribution from the M1 gene only when the M2 gene was present. Cell death could be skewed toward necroptosis when apoptosis was blocked [82] so we hypothesized that T1L could induce Th1 immunity through necroptosis independently of type I IFN. Using mice that lacked the necroptosis executioner mixed lineage kinase domain-like pseudo-kinase (MLKL), we investigated the induction of inflammatory responses in the mLNs upon T1L infection. We observed no defect in viral load (Figure 11 A) or in the ability to upregulate *Mx1* (Figure 11 B). However, we noticed a significant decrease in the frequency of T-bet+ Foxp3- OT-II T cells in the mLNs and a complete absence of these cells in the LP (Figure 11 C, D, E, F). We further investigated in which cell type necroptosis was required. Using a bone marrow chimera strategy, we asked whether the requirement for necroptosis was at the level of hematopoietic cells or non-hematopoietic cells (Figure 12 A). We transferred WT bone marrow into WT or MLKL-/- mice and MLKL-/- bone marrow into WT mice. The absence of MLKL in the hematopoietic compartment did not significantly impact the conversion of OT-II T cells into T-bet+ Foxp3- (Figure 12 B). Only when non-hematopoietic cells lacked MLKL did we observed a significant decrease in the frequency of T-bet+ Foxp3-

T cells (Figure 13 B) suggesting a requirement of necroptosis in the non-hematopoietic compartment.

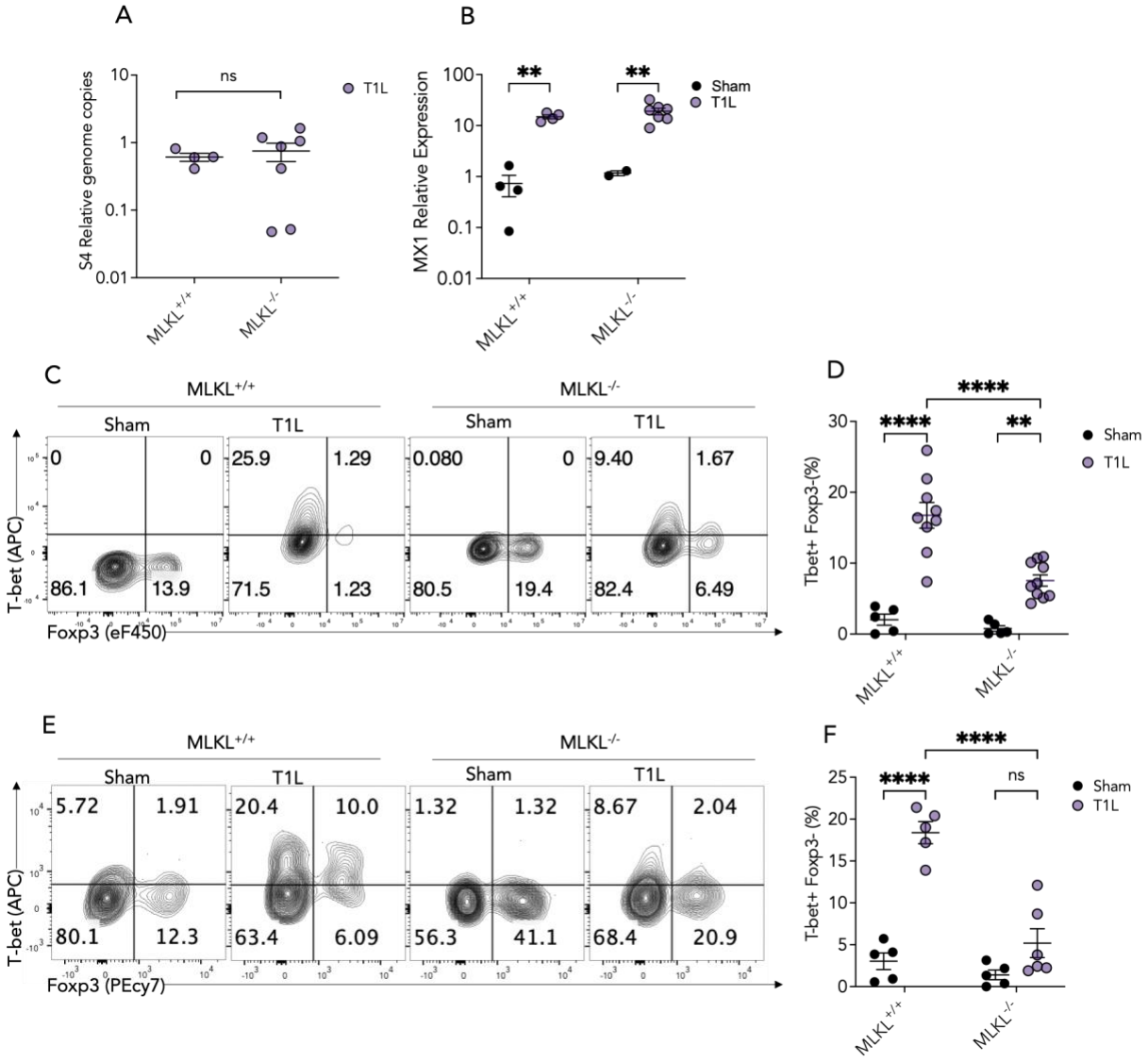


Figure 11: Necroptosis is instrumental in T1L-induced disruption of T cell response to dietary antigens independently of type I IFN

(A and B)WT C57BL/6 CD45.2$^+$ (MLKL+/+) and MLKL-/- CD45.2$^+$ mice were perorally inoculated with 10^8 PFU of T1L or PBS for 2 days. Relative expression of viral S4 genome copies (A) and *MX1* (B) in the mLNs was evaluated by RT-PCR.

(C and D) OTII$^+$ CD45.1$^+$ CD4$^+$ T cells were transferred into CD45.2$^+$ MLKL+/+ and CD45.2$^+$ MLKL-/- CD45.2$^+$ mice. One day post-transfer, mice were inoculated perorally with 10^8 PFU of T1L or PBS and fed an OVA-containing diet for 4 days (mLNs) and 6 days (LP). Representative flow cytometry plots and frequency of T-bet expressing OT-II cells in the mLNs (C) and LP (D) were assessed. Graphs depict three experiments. *P <

0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; one-way analysis of variance (ANOVA)/Tukey's multiple comparison.

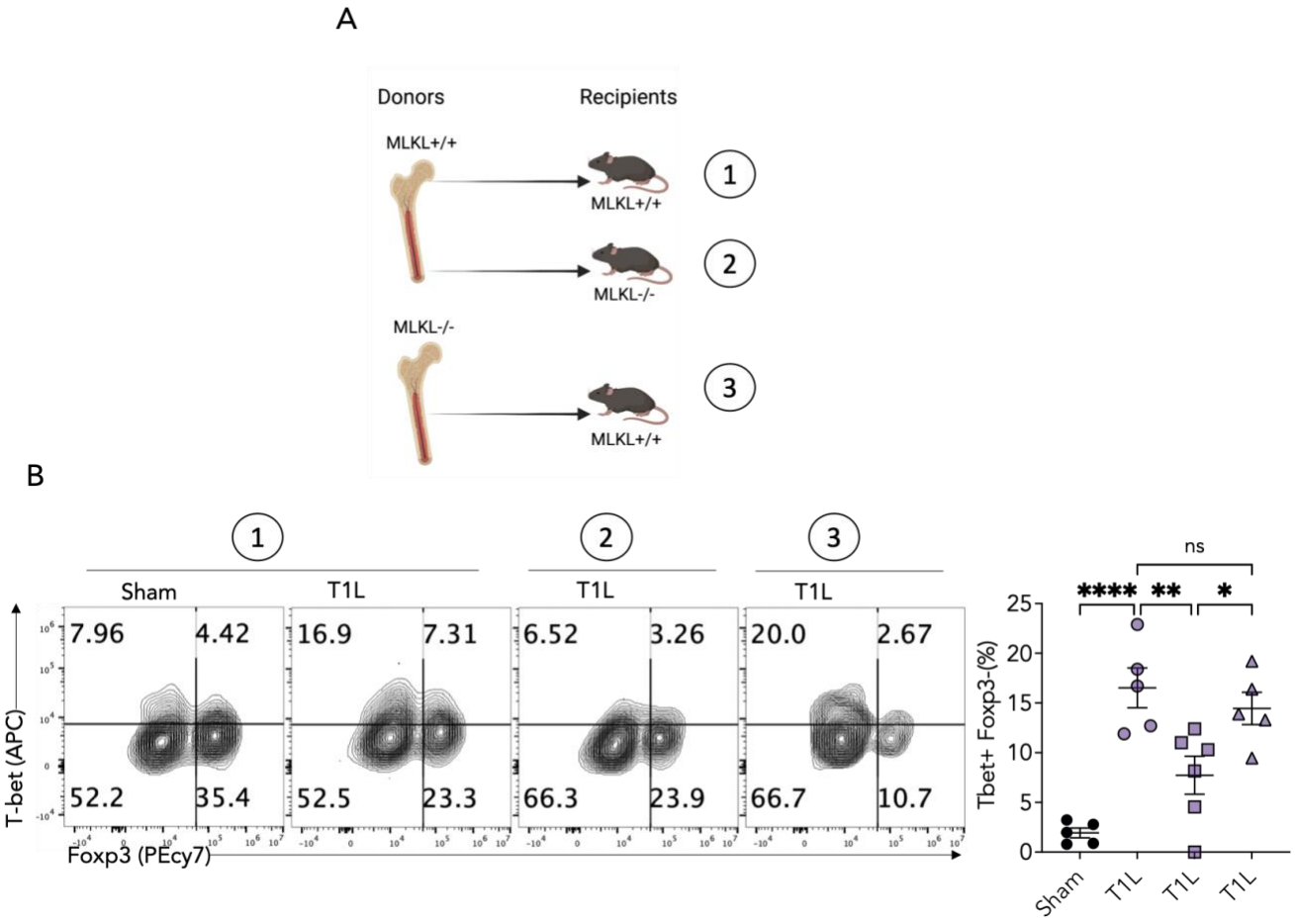


Figure 12 : Necroptosis is required in non-hematopoietic cells
(A) Experimental setup (B) Sub-lethally irradiated MLKL+/+ and mice were reconstituted with 10 x 10^6 bone marrow cells from WT C57BL/6 CD45.1$^+$ and MLKL-/- CD45.2$^+$. 4-5 weeks later, OTII$^+$ CD45.1$^+$ CD4$^+$ T cells were transferred and mice were infected the next day with 10^8 PFU of T1L or PBS for 4 days. The frequency of T-bet and expressing OT-II cells in the LP was assessed. The graph depicts three experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; one-way analysis of variance (ANOVA)/Tukey's multiple comparison.

Migratory DCs are required for Th1 immunity against dietary antigens

DCs are professional antigen-presenting cells, can take cues from the environment, and promote T cell differentiation. Migratory cDC1s in the mLNs especially are required for induction of tolerance to dietary antigens through pTreg induction [83], although in their absence migratory cDC2 can drive pTreg cell differentiation but less efficiently [46]. In the absence of both subsets of migratory DCs, pTregs response to dietary antigens is impaired [46], suggesting that migratory DCs can interact with T cells. Furthermore, although migratory DCs have a high potential to induce tolerance, they also have the ability to induce inflammatory responses based on their expression of inflammatory genes such as IL-15 and Toll-like receptor 9. We set out to investigate the role of migratory DCs in loss of tolerance to dietary antigens during T1L infection using CCR7-KO mice. There was a decrease in the frequency of OT-II in CCR7-KO mice (Figure 13 A, B). This result could be explained by the decrease of cognate TCR signaling in the absence of migratory DCs which are the main cells presenting dietary ovalbumin [4]. OT-II T cells failed to differentiate into Th1 T cells in CCR7-KO mice suggesting a requirement for migratory cells (Figure 13 C, D). Migration of host B and T cells is affected in CCR7-KO mice therefore we cannot definitively conclude off-target effects from the absence of these cells. Furthermore, whether both migratory DC subsets can contribute to Th1 immunity against dietary antigens is unknown.

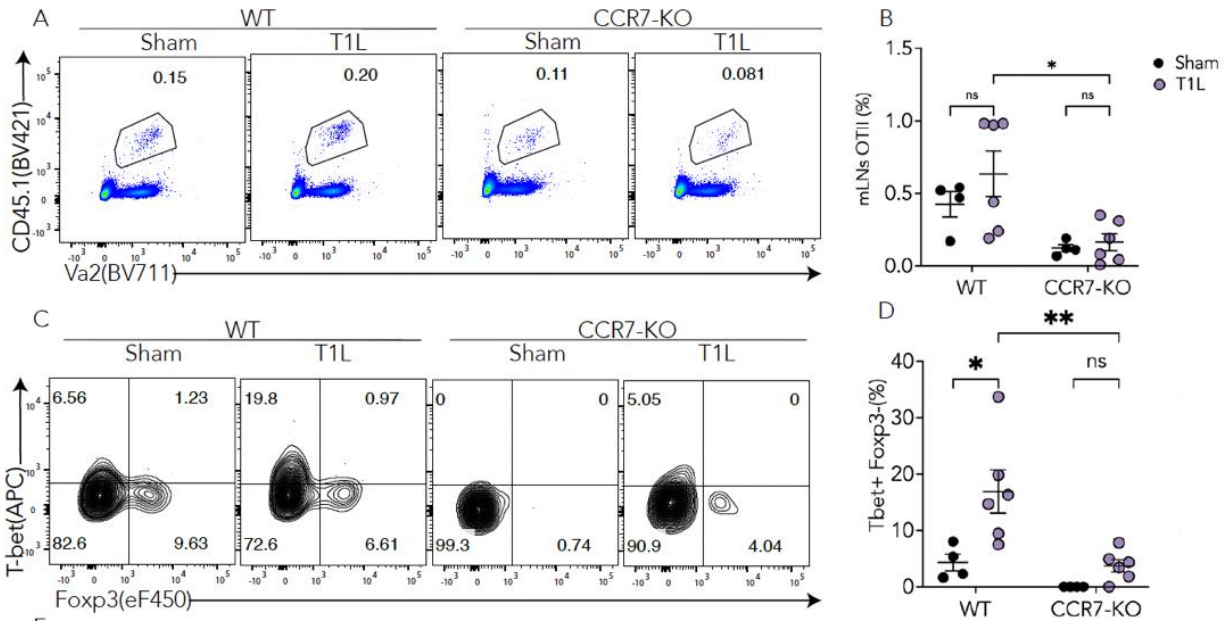


Figure 13: Migratory DCs are required for T1L Induced Th1 Immunity
(A to D) OTII^{+} CD45.1^{+} CD4^{+} T cells were transferred into WT C57BL/6 CD45.2^{+} and CCR7-/- CD45.2^{+} mice. One day post-transfer, mice were inoculated perorally with 10^{8} PFU of T1L or PBS and fed an OVA-containing diet for 3three days. Representative flow cytometry plots (A, C) and frequency (B, D) of OT-II T cells and T-bet+ Foxp3- expressing OT-II cells in the mLNs were assessed. Graphs depict two experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; one-way analysis of variance (ANOVA)/Tukey's multiple comparison.

cDC1s are the key DC subset driving Th1 immunity

To test the role of migratory cDC1s in Th1 immunity against dietary antigens, we transferred OT-II T cells in BATF3-KO mice. Interestingly, unlike in CCR7-KO mice, we did not observe a defect in the recovery of OT-IIs in the mLNs suggesting cognate TCR interaction in the absence of cDC1s provided by other APCs (Figure 14 A, B) and OT-II T cells in BATF-3 KO failed to induce T-bet (Figure 14 C, D, E, F). Interestingly, in the absence of cDC1s, we still observed pTreg blockade suggesting that cDC2s could perhaps compensate for the absence of cDC1s in regulating pTregs (Figure 14 C, D, E, F). Altogether, our findings beg the question of how cDC1s integrate necroptotic signals from non-hematopoietic cells such as intestinal epithelial cells and provide type I IFN to T cells. In order for cDC1 to integrate necroptotic signals, they must be in close contact with IECs to either uptake necroptotic epithelial cells or get indirectly triggered by secreted necroptotic signals from IECs. We generated Rosa26$^{LSL\text{-}Tdtomato}$ Vilin^{creERT2} mice to track the uptake of epithelial cells by migratory cells that traffic to the mLNs. This system is unique and specific in that it allows quantification of the uptake of IECs at a defined period of time. We infected Rosa26$^{LSL\text{-}Tdtomato}$ Vilin^{creERT2} with T1L and labelled IECs at the same time. Two days later, we looked in the mLNs to identify migratory cells that have engulfed labeled IECs from the tissue. Among the migratory cells, only migratory cDC1s were positive for labeled IECs (Figure 15). Interestingly, we did not observe a difference in the uptake of labeled Tdtomato+ IECs cells by migratory cDC1s between sham-infected and T1L-infected mice (Figure 15). These results indicate that uptake of epithelial cells is not dependent on necroptosis. Based on this, we hypothesized that necroptotic IECs might be sensed differently by migratory cDC1s

compared to non-necroptotic IECs. The sensing by cDC1s could be direct through the detection of necroptotic factors on IECs by a specialized receptor on cDC1s or it could be indirect through secretion of a necroptotic mediator that is then sensed by cDC1s. As migratory cDC2s are also in the tissue and near the epithelium [84] they might be affected by the necroptotic mediator. Therefore, we thought the direct sensing hypothesis through a specialized receptor uniquely expressed on cDC1s will be more plausible as the effect will be specific to cDC1s.

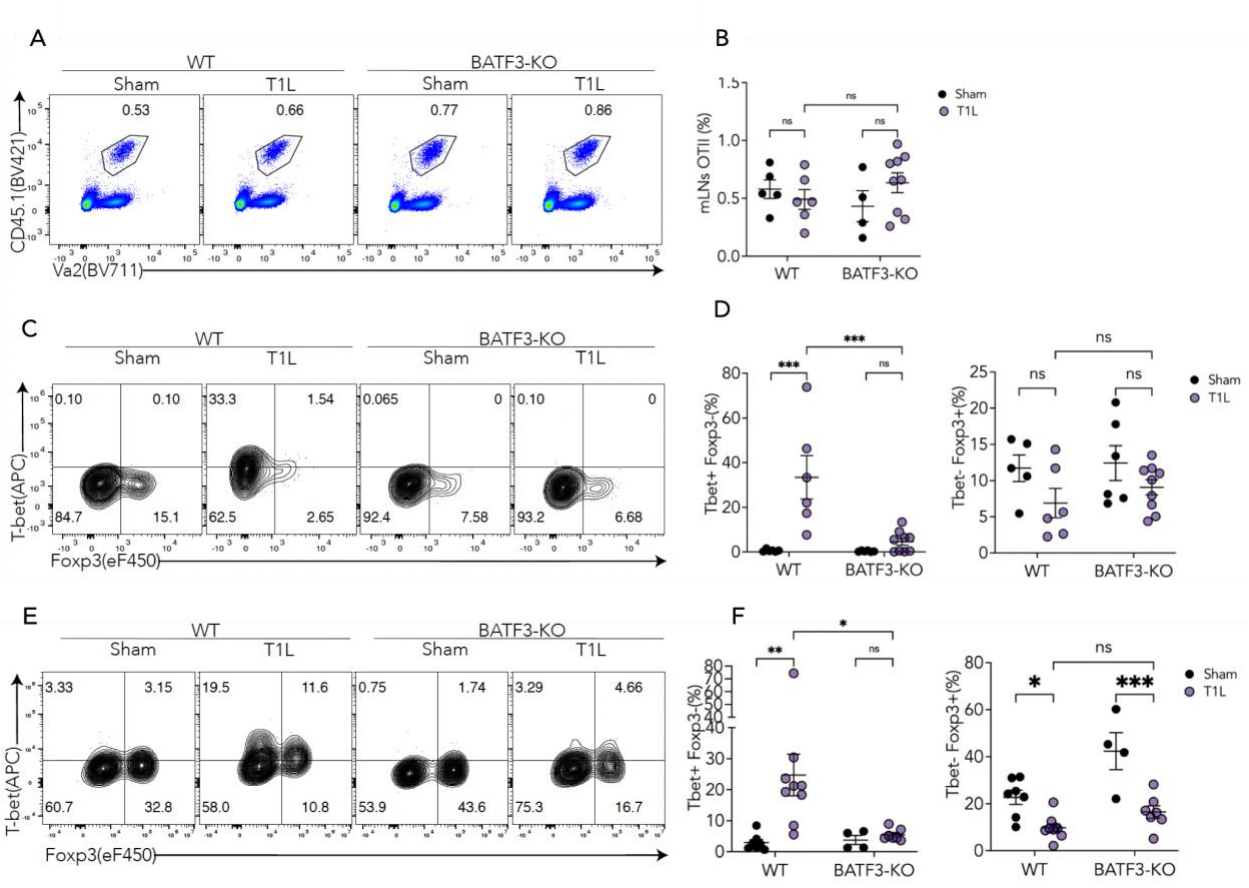


Figure 14: Migratory cDC1s are required for T1L induced Th1 immunity
(A, B, C, D, E and F) OTII^{+} CD45.1^{+} CD4^{+} T cells were transferred into WT C57BL/6 CD45.2^{+} and BATF3-KO CD45.2^{+} mice. One day post-transfer, mice were inoculated perorally with 10^{8} PFU of T1L or PBS and fed an OVA-containing diet for three days. Representative flow cytometry plots (A,C,E) and frequency (B,D,F) of OT-II T cells and T-bet+ Foxp3- expressing OT-II cells in the mLNs (C,D) and LP (E,F) were assessed. Graphs depict two experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; one-way analysis of variance (ANOVA)/Tukey's multiple comparison.

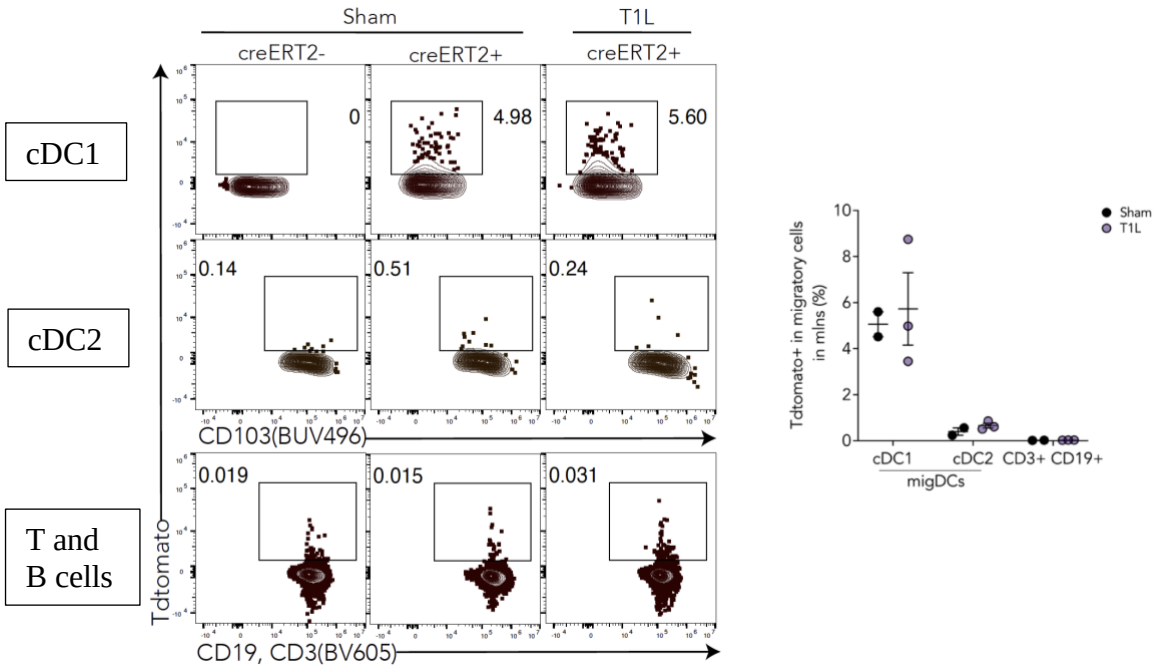


Figure 15: cDC1s are the primary migratory cells that uptake IECs

Rosa26$^{LSL\text{-}Tdtomato}$ Vilin$^{creERT2+}$ and Rosa26$^{LSL\text{-}Tdtomato}$ Vilin$^{creERT2-}$ were injected with tamoxifen twice every day for the duration of the experiment. Mice were infected with sham and T1Lx10^{8} PFU for two days. Representative flow cytometry and frequency of Tdtomato+ cDC1 (CD103+ CD11b-), cDC2 (CD103+ CD11b+) and T and B cells (CD3+ CD19+) was evaluated by flow cytometry. Graphs depict one representative experiment. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; one-way analysis of variance (ANOVA)/Tukey's multiple comparison.

cDC1 expression of Clec9a contributes to Th1 immunity through regulation of the secretion of Th1-inducing cytokines

While migratory DCs express a lot of receptors that enable the clearance of apoptotic cells and necrotic cells [85], only a few of them are specific for cDC1. One of such receptors is Clec9A which is a C-type lectin domain family 9A (Clec9A) also known as DNGR1 is a receptor that recognizes F actin on necrotic cells and is uniquely expressed on cDC1 [51,86]. We evaluated the Th1 response to dietary antigens in T1L-infected Clec9A-KO mice and observed a significant reduction in T-bet+ Foxp3- OT-IIs in the mLNs and LP of these mice (Figure 16 A, B,C, D). We then investigated how Clec9A modulates the cDC1 function. We checked the expression of Clec9A during T1L infection as well as the expression of the co-stimulatory molecule CD86 (Figure 17 A, B, C). Blocking Clec9A was specific to cDC1 and did not impact the expression of CD86 (Figure 17 A, B, and C). cDC1 are unique in their ability to secrete IL-12 [87] which is a Th1 inducing cytokine. To our surprise, blocking Clec9A prevented the expression of IL-12 by cDC1s (Figure 18 A) and the absence of IL-12 alone impaired Th1 immunity (Figure 18 B).

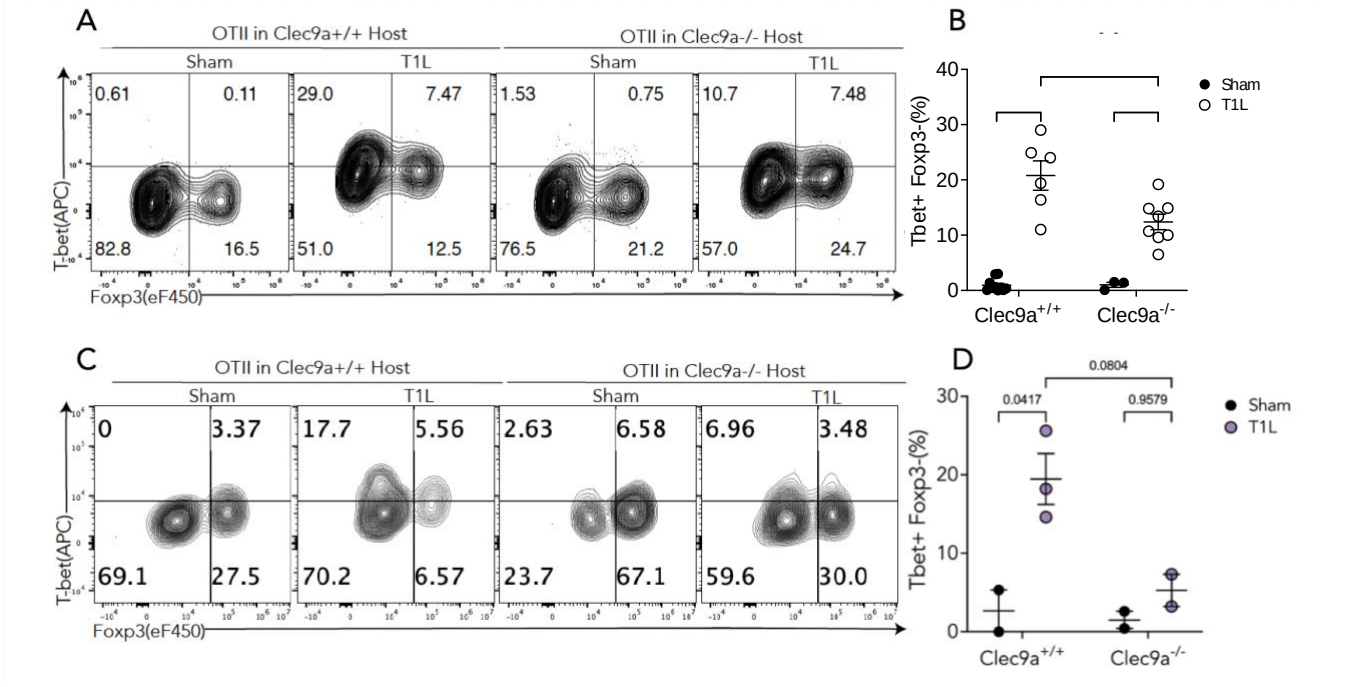


Figure 16 : Absence of Clec9a impairs Th1 T cell differentiation

(A, B, C, D) OTII^{+} CD45.1^{+} CD4^{+} T cells were transferred into WT C57BL/6 CD45.2^{+} and Clec9a-/- CD45.2^{+} mice. One day post-transfer, mice were inoculated perorally with 10^{8} PFU of T1L or PBS and fed an OVA-containing diet for three days (mLNs) and six days (LP). Representative flow cytometry plots (A,C) and frequency (B,D) of T-bet+ Foxp3- expressing OT-II cells in the mLNs (A,B) and LP (C,D) were assessed. Graphs depict five(A,B) and one (C,D) experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; one-way analysis of variance (ANOVA)/Tukey's multiple comparison.

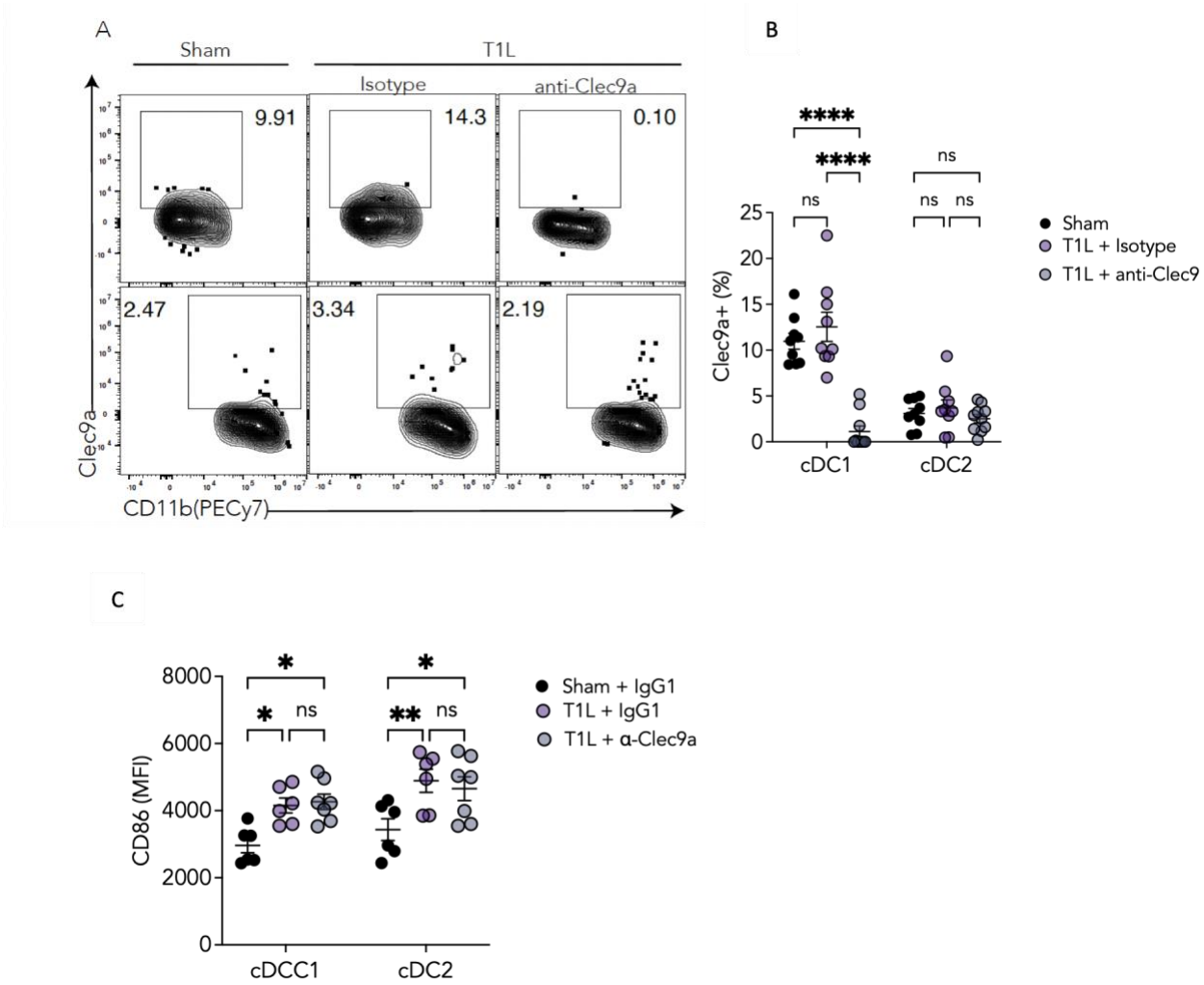


Figure 17: Clec9A is uniquely expressed on cDC1s and does not impact DC maturation

(A,B,C) WT mice were treated with anti-Clec9a (α-Clec9a) antibody and perorally infected with T1L at 10^8 PFU. Two days later, the expression of Clec9a and CD86 on migratory DCs was evaluated by flow cytometry. (A, B) Representative flow cytometry and frequency of Clec9a+ cDC1 and cDC2 were quantified. (C) Expression of CD86 in migratory DCs was evaluated by mean fluorescence intensity. Graphs depict three experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; one-way analysis of variance (ANOVA)/Tukey's multiple comparison.

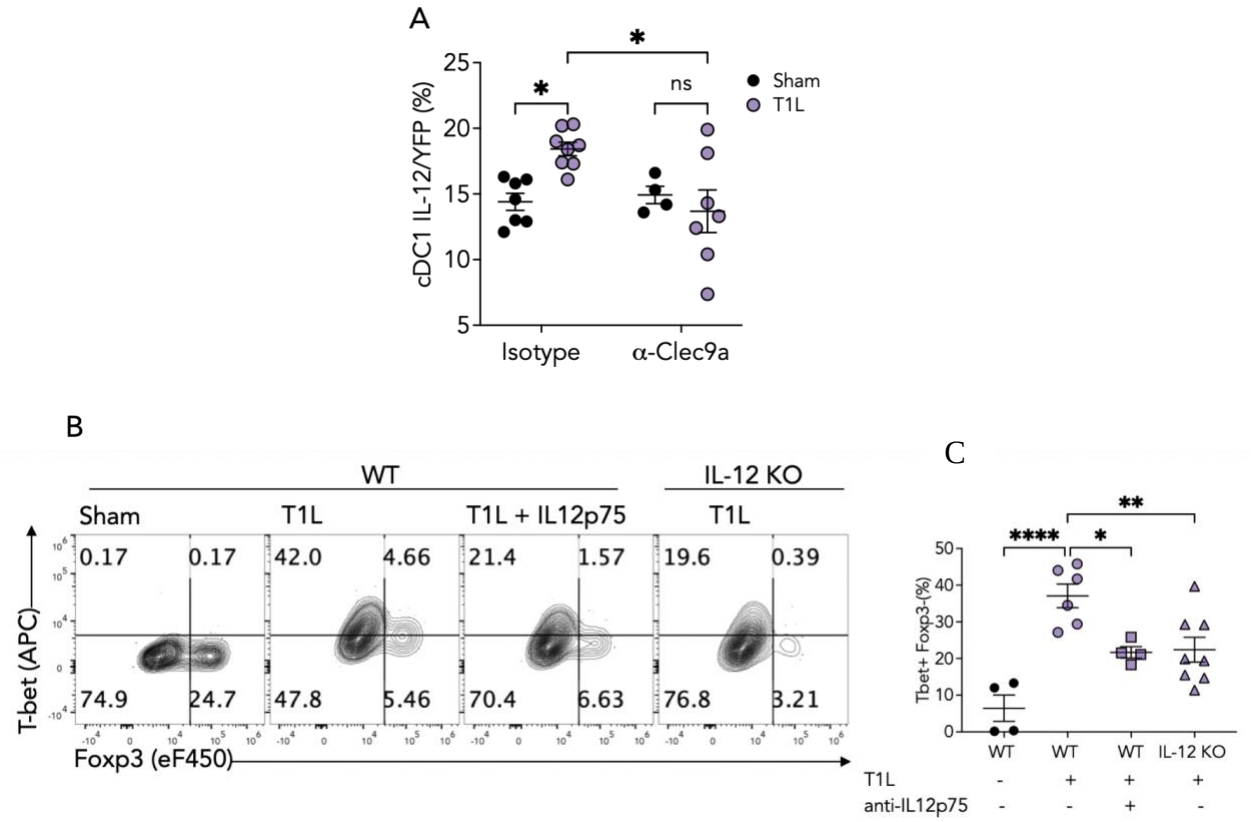


Figure 18: Clec9A contributes to Th1 immunity through secretion of IL-12

(A) IL-12 reporter mice (p40-IRES-eYFP) mice were treated with anti-Clec9a (α-Clec9a) antibody and perorally infected with T1L at 10^8 PFU. Two days later, the expression of IL-12/YFP on migratory cDC1 was evaluated by flow cytometry. (B, C) $OTII^+$ $CD45.1^+$ $CD4^+$ T cells were transferred into WT C57BL/6 $CD45.2^+$ with or without anti-IL12p75 or into IL12-KO $CD45.2^+$ mice. One day post-transfer, mice were inoculated perorally with 10^8 PFU of T1L or PBS and fed an OVA-containing diet for three days. Representative flow cytometry plots and frequency (B,C) of T-bet+ Foxp3- expressing OT-II cells in the mLNs (A,C) was assessed. Graphs depict two experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; one-way analysis of variance (ANOVA)/Tukey's multiple comparison.

Clec9A and type I IFN pathway drive Th1 immunity independently and collaboratively

Blocking Clec9A did not interfere with the expression of ISGs (Figure 19 A) suggesting the Cle9A-IL-12 pathway is independent of the type I IFN pathway. Then, we wanted to investigate how migratory cDC1s integrate type I IFN. We hypothesized that perhaps they support reovirus replication and sense viral particles leading to type I IFN secretion in the mLNs. Unexpectedly, CD11c+ cells did not support reovirus replication (Figure 19 B) but rather phagocytosed infected epithelial cells or viral particles (Figure 19 C). Our data is in line with previous studies in the Peyer's patches showing that DCs do not support reovirus replication but rather uptake dead infected epithelial cells [88]. To test if type I IFN secretion and signaling was dependent on migratory cDC1s, we infected mice lacking BATF3-KO with T1L and examined the induction of ISGs in the mLNs. We did not observe upregulation of *Mx1* in the mLNs of BATF3-KO mice compared to WT mice at 2 days, the peak of infection. (Figure 19 D). However, there was no difference in the expression of *Mx1* in WT and BATF3-KO mice three days post-infection (Figure 19 D). Our data indicate a delay in the type I IFN response in the absence of cDC1s suggesting a compensatory mechanism. These data reinforce the fact that type I IFN alone is not sufficient to induce Th1 immunity and highlight the uniqueness of cDC1s in integrating necroptotic and type I IFN-inducing signals.

Altogether, we identified for the first-time viral determinants in T1L-induced immunopathology. More importantly, we uncovered two checkpoints involving type I IFN and Clec9A-dependent IL-12 needed by viruses to trigger inflammatory responses to dietary antigens.

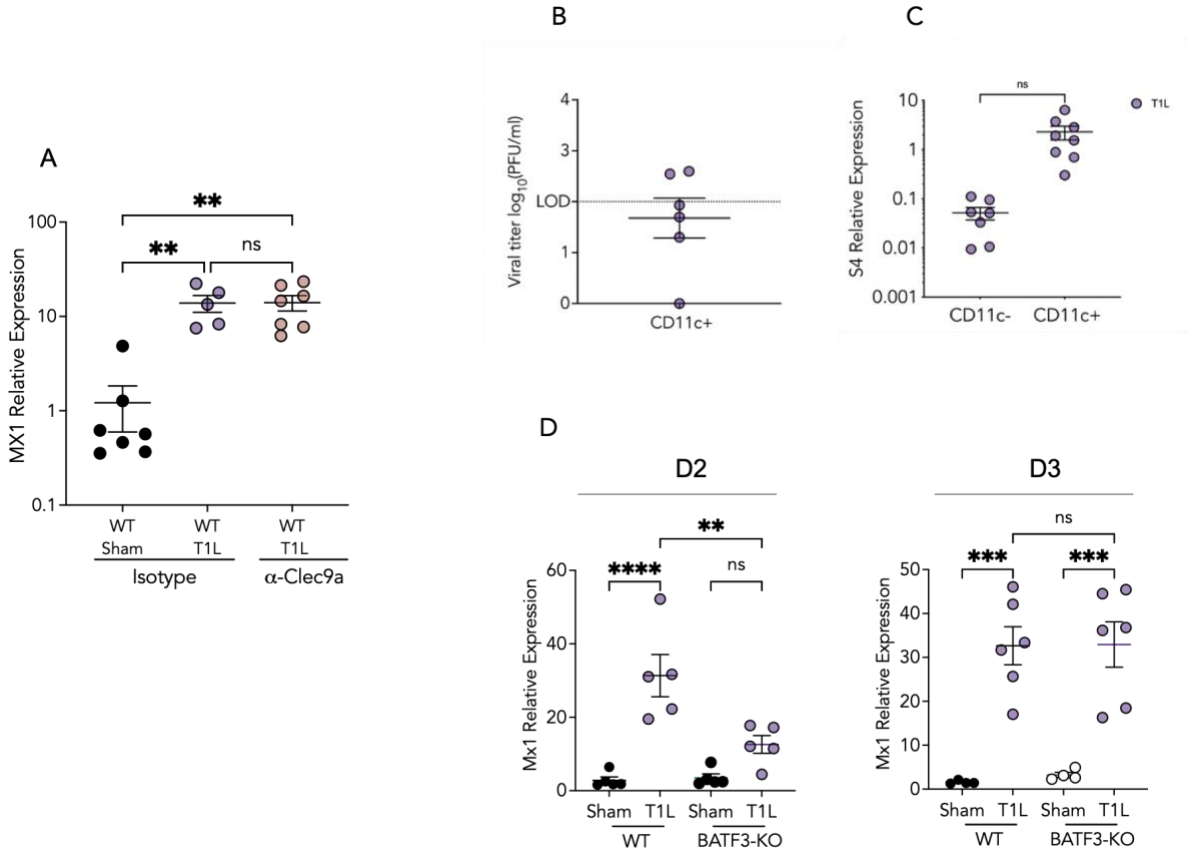


Figure 19: cDC1s upregulate type I IFN-inducing signals independently of Clec9A

(A) WT mice were treated with anti-Clec9a (α-Clec9a) antibody and perorally infected with T1L at 10^8 PFU. Two days later, relative expression of *Mx1* was evaluated by RT-PCR. (B,C) WT mice were infected with T1L 10^8 PFU. Viral titer was quantified by plaque assay in CD11c+ CD11b- cells from the mLNs. (C) WT mice were infected with T1L 10^8 PFU and CD11+ and CD11c- were MACS sorted from the mLNs. Viral S4 relative expression was evaluated by RT-PCR. (D) WT and BATF3-KO mice were infected with T1L 10^8 PFU and relative expression of *Mx1* was evaluated by RT-PCR two days later.

The microbiota does not interfere with T1L-induced Th1 immunity

The microbiota has been shown to modulate the immune response against viruses [70]. A previous study demonstrated that reovirus pathogenesis is altered in antibiotic-treated mice [89]. This suggests interactions between reovirus and the microbiota can impact reovirus pathogenesis. Furthermore, reovirus strains T1L and T3D differ in the capacity to infect the intestine suggesting that T1L may have evolved ways to replicate more efficiently than T3D in a bacteria-rich environment. Although it is known that the microbiota does not interfere with the immune response against food antigens at homeostasis [14], its potential to interfere with reovirus' ability to modulate tolerance to dietary antigens remains to be determined. Here, using germ-free and antibiotic-treated mice, we showed that T1L induced Th1 immunity is microbiota-independent (Figure 20A). Surprisingly the microbiota is required and sufficient to prevent T3D-RV to induce Th1 immunity to dietary antigens (Figure 20 A, B). Contrary to what was shown [89], our data indicated that the microbiota does not interfere with viral replication (Figure 20 C,D). The upregulation of ISGs in T3D-RV-infected mice was not influenced by the microbiota (Figure 20 E). In summary, these data suggest that T1L has evolved mechanisms to subvert the microbiota while T3D-RV has not. However, further investigations are needed to uncover the mechanisms by which the microbiota prevents T3D-RV's ability to disrupt the immune response to dietary antigens.

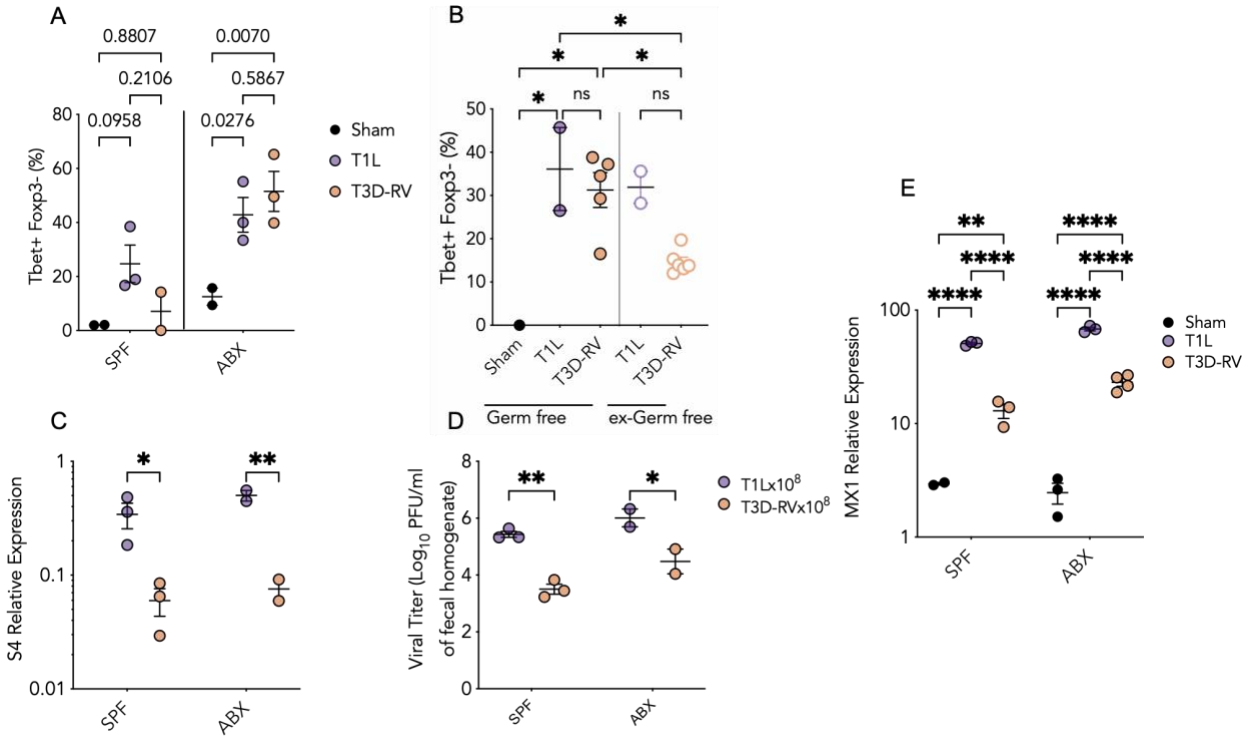


Figure 20 : The gut microbiome does not impact T1L-induced Th1 immunity against dietary antigens

(A) WT mice were treated with an antibiotic cocktail. OTII^{+} CD45.1^{+} CD4^{+} T cells were transferred into WT C57BL/6 CD45.1^{+} mice (SPF and antibiotics (ABX) treated). One day post-transfer, mice were inoculated perorally with 10^{8} PFU of T1L or T3D-RV or PBS and fed an OVA-containing diet for three days. The frequency of T-bet+ Foxp3- expressing OT-II cells in the mLNs was assessed. (B) Germ-free mice were colonized with fecal content from SPF mice or sham colonized. OTII^{+} CD45.1^{+} CD4^{+} T cells were transferred into WT C57BL/6 CD45.1^{+} mice and mice were inoculated perorally with 10^{8} PFU of T1L or T3D-RV or PBS and fed an OVA-containing diet for six days. The frequency of T-bet+ Foxp3- expressing OT-II cells in the LP was assessed. (C, D, and E) WT mice were treated with an antibiotic cocktail. Mice were inoculated perorally with 10^{8} PFU of T1L or T3D-RV or PBS and fed an OVA-containing diet for three days. Two days later, viral S4 relative expression and (E) MX1 relative expression in the mLNs were evaluated by RT-PCR. (D) Viral titer in the feces was quantified by plaque assay. Graphs depict one or two (B only) experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; one-way analysis of variance (ANOVA)/Tukey's multiple comparison.

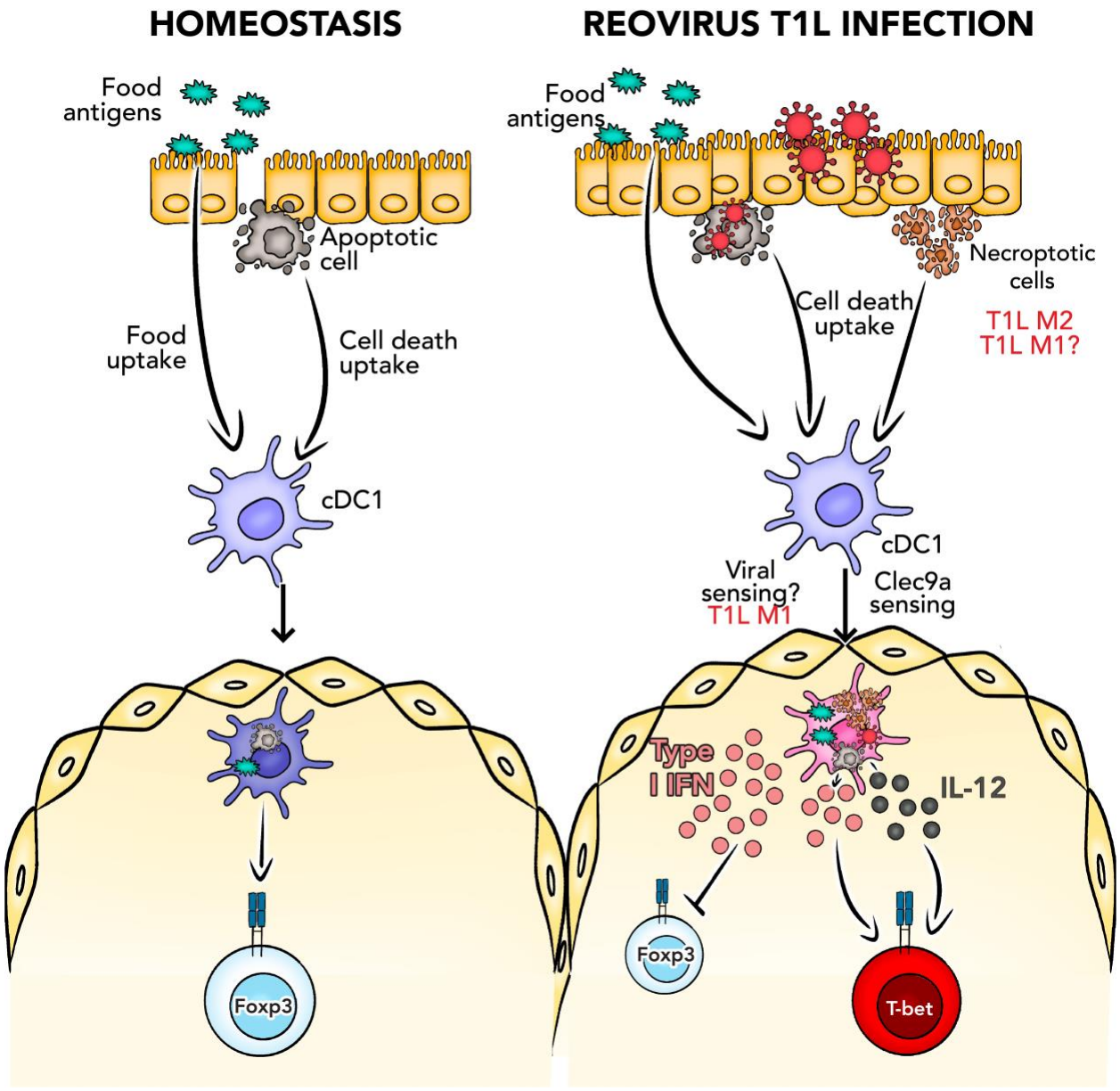


Figure 21: Model of T1L induced Th1 immunity and pTreg blockade against dietary antigens

At homeostasis, migratory cDC1s uptake food antigens or apoptotic epithelial cells leading to a pTreg response. During T1L infection, epithelial cells can undergo apoptosis normally but can also undergo necroptotic cell death in a T1L M2 gene-dependent manner. Migratory cDC1s can uptake infected epithelial cells and sense viral particles from phagocytosed cargo which drive secretion of type I IFN in a T1L M1 dependent manner. However, migratory cDC1s internalize necroptotic cells and through Clec9A sensing of the necroptotic cargo promotes IL-12 secretion. T cells integration of type I IFN and IL-12 drives T-bet expression. The integration of type I IFN alone by T cells and cDC1 blocks Foxp3 expression in T cells.

CHAPTER 4: DISCUSSION

Viruses have long been associated with autoimmune disorders, but how viruses contribute to autoimmunity is poorly understood [90–92]. Our group previously demonstrated that reovirus infection in mice could induce the loss of oral tolerance to dietary antigens [4], but the immunological mechanisms underlying this phenomenon remain to be investigated. Here, we uncovered mechanisms by which reovirus infection disrupts homeostatic responses to dietary antigens by promoting a proinflammatory Th1 T cell response at the expense of a pTreg response. This Th1 T cell response represents the first step in CeD, which later drives the secretion of autoantibodies and licenses the killing of cells by cytotoxic lymphocytes [59]. Through this study, we reveal a novel mechanism for the development of virally induced autoimmune disorders, which could ultimately inform next-generation vaccine strategies.

Using reassortant viruses, we uncovered a unique complex network revolving around two signals required for the induction of Th1 immunity against orally fed ovalbumin antigens. Specifically, we showed that although dendritic cells (DCs) did not support viral replication, migratory cDC1s in particular were able to uptake infected cells and release type I IFN (signal one). Type I IFN at the T cell level was required for the upregulation of T-bet, a transcriptional driver of the Th1 response. However, type I IFN alone was not sufficient to disrupt the T cell response to dietary antigens, suggesting that several checkpoints are required to induce Th1 immunity. In addition to type I IFN, integrating necroptotic signals from infected cells by migratory cDC1s through their unique receptor Clec9A was necessary for the expression of the Th1-inducing cytokine IL-12 (signal two). Furthermore, we identified key reovirus T1L genes that regulated this complex network

leading to Th1 immunity. For the first time, our work highlights a role of necroptosis in Th1 immunity, revealing a new function for Clec9A. Overall, we show that innate mechanisms are instrumental in developing virally-induced autoimmune disorders and propose that several checkpoints are needed to drive Th1 immunity as a way to prevent aberrant immune responses.

4.1 Viral strain-level differences dictate immunopathology

Although reovirus infections are very ubiquitous in nature and clinically silent, previous work from our lab demonstrated that they can induce immunopathology characterized by induction of a pro-inflammatory response against dietary antigens instead of pTregs as seen in CeD [4,63]. Specifically, we identified that the reovirus strain T1L was particularly adept at inducing immunopathology while strain T3D-RV could not, despite differing by only 8 genes. These results indicate that reovirus strain T1L has unique properties that enable the disruption of the immune response to dietary antigens.

Using reverse genetics, we generated reassortant viruses and identified the T1L M1 and M2 genes as the viral determinants underlying T1L-induced Th1 immunity against dietary antigens. Previous studies have shown that the T1L M1 gene is a determinant for strain-specific differences in type I IFN, with both the T1L M1 and M2 genes being determinants for strain-specific differences in cell death. In line with these studies, our data show that reassortant viruses lacking the T1L M1 gene induced levels of type I IFN signaling genes in the mLNs similar to that of wild-type T3D-RV, which correlated with failure to induce T-bet in dietary antigen-specific T cells. While our study found that the T1L M1 gene was required for upregulation of type I IFN in the mLNs of adult mice, the T1L M1 gene product

μ2 has been shown to antagonize type I IFN *in vitro (L929 cells)* and in the heart of neonatal mice through nuclear sequestration of IRF9 [67,68]. Interestingly, the T3D M1 gene promoted type I IFN *in vitro (L929 cells)* and in the heart of neonatal mice while in our study the T3D M1 correlated with a reduced level of type I IFN. The antagonistic property of T1L M1 encoding protein μ2 *in vitro* was due to a single proline substitution at position 208 (P208), while T3D possesses a serine at this position instead.

One possible explanation for strain differences with opposing phenotypes *in vivo* versus *in vitro* may be related to cell-intrinsic and extrinsic differences. It is likely that the T1L M1 gene can still block type I IFN signaling in vivo but in a more complex environment, cell-intrinsic and extrinsic mechanisms might counteract this effect. Perhaps DCs do not support reovirus replication as a way to prevent the shutdown of its type I IFN signaling by T1L to protect the host. In fact, previous studies have shown that the absence of type I IFN signaling in hematopoietic cells is lethal in T1L-infected mice [80]. Alternatively, as only a few cells are being infected in vivo, it is possible that type I IFN secretion by infected cells is sufficient to induce an antiviral state in uninfected cells thereby counteracting defective receptor signaling in infected cells.

Taken together, our results indicate that the blocking of type I IFN signaling by the T1L M1 gene observed in vitro may lead to an increase of type I FN signature in a complex system like the gut and drive pro-inflammatory responses. However, an additional caveat is that infection with the M1 reassortant was associated with reduced viral load. Therefore, we cannot dissociate the impact of T1L M1 on replication from type I IFN. It is possible that the M1 reassortant failed to induce ISGs due to its defect in replication. Future studies using an increasing infectious dose of M1 reassortant or complementing the single M1

reassortant infection with the addition of type I IFN will be important for establishing a correlation between viral load and type I IFN secretion. It is important to note that our data demonstrate that viral load cannot explain T3D-RV's inability to induce Th1 immunity.

In this study, we demonstrated that necroptosis in non-hematopoietic cells is required for the induction of T1L-induced Th1 immunity against dietary antigens. Strain-specific differences between M1 and M2 reovirus genes have been previously associated with cell death. [69,78] The T3D M2 and M1 genes control the induction of apoptosis while T1L M2 represses apoptosis with a stronger effect when combined with the T1L M1 gene in the gut of WT adult mice, gut organoids, and L929 cells [69]. Moreover, another group demonstrated that silencing of T3D M2 in T3D-infected L929 cells led to an increase in necroptosis and accumulation of viral genes [81]. Interestingly, the knockdown of the T3D M2 did not interfere with apoptosis. Integrating data from these studies together would suggest that on one hand, the ability to induce apoptosis is redundant between T3D M1 and M2 while only T3D M2 can repress necroptosis through a unique mechanism [81]. On the other hand, T1L represses apoptosis mainly through its M2 gene [69]. However, whether reovirus T1L M2 impacts the necroptotic pathway is not known. Knowing that preventing apoptosis can induce necroptosis [82], we hypothesized that T1L infection in WT adult mice induced necroptosis. It was experimentally challenging to detect necroptosis *in vivo* due to the unavailability of sensitive antibodies for detecting specific proteins involved in the necroptosis pathway. Therefore, we used mice lacking the necroptosis executioner protein, mixed lineage kinase domain pseudo-kinase (MLKL). To our surprise, the absence of MLKL prevented the induction of Th1 immunity to dietary antigens but did not impact replication and the ability to induce ISGs in T1L-infected mice. These results were

similar to infection with the single M2 reassortant virus and implied that T1L M2 is the viral determinant for necroptosis *in vivo*. To definitively demonstrate that T1L M2 induces necroptosis, further *in vitro* studies with T1L and single M2 reassortants are needed similar to previous studies with single M2 T3D reassortants [69]. Lastly, microscopy strategies aimed at detecting inflammatory cell death *in vivo* using TUNNEL staining in combination with cleaved caspases could be used to detect caspase-negative TUNEL positive cells as a proxy for necroptotic cells.

Finally, our work builds on existing evidence demonstrating that different strains of the same virus can have distinct pathology with varying degrees of severity similar to what has been observed in SARS-CoV-2 infections [93]. Therefore, we propose that strain-level identification of viruses is required to determine their immune properties and mechanisms by which they induce immunopathology. We believe this information is crucial to designing adequate vaccine strategies to prevent disease.

4.2 Integration of type I IFN and necroptosis tips the balance toward Th1 immunity

4.2.1 Migratory cDC1s: master regulators of immunity to dietary antigens

Conventional DCs come in different flavors and have specialized functions [38]. They can be divided into two subsets based on the expression of transcription factors IRF8 and BATF3 for conventional DCs 1 (cDC1) and IRF4 for conventional DCs 2 (cDC2). They can further be divided into migratory versus resident DCs based on their migratory properties and expression of integrin CD103. In the context of immunotolerance to dietary antigens, it was shown that migratory cDC1s have the highest potential to induce pTregs

[46]. However, in their absence, migratory cDC2s could induce pTregs but less efficiently [46]. In addition, migratory cDC1s have a high inflammatory profile as illustrated by the expression of inflammatory genes such as pro-inflammatory cytokines interleukin-12 (IL-12) and interleukin-15 (IL-15). However, whether cDC1s can drive differentiation of Th1 immunity against dietary antigens at the expense of pTregs during T1L infection is not known. Using mice that lack all migratory DCs and mice that specifically lacked only the migratory cDC1 subset we determined migratory cDC1 cells as the subset required for Th1 immunity against dietary antigens. The ability of migratory cDC1s to drive Th1 immunity was unique to their capacity to integrate Th1-inducing signals from the environment. Here we demonstrated that migratory cDC1s could sense necroptotic epithelial cells through the receptor C-type lectin domain family 9 A (Clec9A) and uptake infected epithelial cells. Activation of Clec9A regulated the expression of IL-12 while uptake of infected epithelial cells allowed sensing of viral RNA leading to the secretion of type I IFN. Our results are in line with previous studies demonstrating that migratory cDC1s have the unique ability to induce Th1 immunity due to their unique expression of sensing molecules like TLR3 and TLR11 in the context of the infection [49,52]. Our study contributes to the body of work proposing that although DCs are adaptable, they need to be equipped with the appropriate features to enable the integration of T cell lineage-specific signals.

While T1L infection drives Th1 immunity, it also induces blockade of pTregs [4]. This feature was unique to T1L infection as infection with norovirus CW3, a driver of Th1 immunity, was not able to consistently block pTregs [63]. These data indicate that two distinct

mechanisms may regulate the induction of pro-inflammatory Th1 and blockade of anti-inflammatory pTregs. Our data showed that while Th1 responses were no longer induced, pTregs were still blocked in T1L-infected mice lacking migratory cDC1s. These results were surprising because we expected that cDC1s would be required for both mechanisms. It is possible that similar to what happens under homeostasis, migratory cDC2s could compensate for the absence of migratory cDC1s to block the differentiation of pTregs. This hypothesis is partly supported by evidence suggesting that cDC2s can be further divided into two groups with either pro- or anti-inflammatory properties [94]. In support of these findings, another study showed that under inflammatory conditions, migratory cDC2s could acquire characteristics of migratory cDC1s during viral respiratory infection [95]. Further studies using genetic mouse models lacking migratory cDC2s and cDC1s without affecting the migration of other cells will determine the contribution of both subsets in the blockade of pTregs.

Although T1L infection drives Th1 immunity and pTregs blockade against dietary antigens, the role of pTregs blockade in CeD pathogenesis remains to be investigated. Evidence in humans underscores the importance of pro-inflammatory gluten-specific Th1 T cells at the expense of pTregs in CeD. Expansion of gluten-specific HLA-DQ2 and HLA-DQ8-restricted Th1 T cells was observed in CeD patients, and *ex-vivo* stimulation of these cells with gluten led to secretion of IFNγ and IL-21 [3,96]. Furthermore, exclusion of gluten from the diet is sufficient to clear CeD symptoms in humans, and depletion of CD4 T cells as well as blocking IFNγ in a CeD mouse model is enough to prevent tissue destruction (Abadie & Kim et al., 2020). In sum, our data suggest that migratory cDC1s are non-

redundant APCs in disrupting immune tolerance to dietary antigens, and is supported by previous work in CeD.

4.2.2 cDC1s: the earliest source of type I IFN

In this work, we demonstrated that DCs do not support viral replication but we could amplify viral transcripts from sorted DCs. In addition, a delay in the expression of type I IFN inducible genes (ISGs) in the absence of cDC1s was observed. Of note, a delayed-type I IFN response observed in BATF3-KO mice could imply that T cells are primed very early on and cannot be rescued by late secretion of type I IFN.

In line with other studies [88], our data indicate that DCs do not support viral replication but rather uptake infected epithelial cells, which are known to support reovirus viral replication. Our study goes further and proposes that migratory cDC1s secrete type IFN through sensing of viral particles from phagocytosed infected cells. Nevertheless, viral sensing mechanisms in DCs upon uptake of infected cells remain to be further investigated. Innate sensors such as TLR7 and TLR3 which are expressed on cDC1s and can detect viral RNA could be potential candidates. We hypothesized that if TLR7 is required for sensing viral RNA in cDC1s leading to secretion of type IFNs, then Th1 immunity will be impaired in T1L-infected TLR7-KO mice. We did not observe such a defect in the Th1 response in TLR7-KO mice, however, we noticed a decrease in pSTAT1, which is downstream of the type I IFN receptor, in migratory cDC1s (Figure 22 A, B). This decrease in type I IFN signaling correlated with the loss of T-bet expression in the polyclonal host CD4+ T cell compartment (Figure 22 C). These results suggest that TLR7 is not required for T1L-induced Th1 immunity against dietary antigens but rather in the

host antiviral CD4 T cell response. It is important to note that our conclusions are limited as we are looking at the polyclonal CD4 T cell repertoire as a proxy for the host CD4+ T cell antiviral response. Generation of tetramers specific for reovirus antigens is needed to track the reovirus-specific T cells. The creation of new tools can be complemented with studies aiming at quantifying the antibody-specific response to reovirus in WT and TLR7-KO mice. Nonetheless, our results imply a role for TLR7 in reovirus sensing and suggest that other viral RNA sensors might be involved. For instance, TLR3 in the endosome, RIG-I in the cytosol, or through the recently discovered DC-specific sensor DexDC helicase 9 (DHX9). DHX9 is a cytosolic dsRNA sensor that can induce expression of type I IFN upon activation through MyD88 [97].

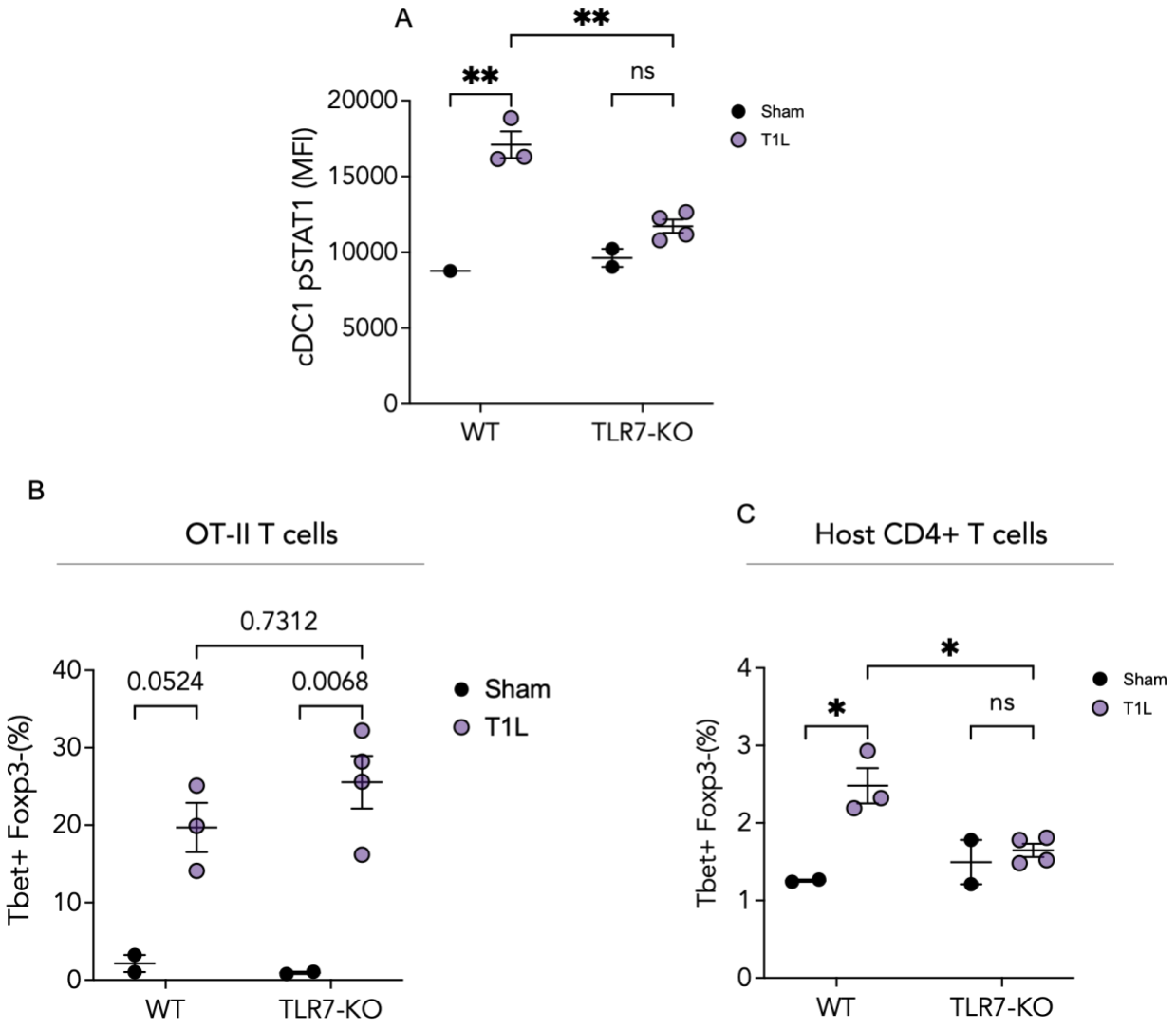


Figure 22 TLR7 seems to be required for type I IFN signaling and Th1 induction in host cells

(A,B,C) OTII^{+} CD45.1^{+} CD4^{+} T cells were transferred into WT C57BL/6 CD45.2^{+} and TLR7-KO mice. One day post-transfer, mice were inoculated perorally with 10^{8} PFU of T1L, Sham and fed an OVA-containing diet for three days. (A) The frequency of pSTAT1 in migratory cDC1 was assessed by Mean Fluorescence Intensity (MFI). (B, C) The frequency of T-bet+ Foxp3- expressing OT-II cells and host (recipients) CD4+ T cells in the mLNs was evaluated by flow cytometry. Graphs depict one representative experiment. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; one-way analysis of variance (ANOVA)/Tukey's multiple comparison.

However, it is unclear how infected epithelial cells cargo that are phagocytosed and shuttled to the lysosome could get into the cytosol making it less likely for the possibility of cytosolic sensors to be activated. An in-depth exploration and analysis via single-cell sequencing of cDC1s that have phagocytosed infected cells as compared to cDC1s that have phagocytosed uninfected cells during T1L infection will enable the identification of sensors involved in the detection of viral RNA in cDC1s.

In summary, our study furthers our understanding of the unique events leading to secretion of type I IFN during T1L infection and the mechanisms by which they regulate DC function and impact adaptive responses against dietary antigens. As type I IFNs have been shown to be involved in not only autoimmunity and infectious diseases, but also in cancer and shock, it is important to understand how the function of type I IFN is modulated depending on the context and the microenvironment.

4.2.3 cDC1s: detectors of necroptosis

Sensing of apoptotic and non-apoptotic cell death can induce tolerogenic and inflammatory immune responses respectively [85]. It was previously shown that uptake of apoptotic epithelial cells by migratory cDC1s induces a tolerogenic program required to prevent inflammatory disorders such as IBD [47]. Mechanistically, during apoptosis, cells release “eat me” signals such as lysophosphatidylcholine (LPC) and phosphatidylserine (Ptdser) which are detected by phagocytes like cDC1s [85]. Activation of these “eat me” sensors activates Ras-related C3 botulinum toxin substrate 1 (RAC1) which will eventually lead to phosphoinositide 3-kinase (PI3K) activation and expression of IL-10 and TGF-β [98]. On the other hand, sensing of non-apoptotic cells such as necroptotic cells

can lead to inflammatory responses [85]. Mechanistically, during necroptotic cell death, membrane integrity is compromised leading to the release of intracellular contents that can activate innate immune receptors on phagocytes. In addition, specific cell surface receptors expressed on phagocytes can recognize components of necroptotic cells. Signaling through necroptotic sensors and innate sensors recognizing necroptotic lead to the expression of inflammatory profile in phagocytes and drive a pro-inflammatory immune response in most cases [85,98].

Here, we demonstrated that necroptosis, a programmed form of necrosis, is required for T1L-induced Th1 immunity against dietary antigens. Specifically, the requirement for necroptosis was at the level of non-hematopoietic cells. Although reovirus infections have been shown to modulate cell death, a role for necroptosis in T1L infection was not known. Furthermore, we showed that induction of necroptosis in the non-hematopoietic epithelial cells did not increase their uptake/ clearance by migratory cDC1s. it was rather the program induced in migratory cDC1s by the sensing of necroptotic cells through Clec9A that was different. This was illustrated by the inability of migratory cDC1 to secrete IL-12 in the absence of Clec9A. Our results are in line with previous studies that have identified a pro-inflammatory role of Clec9A, a C-type lectin receptor uniquely expressed on cDC1s that recognizes F-actin on necrotic cells, in antigen cross-presentation for CD8+ T cell priming in infection and tumor [51,99]. Mechanistically, the signaling of Clec9A recruits tyrosine kinase Syk to promote CD8+ T cells priming [99]. However, Clec9A was shown to have anti-inflammatory properties in a sterile and infectious context [86]. Mechanistically, signaling through SH2 domain-containing phosphatase 1 (SHP-1) prevented neutrophil infiltration by blocking the secretion of chemokine Cxcl2 [86]. Furthermore, the clec9A

mechanisms leading to pro- or anti-inflammatory programs are unknown. Clec9A ligand affinity and avidity have been proposed to be deciding factors between the two pathways. Here we propose that the regulation of Clec9A may be dependent on the DC activation status which directly relates to the microenvironment. In our study, type I IFN signaling in migratory cDC1s may induce DC maturation which in the context of Clec9A sensing will promote a pro-inflammatory program.

Furthermore, molecular mechanisms downstream of Clec9A are still elusive. In the context of cross-presentation, it was proposed that activation of Clec9A induces a local NADPH-dependent oxidative burst that leads to phagosomal membrane rupture. This process then allows internalized cargo to leak out of the phagosome and access the cytosol [50]. Other studies on the other hand have proposed that Clec9A re-routes cargo destined to the lysosome to recycling endocytic compartment [100]. Overall, both mechanisms allow internalized cargo to access the subcellular compartment where antigens can be loaded on MHC molecule but it can also initiate sensing of innate sensors. Depending on the nature of the cargo and triggered innate sensors, cross-priming or cross-tolerance can be induced upon Clec9A signaling. In that same logic, in the context of T1L infection, perhaps necroptotic infected epithelial cells internalized by cDC1s can access the recycling endosome where they activate TLR3 leading to secretion of IL-12 [101].

Regulation of IL-12 by Clec9A could be dependent on Syk. Several studies have demonstrated that Syk can collaborate with TLR3 and control cytokines production [102]. Syk can also interact directly with NFκβ signaling and promote cytokine secretion [103,104].

However, a role for Syk signaling upon Clec9A sensing of necroptotic cells remained to be determined.

Overall, the unique ability of cDC1s to uptake infected epithelial cells, secrete type I IFN, and sense necroptotic cells through the expression of receptor Clec9A are checkpoints that need to be activated in order to disrupt tolerance to dietary antigens. This two-step mechanism may explain that the inability of reovirus T3D-RV and norovirus CR6 to disrupt the immune response to dietary antigens is due to their inability to activate these two pathways despite being able to infect epithelial cells [4,63].

In sum, our findings allow us to model for the first-time mechanistic events by which reovirus T1L induces loss of oral tolerance. Several studies have proposed that viral infections can trigger autoimmunity by serving as adjuvants for the priming of autoreactive cells or through molecular mimicry. Here, we proposed a new concept whereby viruses that can activate the appropriate DC subset through innate mechanisms have the potential to prime autoreactive cells. We argue that the notion of viruses as just adjuvants is too simplistic. It does not consider clinically silent viruses that may be poor adjuvants and are most likely not screened for in studies [105].

Moreover, immune-stimulatory properties of viruses like type I IFNs alone are not sufficient to predict the capacity of a virus to trigger autoreactive responses. Rather, we believe that viruses need to activate several checkpoints in order to break immune homeostasis. Although molecular mimicry is attractive and several reports have shown cross-reactive viral antibodies with self-antigens, it cannot be applied to all autoimmune disorders. For instance, in CeD immune responses to gluten drive autoreactive

antibodies but they are no reports showing that gluten can do so through molecular mimicry. Overall, understanding how viruses can trigger autoimmune conditions could lead to new therapeutic approaches to treat and prevent immune dysregulation.

CHAPTER 5: FUTURE DIRECTIONS

Our findings of the collaborative interaction between type I IFN and inflammatory cell death in virally induced loss of tolerance to dietary antigens triggered a series of interesting scientific questions. The most interesting questions will be reviewed in detail below.

5.1 Molecular mechanisms underlying Th1 immunity

T-bet, encoded by the *Tbx21* gene, is the CD4+ Th1 lineage-defining transcription factor that drives differentiation of CD4+ Th1 T cell. It has been well studied over the years and a lot is known about the factors that regulate its induction. T-bet is thought to be induced in two waves. The first wave is comprised of the synergy between TCR signaling and signals leading to phosphorylation of STAT1 such as IFNg, type I IFN, IL-27, and IL-21. pSTAT1 then binds to the promoter of *Tbx21* and induces the expression of an initial wave of T-bet. This first wave of T-bet is necessary for the T cells to become responsive to IL-12 through upregulation of the IL-12 receptor and signaling of IL-12 through its receptor leads to sustained expression of T-bet needed for commitment to the Th1 lineage [79,106]. Although factors required for Th1 immunity are known, how these factors are integrated by the T cell is not well understood.

In our study, we found that direct type I IFN signaling in T cells was required for induction of T-bet. However, other reports have shown that T-bet can suppress type I IFN signaling in T cells. Furthermore, type I IFN signaling in CD4 T cells in LCMV infection was associated with lethality. Furthermore, type I IFN, one of the triggers of T-bet has been shown to have an inhibitory and immune-stimulatory effect in T cells.

One of the hallmarks of fully differentiated Th1 T cells is their ability to produce IFNγ. However, T-bet independent secretion of IFNγ has been observed in humans [107] and the absence of T-bet in WT mice during *L. major* infection leads to only a reduction of IFNγ [108] suggesting a role for another transcription factor in Th1 differentiation. One potential explanation for these opposing outcomes is believed to be related to fine-tuning of STAT1 [106]. Interestingly, in our work, we found that pSTAT1 was decreased in type I IFN dependent and independent contexts (Figure 6, Figure 23 A, B, C). It was surprising to observe a slight decrease in pSTAT1 in transferred OT-II T cells primed in T1L-infected recipients that either lack necroptosis, Clec9A, or IL-12 because none of these factors have been associated with pSTAT1 inducing signals. These results led us to conclude that increased expression of pSTAT1 was required for induction of Th1 immunity. To test the role of pSTAT1 in T1L-induced Th1 immunity, we generated OT-II T cells that lacked STAT1. Using an *in vivo* T cell conversion assay, we asked whether STAT1 deficient OT-II could differentiate into T-bet+ T cells. A small caveat of this experiment was that STAT1 is necessary for the expression of MHC-I [109]. So, in the absence of STAT1, OT-II T cells are being killed by NK cells. To circumvent this issue, we depleted NK cells for the duration of the experiment. To our surprise, STAT1-deficient OT-II T cells differentiated into T-bet+ T cells (Figure 23 F). This would suggest that there are potentially other STAT proteins that are recruited during Th1 differentiation.

Several approaches could be used to address how CD4+ T cells integrate known Th1-inducing triggers and to identify the T-bet-independent mechanisms leading to IFNγ,

First, we propose an unbiased approach by sorting and sequencing OT-II T cells primed in different contexts (Table 2). We will assess how T cells integrate cell-intrinsic factors by sequencing WT OT-IIs primed in recipients lacking type I IFN signaling, Clec9A, necroptosis, and IL-12. Furthermore, we will take an in-depth look at the integration of cell-intrinsic signals by sequencing STAT1-KO, IFNAR-KO, and T-bet KO OT-II T cells primed in WT hosts (Table 3). Lastly, we will assess how OT-II T cells integrate TCR signaling in combination with the contexts defined above. For that, we have generated mice that lack expression of MHC-II specifically on migratory cDC1 and we will sequence WT and STAT1-KO and IFNAR-KO OT-II primed in these mice.

Second, we could take a biased approach and assess the role of known STAT proteins such as STAT2 and STAT5 which could be activated upon Type I IFN signaling [110].

Finally, we could take a simple approach by using *in vitro* STAT reporter cell culture assays to monitor the expression and activation of several STATs under defined Th1 inducing conditions.

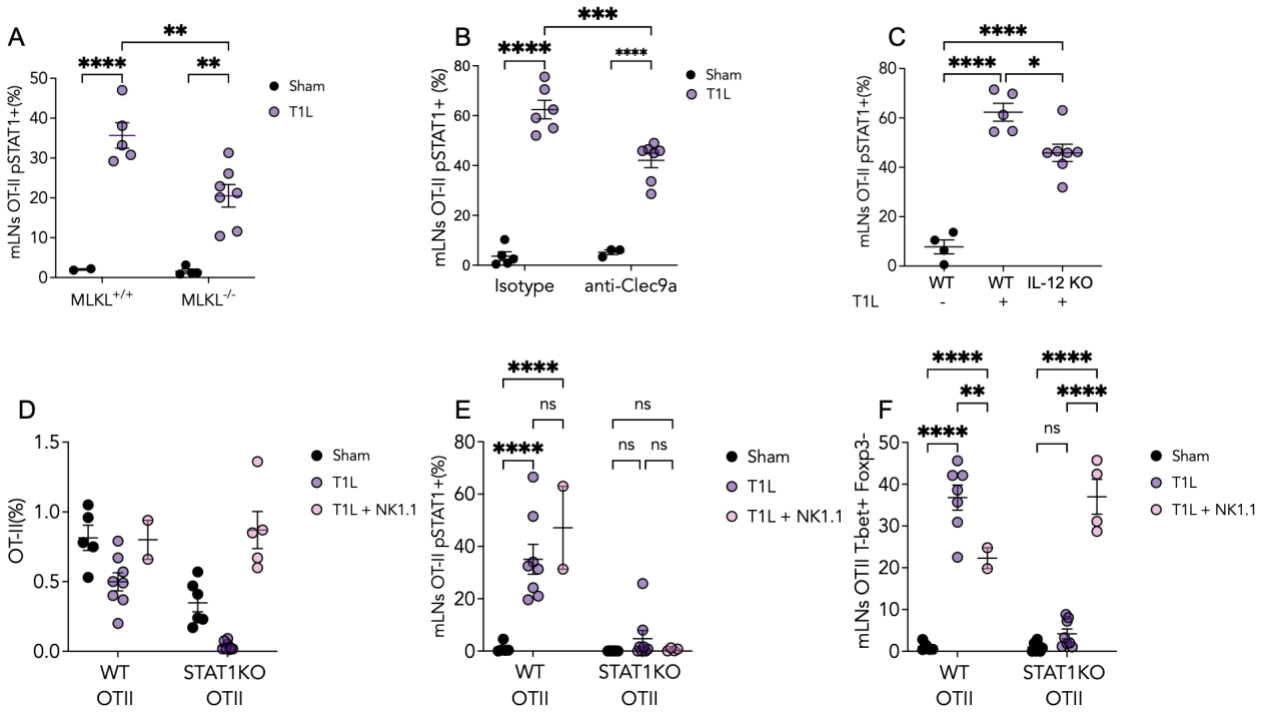


Figure 23 : Various signals control pSTAT1 upregulation, and STAT1 is not required for Th1 immunity

(A) OTII^{+} CD45.1^{+} CD4^{+} T cells were transferred into WT C57BL/6 CD45.2^{+} (MLKL+/+) and MLKL-/- mice. One day post-transfer, mice were inoculated perorally with 10^{8} PFU of T1L, Sham and fed an OVA-containing diet for three days. The frequency of pSTAT1 in OT-II T cells was assessed by flow cytometry. Graphs depict two representative experiments.
(B) OTII^{+} CD45.1^{+} CD4^{+} T cells were transferred into WT C57BL/6 CD45.2^{+} treated with or without anti-Clec9a antibody. One day post-transfer, mice were inoculated perorally with 10^{8} PFU of T1L, Sham and fed an OVA-containing diet for three days. The frequency of pSTAT1 in OT-II T cells was assessed by flow cytometry. Graphs depict two representative experiments.
(C) OTII^{+} CD45.1^{+} CD4^{+} T cells were transferred into WT C57BL/6 CD45.2^{+} and IL-12 KO. One day post-transfer, mice were inoculated perorally with 10^{8} PFU of T1L, Sham and fed an OVA-containing diet for three days. The frequency of pSTAT1 in OT-II T cells was assessed by flow cytometry. Graphs depict two representative experiments.
(D to E) OTII^{+} CD45.1^{+} CD4^{+} T cells were transferred into WT C57BL/6 CD45.2^{+} treated with or without NK1.1 antibody. One day post-transfer, mice were inoculated perorally with 10^{8} PFU of T1L, Sham and fed an OVA-containing diet for three days. The frequency of OTII (D) pSTAT1+ OT-II T cells (E) and Tbet+ Foxp3- OT-II T cells was assessed by flow cytometry. Graphs depict two experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; one-way analysis of variance (ANOVA)/Tukey's multiple comparison.

Table 2: RNA Sequencing strategy for determining T cell-intrinsic signature

	Control group		T cell intrinsic signals group		
OT-II genotype	Wild-type	Wild-type	IFNAR-/-	STAT1-/-	T-bet-/-
Host genotype	Wild-type	Wild-type	Wild-type	Wild-type	Wild-type
Infection status	Sham	T1L	T1L	T1L	T1L

Table 3: RNA Sequencing strategy for determining T cell-extrinsic signature

	Control group		T cell extrinsic signals group			
OT-II genotype	Wild-type	Wild-type	Wild-type	Wild-type	Wild-type	Wild-type
Host genotype	Wild-type	Wild-type	IFNAR-/-	MLKL-/-	Clec9A-/-	IL-12-/-
Infection status	Sham	T1L	T1L	T1L	T1L	T1L

5.2 Cell death-associated signature in migratory cDC1

Clearance of dead cells is an important physiological process required to maintain homeostasis and prevent disease [85,111] Cell death can be divided into two categories: apoptotic and non-apoptotic. Both programs display various molecular cues that instruct phagocytes and direct an immune response [111]. In the gut, especially due to the high turnover of the epithelium, clearance of dead cells by migratory DCs and resident macrophages is constantly required to maintain homeostasis and barrier integrity [85]. In our study, we showed that migratory cDC1s have the unique ability to uptake dead epithelial cells and migrate to the mLNs. Interestingly, previous studies demonstrated that clearance of dead epithelial cells by migratory DCs imparts a tolerogenic profile in DCs with the ability to induce pTregs [47]. Defect in this process has been associated with inflammatory bowel disease susceptibility [12,47]. Furthermore, the disposal of non-apoptotic dead cells can confer a pro-inflammatory immune response. Our work demonstrated that sensing of necroptotic cells through Clec9A expression on migratory cDC1s imparted an inflammatory program that was necessary for Th1 differentiation. Although the display of cell death-specific signals allows DCs to discriminate between dying cells, the integration of these signals is not well understood. For example, the molecular mechanisms regulating the ability of Clec9A to impart an inflammatory response during T1L infection remain to be investigated. Identifying transcriptional signatures associated with apoptotic and non-apoptotic cell death will provide further our understanding of the mechanism underlying the disruption of tolerogenic responses.

Our data and experimental setup put us in a unique position to investigate cell death-associated transcriptional signatures in DCs.

In order to identify the unique transcriptional profile associated with the uptake of apoptotic versus necroptotic dead cells, we would devise two unique strategies.
To establish a cell death signature in DCs, migratory cDC1s (CD103+ CD11b- CD8a+) with high IL-12 potential, migratory cDC1 (CD103+ CD11b- CD8a-) with low potential of IL-12 and migratory cDC2 (CD103+ CD11b+) were sorted from the mLNs of T1L- and Sham-infected MLKL+/+, MLKL+/- and MLKL-/- mice and subjected to single-cell sequencing. Differential gene expression revealed unique genes associated with MLKL (Figure 24). However, this strategy is limited because it cannot distinguish the signal from DCs that have internalized dead cells from the ones that have not.
A targeted sorting strategy is needed to differentiate between DCs that phagocytosed apoptotic versus necroptotic cells under homeostasis and during infection. Using $Rosa26^{LSL\text{-}Tdtomato}$ $Vilin^{creERT2+}$ we would label epithelial cells and infect mice with T1L and a single T1L M2 reassortant that has been associated with cell death. We would then sort tdomato+ cDC1s versus tdtomato- cDC1s. This strategy will enable the identification of three signatures : (1) transcription profile associated with uptake of dead cells at homeostasis (2) transcriptional profile associated with uptake of dead cells during infection where necroptosis is induced (3) transcriptional profile associated with uptake of dead cells during infection where necroptosis is not induced. Furthermore, we would treat the mice with Clec9A blocking antibody to identify the transcriptional profile associated with Clec9A.

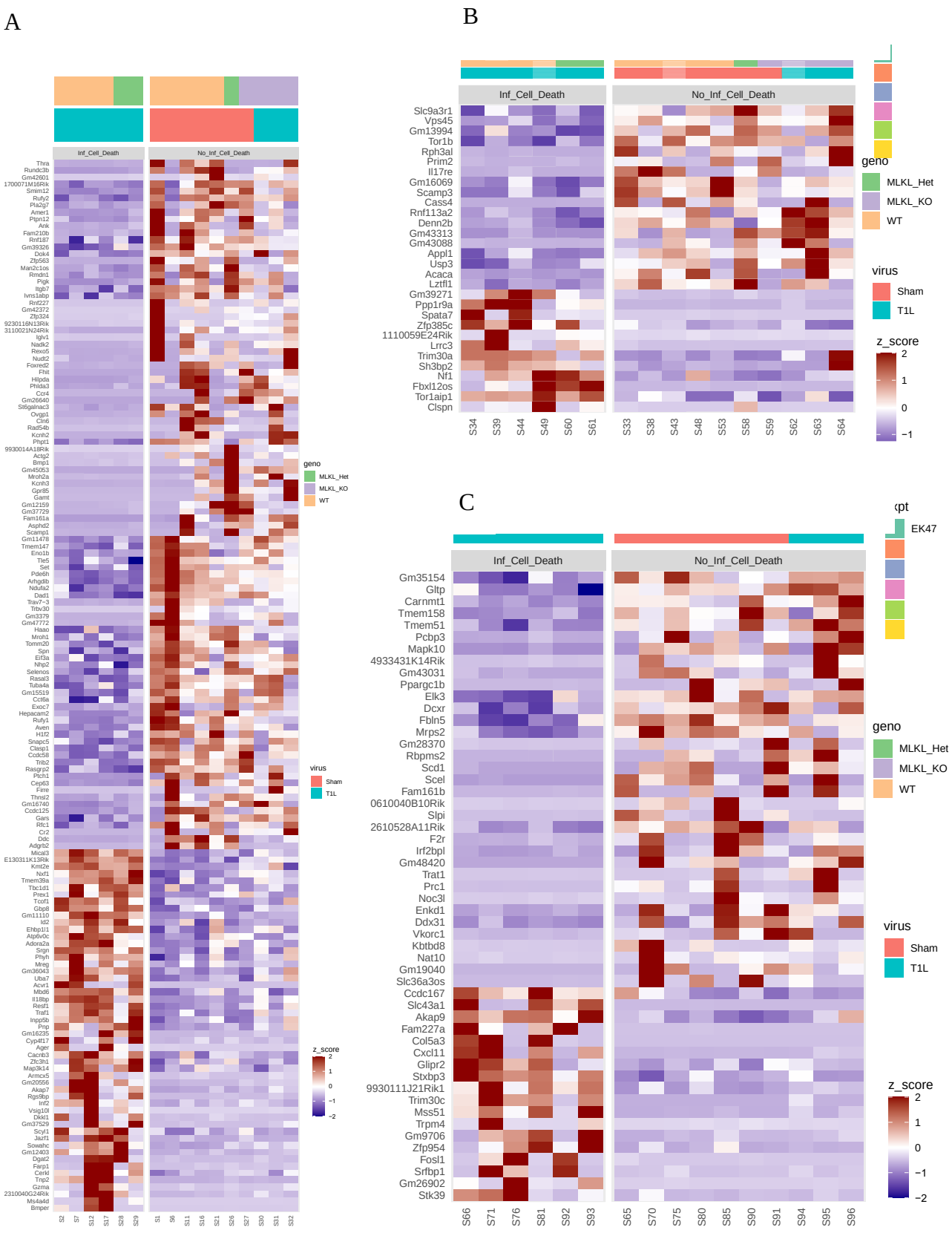

A
B
C
Inf_Cell_Death
No_Inf_Cell_Death
geno
MLKL_Het
MLKL_KO
WT
virus
Sham
T1L
z_score
2
1
0
−1
−2
Slc9a3r1
Vps45
Gm13994
Tor1b
Rph3al
Prim2
Il17re
Gm16069
Scamp3
Cass4
Rnf113a2
Denn2b
Gm43313
Gm43088
Appl1
Usp3
Acaca
Lztfl1
Gm39271
Ppp1r9a
Spata7
Zfp385c
1110059E24Rik
Lrrc3
Trim30a
Sh3bp2
Nf1
Fbxl12os
Tor1aip1
Clspn
S34
S39
S44
S49
S60
S61
S33
S38
S43
S48
S53
S58
S59
S62
S63
S64
pt
EK47
Gm35154
Gltp
Carnmt1
Tmem158
Tmem51
Pcbp3
Mapk10
4933431K14Rik
Gm43031
Ppargc1b
Elk3
Dcxr
Fbln5
Mrps2
Gm28370
Rbpms2
Scd1
Scel
Fam161b
0610040B10Rik
Slpi
2610528A11Rik
F2r
Irf2bpl
Gm48420
Trat1
Prc1
Noc3l
Enkd1
Ddx31
Vkorc1
Kbtbd8
Nat10
Gm19040
Slc36a3os
Ccdc167
Slc43a1
Akap9
Fam227a
Col5a3
Cxcl11
Glipr2
Stxbp3
9930111J21Rik1
Trim30c
Mss51
Trpm4
Gm9706
Zfp954
Fosl1
Srfbp1
Gm26902
Stk39
S66
S71
S76
S81
S92
S93
S65
S70
S75
S80
S85
S90
S91
S94
S95
S96

Figure 25: Migratory DCs display subset-specific gene expression

MLKL+/+, MLKL+/- and MLKL-/- mice were infected with T1L 10^8 PFU or Sham for two days. Heatmaps of genes expression comparing differentially expressed genes between MLKL+/+, MLKL+/- compared to MLKL-/- mice in (A) CD103+ CD11b- CD8a+, (B) CD103+ CD11b- CD8a- and (C) CD103+ CD11b+

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