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DTU Technical University of Denmark

UNDERGRADUATE DISSERTATION

THE ROLE OF TYPE I DENDRITIC CELLS IN THE IMPRINTING OF CD8 T CELL SUBSETS IN RESPONSE TO RSV INFECTION

FUNCION DE LAS CELULAS DENDRITICAS DE TIPO I EN LA IDENTIFICACION DE SUBCONJUNTOS DE LINFOCITOS T CD8 EN RESPUESTA A LA INFECCION POR EL VIRUS VRS

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TECNICHAL UNIVERSITY OF DENMARK (DTU)

GRADO EN BIOTECNOLOGÍA
CURSO 2021- 2022

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ACKNOLEGMENTS

First and foremost, I have to thank my supervisor Katharina Lahl for allowing me into her lab. I am incredibly grateful for the opportunity I was given. Thank you for your support, guidance and for always finding time to answer my questions. Another huge thanks to PhD student Isabel Ulmert, who mentored me and introduced me to the immunology research. Without your help and patience I would not have been able to pursue this project. I will always be thankful for having you as a mentor. I must extend my thanks to other member of the Lahl group, Katrine, Signe and Xiao for their advice, guidance, and help during this project. Moreover, I want to give a special thanks to my tutor in the University of Zaragoza, Pilar Bayona, I cherish your help and guidance during these last months.

Finally, a heartfelt appreciation goes to my family, specially to my parents for the emotional support. Moreover, I want to thank to my friends, Marcos, Marina, Laura and Leyre who always had time to listen to my frustrations and my successes throughout these months. I am truly grateful for you all.

Thank you!

Fernando Andres Martin
Jun 2022

1 ABREVIATIONS

RSV	Respiratory syncytial virus
DCs	Dendritic cells
APC	Antigen presenting cells
cDC	Conventional dendritic cells
TRM	Tissue resident memory
LRTI	Lower respiratory tract infections
ILCs	Innate lymphoid cells
NK	Natural killer
MΦs	Macrophages
PRRs	Pathogen recognition receptors
PAMPs	Pathogen-associated molecular patterns
DAMPs	Danger-associated molecular patterns
SLO	Secondary lymphoid organs
MHC	Major histocompatibility complex
HLA	Human leukocyte antigen
LN	Lymph node
CTL	Cytotoxic T cells
TEM	T effector memory
TCM	T central memory
M protein	RSV matrix protein
L protein	RSV polymerase protein
WT	Wild-type
TG	Transgenic
TCR	T cell receptor

2 ABSTRACT

Respiratory syncytial Virus (RSV) has been known as one of the leading causes of respiratory disease in infants, being nearly all children by the age of two infected at least once by RSV (1).

Dendritic cells (DCs) are antigen-presenting cells (APCs) that are found in both peripheral and lymphatic tissues and serve as sentinels for immune assaults. As a result, DCs serve as intermediaries between innate and adaptive immunity (2).

During this project we aimed to study the effect of the lack of conventional dendritic cells type 1 (cDC1) on two different epitope - specific $CD8^+$ T cell subsets upon RSV infection and reinfection. Thus, trying to identify the role of cDC1 on the activation of those subsets. For that we used XCR1.cre mice as well as wild type mice in three different conditions: control (injected at both timepoints with Mock solution), first infection (injected with Mock and RSV) and reinfection (injected at both timepoints with RSV).

Moreover, we wanted to study the effect of the absence of cDC1 on pathology and immune memory, since both are related to $CD8^+$ T cells function. To do so, we controlled the weight and performed a q-PCR.

This project results showed the importance of cDC1 cross presentation on the activation of CD4 and CD8 T cells. Moreover, the study of two different TCR $CD8^+$ $CD69^+$ suggest a different cDC dependence for activation based on the type of the antigen. All this could serve as a guideline for RSV vaccine development and immunotherapy.

RESUMEN

El virus respiratorio sincitial (VRS) es conocido como una de las principales causas de enfermedad respiratoria en bebés, siendo casi todos los niños de dos años infectados al menos una vez por el VRS.

Las células dendríticas (DCs) son células presentadoras de antígenos (APCs) que se encuentran en los tejidos periféricos y linfáticos y sirven como centinelas de las agresiones inmunológicas. Por ello, las DCs sirven de intermediarias entre la inmunidad innata y la adaptativa.

Durante este proyecto nos propusimos estudiar el efecto de la falta de células dendríticas convencionales tipo I (cDC1) en dos subconjuntos diferentes de células T $CD8^+$ epítopo-específicas tras la infección y la reinfección por el VRS. De este modo, intentamos identificar el papel de cDC1 en la activación de estos subconjuntos. Para ello, utilizamos ratones XCR1.cre y ratones de tipo salvaje en tres condiciones diferentes: control (inyectados en ambos momentos con solución Mock), primera infección (inyectados con Mock y VRS) y reinfección (inyectados en ambos momentos con VRS).

Además, queríamos estudiar el efecto de la ausencia de cDC1 sobre la patología y la memoria inmune, ya que ambas están relacionadas con la función de las células T $CD8^+$. Para ello controlamos el peso y realizamos una q-PCR.

Los resultados de este proyecto mostraron la importancia de la presentación cruzada de cDC1 en la activación de las células T CD4 y CD8. Además, el estudio de dos poblaciones $CD8^+$ $CD69^+$ con diferentes TCR sugiere una dependencia diferente en cDC para la activación en función del tipo de antígeno. Todo esto podría servir de guía para el desarrollo de vacunas e inmunoterapias contra el VRS.

3 INTRODUCTION

Respiratory syncytial Virus (RSV) has been known as one of the leading causes of respiratory disease in infants, being nearly all children by the age of two infected at least once by RSV. Although its infection usually results in only mild disease, sometimes RSV can cause Bronchiolitis and viral pneumonia developing lower respiratory tract infections (LRTI) (1,3).

This project aims to identify cellular player on the immune system for the coordination of protective immunity to RSV. Specially, to study the role of dendritic cells type I (cDC1) on the imprinting of different $CD8^+$ T cells subsets, since $CD8^+$ T cells play an important role in the resolution of acute infection and for virus-specific immunological memory.

3.1 Overview of the immune system

The immune system is formed by multiple organs, cells and molecules which can be divided in two subsystems: the innate and the adaptative immune system.

The innate immune system is nonspecific and the first barrier against pathogens. It is formed by anatomical barriers of the body such as skin and mucosa, which are made by exiting microbiota, acidic pH, surfactants, or antimicrobial peptides. The three types of innate lymphoid cells (ILCs), macrophages, mast cells, neutrophils, eosinophils, basophils, and natural killer (NK) cells are essential in the innate immune response, being macrophages (MΦs), together with DCs and neutrophils the first cell types to encounter the pathogen. These types of cells present pattern recognition receptors (PRRs) which recognize two different kind of patterns, pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPS) (2).

After DCs encounter the pathogen, they are capable of process and present antigens from the infection site to naive T cells in secondary lymphoid organs (SLO). Not only do they present and process antigens, but also they secrete molecules which help T cells in its differentiation. In that way, dendritic cells are crucial in the mediation between the innate and adaptive immune response (2,4).

Naive T cells are divided into large groups, of which one expresses the co-receptor CD8 on its surface and the other bears the co-receptor CD4. $CD8^+$ or $CD4^+$ T cells will receive collected antigens on major histocompatibility complex class I or II (MHCI and II) molecules respectively. As T cells become activated, $CD8^+$ T cells become either cytotoxic T cells or memory T cells. Meanwhile, $CD4^+$ T cells become T helper cells (Th1, Th2, Th17, TFH, or Treg) or their memory analogs (5).

A coordinated series of signals must occur to achieve activation of naive $CD4^+$ T lymphocytes: (a) antigen presentation on MHC molecules (HLA in humans), (b) costimulatory signals, and (c) instructive cell surface and cytokine signals. The first two signals establish a threshold that must be crossed to prime a T cell and thereby help ensure tolerance to self or other innocuous molecules. Although the ability to present antigens on MHC along with costimulatory molecules

is not a property unique to DCs, DCs are often necessary and sufficient in vivo for T cell priming due to their unique ability to migrate from tissues to LNs or within the spleen (5). The third signal instructs T cell differentiation; as we discuss, these signals come from both DCs as well as other cells in the lymph nodes (LNs) microenvironment (5).

3.1.1 Dendritic cells

Dendritic cells (DCs) are antigen-presenting cells (APCs) that are found in both peripheral and lymphatic tissues and serve as sentinels for immune assaults. As a result, DCs serve as intermediaries between innate and adaptive immunity (5).

When naive T cells in T cell zones of secondary lymphoid tissues come into contact with a specific antigen on the surface of an antigen-presenting cell, an adaptive immune response is triggered. Conventional dendritic cells that express the co-stimulatory molecules B7.1 and B7.2 are most often the antigen-presenting cells responsible for activating naive T cells and initiating their clonal growth. Dendritic cells are found not just in lymphoid tissues, but also in the periphery, where they come into contact with pathogens, pick up antigen at infection sites, become activated through innate recognition, and migrate to local lymphoid tissue. The dendritic cell may become a potent direct activator of naive T cells, or it may transfer antigen to dendritic cells resident in secondary lymphoid organs for cross-presentation to naive CD8 T cell (2).

Conventional DCs (cDCs) are the most common DC subset that migrates into LNs and promotes the proliferation of naive T cells. DCs have a lot of functional and phenotypic diversity. For each subset, mice and humans have similar populations. Because the project's practical work is done with mice as the model organism, the DC landscape in mice will be explained in detail (5).

cDCs are divided into two categories: cDC1s and cDC2s. In both mice and humans, cDC1s and cDC2s develop from common DC progenitors in the bone marrow, which differentiate into cDC-restricted progenitors, or pre-cDCs. Pre-cDCs that have decided to become cDC1s or cDC2s move to tissues and lymph organs via the bloodstream to complete their differentiation into cDC1s and cDC2s. In both mice and humans, FLT3L is required for the formation of cDC1 and cDC2 (2,4).

According to their initial seeding location from blood-derived precursors, cDCs in the LN can be separated into resident cDCs and migratory cDCs. Migratory DCs live in tissues and migrate to draining lymph nodes via lymphatics in both steady and inflammatory states. A resident DC, on the other hand, spends its entire existence in lymph nodes; despite their name, resident DCs are nonetheless motile. cDC1s and cDC2s are subtypes of both resident and migratory DCs (4).

Murine cDC1s, are usually classified as Lin$^-$MHC-II$^+$ CD11c$^+$ CD8$^+$ (resident cDC1s) or CD103$^+$ (migratory cDC1s). XCR1, Clec9A, and CADM1 are expressed in nearly all human and mouse cDC1s.

Some dendritic cells can obtain and process exogenous antigens and present them on MHC class I molecules. This process of cross-presentation is important for priming CD8$^+$ T cells to many viral infections (2). The ability of cDC1s to cross-present cell related antigen to prime CD8$^+$ T

lymphocytes is their most recognized function, making them crucial in antiviral and anticancer immune responses. cDC1s also deliver antigen to $CD4^+$ T cells and influence Th cell development, especially during steady-state Treg cell induction and Th1 cell induction during inflammation (5).

3.1.2 $CD8^+$ T cells

T cells mature in the thymus to produce antigen-specific receptors that are rearranged for each antigen. Classic $CD4^+$ and $CD8^+$ T lymphocytes are frequently classified in adaptive immunity based on their helper, cytotoxic, or regulatory functions (2).

They can also be separated into naive, effector, and memory groups based on their activation status. The difference between naive and memory T cells is that naive T cells can respond to a novel antigen, whereas memory T cells can preserve long-term immunity to previous antigens.

After coming into contact with an antigen, a naive T cell proliferates and differentiates into one of several functional types of effector T cells. When an effector T cell detects an antigen, it can engage in one of three types of activity. Cytotoxic T cells kill other cells that are infected with viruses or other intracellular pathogens bearing the antigen. Helper T cells provide signals, often in the form of specific cytokines that activate the functions of other cells, such as B cell production of antibody and macrophage killing of engulfed pathogens. Regulatory T cells suppress the activity of other lymphocytes and help to limit the possible damage of immune responses (2,6,7).

As I mention in the previous section, naive T cells are divided into large groups, of which one express the co-receptor CD8 on its surface and the other bears the co-receptor CD4. As this project discuss the role of CD8 T cells, we are going to focus on this subset.

CD8 T cells become CD8 cytotoxic T cells as they mature (CTLs). They play a crucial role in the fight against viruses. Virus-infected cells display fragments of viral proteins as peptide:MHC class I complexes on their surface, and these are recognized by CTLs.

Because CD8 T cells' effector functions are so damaging, naive CD8 T cells require more co-stimulatory activity than naive CD4 T cells to become activated. This criterion can be satisfied in two ways. The most basic method is priming by activated dendritic cells, which occurs in some viral infections when DC become sufficiently active to drive CD8 T cells to produce the IL-2 essential for differentiation without the assistance of CD4 T cells (2).

Nonetheless, CD8 T-cell activation in most viral infections necessitates the assistance of CD4 effector T cells. APC can be further activated by effector CD4 T cells that have been exposed to an antigen by an antigen presenting cell. B7, which is found in DC, stimulates CD4 T cells to produce IL-2 and CD40 ligand. CD40 ligand attaches to CD40 on dendritic cells, producing an extra signal that causes the dendritic cell to express more B7 and 4-1BBL, providing further co-stimulation to the naive CD8 T cell. The IL-2 generated by activated CD4 T cells also promotes the development of effector CD8 T cells (2,8).

Despite the fact that inducing death in target cells is the most common approach for CTLs to remove infection, most CD8 cytotoxic T cells also release the cytokines IFNg, TNFa, and LTa, which help the host in other ways. IFNg inhibits viral replication directly and induces the increased expression of MHC class I molecules and of other proteins that are involved in peptide loading of these newly synthesized MHC class I in infected cells. This increases the chance that infected cells will be recognized as target cells for cytotoxic attack. Thus, effector CD8 cytotoxic T cells act in a variety of ways to limit the spread of cytosolic pathogens (2).

3.1.2.1 CD8+ T cells markers and subsets: From naive to memory

CD8 T cells comprising the memory pool display considerable heterogeneity, with individual cells differing in phenotype and function. Each subset is defined by distinct surface markers. Antigen-inexperienced T cells express homing receptors CD62L and CCR7 but lack expression of activation markers CD44 and CD95. With ongoing differentiation towards the memory phenotypes, CD62L and CCR7 are down-regulated, while CD44 and CD95 are gradually up-regulated. With progressive differentiation towards the memory phenotype, antigen-dependence, tissue tropism, effector function, and senescence increase (9,10).

Shared features of all CD8 memory T cell subtypes are that they are long-lived and can quickly expand to large numbers of effector T cells upon re-exposure to their cognate antigen, thereby mounting a faster and more potent immune response than the first immune response to a given pathogen. The different subtypes exert different functions and exhibit different properties, such as tissue tropism or capacity for self-renewal, reflecting the specific immune-related circumstances that led to their differentiation into a given memory subtype (11,12).

Compared to naïve cells of the same antigen-specificity, memory CD8 T cells persist in greater numbers; can populate peripheral organs; are poised to immediately proliferate, execute cytotoxic functions, and secrete effector cytokines upon Ag re-encounter; and exist in different metabolic, transcriptional, and epigenetic states. Despite some similarities between effector and memory CD8 T cells at the molecular, epigenetic, metabolic, and functional levels, memory cells persist long-term, while effector cells undergo robust contraction, and unlike effector cells they are capable of vigorous proliferation following Ag re-encounter (7).

Memory CD8 T cells, despite some limitations, have a subset classification which do provide valuable predictive information on the likelihood that cells of a given phenotype will be able to perform a defined function in response to a particular pathogen (7).

Tissue Resident Memory (T_{RM})

Tissue surveillance was a function first ascribed to circulating effector memory cells (T_{EM}). However, elegant parabiosis experiments have made it clear that some cells within tissues are not circulating but are permanent residents. Efforts to identify tissue resident memory T cells (T_{RM}) have shown that, unlike circulating cells, T_{RM} cells are not labeled by intravenous injection of antibodies, with the noted exception of liver T_{RM} cells, which are exposed to the circulation. In addition to tissue residence, T_{RM} cells often are identified based on expression of integrins

CD103 and CD49a, which aid in tissue entry, and CD69, which promotes tissue retention. Responsiveness to TGF-β in most cases is necessary for T_{RM} development, and expression of transcription factors play an important role in promoting TGF-β responsiveness and retention of T_{RM} cells within tissues (7).

T_{RM} cells are generated during acute infection, with precursors moving from lymph nodes to the infection site after first encountering antigen. Local signals increase tissue retention molecules, and T_{RM} cells persist in high numbers after infection, operating as innate-like cells that detect antigen re-encounters quickly. They produce IFN-γ and other cytokines in response to antigen recognition, which attract activated CD8+ T lymphocytes with a variety of specificities, providing an antiviral but proinflammatory environment (13). T_{RM} cells provide protection against diverse microorganisms in an array of tissues including the lungs, salivary glands, female reproductive tract, skin, and liver (7).

3.2 Respiratory Syncytial Virus

RSV is the most prevalent viral cause of significant respiratory illness in children under the age of five years old, and it is the leading cause of infantile bronchiolitis. RSV is a non-segmented enveloped negative-strand RNA virus belonging to the *Paramyxoviridae* family and the *Mononegavirales* order (1,13,14).

The genome of respiratory syncytial virus is 15.2 kb long and has 10 genes that code for 11 proteins (Figure 1). This is because the M2 protein mRNA has two overlapping reading frames (ORFs), which result in two polypeptides, M2-1 and M2-2 (1).

The RNA genome encodes critical internal structural proteins such as matrix protein (M) and nucleoprotein (N), as well as proteins required for the polymerase complex's activity, such as phosphoprotein (P) and polymerase (L), and non-structural proteins involved in evading the innate immune response (NS-1 and NS-2). Transmembrane glycoproteins (small hydrophobic hydrophobic protein (SH), glycoprotein (G), and fusion protein (F)) as well as two regulatory proteins (M2-1 and M2-2 antitermination protein, involved in transcription/replication regulation) are exposed on the outside (14).

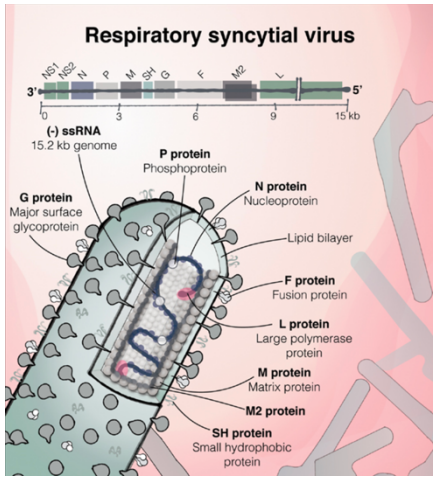

Figure 1. Structure of RSV (1).

3.2.1 Immune response against RSV

RSV is a major cause of respiratory illness in young children. Nearly all children worldwide have experienced at least one infection with RSV. Furthermore, RSV-related acute LRTI is to this day an important cause of childhood mortality in developing countries (14).

Protective defenses against primary RSV infection are known to include innate responses carried out by the resident airway cells (e.g epithelial cells and alveolar macrophages (AMs)) and recruited cells such as neutrophils, monocytes, and NK cells. Moreover, there are antimicrobial secreted proteins. Innate responses impede viral replication and spread to other parts of the respiratory tract (13–16).

Previous research has shown that in established RSV infection, immune responses aid viral clearance and result in partially effective immunological memory from $CD4^+$ and $CD8^+ T_{RM}$ as well as local IgA synthesis in established infection (6,16).

The immediate response by epithelial cells and alveolar macrophages (AMs) induces a cascade of chemotactic factors that recruit a series of other innate (and later adaptive) immune cells. Plasmacytoid and conventional DCs (pDCs and cDCs) are recruited to the nasal mucosa of children with RSV infection. Activation of DCs during RSV infection is partly dependent on autophagy and is regulated by epigenetic modulation of gene transcription. The recruitment of innate cells contributes to a complex network of pro- and anti-inflammatory signals that both helps to clear infection and sets the environment for subsequent adaptive immunity (15,17).

During viral infection, Professional APCs (mainly cDCs) are in charge of presenting peptide antigens derived from proteasomal degradation of external antigens and intracellularly produced antigens in the context of MHC class II and class I, respectively. T cells are required for the clearance of acute infections as well as the development of virus-specific immunological memory (17).

4 PROJECT OBJECTIVES

GENERAL OBJETIVE

The main purpose of this project is to study the effect of the lack of cDC1 on two different epitope - specific $CD8^+$ T cell subsets upon RSV infection and reinfection. Thus, trying to identify the role of cDC1 on the activation of those subsets.

SPECIFIC OBJECTIVES

- To study the effect of infection and reinfection of RSV on the weight of transgenic (XCR1.cre mice) and wild type mice. It is done so by viewing the consequences that an absence of cDC1 will have on the pathology.
- To analyze the effect that a lack of cDC1 will have on neonatally immune memory. For that, we will compare the viral load in infected and reinfected mice as well as mucus related genes.
- To phenotype $CD8^+$ and $CD4^+$ T cell subsets upon RSV infection and to compare the lack of cDC1 on these subsets.
- To study the differences in $CD8^+$ T cell subsets using MHC tetramers in transgenic and wildtype mice.

5 METHODS AND MATERIALS

5.1 Animals: XCR1.cre mice

All animals were housed under specific pathogen-free conditions at the Danish Technical University (Lyngby, Denmark). The experiments were performed under the appropriate national licenses and guidelines for animal care.

C57BL/6 XCR1.cre mice permit to specifically delete floxed gene sections in cDC1 (Figure 2). The deletion of a "lox–stop–lox" sequence in cDC1, leads to the expression of the active domain of the diphtheria toxin (DTA). Thus, Rosa26 lox – stop – lox– DTA (Gt(ROSA)26Sortm1(DTA)Lky) are mice who do not have cDC1.

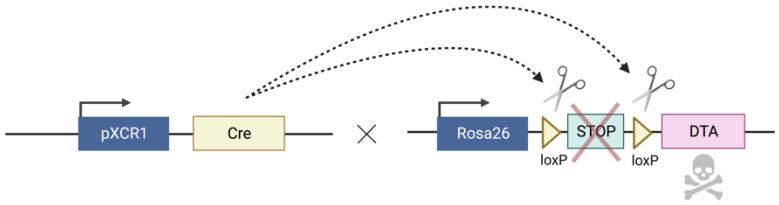

Figure 2. Gt(ROSA)26Sortm1(DTA)Lky mice. Cells that have the promotor pXCR1 (cDC1) will express the recombinase cre leading to the expression of the DTA. Created with BioRender.com

5.2 Experiment design

For the experiment two type of mice were used, a wild type strain and a transgenic strain, XCR1.cre mice. Seven days after birth both wild type (WT) and transgenic (TG) mice were injected with a mock solution or with RSV, the latter resulting in primary infection. Then, those mice were left for 6 to 8 weeks to re-establish a steady state.

After 8 weeks from the first inoculation, mice were again injected with mock solution or with RSV. In total, we chose six conditions: Mock/Mock (MM), Mock/RSV (MR) and RSV/RSV (RR) for wild type and transgenic mice. The MR and the RR conditions will allow us to study primary and secondary infection respectively. The MM condition will serve as a control (Figure 3).

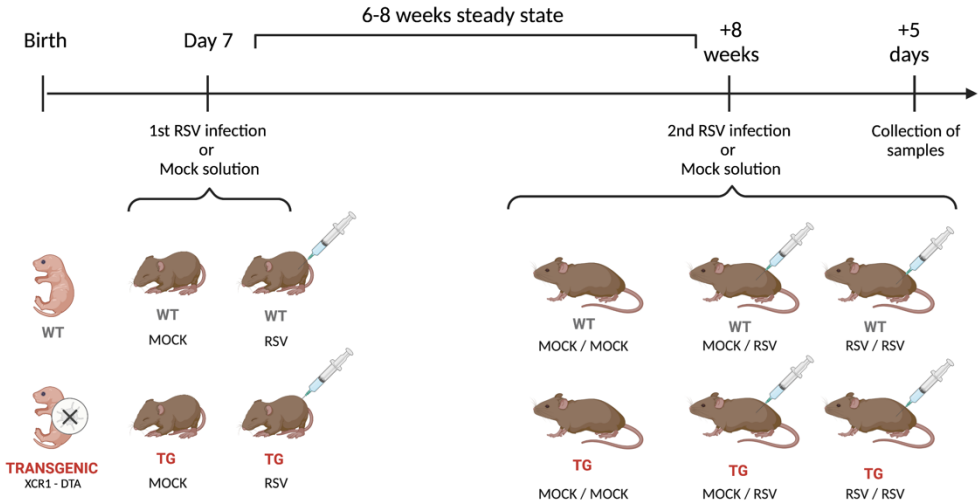

Figure 3. Timeline of experiments Mice are infected 1 week after birth with an RSV or Mock solution. Eight weeks later mice are reinfected with RSV or Mock solution, followed by euthanisation on day five post infection. Created with BioRender.com

5.3 Methods for T cell phenotyping

After euthanisation, an incision was made in the abdomen following a further incision to locate the heart and lungs, subsequently the lungs were perfused by injecting phosphate buffered saline solution (PBS) into the left ventricle of the heart.

Figure 4 shows the different lobes of the murine lung which were used in qPCR and staining. The post-caval lobe was removed and placed in an eppendorf tube containing Allprotect (Qiagen), which protects the integrity of RNA for later use in qPCR experiments. The inferior and superior lobes with the left lung were removed and washed in PBS before being placed in 2 ml of R5 medium (RPMI + 5% FCS) and kept on ice. These lobes were used for phenotyping immune cells in the lungs by flow cytometry.

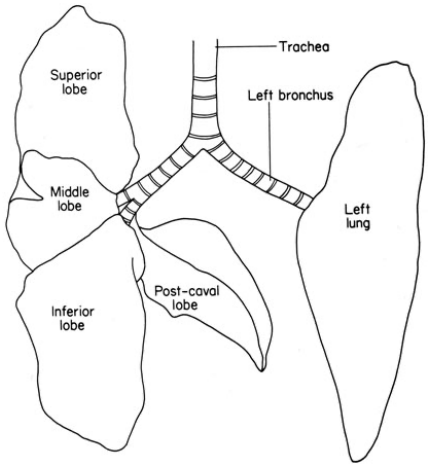

Figure 4. Different lobes of murine lung

Subsequently, inferior, superior lobes and left lung were further dissected into small pieces and digested in 5 ml of R5 medium with the enzymes liberase at a dilution of 1:50, and DNase at a dilution of 1:66. The tissue was placed with a magnet in an incubator at 37^{o}C, spinning at 800 RPM for 45 minutes. After digestion, the tissue was pressed through metal mesh filters into falcon tubes. The solution was centrifuged at 1300 RPM for 7 minutes at room temperature (RT).

In order to isolate lymphocytes, a percoll gradient of 40/70 % was used. The 40/70 gradient will help us to separate lymphatic cells from epithelial cells and alveolar macrophages, placing erythrocytes in a pellet at the bottom of the tube.

Following centrifugation, the solution was resuspended in 5 ml of 40% percoll solution. The 70% percoll solution was carefully underlaid in the same tube at a volume of 2 ml. The gradient was centrifuged at RT with no acceleration and no break at 2500 RPM for 20 minutes. Afterwards, lymphocytes were visible as a white cloudy interface between the 40% and the 70% layer. Cells were harvested with a 1 ml pipette and transferred to a new tube, which was then filled up with R5 medium. Cells were counted using the Sysmex cell counter.

The cell solution was centrifuged, plated in a V-bottom plate, and washed twice. Cells were resuspended in PBS and 25000 123ebeads were added for counting cells in FlowJo during analysis. For surface staining, cells were stained with 50 µl of the viability dyes APC-R700 (BD

Bioscience) at a dilution of 1:1000. Afterwards, the suspension was left covered on ice for 15 minutes. Then, the cells were washed and centrifuged for 4 minutes at 1300 RPM at 4◦C. Subsequently, the cells were resuspended in 50 µl of Fc block (anti-mouse CD16/CD32) at a dilution of 1:100. Following Fc block, the solution was covered, and left for 20 minutes on ice. Finally, the cells were centrifuged under the same conditions as previously described and 50 µl of surface stain was added using the antibody panel for T cells (Table 1). After the antibodies were added, the samples were covered on ice and left for 30 minutes. The cells were collected in a flow cytometer and the data was analyzed using FlowJo.

Table 1. Markers used for FACS

Color	Antigen	Clone	Dilution
BV421	CD103	M290	1:100
BV480	CD8a	53-6.7	1:100
BV605	CD4	RM4-5	1:100
BV650	M187 -197		1:100
FITC	CD44	IM7	1:200
PerCP – Cy5.5	CD11a	M17/4	1:200
PE	ST2	RMST2.2	1:400
PE-Cy7	CD69	H1.2F3	1:200
APC	L2021-2029		1:100
APC-R700	Live / Dead		1:1000
APC-Cy7	TCRbeta	H57-597	1:100
BUV395	CD45	30-F11	1:500
BUV737	CD62L	MEL-14	1:200

5.4 Gene expression analysis: qPCR

RNA Extraction

As mentioned above, the post-caval lobe was used for qPCR. Once the post-caval lobe was removed from the chest cavity, it was placed in a microcentrifuge tube with Allprotect and stored at -80°C. Allprotect allows the long-term storage of tissue while maintaining the integrity of the RNA. The tissue samples were later defrosted and homogenized in buffer and β-mercaptoethanol using the Qiagen Tissue Lyser with 5 mm steel beads. RNA extraction from the tissue was carried out using the Qiagen RNAeasy Quick-Start protocol.

In essence, ethanol was added to the lysate for precipitation of the nucleic acids followed by transferring the solution to a silica membrane collection tube provided by the kit. The silica membrane technology utilized in the QuickStart protocol is used to eliminate DNA. Moreover, DNase digestion was performed on all samples to minimize DNA contamination. Afterwards, the membrane was washed twice with appropriate buffers to remove carbohydrates, fatty acids, and remaining salts. Finally, eluted RNA was collected and either stored at -80°C or used immediately for cDNA synthesis.

cDNA Synthesis

cDNA synthesis was carried out using the BIORAD iScript cDNA synthesis kit. The kit is comprised of a 5x iScript reaction mix, iScript reverse transcriptase, and nuclease-free water. To each PCR tube, in order to make a total of 20 µl solution, 1 µl of eluted RNA was added followed by the calculated amount of iScript reaction mix, nuclease-free water, and 1µl of iScript reverse transcriptase. Two controls were added, a no reverse transcriptase control and a no RNA control, during this step nuclease free water was used in replacement of RNA or reverse transcriptase.

The samples were taken to the thermocycler where the cDNA was incubated according to the protocol: priming for 5 minutes at 25^{o}C, reverse transcriptase for 20 minutes at 46^{o}C, and reverse transcriptase inactivation at 95^{o}C for 1 minute. The samples were collected and stored in the -20^{o}C freezer for later use in qPCR.

qPCR

The genes of interest for qPCR were RSV *L-gene*, *muc5ac*, *gob5* as well as the reference gene *HPRT*, a housekeeping gene.

The forward and reverse primers as well as the probes for both RSV L-gene and HPRT are highlighted in Tables 2,3 and were designed by former Postdoc Anna Hammerich Thysen. The ready-to-use *muc5ac* and *gob5* prime assays based on FAM probe technology, were obtained from BIORAD. The Quantitect Probe PCR kit was used to enable the detection of the target cDNA sequences.

1.6 µl of cDNA from each sample was added to a 96-well PCR plate in duplicates. Following sample addition, 18.4 µl of reaction mix which was added. The reaction mix was composed of Quantitect master mix, water, primers, and probe. The PCR plate was taken to the thermocycler and ran for 50 cycles until completion.

Table 2. Sequences f forward and reverse primers as well as the probe used to detect RSV L-gene in qPCR.

Primer	RSV L-Gene
Forward Primer (5'-3')	GAACTCAGTGTAAGGTAGAATGTTTGCA
Reverse Primer (5'-3')	TTCAGCTATCATTTCTCTGCCAAT
Probe (5'-3')	FAM-TTTGAACCTGTCTGAACATTCCGGTT-BHQ

Table 3. Sequences f forward and reverse primers as well as the probe used to detect RSV HPRT in qPCR.

Primer	HPRT
Forward Primer (5'-3')	GGCCAGACTTTGTTGGATTTG
Reverse Primer (5'-3')	CGCTCATCTTAGGCTTTGTATTTG
Probe (5'-3')	FAM-CCAGACAAG-ZEN-TTTGTTGTTGGATATGCCC-BkFQ

5.5 Flow cytometry

5.5.1 MHC Tetramers

Moreover, during this experiment tetramers were used. Major histocompability complex (MHC) tetramers allow us to identify antigen-specific T cells thanks to its interaction between T cell receptor (TCR) and the MHC:peptide complex.

For its production, MHC heavy chains expressing a bacterial BirA-recognition site are synthesized. The MHC heavy chain is then folded with β2-microglobulin (β2M; the MHC light chain) and synthetic peptide. The enzyme BirA is then added to biotinylate the complex, adding a biotin molecule to each MHC monomer that is formed. In the presence of streptavidin which has four biotin binding sites per molecule, four MHC monomers are joined together to form a tetramer (18).

Whilst single MHC:peptide complexes and TCR bind weakly, MHC tetramers can bind up to four TCRs simultaneously, creating a much stronger interaction. MHC tetramers only bind to the relevant TCR, and tetramers specific for any MHC heavy chain can be produced, coupled with any peptide epitope of choice (e.g. viral, bacterial, tumor and autoimmune antigens) (18).

The streptavidin molecule was linked to fluorochromes such as APC and BV650 (Table 1).

The synthetic peptides used were M187-197 (M peptide) and L2021-2029 (L peptide) based on previous research of RSV $CD8^+$ T cell epitopes in CD57BL/6 mice (Table 4) (19).

Table 4. Sequences of RSV CD8+ T cell epitopes in CD57BL/6 mice.

Protein	Amino Acids	Sequence
M	187-197	NAITNAKIIPY
L	2021-2029	NVVQNAKLI

5.5.2 Gating

Lung tissue from adult mice were dissected at day 5 after second infection, and tissues were prepared for analysis as described in Section 5.3. Cells obtained from inferior and superior lobes with the left lung were stained with antibodies as described in Section 5.3 for flow cytometry analysis. The panel was designed to identify differentiated T cells subsets and included tetramers for RSV-specific T cell characterization.

The T cell response can offer insights into the adaptive immune environment in response to viral insults to lung tissue. The antibody panel shown in Table 1 outlines markers used to detect T cell subsets, and the gating strategy is illustrated in Figure 5. After gating out debris, doublets, and dead cells, $CD45^+$ TCR β^+ immune cells were gated, which will include all T cells. TCR β^+ $CD45^+$ cells were divided into $CD8^+$ and $CD4^+$ T cells. The $CD8^+$ cells were further investigated by examining the TCR specific populations, L $peptide^+$ (Figure 5A) and M $peptide^+$ (Figure 5B).

Moreover, inside each population, the presence of effector CD8 T cells, which express CD44, were gated.

Within the effector specific TCR $CD8^+$ T cell population it was possible to analyze the expression of CD69 and CD62L which will be further discussed in the results section.

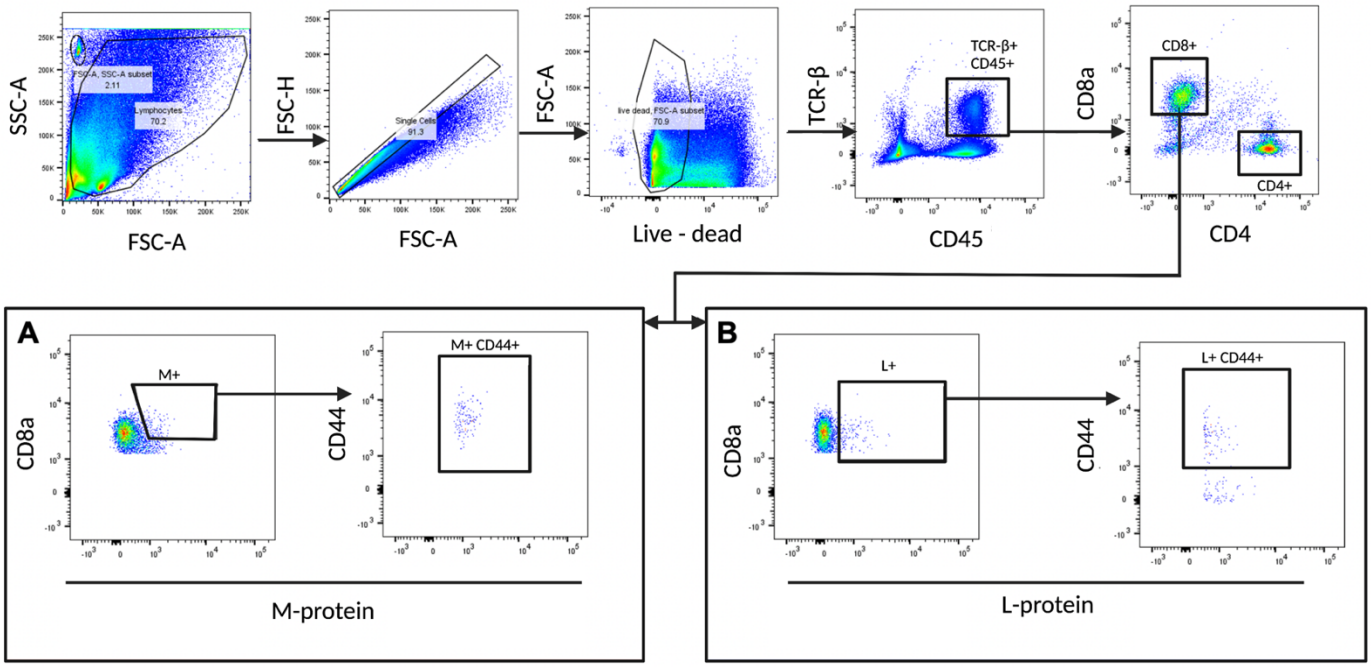

Figure 5. Gating strategy for the T cell panel based on a representative sample from one experiment. The same gating strategy was followed for all the samples. A; M peptide. B; L peptide. Created with BioRender.com

6 RESULTS

6.1 Study of weight in RSV infected and reinfected XCR1.cre and wild type mice

To study the effect of infection and reinfection of RSV on the weight in transgenic and wild type mice, all mice were weighted at the day of the second injection (0) and 2, 4 and 6 days after. (Figure 6).

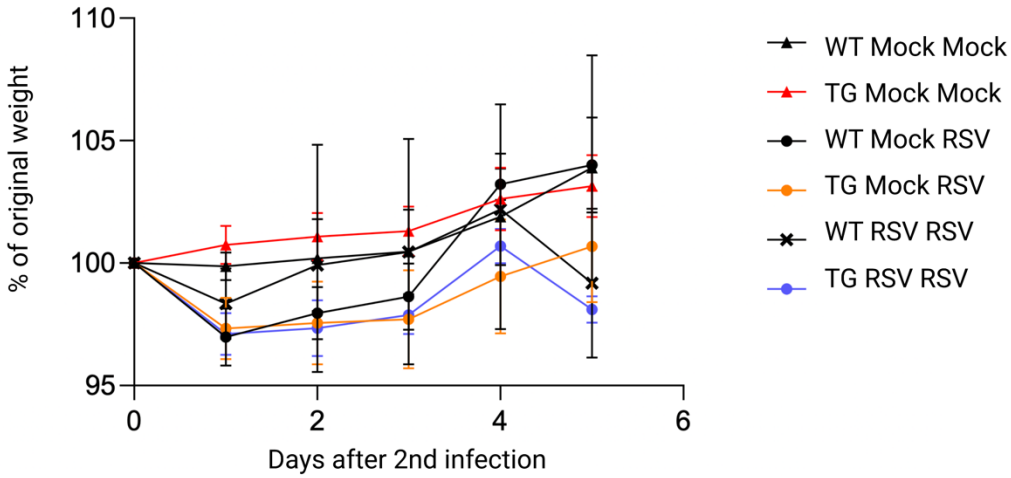

Figure 6. Weight graphic showing the percent of loss according to the original weight. Mice were initially infected with RSV (n=4-5/group) at 7-days-old and reinfected 8 weeks later; controls received sham (mock) inoculation (n= 4-5/group). WT = wild type ; TG = transgenic.

In both mice, wild type, and transgenic in which we injected mock solution at both timepoints (Mock/Mock), there were not any weight loss. In wildtype mice that were injected with a mock solution and RSV (Mock/RSV), corresponding to a first infection condition, there were a loss of weight during the first 3 days, but then they went back to their normal weight or even higher. This tendency was also observed for the transgenic mice. However, the transgenic mice did not gain as much weight.

In mice infected at both timepoints (RSV/RSV), there were a slightly weight loss the first day and after 5 days.

What happens to the weight in this setting is complicated to interpret, or even predict because weight loss in RSV is driven by $CD8^+$ T cell activity. XCR1.cre mice develop more Th2 pathology, but also have a deficiency in CD8 T cell priming.

6.2 Study of pulmonary pathology and neonatally immune memory by q-PCR in infected XCR1.cre and wild type mice

We infected adult mice (8-weeks-old) to explore the effect of neonatally immune memory on disease severity. At 5 days post-reinfection, we euthanized the mice and evaluated the

pulmonary pathology. For this purpose, we performed a qPCR of an RSV gene (*L-gene)* and mucus genes (*gob5* and *muc5ac)* (Figure 7).

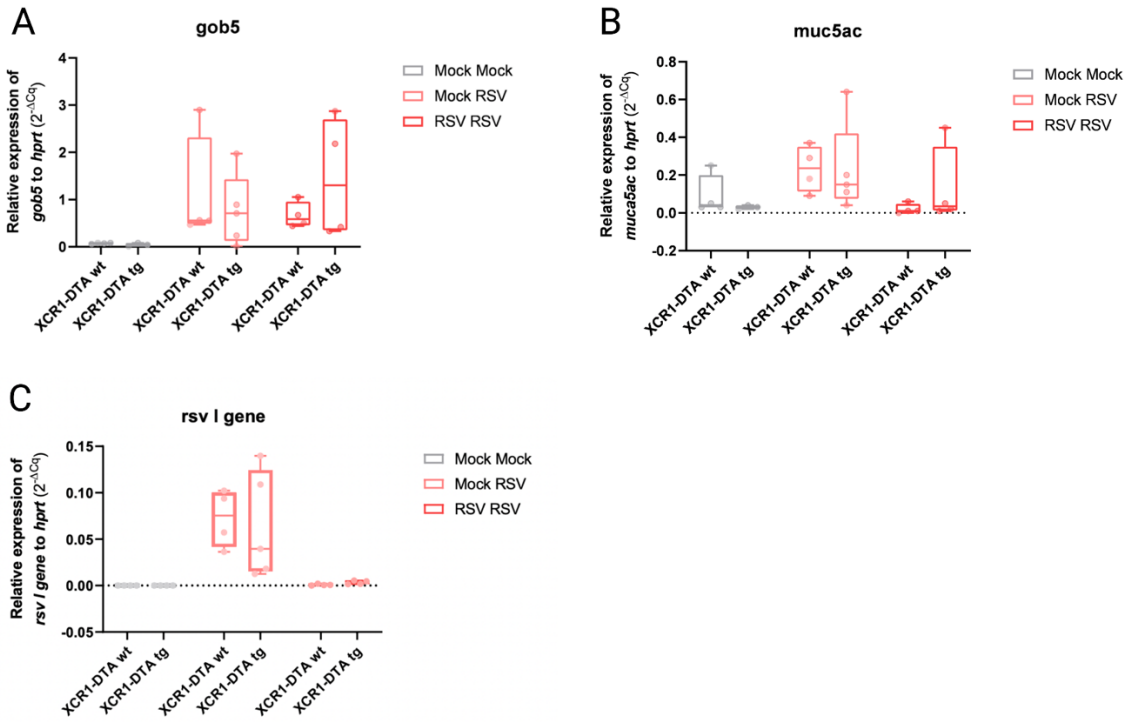

Figure 7. qPCR results for expression of genes relative to the housekeeping gene HPRT. Mice were initially infected with RSV (n=4-5/group) at 7-days-old and reinfected 8 weeks later; controls received sham (mock) inoculation (n= 4-5/group). A) Gob5 gene expression. B) Muc5ac gene expression. C) RSV L gene expression.

L-gene encodes the subunit L of large polymerase, a polymerase protein of RSV virus. Both, *muc5ac* and *gob5* were used for the detection of mucous related genes. *Muc5ac* encodes for mucin 5AC and is linked to mucous production. Moreover, *gob5* is also associated with mucous hyperproduction and goblet cell metaplasia (20).

In this experimental setting, the qPCR results reveal that acute adult infection (Mock/RSV) or reinfections (RSV/RSV) did not enhance the expression of mucous related genes, *muc5ac* and *gob5* (Figure 7A and 7B). These results imply a low mucous inducing environment in response to RSV, especially in reinfected mice.

The viral load after acute infection (Mock/RSV) and reinfection (RSV/RSV) was also studied (results shown Figure 7C). RSV *L-gene* was highest in Mock/RSV adult mice compared with reinfected RSV/RSV. The low expression levels of RSV *L-gene* in the reinfected groups points to a level of immunological memory and faster viral clearance. In this way, the severe disease experienced in mice upon reinfection is likely due to immunopathology rather than the virus itself.

6.3 T cell phenotyping of lungs in infected mice

CD4 and CD8 T cells are important for the clearance of RSV during primary infection, but also contribute to the immunopathogenesis (21). Effector T cells are activated T cells that migrate

from the lymph nodes to the inflamed tissue and function by secreting cytokines that contribute to pathogen clearance as well as enhancing macrophage killing mechanisms (11,22). Moreover, CD8 effector T cells can target and kill infected cells (23). We confirmed the presence of effector T cells by isolating $CD44^+$ $CD4^+/CD8^+$ T cells, providing information on the adaptive immune response to RSV infections (Figure 8C and 8D).

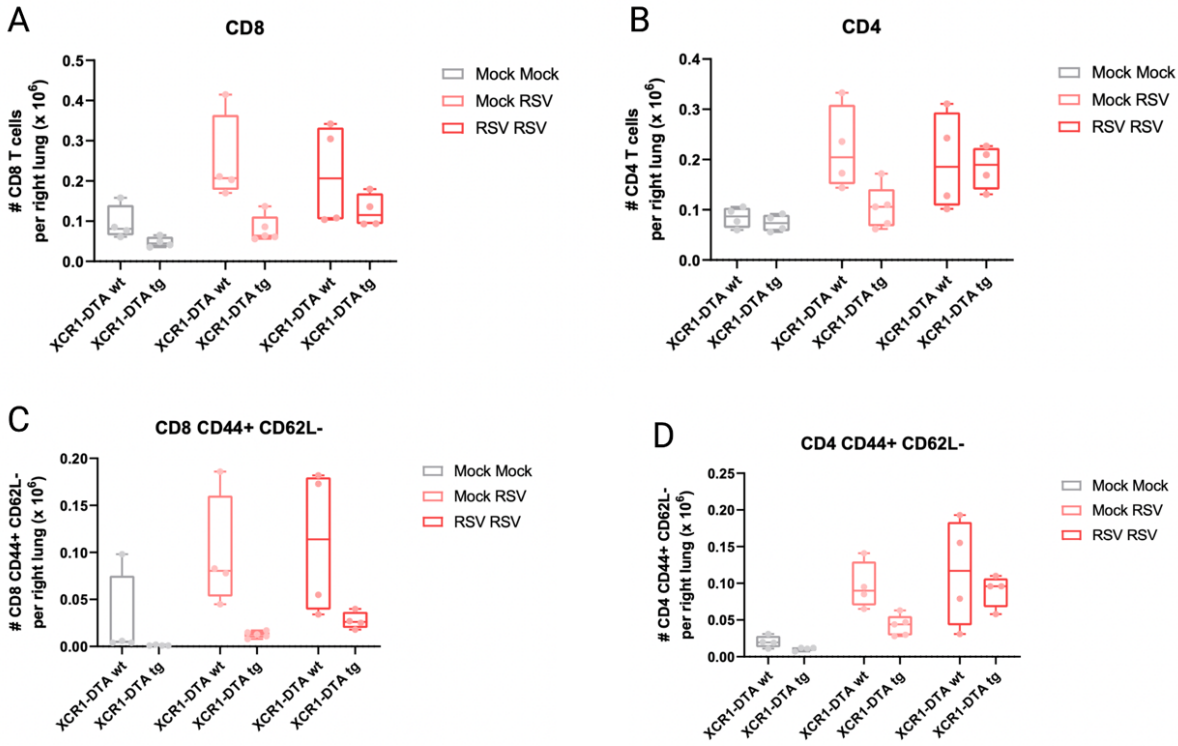

Figure 8. CD4 and CD8 T cell response to RSV reinfection: A) Total number of CD8+ T cells per right lung B) Total number of CD4+ T cells per right lung C) Number of CD8+ CD44+ effector T cells D) Number of CD4+ CD44+ effector T cells. Mice were initially infected with RSV (n=4- 5/group) at 7-days-old and reinfected 8 weeks later; controls received sham inoculation (n=4-5/group). Lung cell counts were assessed by flow cytometry on day 5 post infection.

Our results show that reinfected mice (RSV/RSV) do not display higher levels of T cells later on in life (Figure 8A and 8B). Moreover, reinfected mice seem to have more recruitment of $CD44^+$ CD4 and CD8 T cells, with a most significant increase occurring in the effector CD4 T cells (Figure 8C and 8D). In addition, in the transgenic mice in comparation with wild type mice, the lack of cDC1 leads to a decrease in total numbers of cells in both adult acute infection (Mock/RSV) and in reinfection (RSV/RSV).

6.3.1 Tissue Resident Memory T cells

Tissue resident memory T cells are capable of activating both innate and adaptive immune response by producing large amounts of effector molecules. Their innate-like function makes them an important cell type during secondary infections. CD69 and CD103 are markers used for detection of T_{RM} cells within the $CD44^+$ effector CD8 and CD4 T cell population (8,23–25).

Our results in Figure 9 show an accumulation of T_{RM} T cells in lungs of reinfected mice (RSV/RSV). The number of T_{RM} T cells were higher in wildtype mice than in transgenic mice. Moreover, the number of CD8 T_{RM} cells is much higher than their CD4 analog.

In addition to that, when comparing the decrease between reinfected (RSV/RSV) wild type and transgenic mice, there is a greater decrease in the $CD8^+$ population, suggesting that cDC1 drive $CD8^+$ T_{RM} formation in the lungs (Figure 9B).

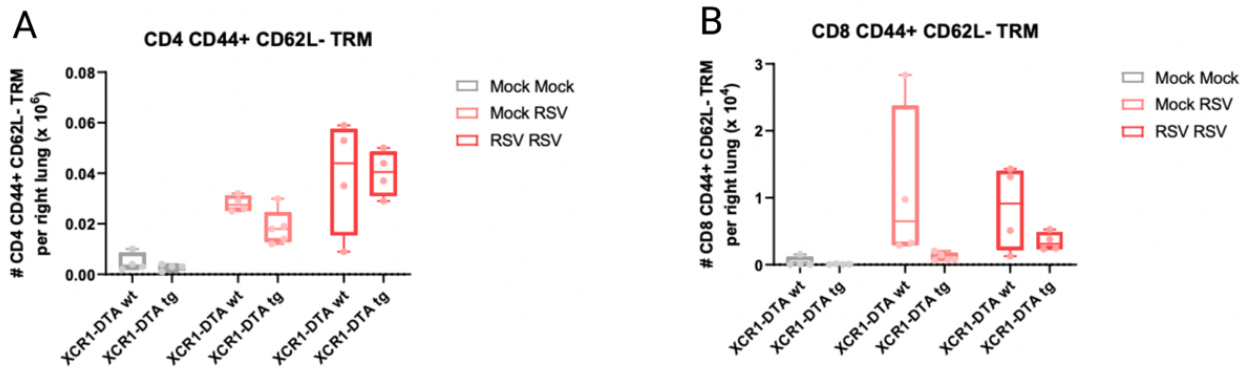

Figure 9. Tissue resident memory T cell response during RSV reinfection: A) Total numbers CD4+ CD44+ CD69+ CD103+ TRM in the right lung lobe. B) Total numbers CD8+ CD44+ CD69+ CD103+ TRM in the right lung lobe. Mice were initially infected with RSV (n=4-5/group) at 7-days-old and reinfected 8 weeks later; controls received sham inoculation (n=4-5/group).

6.3.2 RSV-specific $CD8^+$ dependent cell response

We next studied the RSV-specific $CD8^+$ T cell response for which we used MHC tetramers with immunogenic peptides; one with the L-peptide (L-tetramer) which is a peptide from the RSV polymerase and another with M-peptide (M187-196-tetramer) which a peptide from the matrix protein of the RSV capsid (Figure 10).

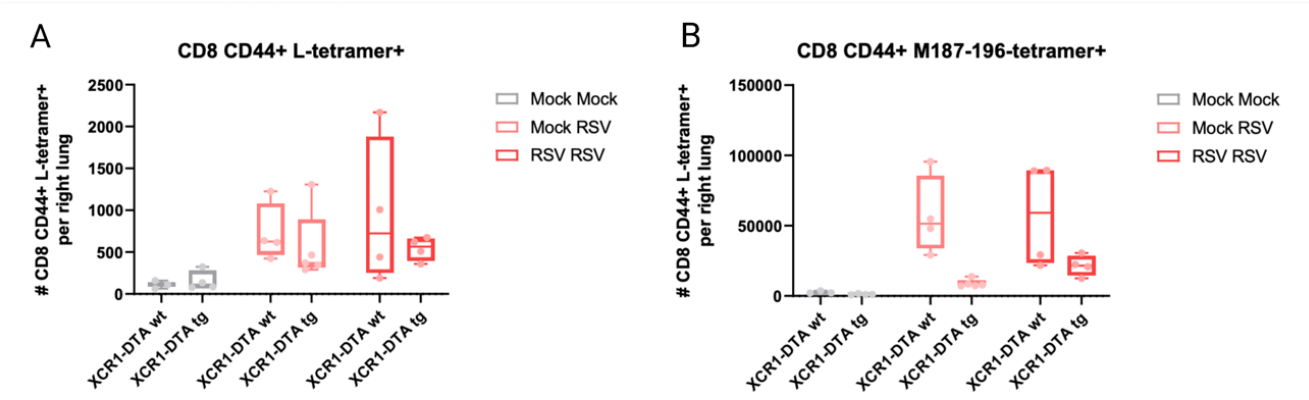

Figure 10. CD8 T cell TCR dependent response to RSV reinfection: A) Total number of CD8+ CD44+ L-tetramer+ T cells per right lung B) Total number of CD8+ CD44+ M187-196-tetramer+ T cells per right lung. Mice were initially infected with RSV (n=4-5/group) at 7-days-old and reinfected 8 weeks later; controls received sham inoculation (n=4-5/group).

There is a decrease in $CD8^+$ T cell subsets in XCR1.cre mice regarding wild type mice in both conditions, infection (Mock/RSV) and reinfection (RSV/RSV). This tendency is observed

independently of the tetramer used, L-tetramer^{+} (Figure 10A) and M187-196-tetramer^{+} (Figure 10B). This states the importance of cDC1s in CD8^{+} T cell activation.

In addition to that, there is a considerable difference in the total number of cells between L-tetramer^{+} (Figure 10A) and M187-196-tetramer^{+} subsets (Figure 10B). These results correspond to the immunodominance of the M-peptide in relation to L-peptide.

Differences between CD8^{+} TCR dependent subsets were studied using CD69, which is a lectin that prevents the cells from leaving the tissue, characteristic of the T_{RM} subset (Figure 11).

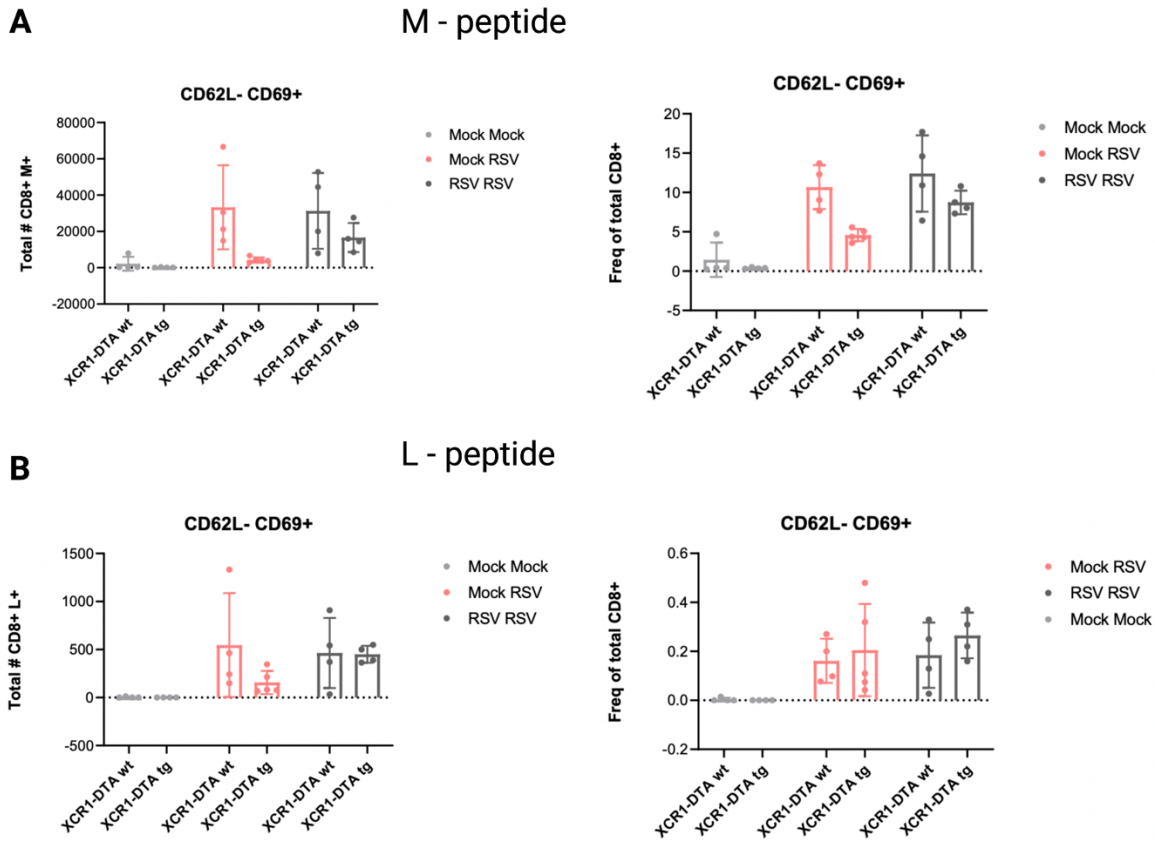

Figure 11. CD8 T cell TCR dependent response to RSV reinfection: Right total number of cells per right lung; Left frequency of CD8+ T cells. A) CD8+ CD44+ M187-196-tetramer+ B) CD8+ CD44+ L-peptide-tetramer+.

The CD8^{+} CD62L^{-} CD69^{+} M-peptide^{+} subset (Figure 11A) follows the same tendency of the other subsets where the transgenic mice have lower numbers than the wildtype, indicating the importance of cDC1 cells in CD8 T_{RM} cells activation.

Nevertheless, the results show that the frequency of CD8^{+} CD62L^{-} CD69^{+} L-peptide^{+} was higher in the transgenic reinfected (RSV/RSV) mice in comparation to the wildtype which is not consistent with the CD8^{+} CD62L^{-} CD69^{+} M-peptide^{+} subset and with the other subsets.

7 DISCUSSION

RSV is a major cause of respiratory illness in young children. Nearly all children worldwide have experienced at least one infection with RSV. Furthermore, RSV-related acute LRTI is to this day an important cause of childhood mortality in developing countries (1,13).

In mice, RSV induces a typical antiviral adaptive immune response, with resolution of primary infection resulting in high titers of virus-specific antibodies and large numbers of antigen-specific T cells (1,13,14), which align with this dissertation results (Figure 8). This limits infection during secondary infection (Figure 7C), so that reinfection leads to only low levels of transient virus replication with little associated disease except under circumstances in which narrowly focused immunity enhances disease severity (26).

Previous studies states that $CD4^+$ T cells are critical for an efficient host response since they aid B cells in producing high-affinity antibodies and maintaining optimal $CD8^+$ T cell memory (13), which can be seen in this dissertation results, when a decrease of $CD4^+$ T cell (Figure 8B) in the acute infection of transgenic mice leads to higher viral load (Figure 7C) and a decrease in the $CD8^+$ T cell population (Figure 8A). Moreover $CD4^+$ T cells do, however, have direct antiviral effector capabilities, and improper stimulation of $CD4^+$ T cell responses may play a role in acute RSV sickness (22,23).

In RSV, $CD8^+$ T lymphocytes from mice detect a hierarchy of dominant and subdominant epitopes, which is also visible in humans (11,19,23). This can be seen in Figure 10, were a high number of $CD8^+$ TCR specific to M-peptide were seen in comparation with the L-peptide.

Previous research using infected adult human volunteers reported that RSV-specific $CD8^+$ T cells were common in the lower respiratory tract, with up to 20% of $CD8^+$ T cells recognizing a single RSV epitope in some cases (23). Those, RSV-specific $CD8^+$ T cells in the respiratory tract invariably displayed characteristics of T_{RM} cells, such as strong CD69 and CD103 expression (8,23,25). There results correlate with what it is shown in Figure 11A, where $CD8^+$ $CD69^+$ $CD44^+$ T cells are almost 15% of the total $CD8^+$ T cells in both acute and reinfection.

Moreover, $CD8^+$ T_{RM} are known to be critical in conferring protection from pathogen reinfection, being related in humans the presence of lung $CD8^+$ T_{RM} with a better outcome (27). As Figure 9 shows, the presence of T_{RM} in reinfection is related with less viral load (Figure 7C).

The requirements for generating and maintaining lung T_{RM} cells during RSV infection still under investigation (27). Previous research states that cDCs are dispensable for lung $CD8^+$ T_{RM} reactivation (16) which correlates with our findings, where in RSV reinfection in XCR1.cre mice the frequency of $CD69^+$ $CD62L^-$ $CD8^+$ T cells recognizing L-peptide is higher than in the wild type mice (figure 11B). Nevertheless, this is not what it is seen in the TCR specific to M-peptide subtype (figure 11A) and in the $CD8^+$ T_{RM} with undefined TCR specificity (Figure 9B).

Low J. et al. (24) revealed that different $CD8^+$ T_{RM} cell functional responses can be modulated by the nature of antigen presenting partners. A prominent feature of DC–T cell interactions is

costimulation (4,5). Altogether with these results, suggest that some types of antigens will display different cDCs dependency for reactivation and activation of T_{RM}.

Little is known about how T_{RM} are initiated and regulated. More research has to be done, specially to study if this tendency observed in the $CD8^+CD44^+CD69^+$ subset in the lung is the same in the lymph nodes, given the distinct tissue specific compartmentalization of the functional recall responses between memory T cells in the lung vs. lymph nodes and the existence of $CD69^+$ resident memory T cells in the draining lymph nodes (16).

Studies uncovering innate immune regulatory mechanisms of the memory T cell response to viral infection are important for an application in vaccination and therapy. The importance of eliciting long-lasting tissue resident memory response after natural or artificial immunization has been suggested by some as a mean to avoid future infections (8,16,23,24).

Furthermore, Liu J. et al. (28) reported that although highly immunodominant epitopes that elicit large epitope specific $CD8^+$ T cell responses have been observed in inbred mice, it has been reported that those recognizing subdominant epitopes are the most protective and less pathogenic, but the relevance of these findings to human infection has not been confirmed. All this together with this finding of a difference $CD8^+$ T cell memory subset induction depending on cDC1 may serve as a guideline for vaccine development.

8 CONCLUSIONS

- To study the consequences on the pathology that an absence of cDC1 will have, we observed the weight of infected XCR1.cre and wild type mice. No tendency in weight loss after infection and reinfection with RSV was observed.

- The qPCR analysis of mucus related genes, *muc5ac* and *gob5ac* showed that RSV did not induce a mucous environment, especially in reinfected mice. The analysis of *L-gene* showed that despite the absence of cDC1 there was an immunological memory in reinfected mice.

- The lack of cDC1 led to a decrease in CD4 and CD8 T cell subsets. This is because cDC1 cross-presentation function play a crucial role in priming CD4 and CD8 T cells.

- Studying the differences in $CD8^+$ T cell subsets using MHC tetramers allowed us to identify an immunodominance of the M-peptide in relation to L-peptide.

- $CD8^+$ T_{RM} M-peptide$^+$ subset in reinfected mice have a higher dependency on cDC1 than $CD8^+$ T_{RM} L-peptide$^+$ subset. This suggests that some types of antigens will display different cDCs dependency for reactivation and activation.

However, to determine the significance of these results, additional experiments and repeats are needed to verify these results as well as statistical studies.

CONCLUSIONES

- Para estudiar las consecuencias en la patología que tendrá la ausencia de cDC1, observamos el peso de los ratos transgénicos y de los de tipo salvaje infectados. No se observó ninguna tendencia en la pérdida de peso tras la infección y reinfección con el VRS.

- El análisis por qPCR de los genes relacionados con las mucosas, muc5ac y gob5ac, mostró que el VRS no indujo un ambiente mucoso, especialmente en los ratones reinfectados. El análisis del gen L mostró que, a pesar de la ausencia de cDC1, había una memoria inmunológica en los ratones reinfectados.

- La falta de cDC1 provocó una disminución de los subconjuntos de células T CD4 y CD8. Esto se debe a que la función de presentación cruzada de cDC1 desempeña un papel crucial en el cebado de las células T CD4 y CD8.

- El estudio de las diferencias en los subconjuntos de células T $CD8^+$ utilizando tetrámeros del MHC nos permitió identificar una inmunodominancia del péptido M en relación con el péptido L.

- El subconjunto $CD8^+$ T_{RM} M-péptido$^+$ en ratones reinfectados tiene una mayor dependencia de cDC1 que el subconjunto $CD8^+$ T_{RM} L-péptido$^+$. Esto sugiere que algunos tipos de

antígenos muestran una dependencia diferente de los cDC para la reactivación y la activación.

Sin embargo, para determinar la importancia de estos resultados, se necesitan experimentos adicionales y repeticiones para verificar estos resultados, así como estudios estadísticos.

9 BIBLIOGRAPHY

1. Lambert L, Sagfors AM, Openshaw PJM, Culley FJ. Immunity to RSV in early-life. Vol. 5, Frontiers in Immunology. Frontiers Media S.A.; 2014.
2. Murphy K (Kenneth M), Weaver C, Janeway C. Janeway's immunobiology. Vol. 9. 2017. 345–399 p.
3. Openshaw PJM, Chiu C, Culley FJ, Johansson C. Protective and harmful immunity to RSV infection. Annual Review of Immunology. 2017;35:501–32.
4. Cabeza-Cabrerizo M, Cardoso A, Minutti CM, Pereira da Costa M, Reis Sousa C. Dendritic Cells Revisited. Annual Review of Immunology . 2021;39:131–66.
5. Yin X, Chen S, Eisenbarth SC. Dendritic Cell Regulation of T Helper Cells. Annual Review of Immunology. 2021;39:759–90.
6. Schmidt ME, Varga SM. The CD8 T cell response to respiratory virus infections. Vol. 9, Frontiers in Immunology. Frontiers Media S.A.; 2018.
7. Martin MD, Badovinac VP. Defining memory CD8 T cell. Frontiers in Immunology. 2018 Nov 20;9(2692).
8. Takamura S, Yagi H, Hakata Y, Motozono C, McMaster SR, Masumoto T, et al. Specific niches for lung-resident memory CD8+ T cells at the site of tissue regeneration enable CD69-independent maintenance. Journal of Experimental Medicine. 2016 Dec 12;213(13):3057–73.
9. Golubovskaya V, Wu L. Different subsets of T cells, memory, effector functions, and CAR-T immunotherapy. Cancers (Basel). 2016 Mar 15;8(36).
10. Mahnke YD, Brodie TM, Sallusto F, Roederer M, Lugli E. The who's who of T-cell differentiation: Human memory T-cell subsets. European Journal of Immunology. 2013 Nov;43(11):2797–809.
11. Moon JJ, Chu HH, Pepper M, Mcsorley SJ, Jameson SC, Kedl RM, et al. Naïve CD4+ T cell frequency varies for different epitopes and predicts repertoire diversity and response magnitude. Immunity. 2007;27(2):203–13.
12. Jenkins MK, Moon JJ. The Role of Naive T Cell Precursor Frequency and Recruitment in Dictating Immune Response Magnitude. The Journal of Immunology. 2012 May 1;188(9):4135–40.
13. Openshaw PJM, Chiu C, Culley FJ, Johansson C. Protective and Harmful Immunity to RSV Infection. Annual Review of Immunology. 2017;35:501–32.
14. Griffiths C, Drews SJ, Marchant DJ. Respiratory syncytial virus: Infection, detection, and new options for prevention and treatment. Clinical Microbiology Reviews. 2017 Jan 1;30(1):277–319.
15. Lambrecht BN, Hammad H. Lung dendritic cells in respiratory viral infection and asthma: From protection to immunopathology. Annual Review of Immunology. 2012 Apr;30:243–70.

16. Varese A, Nakawesi J, Farias A, Kirsebom FCM, Paulsen M, Nuriev R, et al. Type I interferons and MAVS signaling are necessary for tissue resident memory CD8+ T cell responses to RSV infection. PLoS Pathogens. 2022 Feb 1;18(2).

17. Lukens M v., Kruijsen D, Coenjaerts FEJ, Kimpen JLL, van Bleek GM. Respiratory Syncytial Virus-Induced Activation and Migration of Respiratory Dendritic Cells and Subsequent Antigen Presentation in the Lung-Draining Lymph Node. Journal of Virology. 2009 Jul 15;83(14):7235–43.

18. Horlock C. Production of MHC Class I Tetramers [Internet]. British Society for immunology. 2020 [cited 2022 Jun 21]. Available from: https://www.immunology.org/public-information/bitesized-immunology/experimental-techniques/production-mhc-class-i-tetramers

19. Schmidt ME, Varga SM. Identification of Novel Respiratory Syncytial Virus CD4 + and CD8 + T Cell Epitopes in C57BL/6 Mice . Immunohorizons. 2019 Jan 1;3(1):1–12.

20. Nakanishi A, Morita S, Iwashita H, Sagiya Y, Ashida Y, Shirafuji H, et al. Role of gob-5 in mucus overproduction and airway hyperresponsiveness in asthma. Proceedings of the National Academy of Sciences. 2001;98(9):5175–80.

21. Tregoning JS, Yamaguchi Y, Harker J, Wang B, Openshaw PJM. The Role of T Cells in the Enhancement of Respiratory Syncytial Virus Infection Severity during Adult Reinfection of Neonatally Sensitized Mice. Journal of Virology. 2008 Apr 15;82(8):4115–24.

22. Chiu C, Openshaw PJ. Antiviral B cell and T cell immunity in the lungs. Nature Immunology. 2015 Dec 18;16(1):18–26.

23. Jozwik A, Habibi MS, Paras A, Zhu J, Guvenel A, Dhariwal J, et al. RSV-specific airway resident memory CD8+ T cells and differential disease severity after experimental human infection. Nature Communications. 2015 Dec 21;6(10224).

24. Low JS, Farsakoglu Y, Amezcua Vesely MC, Sefik E, Kelly JB, Harman CCD, et al. Tissue-resident memory T cell reactivation by diverse antigen-presenting cells imparts distinct functional responses. Journal of Experimental Medicine. 2020 Aug 3;217(8).

25. Uss E, Rowshani AT, Hooibrink B, Lardy NM, van Lier RAW, ten Berge IJM. CD103 Is a Marker for Alloantigen-Induced Regulatory CD8 + T Cells . The Journal of Immunology. 2006 Sep 1;177(5):2775–83.

26. Blum JS, Wearsch PA, Cresswell P. Pathways of antigen processing. Annual Review of Immunology. 2013 Mar;31:443–73.

27. DiNapoli JM, Murphy BR, Collins PL, Bukreyev A. Impairment of the CD8+ T cell response in lungs following infection with human respiratory syncytial virus is specific to the anatomical site rather than the virus, antigen, or route of infection. Virology Journal. 2008;5(105).

28. Liu J, Haddad EK, Marceau J, Morabito KM, Rao SS, Filali-Mouhim A, et al. A Numerically Subdominant CD8 T Cell Response to Matrix Protein of Respiratory Syncytial Virus Controls Infection with Limited Immunopathology. PLoS Pathogens. 2016 Mar 1;12(3).