2024

Using Tamoxifen-Induced fate reporting to investigate the effect of CD4 T cell age on responses to influenza

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Contents

Abstract
Influenza is a pathogen viewed differently across different patient demographics. Amongst the elderly, influenza poses a large threat, so much so regular immunisations are warranted for those deemed at risk. Amongst the young and non-immunocompromised, influenza is brief spell of a chesty cough and runny nose. The reason for this difference in immunity is not very well understood on the cellular level. The purpose of this experiment was to investigate if the age of CD4+ T cells impacted the immune response to influenza. We used a tamoxifen inducible fate reporter system to label older and younger CD4+ T cells We measured flu-specific CD4+ effector memory (EM) and CD4+ T cell counts as well as their Ki67+ frequency, thus, allowing us to compare responses of older CD4+ cells and younger cells. Our observations suggest that cellular age did not impact the response of tetramer positive flu-specific CD4+ EM, however, when looking at tetramer negative bulk EM, the older CD4+ T cells responded better.

1. Introduction

1.1 The Role of CD4 Cells in the immune response

CD4+ T cells, also known as T Helper Cells, are essential to the body's immune system and have multiple functions that help fight disease. They produce cytokines, activate B cells and CD8+ T cells, as well as provide long-lasting immunity by differentiating into memory cells [9]. To be activated, CD4+ T cells require several key stimuli, the first of which, is antigen presentation by major histocompatibility complex (MHC) class II molecules on professional antigen presenting cells. In addition, co-stimulation is required by specific co-stimulatory molecules, such as CD80 [9]. CD4+ T cells are involved in both primary and memory responses to infection however these responses are not equal. Memory CD4+ T cells respond much quicker than naïve CD4+ T cells due to antigen-induced alterations which pre-prime them to deal with the pathogen. Memory T cells of a specific antigen specificity are also present in much greater numbers compared to naïve t cells with corresponding specificity reference. Memory responses will typically occur within hours to a few days compared to the weeks it would take for naïve CD4+ T cells [9]. Furthermore, Memory CD4+ T cells will efficiently produce cytokines which are specific to the pathogen. On the other hand, Naïve CD4+ T cells release a broad range of cytokines during the primary response which are less tailored to specific pathogens. Memory CD4+ T cells can also proliferate and differentiate into effector cells more rapidly than naïve CD4+ T cells resulting in a more robust response [9].

1.2 Flu Response in the Old and Young

Flu is much more of a concern for the older population than the young [10]. Those over the age of 65 have a higher risk of developing severe complications from influenza due to age related decline in immune function, known as immunosenescence [10]. Decline in T cell function and numbers, as well as lowered antibody production by B cells hinders the body's capability to recognise and respond to the viral infection [11]. Furthermore, there is often an accumulation of Memory T cells instead of naïve T cells which hinders the person's ability to respond effectively to a new pathogen, such as a new strain of influenza [11]. Aging also affects signalling pathways necessary for T cell activation [11]. For example, co-stimulatory molecule (e.g. CD28) expression is reduced resulting in lower T cell activation and weakening of the immune response [11]. Natural Killer Cells and Macrophages also suffer from a reduced functional output reducing the body's ability to control viral infections [11]. Older adults often suffer from inflammaging; a chronic state of low-level inflammation [11]. This can not only impair the immune response, but also causes an exaggerated inflammatory response which contributes to complications, such as pneumonia in the case of influenza [11]. Immunosenescence also causes the elderly to respond poorly to vaccination leaving them less protected against flu due to lowered antibody production [2]. There is also a discrepancy between the cytokine response of younger and older populations, with the former producing a more robust response [3]. Thus, the ability of the elderly to clear the virus is compromised [3].

1.3 Impact of T cell age on cellular function

Whilst the impact of host age on responses to infection is well appreciated, little is known about the relationship between T cell age and function. However, studies have shown that the age of CD4+ naïve T cells does affect their survival. As CD4 + naïve T cells age in the circulation, they gain a survival advantage [5]. It is thought this may be due to intrinsic changes allowing them to specifically survive better in the periphery [5]. However, functional impairment is also thought to occur as the cells age [5]. CD4+ naïve T cells from older donors exhibit decreased effectiveness in responding to infections compared with those from younger donors [6]. Overall, while the ability of these cells to survive improves with the aging of the cell, the suspected functional impairment means that the elderly may harbour significant populations of long lived CD4+ naïve T cells, which are less effective at responding to infection, resulting in a weaker immune response. In addition, as CD4+ memory T cells age, their effectiveness as part of the immune response declines [12]. Older CD4+ memory T cells experience a hindrance in their effector function such as reduced cytokine production and proliferation in response to a pathogen [12]. This in tandem with the changes experienced by aging naïve CD4 T cells leaves a weakened immune response in older individuals.

Aim: To investigate if aging flu-specific CD4+ T cells exhibited a change in response to intra-nasal infection with Influenza, compared to younger flu-specific CD4+ T cells.

2. Methods

2.1 Mice preparation

Mice 7-8 weeks of age were each fed tamoxifen on day 0. Tamoxifen activates inactive cre recombinase **(figure 1)** which is expressed in all cells expressing CD4. Active Cre

recombinase removes a stop codon downstream of the Rosa26 gene which permits expression of the mTOM fluorescent reporter **(figure 1)**. As this is a constitutively expressed gene, Cells present at the time of tamoxifen treatment will be permanently labelled with mTOM. Any cells produced after treatment, will not have experienced tamoxifen and thus will remain unlabelled. This gives rise to a population of labelled older cells and unlabelled younger cells.7-8 weeks after tamoxifen treatment, mice were intranasally treated with flu (strain X31) or PBS. 3 minutes before culling the mice, they were given an intravenous injection of anti-Thy1 antibody, which labels cells in circulation.

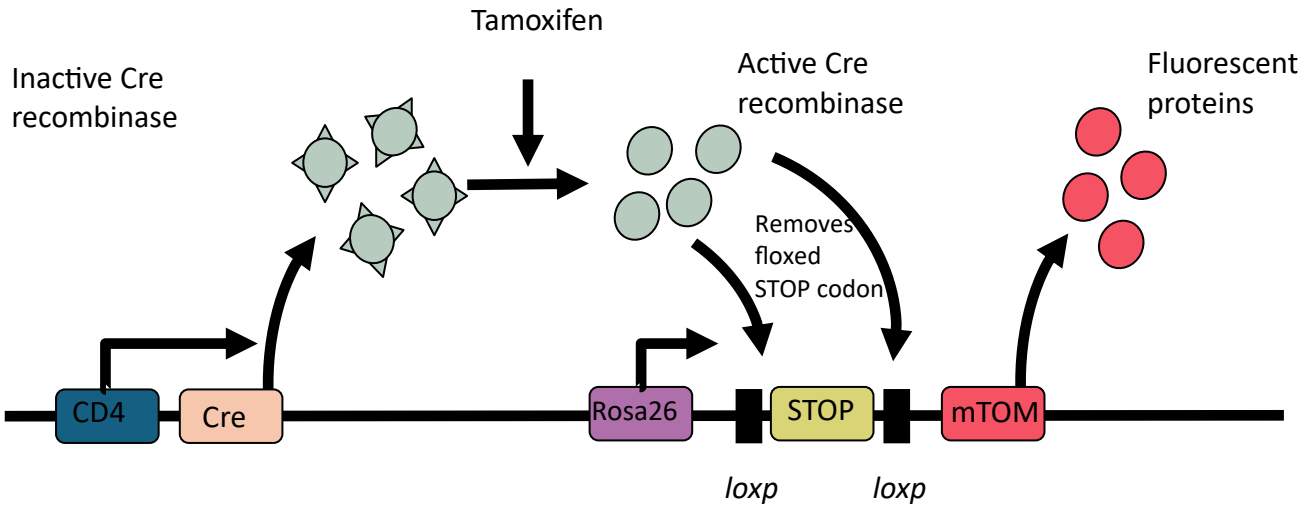


Figure 1: Schematic showing the genetic make-up of the CD4-CreERT2 Rosa26RmTom mouse used in this study. Tamoxifen activates inactive Cre recombinase, which removes the STOP codon downstream of Rosa26, allowing expression of mTOM fluorescent reporter

2.2 Obtaining single cell suspensions from lung tissue.

Digestive medium was made up in two separate bijou tubes. The contents consisted of 4.94 ml of Roswell Park Memorial Institute (RPMI) medium with 10% Foetal calf serum (FCS), 50 µl of Colaginase IV and 6 µl of DNAse I. 1 ml of digestive medium was taken from each bijou and added to two separate bijous. Lungs from each mouse were added to a bijou containing 1 ml of digestive medium. Scissors were used to cut the lung until the mixture became homogenous. Shredded tissue was added back into the appropriate bijou containing the rest of the digestive medium. Suspensions were placed in a shake incubater at 200 rpm and 37 °C for 1 hour. Once finished, 20 µl of EDTA was added to each sample and vortexed. Each suspension was then filtered into 50 ml falcon tubes using a 70 µl filter. The filters were rinsed with 15 ml of RPMI + FCS medium into the falcons. The remaining tissue on the filter was then crushed using a syringe plunger and the filter was rinsed with 5 ml of RPMI medium + FCS.

2.3 Obtaining single cell suspensions from lymphoid tissues

Mediastinal lymph nodes (MLN), pan-lymph nodes (LN) and spleens were placed separately between two pieces of mesh in a petri dish. Tissues were mashed until homogenous. Both layers of mesh were rinsed RPMI + FCS and the solution was filtered into the appropriately labelled falcon through a 30 µl filter. RPMI + FCS was then used to rinse the petri dish and the filter.

2.4 Cell counting

All samples were centrifuged for 5 minutes at 4 °C, 1500 rpm and an acceleration of 9. The supernatant was poured discarded, and all samples were 10 ml. Importantly, lung tissue was filtered through a 30µm filter at this point. Samples were suspended in PBS. 10 µl of each sample was added to the appropriate Casy tubes and the number of cells per ml was recorded using the Casy counter. 5x10(6) cells were taken for staining.

2.5 Tetramer Staining

Cells were washed with PBS and stained with I-A(b) influenza A NP 311-325 and H-2D(b) influenza A NP 366-374 tetramers in RPMI + FCS for 30 minutes at room temperature. The samples were then washed with PBS twice and supernatant discarded.

2.6 Surface Staining

Cells were then surface stained on ice for 30 minutes with a cocktail of surface antibodies in PBS. Cells were then washed twice in FACS buffer. Next, cells were fixed in Invitrogen Fixation/Perm Concentrate and Fixation/Perm Diluent in a 1:3 ratio. 300 µl of fixative was added to each tube and vortexed before incubating on ice in the dark for 20 minutes. After incubating, 1 part perm buffer, was added to 9 parts PBS to make up perm buffer which was added to each sample. Samples were centrifuged and resuspended in 400 µl FACS for overnight storage on ice.

2.7 Intracellular Staining

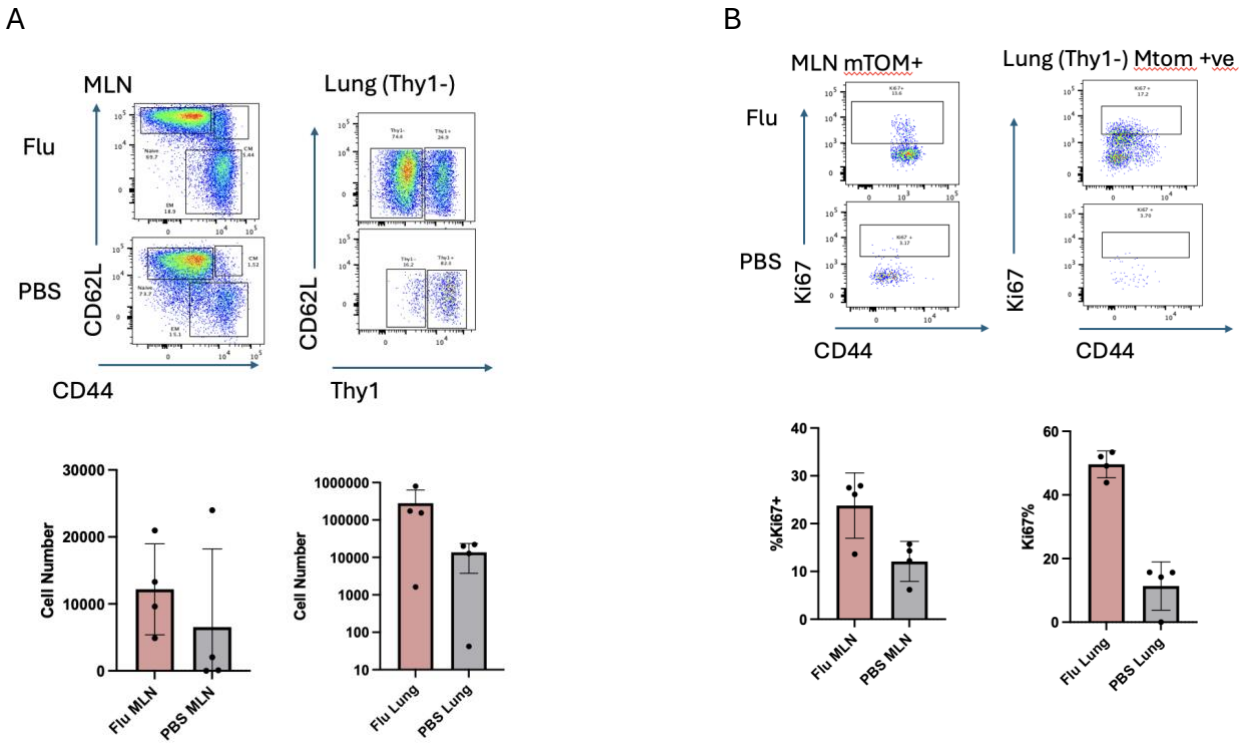
Samples were washed in 1 ml of the perm buffer and stained with a cocktail of antibodies suspended in perm buffer for 1 hour on ice. Cells were washed twice in perm buffer and re-suspended in 400µl of FACS buffer for acquisition by flow cytometry. Immediately before flow cytometry of the lung and MLN samples, 10000 Accu-Chek counting beads were added.

3. Results

3.1 Identifying Flu Responses in Vivo

As mice were given s sub-lethal dose of influenza, we could not assess the success of infection by weight loss, instead we analysed cellular responses in the mediastinal lymph

nodes (MLN) and lung, tissues where we suspected a response would occur. Infection was confirmed using several readouts. Firstly, it is known the CD4+ naïve T cells differentiate into effector memory T cells in response to an overt challenge. Therefore, we measured to total number of effector memory CD4+ T cells in the MLN and lung and as seen in **figure 2a** both tissues showed an increased number of CD4+ EM in response to flu infection compared to the PBS control. Similarly, we measured Ki67, a marker of proliferation, in CD4+ EM and observed greater frequencies of Ki67+ cells in response to flu infection compared to PBS **(Figure 2b).** Finally, we also measured the frequency of CD4+ EM that stained negative for Thy1. 3 minutes before culling the mice an intravenous injection of fluorochrome labelled anti-Thy1 antibody was injected. This was used to distinguish between cells circulating in the blood (labelled) and those deeper within the tissue (unlabelled). This was particularly important when identifying flu responding T cells in the lungs as this tissue is heavily vascularised and the responding cells are those that have penetrated deep into the tissue. As seen in **figure 2c**, there was a marked increase in the frequency of CD4+ EM that were Thy1 negative in response to flu infection. Combined, these data highlight several readouts that define a response to our model of flu infection.



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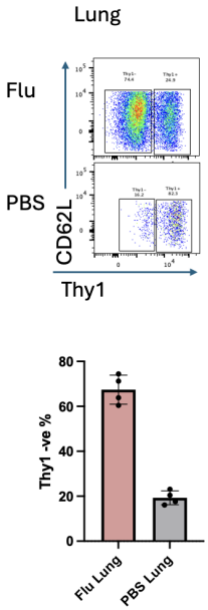
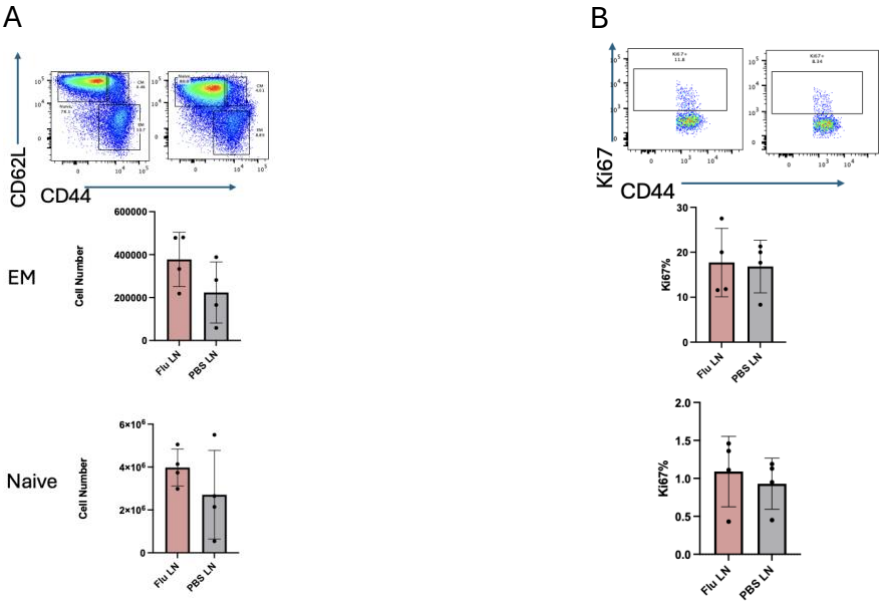


Figure 2: CD4+ EM cell counts, Ki67 expression and protection from Thy1 staining identify T cells that are responding to flu infection. A) FACS plots and graphs showing number of CD4+ EM T cells in the lungs and MLN **B)** FACS plots and graphs showing the frequency of CD4+ EM expressing Ki67 in the lungs and MLN **C)** FACS plot and graph showing the frequency of CD4+ EM protected from Thy1 staining in the MLN and lungs of PBS treated and flu infected mice n=4.

3.2 Identifying a control population of T cells, not impacted by flu treatment

There are two controls that were used in these experiments, the first is an extrinsic control, where mice were treated with PBS instead of flu and the second was an intrinsic control, where we identify a population of cells within the flu treated mouse in a location where no response to the pathogen has likely occurred. We did not expect the spleen and LN to participate in the repones to influenza. When we measured Effector Memory (EM) cell number and proliferation and compared to its PBS counterpart we observed comparable numbers and levels of ki67 expression. This suggests that flu infection is not impacting cells in the spleen or LN and that the mTOM content in naïve and EM populations may be used as a control.



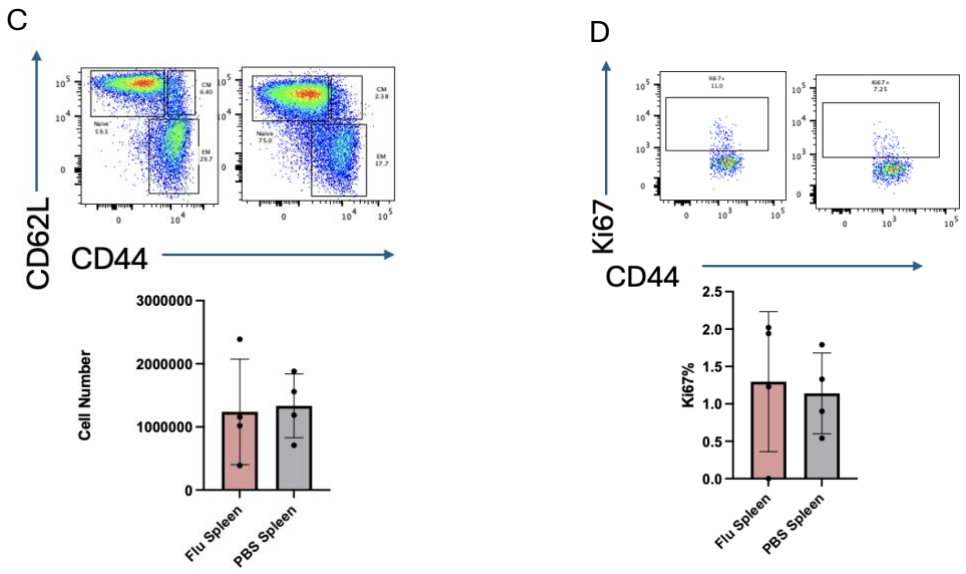


Figure 3: Cells within the spleen and LN do not appear to participate in the response to flu infection. **A)** FACS plots and Graphs showing number of CD4+ EM and naïve T cells in the LN and Spleens. **B)** FACS plots and graphs showing Ki67+ EM and Ki67+ Naïve cell frequency in the LN. **C)** FACS plots and graph showing the number of CD4+ EM in the spleen. **D)** FACS plots and graphs showing CD4+ Naïve cell Ki67 expression in the spleen n=4.

3.3 Effector Memory CD4+ T Cells in the lung appear to be derived from older precursors

Studies have already shown that CD4+ EMs are derived from CD4+ naïve T cells [7]. These naïve cells are present in the draining LN and following activation, proliferate and differentiate and migrate to tissues at the site of infection, in this case, the lungs [8]. mTOM+ frequency of Thy1- CD4+ EMs in the lungs was more than double that of the LN naïve CD4+ cells **(figure 4a)**. From this we can hypothesise that older cells are more effectively being recruited into the response as if this were not the case, the mTOM content in the EM would be equal to or lesser than the naïve precursor population.

Whilst Thy1- EM in the lung are responding to flu, we also sought to identify flu-specific T cells more robustly by staining for tetramer positive cells. However, in contrast to bulk EM, the mTOM+ content among tetramer positive lung EMs is the same as the mTOM+ frequency among the LN naïve CD4+ T cells in both flu treated and PBS treated mice. Thus, this population of flu-specific T cells did not appear to show a cell age related effect.

Here are some hypotheses;

- High affinity TCR T cells are not impacted by age, but lower affinity TCR might be Or
- Flu responsive T cells may be derived from memory phenotype EM which would have higher mTOM content.

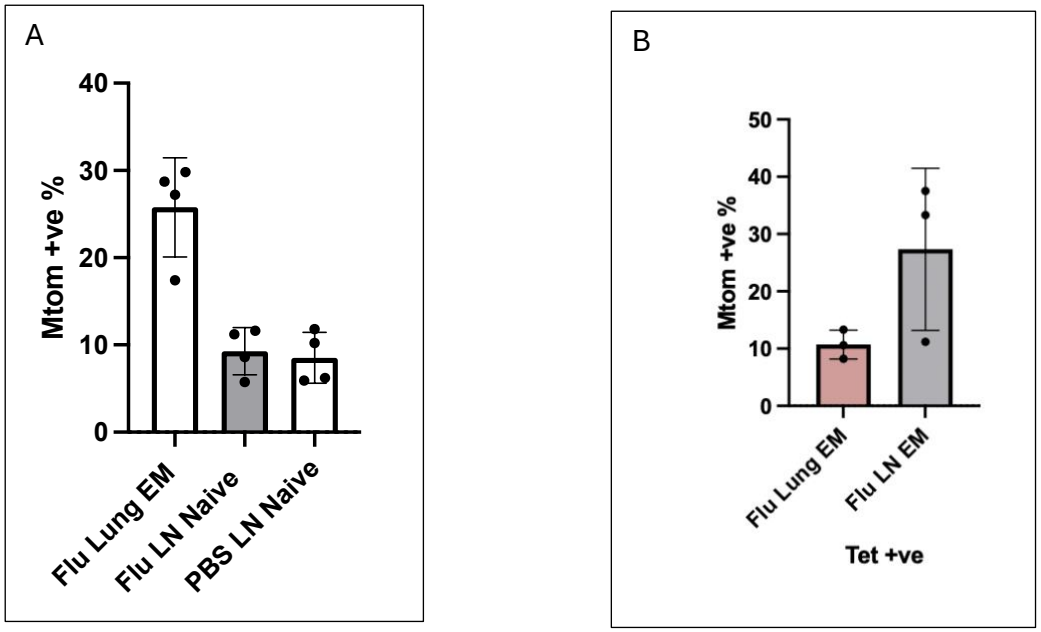


Figure 4: Mtom+ frequencies among CD4+ EM T cells in the lungs and Naïve CD4+ T cells in the LN as well as among Tetramer positive lung and LN EMs. **A)** Graph showing Mtom+ frequency among Flu lung CD4+ EM and LN CD4+ naive. **B)** Graph showing Mtom+ frequency of tetramer positive CD4+ EMs in the flu lung and LN

Comparing the responsiveness of younger and older responses

We specifically looked at Mtom+ and Mtom- Thy 1- CD4+ EMs of the lungs, where the site of infection is. To compare the responsiveness of each population, we looked at Ki67+ frequency. Ki67 expression is a marker of proliferation so can serve as an indicator of responsiveness. Both Mtom positive **(Figure 5a)** and negative **(Figure 5b)** populations demonstrated similar levels of increased Ki67+ frequency compared to their PBS counterparts. From this, we can deduce that cell age does not have a significant effect on the magnitude of response to influenza infection in the lungs as there is not an increase in proliferation of CD4+ EM T cells within the tissues at the site of infection.

Figure 5

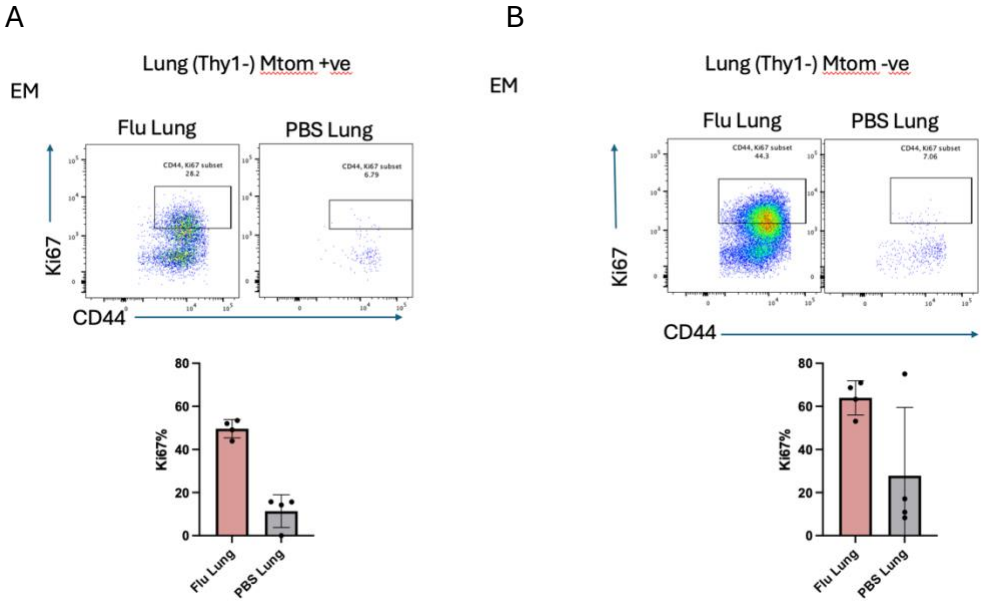


Figure 5: Ki67+ frequency showing increased proliferation levels of CD4+ EM T cells in the flu lungs. A) FACs plot and graph showing Ki67 expression of Mtom+ CD4+ EMs in the lungs **B)** Facs plot and graph showing Mtom- CD4+ EMs in the lungs.

4. Discussion

It is well established that upon infection, naïve CD4+ T cells become activated and proliferate [7]. Following these initial steps, they can differentiate into memory and effector memory CD4+ T cells [7]. This process is crucial for the adaptive immune response which is the specific tailored defence mechanism of the body that eliminates pathogens and establishes long term immunity.

Recently, there have also been studies investigating the impact of naïve CD4+ T cell age on their function and ability to survive. It was found that their ability to persist increases with age, however there may be a functional impairment with older naïve CD4+ T cells [5]. In our study, we sought to unlock another piece to this puzzle. At first, we found that the proportion of Mtom+ lung EM cells was drastically higher than Mtom+ LN naïve cells **(Figure 3a)**. This initially told us that the older cells were in fact responding better, seeing that the older naïve CD4+ T cells had differentiated, giving rise to a higher number of EMs than there were older naïve cells. However, the Mtom+ proportion of the flu-specific EM cells in the lungs was the same as the naïve CD4+ T cells in the LN **(Figure 3b)**. Therefore, we could safely assume that these Mtom+ flu-specifc EMs in the lungs were derived from the Mtom+ naïve cells in the LN. This informs us that cell age in fact does not influence flu response because the older naïve cells differentiated into flu specific EMs at a roughly 1:1 rate just like the Mtom -ve naïve cells.

A potential next question to ask is regarding the increased proportion of Mtom+ general EMs. Potentially we want to know why we saw an increase in them and not in the flu specific EMs and what mechanisms contribute to this. We can also ask what overall effect it has on immune responsiveness.

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