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Antigen presenting cells and cytokines in the differentiation of effector CD8+ T cell and tissue resident CD8+ T Cell responses to influenza.

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Antigen presenting cells and cytokines in the differentiation of effector CD8+ T cell and tissue resident CD8+ T Cell responses to influenza.

By

Paul Richard Dunbar

B.S., University of North Carolina, Chapel Hill 2011

Advisor: Dr. Jacob Kohlmeier PhD

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Doctor of Philosophy

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Immunology and Molecular Pathogenesis

2019

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CD8 effector T cell responses, and in particular lung resident memory CD8 T cells (T_{RM}) are critical for protection against respiratory viruses, but the cellular and cytokine interactions required for their development are poorly understood. Herein we describe roles for APCs and cytokines for the development of effector and tissue resident CD8 responses to influenza. Chapter 2 demonstrates the necessity of classical monocytes for the establishment of lung T_{RM} following influenza infection. We find that, during the initial appearance of lung T_{RM}, monocytes and dendritic cells are the most numerous influenza antigen-bearing APCs in the lung. Surprisingly, depletion of DCs after initial T cell priming did not impact lung T_{RM} development or maintenance. In contrast, a monocyte deficient pulmonary environment in $CCR2^{-/-}$ mice results in significantly less lung T_{RM} following influenza infection, despite no defect in the antiviral effector response or in the peripheral memory pool. IL-27 has pleiotropic effects on a range of immune cells, but the impact of IL-27 signaling on antigen-specific CD8 T cells responding to a respiratory viral infection has not been thoroughly studied. We utilized a direct competition model to compare antiviral CD8 T cell response in the presence and absence of IL-27 signaling following influenza infection. We find that while CD8 T cells lacking the IL-27 receptor (IL-27R) are competent to expand following stimulation *in vitro*, they exhibit a severe accumulation defect in both peripheral and lymphoid tissues during acute infection. Although this defect was supported by decreased proliferation in the lung and the development of fewer terminally-differentiated effector cells in the absence of IL-27 signaling, these observations could not fully account for the impaired expansion of virus-specific $IL\text{-}27R^{-/-}$ CD8 T cells. However, RNA-sequencing analysis of WT and $IL27R^{-/-}$ CD8 T cells revealed differential expression of genes involved in apoptosis. Furthermore, we observed that $IL\text{-}27R^{-/-}$ CD8 T cells entered apoptosis at a greater rate than their WT counterparts in both peripheral and lymphoid tissues. Together, these findings have identified novel mechanisms regulating the establishment and protective efficacy of CD8 populations. Applying these findings may aid in the development of a new vaccination strategy for enhanced CD8 T cell establishment and protection.

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Chapter 1:

The Role of APC's in the differentiation of Lung Tissue Resident Memory T Cells

The Adaptive Immune System

The adaptive immune system can mediate a protective response against pathogen challenges. Beyond just providing the initial clearance of a pathogen, the adaptive immune system mediates protection against future pathogen challenges expressing the same antigens. This enables the body to not only persevere through current insults, but prevent future ones from occurring. One well studied aspect of the adaptive immune response is humoral immunity, which produces antibodies specific to pathogen antigens (Chen et al., 2018). This is accomplished by a multistep process beginning with the development of a variety of B cells with differing antigen specificities followed by their maturation through a selection process eliminating self-reactive B cells (Hardy and Hayakawa, 2001). Their later activation is dependent on recognition of their cognate antigen in the presence of an inflammatory stimulus, and their ability to further refine their antigen affinity and class switching is dependent on receiving cytokine and surface signals from CD4 T cells specific for the same antigen (Barrington et al., 2005). This process produces a collection of antibodies specific for the pathogen that help clear the current pathogen and may protect against future infections. This is accomplished via multiple mechanisms including 1) Blocking the pathogen from infecting host cells, generally via steric hindrance of receptor binding sites required for pathogen entry into host cells, 2) Antibody-dependent cell mediated cytotoxicity which can lead to degranulation of macrophages, NK cells, neutrophils and eosinophils to effect direct cell

lysis; 3) Opsonization (coming from the Greek opsos to prepare for eating, or to spread with butter) of the pathogen leading to its uptake into a macrophage or neutrophil via phagocytosis; and 4) Inducing the complement cascade via the formation of an active site for the cleavage of complement proteins leading to their binding to the pathogen surface, opsonizing it further, as well as the release of factors to recruit more immune cells to the site (Ward and Ghetie, 1995). However, some pathogens, such as influenza, have a high rate of mutation. This high rate of mutation may be driven by the fact that influenza is a negative sense RNA virus, and thus lacks proof-reading mechanisms (Parvin et al., 1986). In the context of an adaptive immune response to the virus, this leads to an environment that applies selective pressure against viruses still bearing surface epitopes that the antibodies can bind to. Consequently, there is a selective advantage in favor of those that have mutated these surface epitopes such that antibodies no longer bind them. Thus, the pathogen may quickly escape this immune response and re-infect an individual (Yu et al., 2008).

Cellular Immunity

A further defense against such a possibility is mediated by the other arm of the adaptive immune response, cellular immunity. This method of defense whereby T cells identify and destroy cells bearing intracellular pathogens or their components is particularly important in the defense against viral pathogens, which must replicate within a host cell, only venturing outside of this area that is relatively shielded from immune surveillance to disseminate to other cells. Thus, it is of particular importance that these antigens are sampled by the host cell, with protein antigens being processed into short peptide sequences by the proteasome, and then loaded into major

histocompatibility complex one (MHC-I) in the endoplasmic reticulum (ER) for export to the cell surface where they can be displayed for recognition by T cell receptors (TCR) (Stanfield and Wilson, 1995; Williams et al., 2002). The importance of this immunity that gives it specific advantages over humoral immunity is that the peptides recognized can be internal, or non-structural to the virus, and thus can be from highly conserved sequences such as RNA polymerases or proteins used for coating the viruses genetic material. These proteins, which require certain sites for their function, are less likely to undergo the mutations required to evade this detection. This provides some protection against re-challenge by related but non-identical viral strains, (heterologous challenge) so long as the challenge expresses these same peptide sequences (Smith et al., 2019).

T Cell Development

Prior to reacting to a viral challenge however, T cells must be generated in a specialized process. For T cell development, thymocytes must first undergo a TCR-rearrangement so that each T cell produces a single TCR, producing a vast array of TCR sequences. This is accomplished both by the recombination of segments of the genome as well as random mutation of the joining sections of these segments. This creates a highly diverse repertoire, enabling the production of TCR able to detect a vast array of peptides. These precursor cells are produced in the bone marrow and then travel to the thymus where they undergo positive selection to ensure that they can recognize self MHC molecules, as well as negative selection to ensure that they will not become activated by MHC presenting host antigen and thus have an aberrant auto immune response. If a thymocyte does not receive the signal for positive selection it will cease developing and die from neglect, while thymocytes that respond to self MHC too

strongly, or MHC-host peptide complexes will undergo negative selection and apoptose, deleting them from the repertoire. While in the thymus, the T cell will also chose to express CD4 or CD8, based on which co-receptor gives it the proper MHC class binding affinity (Anderson et al., 1996). After this process, it will exit the thymus and begin circulating through the circulatory and lymphatic systems, scanning for presented antigen in lymph nodes. This selection against potentially harmful T cells is further refined by T cells in the periphery encountering their cognate MHC-Ag in the absence of an inflammatory signal, and becoming apoptotic or anergic. This removes the self-reactive T cell from the response pool. If however the antigen is recognized in the presence of an inflammatory signal, then that will constitute the second of three signals that will induce the T cell to activate and differentiate. The first signal being the TCR binding to antigen presented on an MHC, and the third being the detection of various cytokines that drive the differentiation of the T cell into a variety of subtypes (Starr et al., 2003).

T Cells may be divided into two major categories, the T helper cell CD4's and the cytotoxic CD8's. CD4 T cells recognize their cognate MHC-ag in the context of MHC class II, which limits their recognition of antigen to those displayed on professional antigen presenting cells and endothelial cells (Villadangos, 2001). CD4 T cells are not as directly cytolytic as CD8s, though they have been found to directly kill some target cells (Brown et al., 2016). They do produce a wide variety of cytokines to differentiate other cells, recruit more immune cells, and induce an anti-viral state in potential host cells by downregulating their transcriptional machinery. They not only produce a wider variety of these cytokines, but also are responsible for a larger proportion of their total

production than CD8s. These helper T cells are further subdivided into variety of specializations defined by the cytokines that induce them, the cytokines they produce, and the transcription factors that characterize their phenotype. These serve to direct specialized immune responses when specific sorts of threats are detected, allowing for a different response to be mounted against an intracellular virus versus an extracellular multicellular parasitic worm, allowing for greater efficacy and efficiency of immune response. These CD4 T cell subsets are summarized in Table 1.

CD4 T Cell Subset	**Inducing Cytokines**	**Characteristic Transcription Factor**	**Cytokines Produced**	**General Function**
Th1	IL-12	T-bet	it- γ, TNF- **α**	Defense against intracellular pathogens
Th2	IL-4	GATA-3	IL-4, IL-5, IL-13	Defend against multicellular parasites
Th9	TGF- β, IL-4	PU.1	IL-9	Protect from extracellular parasites
Th17	TGF- β, IL-6, IL-23	RORC2	IL-17, IL-21, IL-22, IL-25, IL-26	Protect from extracellular bacteria and fungi
Th22	IL-6, TNF- **α**	AHR	IL-22	Restrict commensals to their niche
Treg	TGF- β	FOXP3	IL-10, TGF- β	Maintain self-tolerance
Tfh	IL-6, IL-21	BCL6	IL-21	B Cell help

Table 1: Description of known helper CD4 subsets; adapted from Geginat, J *et al.* 2014 Frontiers in Immunology & Raphael, I *et al.* 2014, Cytokine (Geginat et al., 2014; Raphael et al., 2015).

The other major division of T cells are the CD8 cytotoxic T lymphocytes (CTLs). As their name implies, these cells seek out and cause the death of target host cells. Unlike CD4 T cells, CD8 T cells recognize their cognate MHC-Ag in the context of Major Histocompatibility Complex I (MHC-I) which is expressed by nearly all cell types, with the notable exception of erythrocytes. One of the advantages of this is that by being able to recognize conserved structures internal to a pathogen, a response may be established that is more broadly protective than that offered by humoral immunity. This reduces pathogen escape by mediating effective responses (Cheuk and Chamberlain, 2005; Reemers et al., 2012; Townsend et al., 1986). After being activated by recognition of their cognate MHC-Ag in an inflammatory context, a CD8 cytotoxic T cell will then go on to kill future cells it recognizes in this way, even in the absence of another inflammatory signal. This cytotoxic effect can be brought about via two different paths. 1) A synapse may form between the CD8 and the target cell, and degranulation of the CD8 cell at this synapse will release both perforin and granzyme B, with the perforin self-assembling in the target cell membrane to mediate the entry of the serine protease granzyme B into the cell where it will directly induce the apoptosis of the target cell (Braciale, 1977; Eichelberger et al., 1991; Yewdell et al., 1985). 2) CTLs can also upregulate FAS ligand on their surface, which, when bound to FAS receptor on a target cell will induce the cleavage of caspase 1 to expose its active site, leading to the apoptosis of the cell. This apoptotic process will also result in the release of IL-1β a danger signal that may further inflame the local environment and mediate a further response (Russell and Ley, 2002). Lastly CTLs also produce cytokines that affect the activation state, anti-viral state and recruitment of other cells, they produce less of these cytokines than their

CD4 counterparts, but are still important in the development and modulation of immune responses.

T Cell Activation

If a T cell is to be activated to mediate a protective response and not become anergic, its activation is a multi-step process with multiple checkpoints to ensure that aberrant or harmful activation does not occur. In the context of an acute infection that has already activated APCs via inflammatory cytokines or their own pathogen sensing proteins, (discussed later) antigens will have been taken into the APCs via phagocytosis, processed in the phagosome and presented on MHC-II. Antigens may also be "cross-presented" whereby they are released into the cytosol for processing by the proteasome for loading onto MHC-I and presented (Barnaba, 2013). If a naive T cell TCR binds a MHC-Ag complex this will provide signal one of the activation sequence (Dustin, 2008; van der Merwe and Davis, 2003). If this is the only signal, the T cell will apoptose or become anergic. If however the T cell is co-stimulated via T cell surface CD28 interacting with surface CD80 or CD86 on an activated APC, then this will be sufficient to activate the T cell to both gain effector function and proliferate. This is the second signal of T cell activation, and is necessary for the creation of long lived memory T cells (Acuto and Michel, 2003). At this point CD4 cells produce IL-2, which as pro-proliferative cytokine makes a positive signaling loop whereby the cells divide and produce more IL-2, inducing more division (Weninger et al., 2002).

After the activation and proliferation of T cells comes the next phase, contraction of the T cell pool. The initial activation creates many short-lived effector T cells (SLECs) characterized by their expression of the terminal differentiation marker KLRG-1 as well

as the absence of cytokine receptors for homeostatic interleukins. As these cells will lack the ability to detect homeostatic signals, these will be the first cells to apoptose during the resolution of an immune response (King et al., 2012). In contrast, the memory precursor effector T cells (MPECs) are characterized by their expression of CD127 (IL-7 receptor), and will further differentiate into multiple memory T cell subsets (Joshi et al., 2007). These subsets include central memory, effector memory, and possibly resident memory, though this last point is currently unknown; it is a reasonable assertion. Central memory T (T_{CM}) cells, which express the chemokine localization factor CCR7 as well as CD62L, will home to and reside in the secondary lymphoid tissues. Most of these central memory T cells will accumulate in the draining lymph node of the site of infection. Some T_{CM} will also reside in the white pulp of the spleen (Sallusto et al., 2004; Sallusto et al., 1999). These cells have a great proliferative capacity upon re-encountering their antigen and will provide a robust response upon re-challenge. In contrast, effector memory T cells express markers of antigen experience such as CD44 like central memory T cells, but lack the localization factors like CCR7 and CD62L, and thus circulate through the blood and lymph systems. This provides peripheral surveillance in the case of re-infection (Sallusto et al., 2004). They will also transiently enter tissues, especially inflamed ones, which is important for early responses to infection. These cells have poor proliferative capacity, but upon activation display high cytolytic activity as well as high cytokine production, effecting a rapid response upon re-infection that contain the spread of the pathogen to the initial site (Mueller et al., 2013; Schenkel et al., 2013). In addition, recent findings from the Von Adrian lab indicate that there may be another “peripheral vasculature” anatomical compartment for T cells that are limited to peripheral circulation. These T cells are typified by intermediate CX3CR1

expression, in contrast to effector memory with high CX3CR1 expression. Though what immunologic advantage such as anatomic limitation may provide is less clear. These T cells limited to the vasculature may mediate some peripheral surveillance, or surveillance specific to endothelial cells (Gerlach et al., 2016). A schematic of T cell differentiation into these subsets and some key factors in driving these individual fates is provided in Figure 1 below.

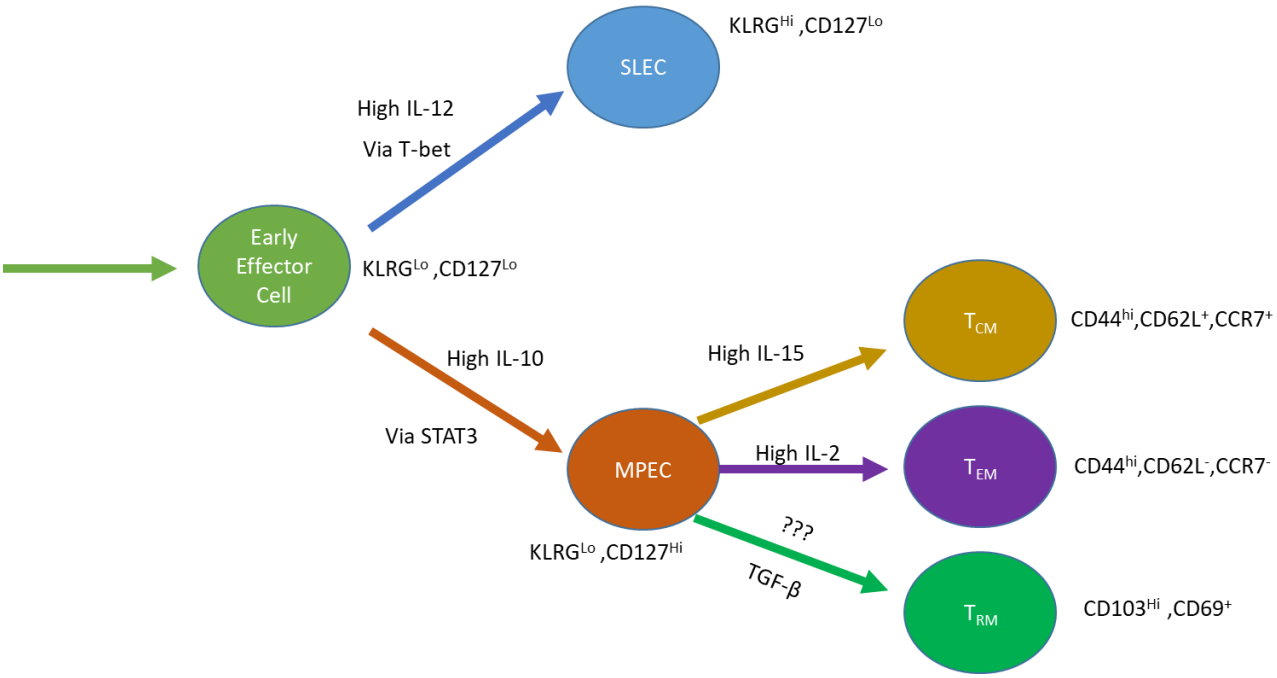

Figure 1. Schematic of T cell differentiation and major cytokine factors governing this differentiation. Figure adapted from information in Klonowski K.D. et al. Journal of Immunology 2006.

IL-27 as a cytokine of interest

IL-27 is a heterodimeric cytokine in the IL-27 cytokine family. It is composed of two distinct genes, Epstein-Barr virus-induced gene 3 and IL-27p28 (Villarino and Hunter, 2004). IL-27 appears to be produced by monocytes and

dendritic cells in response to a variety of TLR stimulation. TLR9 engagement induces the expression if IL-27, though it also is produced in response to LPS signaling on TLR2, indicating it may be produced in response to most TLR activity (Hu et al., 2008).

In parainfluenza Sendai infections, IL-27 has been found to be present in the lung days 6-10 post infection with a sharp peak around day 7 (Muallem et al., 2017). The IL-27 receptor is a heterodimeric receptor consisting of IL-27α and gp130. Engagement of the receptor with IL-27 leads to signaling via Jak1, Tyk2, Jak2 and eventual signaling through STAT1, STAT3, and STAT5, leading to a variety of downstream gene expression patterns that appears to be highly dependent on the infectious context (Chen et al., 2019). This context is informed in part by what cells are present during the production of IL-27 as its receptor is known to be expressed on monocytes, dendritic cells, NK cells, CD4 T cells and CD8 T cells(Chong et al., 2015; Laroni et al., 2011).

While our focus in Chapter 3 is on the action of IL-27 on CD8 T cells, far more is known about the function of IL-27 in signaling on CD4 T cells. Broadly, IL-27 acts to both drive differentiation as well as immune-suppression of CD4s. It does this by augmenting T-bet expression, leading to an increase in Th1 polarization and a concomitant decrease in Th17 differentiation (Fitzgerald et al., 2007; Peters et al., 2015). As might be expected from these contradictory roles, the desirability of IL-27 action on CD4s during an infection is context dependent. In the case of tuberculosis infection, IL-27 appears to help prevent clearance of the bacteria, contributing to chronic infection (Abdalla et al., 2015). However in parainfluenza Sendai infection models IL-27 appears to lower CD4 activation and consequently reduce neutrophil influx, preventing excessive immunopathology while not affecting clearance of virus (Liu et al., 2014; Muallem et al.,

2017). In our models, IL-27 signaling appears to have no effect on clearance of influenza virus. In the context of chronic antigen stimulation such as cancer, IL-27 may be important for allowing continued division of CD8 and CD4 T cells via upregulation of IRF1 to avoid cell death, though as seen in Chapter 3 we do not find that to be the case in an acute infection model(Karwacz et al., 2017).

Tissue resident memory T cell

While the effector and central memory subsets have been the most studied in T cell memory, it has emerged that there exists a third distinct subset, that of the tissue resident T memory cell (T_{RM}). This cell is distinguished by the fact that it neither resides in lymphatic tissues nor circulates through the periphery, but remains in the tissue at the site of infection (Ibraghimov and Lynch, 1994; Masopust et al., 2010). Depending on the tissue, these T_{RM} may be long lived, presumably homeostatically proliferating, or the population may decline over time (Park et al., 2018). Given their localization, these cells would be ideally placed to be sentinels or first responders to re-infection. This is a simple but powerful idea best illustrated by the contrast between a T cell, already localized in the tissue being able to survey and respond to newly infected epithelial cells versus a T cell of the same specificity that must first be drawn into the tissue by the inflammation driven by an already developed infection. This makes it obvious that for T cells, lacking the action at a distance capability of B cells, location is of supreme importance. And indeed they have been found to have a role in limiting the expansion of pathogens past their initial entry point as well as reducing immunopathology (Mueller et al., 2013).

It is already known that CD8 T_{RM} are correlates of protection against infection of the tissues they reside in. Multiple studies have demonstrated that the number of CD8 T_{RM} present in a tissue is predictive of the ability to reduce pathogen load upon re-challenge (Ariotti et al., 2012; Masopust and Schenkel, 2013; Schenkel et al., 2013). Depending on the tissue, T_{RM} have specific chemokine receptors or adhesion molecules including integrins that determine their tissue specificity and allow their extravasation into that tissue. These may also be factors in the T_{RM} residing in the tissue long term (Masopust et al., 2010). The two best studied examples of this phenomenon in specific tissues are the gut and the skin. Gut T_{RM} express integrin α4β7 and the chemokine receptor CCR9, while skin T_{RM} express the chemokine receptor CCR10 and adhesion molecule cutaneous lymphocyte-associated antigen (CLA). Gut localization of T cells is mediated by interactions between integrin α4β7 and its ligand MAdCAM-1. The interaction between integrin α4β7 and its ligand MAdCAM-1 has been found to important in mediating pathology in HIV infected individuals. Skin T_{RM} have been found to protect against local infections including herpes simplex virus. In addition, these specific tissue homing markers are induced by specific signals. In the case of gut localizing T cells, dendritic cells expressing retinaldehyde dehydrogenase (RALDH) produce retinoic acid (RA) via their action on Vitamin A. This signal induces the upregulation of integrin α4β7 on target cells. Likewise, T cells exposed to active vitamin D (calcitriol) will upregulate CCR10 and acquire a skin homing phenotype. This points to the importance of immune signaling in the establishment of specific T_{RM}, a topic that will be addressed in detail below (Berg et al., 1991; Gebhardt et al., 2009; Sigmundsdottir and Butcher, 2008). There are also tissues for which we know T_{RM} are important, but have an incomplete understanding of their tissue specific induction. In

the female reproductive tract (FRT), it has been found that CXCR3 is important for CD8 T_{RM} formation, but what molecule is signaling to this receptor, or any receptors specific for FRT localization have yet to be found (Singh et al., 2016; Zaric et al., 2019).

Lung T_{RM}

Despite all of these studies of T_{RM}, there is much we do not know about lung T_{RM} specifically. While specific homing factors and chemokine receptors have been found to characterize both gut and skin T_{RM}, no specific surface marker has been found to differentiate lung T_{RM}. Instead lung T_{RM} are characterized via their expression of CD69 and CD103, though parabiosis studies have shown that these are not expressed on all lung T_{RM} (Bankovich et al., 2010). Another method for identifying lung T_{RM} is intravenous antibody labeling, whereby fluorescently conjugated antibodies are injected intravenously into the mouse before the harvest of the lungs, which leads to cells in circulation and more importantly in the micro-vasculature of the lungs to be labeled and later detected via flow-cytometry. This allows lung T_{RM} to be distinguished despite the fact that many systemic T cells are found in the lung as it is a highly vascularized organ. Many T cells in the lung may express some residency markers but actually be in the capillary beds. As these are accessible to the intravenous antibody they will be labeled, enabling this technique to distinguish them from bona-fide tissue resident cells. Likewise, a T cell may not express residency markers but may prove to be enmeshed in the tissue and thus inaccessible to the antibody (Anderson et al., 2012). This shows the power of this technique to delineate tissue resident versus non tissue resident T cells, especially without prior knowledge of tissue specific markers. T cells may also be located in the airway itself and harvested in bronchiolar lavage fluid, and previous studies have

indicated that T cells go to the airway as a one way trip (Skon et al., 2013). These airway resident T cells are isolated by inserting a catheter into the mouse trachea and repeatedly filling the lungs with about 1 microliter of cell growth media, then withdrawing that media into a syringe for collection, thus physically washing the cells out of the airway. The gold standard for the determination of T_{RM} of any sort are still parabiosis experiments, where the vasculature of one mouse is joined to another congenic mouse, and thus their circulating cells will reach equilibrium, while tissue resident memory cells will not be found in the congenic partner (Jungi and Jungi, 1981).

In studies performed before IV labelling became an accepted technique for distinguishing tissue resident cells, the two markers most commonly used to define tissue residency were CD69 and CD103. CD69 is a C-type lectin marker that is expressed quickly upon T cell activation (Casey et al., 2012; Park et al., 2016). On all T cells this has been found to be important for CD69's antagonistic effect on sphingosine 1-phosphate receptor-1 (S1P1), which mediates extravasation and lymphoid homing. In T_{RM} especially, S1P1 expression would likely lead to the cell mobilizing out of the tissue and back into the lymphatics, and it follows that CD69 expression is constant on most T_{RM}. Though CD69 is not required for the formation of all T_{RM} (Walsh et al., 2019). CD69 antagonizes S1P1 via the down regulation of S1pr1, which is the gene encoding S1P1 (Skon et al., 2013). This allows for stringent control at the transcriptional level. The transcription factor Kruppel-like factor 2 (KLF-2) also plays a role, being downregulated upon activation and leading to a reduction in S1P1 as well as allowing CD69 expression. In the case of T_{EM} and T_{CM}, KLF-2 will increase, allowing the cell to exit the tissue once more, but in resident T cells this increase does not appear to occur, leaving the cell

resident in the tissue. Expression of CD103, the integrin subunit αe, leads to the formation of a heterodimer with integrin β7 in mucosal tissues that allows the cell to bind to E-cadherins in adherin junctions on the basal side of the epithelium (Pauls et al., 2001). But as later studies using intravenous labeling have discovered, not all tissue resident T cells express these markers, some express CD69 alone, some express CD103 alone and some have neither (Steinert et al., 2015). Despite this, our lab finds that T cells expressing the surface markers: CD8, CD44, CD103 and CD69 are found only in the resident compartment that is protected from intra-vascular staining, and not systemic compartments that stain with intra-vascular labels (Dunbar et al., 2019). This allows the use of these markers for particularly stringent analysis of tissue resident memory cells. This is in contrast to lung CD4 T_{RM} which lack the high expression of CD103 found on their CD8 counterparts (Wilk et al., 2017). It has been found that in other tissues such as the brain and kidney, CD8 T_{RM} also lack CD103 expression, though more study may be required to find if this is truly generalizable to the tissue as a whole, or the specific stimulus used to induce CD8 T_{RM} differentiation (Walsh et al., 2019). This difference could be due to the specific milieu of adherens junctions present in the tissue, as micro-environments low in E-cadherins offer no ligand for CD103. Or it may indicate the reliance on other factors for tissue residency in those tissues. It is also of note that there is the possibility of a population of tissue resident memory cells in the lymphoid tissues such as the lymph nodes and spleen, however these are particularly difficult to discern via intravenous labeling due to the lack of vascularization of the former and the effective hyper vascularization of the latter. However, memory T cells expressing CD69 and CD103 are found in these organs, making the idea reasonable. In addition, parabiosis experiments have shown that memory CD8 T cells in these tissues

can *lack* CD103 and CD69 expression but still exhibit residency at the same rate as their counterparts that express both(Beura et al., 2018; Schenkel et al., 2014). In sum this leads to our current understanding that while cells positive for CD103 and CD69 are generally resident T cells, cells lacking these markers are not necessarily systemic.

As discussed, the pairing of chemokine receptors and adhesion molecules can mark tissue specificity such as in the gut or skin but these factors are as yet unknown in many other tissues. However a recent characterization of transcription factor expression has suggested that KLF2 (Kruppel-like factor 2), a zinc finger which regulates expression of CD62L and S1P1 is down-regulated in T_{RM} across all examined tissues, which may offer another way to identify tissue resident T cells without needing a parabiosis model (Mackay et al., 2013; Skon et al., 2013).

As always, beyond phenotypic markers what really matters is the effect of a cell during an infection, and this offers another way to define T_{RM} populations. Direct transfer of cells into their supposed tissue of residency may allow for the demonstration of sufficiency of protection, and this has been done for airway CD8 T_{RM} (Mackay et al., 2012). However, there are a series of significant technical hurdles that must be overcome with this technique for each tissue, beginning with the efficient isolation of supposed tissue resident cells from an organ in sufficient numbers, and without altering their characteristics through the isolation process. The second hurdle is in the ensuring their localization, as a terminally differentiated tissue resident memory cell may not be able to traffic back to its tissue, or the location may be difficult to apply cells to in an animal model. Studies on relatively easy to access populations such as the lung airways and FRT have indicated that in both models, antigen specific protection is mediated by

these cells producing effector chemokines and cytokines to establish an effective anti-viral state upon secondary challenge (Schluns and Klonowski, 2015). While this work has many hurdles to overcome, it is fertile ground for more detailed studies of T_{RM}.

Establishment and Maintenance of T_{RM} Cells:

As discussed earlier, during an immune response, effector T cells that were activated in the draining lymph node are drawn into peripheral tissues, moving along chemokine gradients that have been produced by innate immune cells in response to inflammation and pathogen and damage associated molecular patterns (PAMPs and DAMPs) (von Andrian and Mackay, 2000). Over the course of infection as more immune-suppressing factors come to bear this number of T cells recruited to the tissues will wane along with inflammation and hopefully the local infection. In contrast to this general theme, a sub population of T cells which will go on to become T_{RM} cells traffic to the tissue, maintain themselves locally via homeostatic proliferation and do not return to the circulating pool (Bevan, 2011; Kundig et al., 1996). At present it is known that T_{RM} are generated via an imprinting, licensing or education process by which they gain a unique set of chemokine and adhesion molecules that will mediate their homing and residence in a particular peripheral tissue. It appears that a combination of exposure to factors in the tissue itself (the previously mentioned imprinting) as well as specific signals given by antigen presenting cells that vary based on the tissue they migrated from (licensing and education) combine to direct T cells to tissue specific fates.

However, understanding how this occurs in the case of each tissue represents a fundamental gap in the knowledge of the field, and no general theme appears to be true for all or even most T_{RM} subsets. To more effectively study this issue, the strategy of

"pulling" systemic effectors to areas where resident memory formation is to be studied have been developed and used by our lab to ascertain what signals the T cells need to differentiate into T_{RM}. This is a system where effector T cells may be generated in the periphery, then drawn into the tissue of interest by giving an inflammatory stimulus to the tissue. This inflammation may be combined with other signals such as cognate or irrelevant antigen to test the effect of further signals on the differentiation of CD8 T_{RM} (McMaster et al., 2018).

As an example of the model in use, studies of the FRT T_{RM} demonstrated that transient chemokine signaling to systemic effectors was enough to both draw them to the tissue and have them establish residency, even without their specific antigen or the tissue being inflamed(Casey et al., 2012). This contrasts with models in the skin where a long lived T_{RM} population could be established, but it was found that inflammation was necessary to draw the systemic effectors to the site, have them establish residency and mediate protection. Currently the full suite of required stimuli for the induction of lung parenchyma and airway T_{RM} are unknown, but some of our recently published data are discussed later in reference to this issue (Mackay et al., 2012).

Lung and Airways:

The lung and its functions are critical to life, but its highly organized and specialized structure is also delicate, both physically and in terms of the damage that pathogens or inflammation may cause. Further compounding this, is that the lung is topologically on the outside of the body, a mucosal surface that interacts with the environment with every breath. Each breath has the potential to bring injurious material into the lung, but the lung needs to avoid unnecessary inflammation and

immune mediated pathologies. Thus, the responses in the lung must be simultaneously robust and tightly controlled (Crosby and Waters, 2010; Ternesten-Hasseus et al., 2011). Airway and tissue resident CD8 T cells represent one way that these goals can be met simultaneously, to effect rapid control of a pathogen, limit its spread and prevent aberrant inflammation. However the existence of these cells at all poses many questions, as the lung airway is a difficult environment for cells to exist in, because despite the lungs being highly vascularized for the exchange of oxygen and carbon dioxide, the airways are nutritionally poor and energetically poor, featuring much lower glucose concentrations than the blood (Baker et al., 2007; Helassa et al., 2014). Thus, the metabolism and maintenance of lung T_{RM} pose many problems, making autophagy a likely source of energy during the lifetime of a cell in the airways, and effectively limiting their time there. Corresponding to this, airway T cells have been found to have lower cytolytic function, but this function may be recovered if the cells are put at general physiological oxygen tension, indicating the difficulties of the airway in terms of high oxygen tension (Vuillefroy de Silly et al., 2015). Even some innate host defenses such as the surfactants found in the mucus that forms a protective layer for the lung suppresses T cell proliferation. Despite all of these difficulties and as yet poorly understood factors lung T_{RM} appear to play a key role in defense against viral challenges (Wilsher et al., 1988a, b, c; Wilsher et al., 1990; Yao et al., 2001).

Health impact and pathogenesis of influenza virus

Respiratory viral infections are a serious source of morbidity and mortality the world over, with influenza virus hospitalizing 200,000, and killing 30,000 people per year in the United States alone. Beyond this, influenza also exacerbates a variety of other

conditions including asthma, cystic fibrosis, and chronic obstructive pulmonary disease (Glezen, 1982). This already significant problem also appears to be worsening, with the current economic burden of yearly influenza epidemics reaching 87.1 billion per year. More recently, the 2009 pandemic H1N1 swine flu infected 11-21% of the world's population before vaccination efforts (Centers for Disease and Prevention, 2010; Dulek and Peebles, 2011; Hsu et al., 2012; Thompson et al., 2004; Wat et al., 2008; Zhou et al., 2012). As global travel and transport of goods increase it is reasonable to expect that the rapid dissemination of novel viruses is only going to increase. This demonstrates the importance of research into all aspects of how defenses may be erected against these diseases, and in particular highlights the need for a broadly applicable protective strategy, as opposed to yearly vaccinations against predicted strains. This more broadly protective response may be mediated by T cells as described above, and in particular the establishment of tissue resident memory T cells in the lung to mediate a rapid response (Armstrong et al., 1999; Molinari et al., 2007; Simonsen et al., 1998).

Influenza as a tool for studying T cell activation and differentiation

For our purposes, influenza virus in mice is used to elicit an anti-viral CD8+ T cell response, and some context for the viruses adaptation to mice and the different viral strains used will be useful. While not all influenza strains will infect commonly used lab mice, both lab adapted H1N1 A/Puerto Rico/8/1934 (PR8) as well as H3N2 A/Hong Kong/1/1968 (X31) will infect C57BL/6 mice. In our experimental system X31 influenza virus produces significant but not fatal disease, causing significant weight loss in infected C57BL/6 mice. PR8 influenza is more highly pathogenic, capable of causing the death of naïve mice at relatively low doses, and providing an immune challenge suitable

for death curves with vaccinated mice at higher doses. It is notable that influenza virus attaches to sialic acid moieties with an (alpha)2,6 linkage, and these viral attachment sites are in the upper airways in humans. In mice however, these sites are abundant in the airways, which may lead to a difference in pathology seen in mouse models of influenza, as the influenza infects lower in the respiratory tract than in human influenza cases. In addition the susceptibility of the mice to influenza in general may be due to their lack of Mx1, an important anti-viral protein that has a role in preventing influenza infection of cells (Bouvier and Lowen, 2010). For the context of this work it is important to note that infection of mice with intra-nasal X31 influenza leads to the production of CD8 T cells protective against virus containing the Flu nucleo-protein epitope. This epitope is shared by PR8 influenza. This allows a system where infection with X31 influenza leads to protection against PR8 influenza, despite the fact that X31 influenza infection does not generate effective antibodies against PR8 influenza (McMaster et al., 2018). This allows for a system of generating and testing the protective response provided by T cells specifically.

Establishment of Airway and Lung Tissue CD8 T_{RM}:

The similarity of surface markers found on both tissue resident and airway CD8 T_{RM} lead us to suppose that they are from a similar origin rather than independently arising populations. But currently it is unclear whether their differentiation is occurring in the lung tissue itself, or if they are being conditioned in the mediastinal lymph node for later travel to and residency in the lung. However, some preliminary data appears to indicate that airway CD8 T_{RM} may be coming from the tissue CD8 T_{RM} (Hogan et al., 2001; Kohlmeier et al., 2007; Kohlmeier and Woodland, 2009; Marshall et al., 2001).

The challenges for vaccine design lie in elucidating the factors that lead to the induction of cells that can localize to likely sites of infection. To find these, experiments must look for the sufficiency and necessity of certain stimuli to lead to long lived T_{RM} populations. It has been found by our lab that the route of infection is of particular importance for lung tissue and airway T_{RM} to be established (McMaster et al., 2018; Takamura et al., 2010). While mice infected intramuscularly or intraperitoneally with influenza virus do develop a robust and antigen-specific T cell response to the virus, this is insufficient to generate lung and airway CD8 T_{RM}. However, if this peripheral infection is followed by an intranasal infection with the same influenza virus, lung and airway CD8 T_{RM} will be established. This shows that original antigenic sin is not preventing the establishment of these cells in a new tissue despite the previously generated systemic effectors. In addition, memory CD8 T cells from an intranasally infected mouse will preferentially localize to the lung and airways compared to CD8 memory T cells generated in an intraperitoneally infected mouse. These influenza specific CD8 T cells generated by intra-nasal infection establish populations in the lung sufficient for a long- lived memory response, permitting continual steady-state cell recruitment to the airways independent of the presence of their cognate antigen. The influenza virus will be cleared by day fourteen post-infection, but the influenza specific T cells in the tissue are detectible at least ninety days post infection. This data from mouse studies is backed up with multiple studies showing that T cells specific for respiratory viruses are more prevalent in the lung than T cells for non-respiratory pathogens in humans (Liang et al., 1994; Wu et al., 2014).

In addition to the roles described for both CD69 and CD103 in the formation of tissue resident memory CD8 T cells, CD49a (VLA-1) has been proven necessary for the CD8 T_{RM} to provide a protective response in the lung parenchyma (LP) and airways on secondary virus challenge, but its mechanism of action in this role is unknown (Ray et al., 2004).

The innate immune systems links to CD8 T cell activation and differentiation

The innate immune system guides the development of the adaptive response, and our interest lies particularly in APC's in their role in activating and differentiating T cells (Jain and Pasare, 2017). While other cell types can activate CD8 T cells, only APCs express the MHC-II required for the activation of CD4s. In addition, while CD8's can be activated by other cell types, APCs do so more efficiently, and in general are more responsible for driving CD8's to particular fates other than short term effector function (Feau et al., 2012). Given their importance for T cell activation and memory formation it would be reasonable to suspect that APCs have a role in the formation of T_{RM} as well. In addition to this, our finding that antigen is required for the establishment of lung CD8 T_{RM} further indicates that APCs are likely to be involved in the differentiation of T_{RM}. Further, the timeline indicates that it is likely this presentation is occurring in either the lung or the lung draining mediastinal lymph node. Other groups have found that the establishment of T_{RM} is dependent on TGF-β signaling inducing down regulation of T-bet and contemporaneous up-regulation of CD103. This appears to happen independent of the canonical TGF-β signaling pathway which goes through SMAD4 (Sma- and Mad-related protein 4) leaving it an open question what receptor/s

and signaling mechanisms mediate this effect (Hu et al., 2015). In addition interaction with APCs which generally display a higher density of antigen-MHC complexes better induce up regulation of CD69, which has a well described role preventing T cell return to the lymphatics (Skon et al., 2013). CCR7 is also down regulated via interaction with APCs and these factors combined inhibit chemotaxis to the lymph nodes, thus increasing tissue retention of these cells. In the lung antigen specific CD8's enter the tissue between days 6 and 10 post infection with upregulation of CD69 and CD103 occurring in the latter portion of this time period (Chapter 2).

Dendritic Cells:

As will be discussed in much greater detail in Chapter 2, we felt it likely that dendritic cells could play a key role in the generation of lung CD8 T_{RM} given that dendritic cells are the most efficient stimulators of T cells, as well as a cell type that already has a known role in promoting the effective response to an influenza infection as described by work in the Braciele lab (Braciale and Kim, 2011; Hao et al., 2008; Kim and Braciale, 2009; Steinman, 2001). Three main subsets of DC's in the lungs appear in the literature, and each appears to have a particular importance to the control, resolution, and defense against influenza. CD103+ DC's as may be inferred by their expression of CD103 tend to localize in the lung tissue itself, and have been found to be of particular importance for trafficking antigen from the lung back to the mediastinal lymph node for presentation (Kim and Braciale, 2009). In the absence of these cells the magnitude of the T cell response is reduced, and viral clearance is inhibited. In addition to this, in vitro studies have found that CD103+ DCs are efficient promotors of CD103 expression in the T cells they activate, though this has not been demonstrated in vivo. CD103+ DC's

also displayed elevated levels of CD24, and this contributed to the activation of T cells into T effector cells via HMGB-1 mediated engagement of T cell receptor for advanced glycation end-products (RAGE) (Kim et al., 2014). In addition, it is this CD103+ subset that delivers non-infectious viral vaccine antigen to the lymph node for a response to be induced, making them of particular importance in vaccine design. In the lung itself another DC subset, $CD11b^{hi}$ DCs are considered potent primers of CD8 effectors against influenza, though their lower expression of CD24 as compared to CD103+ DC may contribute to the fact that $CD11b^{hi}$ DC stimulated T cells are more prone to adopting a central memory T cell surface expression pattern. Lastly monocyte like respiratory dendritic cells have been noticed and described in the lungs based on their low expression of MHC-II (Kim and Braciale, 2009; Kim et al., 2014). Our lab has found that they contain a significant amount of FluNP antigen during infection (Chapter 2), but currently their specific role in influenza infection is unknown. In addition, all of the dendritic cells discussed have been found to support influenza infection themselves, though not to the same degree. Though presumably the virus may cause some dysfunction of the DC, it is also possible that the DC's detection of its own infection may enable it to more effectively stimulate T cells to an appropriate anti-viral action. Likewise, infected dendritic cell would be able to present viral proteins on MHC-I without the added step of cross presentation via autophagy or other methods. However, the action of the virus upon the dendritic cell may make infected DCs less effective at creating a potent CD8 response (Smed-Sorensen et al., 2012). All of the cell subsets discussed may have a role in providing the signals for CD8's to differentiate into long lived lung tissue resident memory, but it is still unclear which subsets may be the most important or what signals beyond TGF-β they may be providing.

Previously, a developing idea in the field was that of dendritic cell vaccines, whereby a person could have their dendritic cells or other APC's isolated from their blood, and cultured with antigen and cytokines in such a way as to provide a potent dose of efficient immune activating cells. This would avoid the need for them to see inflammatory signals in the host, limiting immune pathology, and entirely bypassing the potential need for an attenuated virus. This would have combined the benefits of a killed virus or peptide-based vaccine approach (low risk to the host) with the benefits of attenuated virus vaccines which generate adequate T cell responses in addition to B cell responses (Mastelic-Gavillet et al., 2019). This idea has fallen out of favor mostly due to the still high cost of personalized medicine that the use of autologous cells demands. However, a recent study has found another angle on this idea, the use and manipulation of DC's in a vaccine. In this work dendritic cells were targeted with antibodies that had antigen attached to their Fc segment. This resulted both in efficient delivery of antigen to the DCs as well as a robust T cell response (Wakim et al., 2015). This proof of principle may lead to more refined methods in the future, whereby more than just delivery of antigen may be manipulated, but signals influencing cell activation differentiation may also be incorporated.

Monocytes:

Monocytes circulate systemically through the blood, which differentiates them from macrophages which are defined as residing in the tissues. While some monocytes may develop into macrophages, most macrophage populations are self-replenishing, relying on homeostatic proliferation in the tissues rather than recruitment from the blood. Inflamed tissues may release chemotactic signals such as CCL2, activating

circulating monocytes that are then recruited as pro inflammatory monocytes typified by the expression of Ly6c and CCR2 into inflamed tissues where they mediate multiple roles including the production of pro-inflammatory cytokines, phagocytosis of opsonized bacteria and viruses, production of ROS, and possibly antigen presentation to T cells drawn into the inflamed tissues. The monocytes may transition into an anti-inflammatory phenotype as the immune response progresses. In this they will begin to express fractalkine receptor (CX3CR1) and become less chemotactic to CCL2 as they lose CCR2 expression (Geissmann et al., 2010). This will solidify their localization in the inflamed tissue as they switch from the production of pro-inflammatory cytokines to TGF-β which can drive Treg differentiation and expression of IL-10. In this way they help in both the initiation and driving of an immune response as well as its resolution, and as is discussed below may play a role in the transition of T cells to a long-lived memory phenotype. In murine models, monocytes may be broadly classified into two subsets as either classical pro-inflammatory CCR2+/Cx3CR1- $Ly6c^{Hi}$ or non-classical anti-inflammatory CCR2-/CX3CR1+ $Ly6c^{Lo}$. [14] These monocytes, in particular classical monocytes, have been found to be drivers of immune mediated pathology during influenza infection, but have not been found to be of particular importance for the clearance of influenza virus (Lin et al., 2008; Wareing et al., 2007). Inflammatory monocytes mediate immune pathology via the production of a variety of pro inflammatory cytokines as well as facilitating the recruitment of immune cells including neutrophils. However previous studies have only examined the acute response of *Ccr2*$^{-/-}$ mice, which are deficient in the recruitment of classical monocytes to the lung following influenza infection. In addition, these studies did not look in detail at the phenotypes of T cells produced, nor their tissue residency state. Thus, the potential role of monocytes

on the generation of lung-resident memory T cells is unexplored (Jakubzick et al., 2017; Larson et al., 2016). Given this gap, as well as our preliminary data indicating that monocytes are the primary influenza antigen-bearing cells in the lung during the appearance of T_{RM}, we have performed preliminary experiments in *Ccr2*$^{-/-}$ mice to investigate the establishment of lung T_{RM}. Wild-type (WT) and *Ccr2*$^{-/-}$ mice showed equal numbers of flu-specific CD8 effector T cells in all tissues at the peak of the acute response at day 10 post infection. In contrast, at day 45 post-infection there is a significant decrease in the number of flu-specific memory CD8 T cells in the lung-resident compartments of *Ccr2*$^{-/-}$ mice compared to WT mice. Importantly, there is no difference when we compare the number of flu-specific memory CD8 T cells in the systemic (circulating) compartment of the lung or the MLN. This finding indicates that monocytes are likely an important driver of the differentiation of T_{RM} in the lung and warrant further investigation. In addition, non-classical monocytes which express factalkine receptor CX3CR1 are known producers of TGF-β , aligning them with a potential role in the canonical story of TGF-β inducing CD103 expression on T cells, providing an important componet of their resident phenotype (van Leeuwen-Kerkhoff et al., 2017).

Tools for Studying Lung T_{RM}

The techniques that can be brought to bear for the study of lung tissue and airway T_{RM} have grown by leaps and bounds in the past few years. Multiple in vivo models have been developed, and they have the advantages of including the full mileu of signals that the T cell would receive as it enters lung tissue, which is itself made of a variety of cell types, and interacts with multiple types of innate immune cells. In vivo models are important precisely because the actual system is so complex that any in vitro models are

inherently grossly reductive. While parabiosis remains the gold standard experimental technique for identifying T_{RM}, these studies have demonstrated that other markers such as CD69 and CD103 can be used in place of the difficult and time consuming parabiosis process. Since these studies also helped determine that CD69 and CD103 were not by themselves perfect predictors of tissue residency or systemic localization, the use of intravenously injecting a fluorophore-conjugated antibody prior to animal sacrifice and organ harvest has been important in delineating truly tissue resident cells (Steinert et al., 2015). This technique has given similar results to parabiosis models, delineating cells that are in the tissue versus those in either the systemic circulation or the lung microvasculature such as the capillary beds. It should be noted however that recent studies using fluorescence microscopy and parabiotic models have indicated that techniques relying on organ harvest and single cell isolation have vastly underestimated the number of T_{RM} present in lung tissues (Anderson et al., 2012; Hsu et al., 2012). This is worth keeping in mind when interpreting data from such studies that they can only access a portion of the true population, and other biases may arise from the collection methods used. Bone marrow chimeras have become an increasingly poewrful tool for the comparison of different hematopoetic lineages in a head to head manner controlled within each indivdual mouse. Likewise, chimeras have enabled the creation of mice deficient for receptors that in a whole animal knockout would affect development or non-immune cells (Kohlmeier et al., 2010). Of particular use have been bone marrow chimeras generated from mice expressing a diptheria toxin receptor under the control of promoters specific to different APC subsets such as the Itgax-DTR mice. These mice express DTR on cells that express CD11c, the classic dendritic cell marker and thus their cells may be inducibly depleted via the administration of diptheria toxin without other

toxic effects on the mouse. Whole body Itgax-DTR mice do poorly upon administration of diptheria toxin as some non immune cells also express CD11c, and the loss of these leads to weight loss and death. However bone marrow chimeras tolerate diptheria toxin treatment quite well, with large amounts of toxin given resulting in thorough depletion of the target cell types with no apparent pathology (Jung et al., 2002; van Rijt et al., 2005). Complementing these strategies are the use of adoptive transfer strategies, or even dual adoptive cell transfers. This allows the generation of a normal, polyclonal T cell response in a wild type mouse, for those T cells to then be sorted and transferred into a host deficient for APC function to examine the establishment of T_{RM} from the systemic pool without the confounding variable that mice deficient for APC function may have a diminished T cell response overall, which would prevent interpretation of their T_{RM} response compared to a wild type mouse (Knudson et al., 2014). An important variant on the use of adoptive transfer strategies that we exploit in Chapter 2 and 3 is the use of transgenic OT-I CD8 T cells specific for OVA peptide. The use of these T cells of a defined specificity and genotype along with the use of OVA expressing virus allows us to use whole body knock out models with adoptively transferred OT-I CD8s, allowing us to circumvent a potential confounder when the protein of interest is expressed on CD8s as well as other cells.

Advancements in microscopy including in situ imaging allow direct looks at what cells are intereacting, in what tissue context and when, providing the context that the phenotypic data of flow cytometry lack. A snapshot using immunofluorescene of the lung and airway can also look at the surface markers expressed on cells of interest as well as that of the vasculature or endothelium itself. Progress is also continuing in intravital imaging, which would eventually allow for cells in lungs and their trafficing to

be observed not in fixed sections but as they actually move in real time (Rodriguez-Tirado et al., 2016).

This field is ultimately focused on the production and validation of new vaccination strategies through the advancement of basic biology. Thus correlates of protection in the lung tissue and airways are important. Novel vaccination techniques such as the "pulling" of previously produced systemic effectors into a delicate tissue to establish residency without undue harm to the target tissue can be tested with in vivo models and challenge systems looking at reduction of viral titers, gross pathology as seen through weight loss studies, and molecular detection of viral loads(McMaster et al., 2018).

As previously discussed the lung presents a uniquely harsh metabolic environment for an immune cell to persist in, oxygen tension and nutrient availability both being poor(Wilsher et al., 1988b). Thus thorough understanding of the metabolism of these cells will be critical, particularly as we look for ways to effect their longevity. Fortunately great strides have been made in the use of a Seahorse Extracellular Flux analyzer to measure multiple metabolic paramaters including the use of aerobic, anaerobic matabolism, or catatbolic autophagy energy acquisition. Resident T cells may be sorted using flow cytometry after intravascular staining and cultured in these systems to compare them to T cells isolated from the periphery or secondary lymphoid organs (de Bree et al., 2005; Hu et al., 2015). Lastly there is great promise in the advancement of measuring transcription factors and the production of mRNA to obtain a more complete picture of the activity of lung T_{RM} and how they differ from systemic T cells and in particular how they differ from resident cells in other tissues. This comparative

analysis could yield data on the specific factors that promote lung homing and the persistence of T cells in the lungs and airways (Mackay et al., 2013).

As evidence of the particular importance of the unique metabolism of long-lived T cells, it has recently been found that expression of aquaporin 9 is induced by IL-7 signaling, but only in activated CD8 T cells. Further, this AQP9 is required for long term survival, its absence being marked by impaired glycerol import. This is a first step to understanding how more durable responses may be achieved (Cui et al., 2015).

CD8 T_{RM} have been found by our lab to be of particular importance in mitigating immune mediated damage during influenza infection, this protective effect is dependent on their ability to rapidly produce IFN-γ upon re-challenge (McMaster et al., 2015). It is thought that this is mediated via the rapid recruitment of effector cells to facilitate efficient clearance of the virus.

Recent models of brain resident T cells also shed some light on possible general phenomena of tissue resident T cells. In the brain, depletion of Tregs lead to larger CD4 and CD8 responses, increased inflammation and immune driven pathology, but surprisingly less expression of CD103, at least in the context of murine cytomegalovirus infection. Furthering this unexpected phenotype, the T_{RM} present in Treg sufficient mice were able to produce more granzyme B than their counterparts from the Treg depleted mice. These findings would seem to indicate that Tregs are important for the establishment of T_{RM} in the brain, and that inflammation may not be a driver of Treg differentiation, though it may be necessary to pull effector T cells into the tissue (Graham et al., 2014; Prasad et al., 2015).

Supporting these ideas are studies that link IL-10, a cytokine characteristically produced by Tregs, with the induction of memory CD8s (Laidlaw et al., 2015). This highlights the links between the resolution of the immune response and the transition into memory. This also is supported by other studies that found CD4s were necessary for the generation of CD8 T_{RM} in the lung (Laidlaw et al., 2016; Laidlaw et al., 2014). While Laidlaw et al. proposed IFN-γ as the mediator of that effect, we feel that it is more likely to be due to the loss of Tregs with the depletion of all CD4s, as IFN-γ can come from a variety of cell types. No matter the specifics of the signal, the case for the necessity of CD4s further outlines the important role APCs likely play in the generation of T_{RM}, as only APCs can activate CD4s via their interaction with MHC-II presented antigens. Though we hypothesize an APC and CD4 driven model for the lung, this is likely to differ from other tissues. For a specific case there is the recent study of T_{RM} in the salivary glands during murine cytomegalovirus infection. It was found that while CD4 T_{RM} generation required antigen, CD8 T_{RM} generation in the salivary gland did not require local antigen encounter. They also find that despite MCMV downregulating MHC-I, the resident CD8 T cells were able to confer effective protection against secondary challenge. This may further support the idea that resident CD8s mediate their protection via the rapid production of IFN-γ, whether in an antigen dependent or TLR or other innate viral detection method dependent manner. This rapid release of IFN-γ could delay viral onset long enough for an effective response to be mounted before the virus can establish itself and replicate (Thom et al., 2015).

It should also be noted that CD8s have been found to have a role in mediating the efficacy of non-neutralizing antibodies, further delineating their importance in an

antiviral response. It is possible that this is due to the recognition of conserved epitopes by the T_{RM}, which lead to the selection of B cells specific for these same conserved epitopes, also leading to them undergoing the affinity maturation process that may be required to produce effective neutralizing antibody responses (Laidlaw et al., 2013).

Further bolstering both the important role T_{RM} may play in providing a defense against disease, as well as further developing knowledge of the role of TGF-β in T_{RM} differentiation, was a recent study that found T-Box transcription factors Eomes and T-bet were downregulated by TGF-β signaling. This downregulation appears to be required for CD103 expression on CD8 T cells. Adding nuance to this study however, was the fact that some T-bet remained, and further this T-bet expression was required for surface expression of the IL-15 receptor, and thus controlled the cells ability to detect this homeostatic cytokine. Thus, these transcription factors, and the signals that control them may offer a rich suite of options for both further experimentation as well as future vaccine design (Mackay et al., 2015).

Summary:

Cellular immunity against respiratory viruses including influenza virus offers the possibility of generating durable, broad, protective immune responses. This would recognize and limit infections from multiple influenza strains that lack shared hemagglutinin and neuraminidase epitopes, making them particularly unsuited to clearance or neutralization by previously generated antibody responses. Both human and animal models have shown that memory T cells can protect against heterologous

influenza challenge in a specific antigen dependent manner. The caveat however is that thus far these responses generated have been limited in duration, with protection falling off sharply after 90 days. Likewise, the role different T cell subsets may be playing is currently poorly understood. However, lung tissue resident memory CD8 T cells are correlated with protection, it is reasonable to suspect that they are a key player. This relatively newly recognized subset is currently poorly understood itself. Particularly pertinent questions include how they are differentiated and how they are maintained. As recent data indicates that their formation in the lung is antigen dependent is it reasonable to suppose that antigen presenting cells are responsible for their development. In addition, the unique and harsh environment of the airways represents a unique challenge for the maintenance of T cells. As recent studies have shown the importance of metabolic switches in memory T cells it is likely that tissue resident, and airway resident T cells are further metabolically differentiated to deal with the low glucose and high oxygen tension present at the lung mucosa. Though it is possible that both those groups will be similar metabolically, as it is hypothesized that the airway T cells are being replenished from homeostatically proliferating lung tissue resident T cells. For both their induction and maintenance the signals that drive their differentiation must be better delineated. Though TGF-β is a canonical driver of CD103 expression this is insufficient as a marker of residency, and does not explain the trafficking of cells to the lung specifically. Comparative analysis of the effect of various APC subsets on T cells, and the signals used by the APCs to mediate these effects may yield these specific signals. Likewise, comparison of lung resident T cells to other tissues in the periphery may yield further answers as to specific factors of lung homing. This

dissertation will aim to further explore the links between innate and adaptive immunity, particularly the role of APCs in driving the differentiation of resident T cells.

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Pulmonary Monocytes Interact with Effector T Cells in the Lung Tissue to Drive T_{RM} Differentiation Following Viral Infection

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ABSTRACT

Lung resident memory CD8 T cells (T_{RM}) are critical for protection against respiratory viruses, but the cellular interactions required for their development are poorly understood. Herein we describe the necessity of classical monocytes for the establishment of lung T_{RM} following influenza infection. We find that, during the initial appearance of lung T_{RM}, monocytes and dendritic cells are the most numerous influenza antigen-bearing APCs in the lung. Surprisingly, depletion of DCs after initial T cell priming did not impact lung T_{RM} development or maintenance. In contrast, a monocyte deficient pulmonary environment in $CCR2^{-/-}$ mice results in significantly less lung T_{RM} following influenza infection, despite no defect in the antiviral effector response or in the peripheral memory pool. Imaging shows direct interaction of antigen-specific T cells with antigen-bearing monocytes in the lung, and pulmonary classical monocytes from the lungs of influenza infected mice are sufficient to drive differentiation of T cells *in vitro*. These data describe a novel role for pulmonary monocytes in mediating lung T_{RM} development through direct interaction with T cells in the lung.

INTRODUCTION

During respiratory virus infections, effector CD8 T cells are primed in the lung-draining lymph nodes by antigen presenting cells (APCs) that have migrated from the infected lung (Banchereau and Steinman, 1998; Henri et al., 2001; Villadangos and Heath, 2005). Following a program of proliferation and differentiation that is regulated by antigen encounter, co-stimulation receptor engagement, and the local cytokine environment, virus-specific effector CD8 T cells then traffic back to the infected lung to mediate their effector functions (Kim and Braciale, 2009; Kohlmeier et al., 2009). Upon entry into the lung tissue, virus-specific effector CD8 T cells may re-encounter antigen presented by local APCs, including monocytes, macrophages, dendritic cells, and infected epithelial cells. In addition to antigen re-encounter, cues from the local microenvironment can further influence the differentiation of virus-specific CD8 T cells into short-lived effector or long-lived memory cells, ultimately directing cell fate (Cui et al., 2015; Laidlaw et al., 2015; Poholek et al., 2016). Despite the significance of these cell fate decisions for pathogen clearance and the establishment of immune memory, the importance of the local microenvironment on T cell differentiation in the tissue and the roles of individual tissue-resident APC subsets that provide these signals for the development of T cell memory are not well understood.

Following respiratory virus clearance, subsets of the CD8 memory precursor cells in the lung will differentiate into tissue-resident memory T cells (T_{RM}). Lung T_{RM} have been shown to be critical for protective cellular immunity against secondary heterosubtypic respiratory infections, enabling the rapid detection of the invading pathogen and thereby limiting pathogen replication and immunopathology (Anderson

and Masopust, 2014; McMaster et al., 2015; Schenkel et al., 2014). The programming of CD8 T_{RM} has been extensively investigated in recent years, and key cytokines such as TGF-β and IL-15 have been shown to be important for their development (Jung et al., 2016; Mohammed et al., 2016; Sandau et al., 2010). Although antigen stimulation is required to initiate the effector T cell response in the lymph nodes, its role in T_{RM} development in the tissue had been less well characterized. Recently, it has been demonstrated that differentiation of lung T_{RM} during influenza virus infection requires virus-specific T cells to re-encounter antigen in the lung tissue (McMaster et al., 2018; Pizzolla et al., 2017). However, it is not clear if this requires interaction between effector T cells and specific APC subsets in the lung. Previous reports have shown that targeting vaccines to pulmonary $CD103^+$ dendritic cells or alveolar macrophages promotes the establishment of lung T_{RM}, but it is unclear whether this was due to the ability of these APC subsets to promote T_{RM} programming during initial priming in the lymph node, or whether these APC subsets regulate lung T_{RM} establishment through antigen re-encounter in the lung itself (Vega-Ramos and Villadangos, 2013; Wakim et al., 2015). Given the importance of T_{RM} for protective cellular immunity in the lung, it is critical to define the cellular and molecular requirements for their establishment and identify new approaches for optimizing vaccines against respiratory pathogens.

To better define the factors that promote T_{RM} in the lung, we investigated the role of different lung-resident APC subsets in virus-specific CD8 T_{RM} development. Although essential for initial virus-specific CD8 T cell activation, depletion of $CD11c^+$ dendritic cells during the peak of the effector T cell response did not impact the number of virus-specific lung CD8 T_{RM} following influenza infection. Surprisingly, analysis of pulmonary APC subsets around the time of viral clearance showed that monocytes were among the

most numerous lung APC subsets harboring influenza antigens, and this correlated with the initial appearance of virus-specific lung CD8 T_{RM}. While monocytes have been investigated for their pro-inflammatory roles in innate immunity, their ability to influence T cell responses through direct interactions with virus-specific CD8 T cells has not been as extensively investigated (Astiz et al., 1996; Carlsen et al., 2004; Tsou et al., 2007). Using CCR2-deficient mice, which are defective in monocyte trafficking to the lung during influenza infection (Aldridge et al., 2009), we observed a significant decrease in the number of virus-specific lung CD8 T_{RM} in both the parenchyma and airways, but there was no effect on the number of circulating virus-specific memory CD8 T cells in the spleen. Notably, there were no differences in the number of virus-specific effector CD8 T cells or virus-specific memory CD8 precursor cells generated in the lung at the time of viral clearance when comparing wild-type and $CCR2^{-/-}$ mice, demonstrating the role for monocytes in T_{RM} development was restricted to antigen re-encounter in the tissue and not initial T cell priming. In support of this, imaging of the lung revealed a close interaction between virus-specific CD8 T cells and monocytes. Furthermore, pulmonary monocytes sorted from infected lungs were sufficient to activate naive antigen specific T cells, as well as induce their expression of CD103 on a subset of highly-divided cells *in vitro*. Together, these data define a novel role for lung tissue-resident monocytes as critical mediators in the establishment of lung CD8 T_{RM} but not circulating T cell memory following respiratory infection, through presentation of viral antigens to T cells in the infected lung.

MATERIALS AND METHODS

Mice and infections

C57BL/6J (WT), B6.PL-Thy1^{a}/CyJ (CD90.1), B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ (CD45.1), B6.129S4-*Ccr2^{tm1Ifc}*/J (CCR2$^{-/-}$), B6.129P-CX3CR1^{tm1Litt}/J (CX3CR1-GFP), B6.129(Cg)-CCR2$^{tm2.1Ifc}$/J (CCR2-RFP), C57BL/6-Tg(Nr4a1-EGFP/cre)820Khog/J (Nur77-GFP), B6N.129P2-Cx3cr1$^{tm3(DTR)Litt}$/J (CX3CR1-DTR), and B6.FVB-*1700016L21Rik$^{Tg(Itgax-DTR/EGFP)57Lan}$*/J (CD11c-DTR) mice from The Jackson Laboratory were housed under SPF conditions at Emory University and Kindai University. B6.129P-CX3CR1^{tm1Litt}/J and B6.129(Cg)-CCR2$^{tm2.1Ifc}$/J mice were crossed to generate F1 dual reporter mice (CX3CR1$^{+/GFP}$ CCR2$^{+/RFP}$) for imaging. Intranasal infection with influenza A/HKx31 (H3N2) at 30,000 50% egg infectious doses (EID_{50}), A/HKx31-OVAI expressing SIINFEKL peptide at 30,000 EID_{50}, and influenza A/PR8-OVAI expressing SIINFEKL peptide (H1N1) at 6,000 EID_{50} were performed as previously described (Kohlmeier et al., 2010). In some experiments, 10^4 CD90.1$^+$ naïve OT-I CD8 T cells were injected i.v. into recipient mice one day prior to infection. In protection experiments, mice were injected daily i.p. with 150 μg FTY720 (Cayman Chemical) suspended in PBS. All experiments were completed in accordance with the Institutional Animal Care and Use Committee guidelines of Emory University.

Generation of CD11c-DTR and CX3CR1-DTR chimeras

Recipient CD45.1 mice were injected i.p. with 600 μg of busulfan (Otsuka Pharmaceutical). The next day and 5×10^6 BM cells isolated from CD11c-DTR (Itgax-DTR) or CX3CR1-DTR mice were injected intravenously. Chimeras were rested for 6 weeks for reconstitution, and were bled to confirm the presence of the donor CD45.2$^+$ cells prior to virus infection. Some groups of mice were injected i.n. with 60 μg of

Diphtheria Toxin (DTx) (Sigma-Aldrich) or PBS following infection with influenza x31 or x31-OVA.

Tissue collection and flow cytometry

Intravital staining was performed immediately before mouse euthanasia and tissue harvest as previously described (Anderson et al., 2012). Briefly, to identify T cells resident in various tissues, including the lung parenchyma, 1.5µg of fluorophore-conjugated α-CD45.2 antibody in 200λ 1× PBS was intravenously injected into the tail vein of mice; five minutes post-injection, mice were euthanized with Avertin (2,2,2-Tribromoethanol, Sigma) and exsanguinated prior to harvest of BAL and other tissues. Cells in the lung airways were recovered by lavage with 5 × 1 ml R10 media. Lung tissues were digested by collagenase D (Roche) for 30 min at 37°C and enriched by centrifugation in 40/80% Percoll gradient. Splenocytes were obtained by straining through nylon mesh, followed by RBC lysis in buffered ammonium chloride. Cells were blocked first with mAbs to FcRγIII/II and then stained with APC-conjugated influenza $NP_{366–374}/D^b$ tetramer. Tetramer-labeled cells were washed and stained with fluorophore-conjugated reagents purchased from BD Biosciences (CD103, CD11c, Ly6C, Siglec-F), BioLegend (CD103, CD11b, CD127, CD69, CD8a, CD90.1, KLRG-1, Ly6G, I-A/I-E, CD45.2), eBiosciences (CD4, CD44, H2KB-OVA), anti influenza NP (Abcam) and R&D (CCR2)**.** Intracellular staining was performed using the Cytofix/Cytoperm Kit according to manufacturer protocol (BD Biosciences). Tetramers were generated by the NIH Tetramer Core at Emory University. Samples were run on LSRFortessa and LSR-II flow cytometers (BD), and data were analyzed using FlowJo software (Tree Star). Cell

sorting was performed on an SH800 (Sony) or FACSAria III cell sorter (BD Biosciences).

Fluorescence and confocal microscopy

Mouse lungs were inflated by intratracheal administration of optimal cutting temperature (OCT) media to preserve lung morphology followed by snap freezing in liquid nitrogen. Six or Seven -μm-thick cryosections were fixed for 2 min with acetone / ethanol, and blocked with combined rat serum, donkey serum, mouse serum and FcBlock (anti CD16/32 2.4G2) or Blocking One reagent (Nacalai Tesque) followed by blocking with endogenous avidin and biotin blocking system (Abcam). Sections were then stained with antibodies purchased from Biolegend (CD90.1, anti-GFP, donkey anti rabbit IgG, CD8a, CD11b, CD11c, B220), F4/80 (Bay Bioscience), pan-Cytokeratin (Bioss Antibodies), Abcam (fluNP), Lifetech (streptavidin Alexa Fluor 405), Invitrogen (a-RFP rabbit polyclonal) and mounted with ProLong™ Diamond Antifade (Thermo Fisher). Images were acquired on an AxioObserver.Z1 (Zeiss) using a 100x oil objective at room temperature or a C2si confocal microscope (Nikon). Images were processed using Zen 2.3 blue edition software.

Monocyte and T Cell in vitro co-culture

Classical and non-classical monocytes were sorted from lungs of day 10 x31 influenza infected mice ($CD45^+$, $CD11b^+$, MHC-II^-, $Ly6g^-$, $Ly6c^+$, $CCR2^+$ classical monocytes; $CD45^+$, $CD11b^+$, MHC-II^-, $Ly6g^-$, $Ly6c^-$, $CCR2^-$ non-classical monocytes). Sorted monocytes were pulsed for 2 hours with 1μM OVA peptide (SIINFEKL) in round bottom

plates at 37°C. and cultured with naive CD8 OT-I T cells isolated from spleens using the EasySep™ Mouse CD8+ T Cell Isolation Kit (Stem Cell Technologies) and stained with Cell Trace Violet (ThermoFisher) Co-cultures were performed at a 1:2 monocyte to T cell ratio for 3 days prior to analysis (Lewis et al., 2015).

Statistical analysis

Statistical analysis was performed using Prism 5 (GraphPad Software), and significance was determined by an unpaired two-tailed Student's *t* test unless otherwise noted in the figure legend.

P-values less than 0.05 were considered significant.

RESULTS

Lung CD8 T_{RM} develop immediately following viral clearance

While there is growing appreciation for the role of tissue resident memory T cells in protection against viral challenges at mucosal surfaces, less is known about their ontogeny. To determine the cellular interactions critical for lung T_{RM} development, we first sought to define the kinetics of the appearance of CD8 T_{RM} in the lung following influenza infection. Flu-specific CD8 T cells were identified in both the lung vasculature and lung parenchyma following intravital labeling. The appearance of cells with a tissue-resident phenotype ($CD69^+$ $CD103^+$) was observed in the lung parenchyma beginning on day 8 post infection (Fig. 1A and 1B), just preceding the peak of the effector CD8 T cell response at day 10 post infection, as well as influenza viral clearance (Kohlmeier and Woodland, 2009; Li et al., 2012). Over the course of the effector T cell response in the lung, the frequency of vascular versus tissue-resident flu-specific CD8 T cells remains relatively constant. However, the frequency of $CD69^+$ $CD103^+$ flu-specific CD8 T cells steadily increases within the resident population from day 8-14 post-infection (Fig. 1C). We see a similar pattern in the number of lung T_{RM}, with a peak at D10 and a decline following viral clearance that mimics the kinetics of the total effector response (Fig. 1c). Similar to polyclonal flu-specific T cells, transgenic OT-I T cells resident in the lung show an increase in the frequency of $CD69^+$ $CD103^+$ cells from days 8-14 following infection with influenza x31-OVA (Fig. 1D), and numbers of lung OT-I T_{RM} matched the kinetics of the overall effector T cell response. Coincident with the expansion of $CD69^+$ $CD103^+$ cells we observed continued antigen stimulation, as measured by Nur77-GFP expression, in flu-specific CD8 T cells resident in the lung, but not in the lung vasculature or the spleen, on day 10 post-infection (Fig. 1E and 1F). Increased Nur77-

GFP expression in lung flu-specific CD8 T cells continued through day 14 post-infection, but was largely absent by day 30 post-infection. In addition, there were increased numbers of flu-specific $CD69^+$ $CD103^+$ cells in the lung at day 14 post-infection (Figure 1G). Together, these data show the seeding of the lung-resident T cell pool occurs rapidly following the resolution of influenza infection and is associated with continued antigen recognition by flu-specific T cells in the lung tissue.

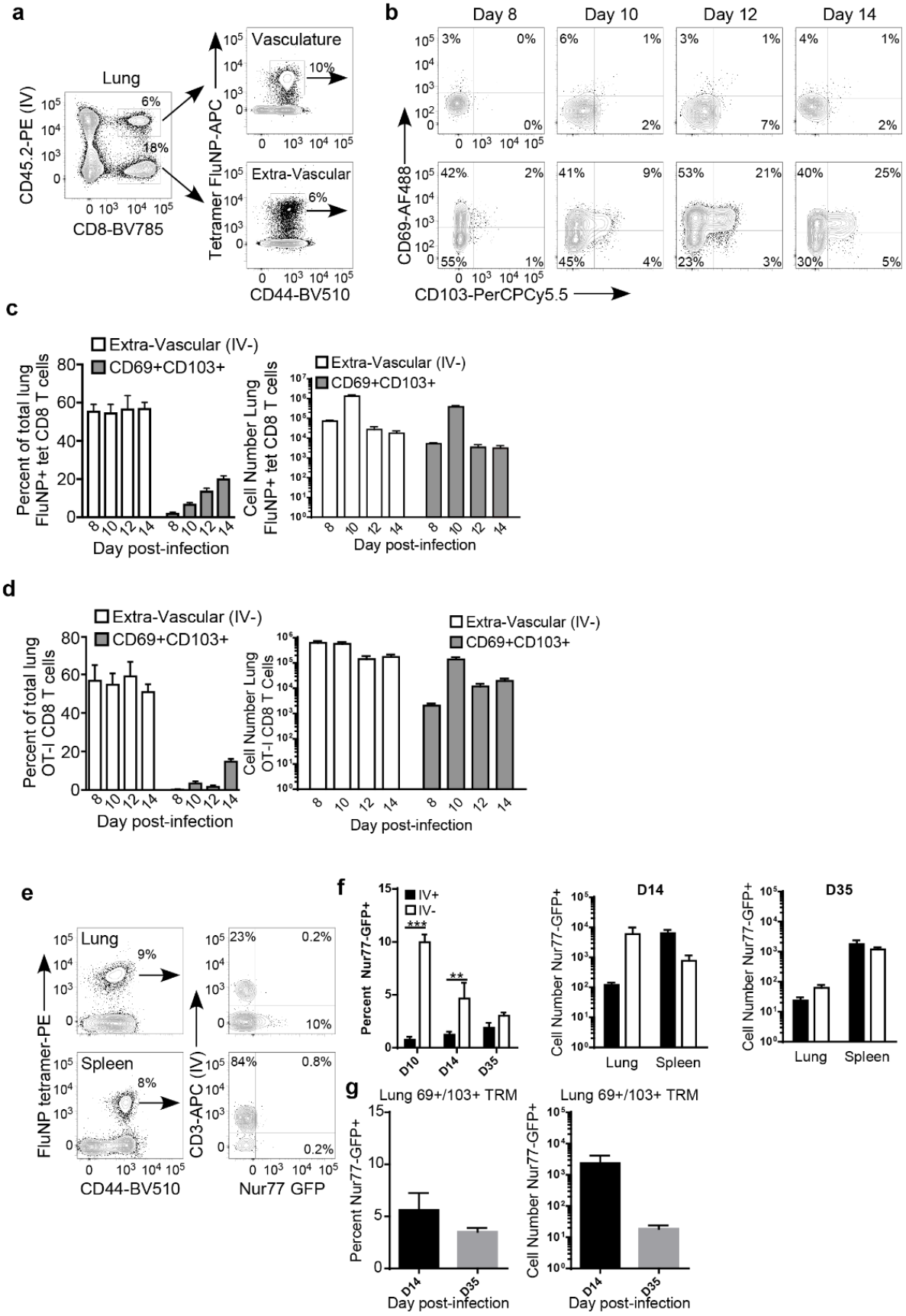
a
Lung
CD45.2-PE (IV)
CD8-BV785
6%
18%
Vasculature
10%
Extra-Vascular
6%
Tetramer FluNP-APC
CD44-BV510
b
Day 8
Day 10
Day 12
Day 14
CD69-AF488
CD103-PerCPCy5.5
c
Extra-Vascular (IV-)
CD69+CD103+
Percent of total lung FluNP+ tet CD8 T cells
Cell Number Lung FluNP+ tet CD8 T cells
Day post-infection
d
Percent of total lung OT-I CD8 T cells
Cell Number Lung OT-I CD8 T Cells
e
Lung
Spleen
FluNP tetramer-PE
CD44-BV510
CD3-APC (IV)
Nur77 GFP
f
IV+
IV-
Percent Nur77-GFP+
D10
D14
D35
D14
D35
Cell Number Nur77-GFP+
Lung
Spleen
g
Lung 69+/103+ TRM
Percent Nur77-GFP+
Cell Number Nur77-GFP+
Day post-infection

Figure 1. Rapid appearance of lung resident CD8 T cells following influenza infection. (A) Gating strategy and representative flow plots for the intravital labeling of FluNP-specific CD8 T cells in the lung. (B) Representative flow plots for CD69 and CD103 staining on FluNP-specific CD8 T cells in the lung vasculature (top row) or lung extra-vascular compartments (bottom row). (C) Frequency and number of total extra-vascular FluNP-specific CD8 T cells and $CD69^+$ $CD103^+$ resident FluNP-specific CD8 T cells among total lung FluNP-specific CD8 T cells over time. (D) Frequency and number of total extra-vascular OT-I CD8 T cells and $CD69^+$ $CD103^+$ resident OT-I CD8 T cells among total lung OT-I CD8 T cells over time. (E) Representative staining of Nur77-GFP expression in FluNP-specific CD8 T cells. (F) Frequency and number of Nur77-GFP^+ FluNP-specific CD8 T cells that are circulating (IV+) or extra-vascular (IV-) in the lung and spleen on days 10, 14, and 35 post-infection. (G) Frequency and number of Nur77-GFP^+ FluNP-specific tissue-resident CD8 T cells expressing both CD69 and CD103 in the lung extra-vascular population. *** $p<0.001$ (two-tailed Student's *t*-test) All graphs error bars are S.E.M. Data are representative of 3 independent experiments with 5 mice per time point (A-C and G) or 2 independent experiments with 4 mice each (E and F).

Depletion of lung dendritic cells after initial T cell priming does not alter lung CD8 T_{RM} establishment

Previous studies have shown that the establishment of lung-resident T cell memory requires antigen re-encounter in the lung (McMaster et al., 2018), and flu-specific T cells continue to receive antigen stimulation in the lung following viral clearance (Hamilton et al., 2016; Kim et al., 2010). Dendritic cells, specifically, are appreciated as efficient mediators of T cell activation and differentiation. As well, the importance of

dendritic cells in the initiation of a T cell response against influenza infection has been well documented (Aldridge et al., 2009; Kim and Braciale, 2009; Mikhak et al., 2013; Richert et al., 2013; Wei et al., 2013), but the role pulmonary DCs may be playing in the differentiation of flu-specific T_{RM} in the tissue after initial priming is less well understood. To address this question, we investigated the establishment of lung T_{RM} following depletion of DCs after initial T cell priming (Fig. 2A). Treatment of CD11c-DTR (Itgax-DTR) chimeras with diphtheria toxin (DTx) beginning on day 5 post-infection was sufficient to deplete the majority of $CD11c^+$ cells in the lung, which include both DCs and alveolar macrophages, while leaving the $CD11b^+$ cells intact (Fig. 2B, 2C, and 3). Surprisingly, depletion of $CD11c^+$ cells before T_{RM} generation but after initial T cell activation had no effect on the phenotype or numbers of flu-specific lung T_{RM} in the airways or parenchyma on day 14 post-infection (Fig. 3D and 3E), or at memory (Fig. 2D and 2E). Therefore, despite their importance for the initial priming of naïve CD8 T cells, interactions between DCs and virus-specific CD8 T cells in the tissue are not required for the establishment of lung T_{RM}.

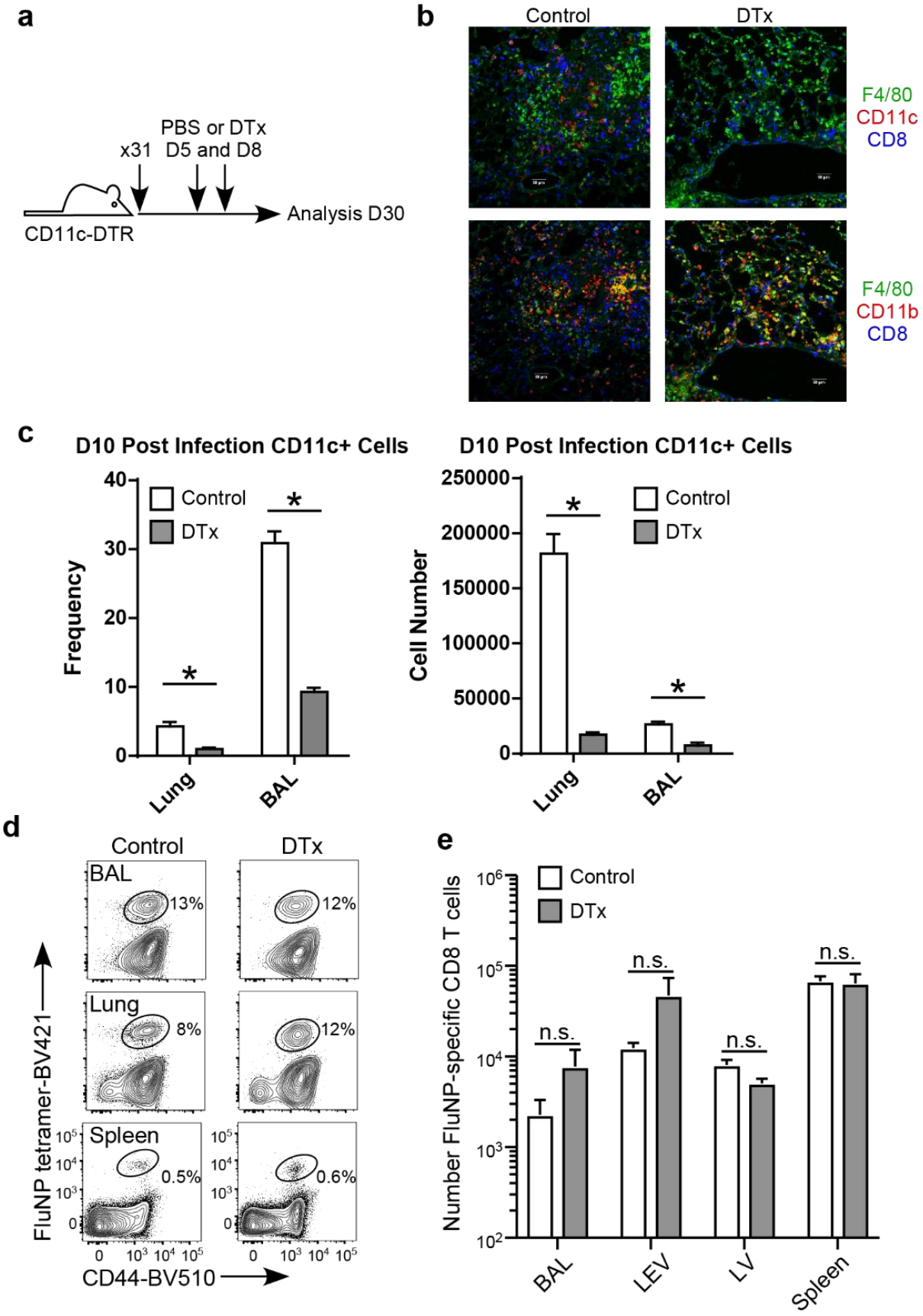
a
PBS or DTx
x31 D5 and D8
Analysis D30
CD11c-DTR
b
Control
DTx
F4/80
CD11c
CD8
F4/80
CD11b
CD8
c
D10 Post Infection CD11c+ Cells
Frequency
Control
DTx
Lung
BAL
D10 Post Infection CD11c+ Cells
Cell Number
Control
DTx
Lung
BAL
d
Control
DTx
BAL
13%
12%
Lung
8%
12%
Spleen
0.5%
0.6%
FluNP tetramer-BV421
CD44-BV510
e
Number FluNP-specific CD8 T cells
Control
DTx
n.s.
n.s.
n.s.
n.s.
BAL
LEV
LV
Spleen

Figure 2. Depletion of DCs after initial priming does not affect establishment of lung T_{RM}. (A) Experimental diagram of DC depletion using CD11c-DTR (Itgax-DTR) chimeras. (B) Representative images from the lung on day 11 post-infection (day 3 post-depletion) demonstrating the specific depletion of $CD11c^+$ cells. (C) Frequency and number of total CD11c expressing cells in the lung and BAL at D10 post infection after DTX or PBS treatment. * $p<0.05$ (two-tailed Student's *t*-test) (D) Representative staining of FluNP-specific CD8 T cells 30 days post-infection in control or DTx-treated mice. BAL and lung plots are gated on extra-vascular (IV-) cells. (E) Number of FluNP-specific CD8 T cells in BAL, lung extra-vascular (LEV), lung vasculature (LV), and spleen 30 days post-infection in control or DTx-treated mice. Data are representative of three independent experiments with 3-4 mice per group. All graphs error bars are S.E.M.

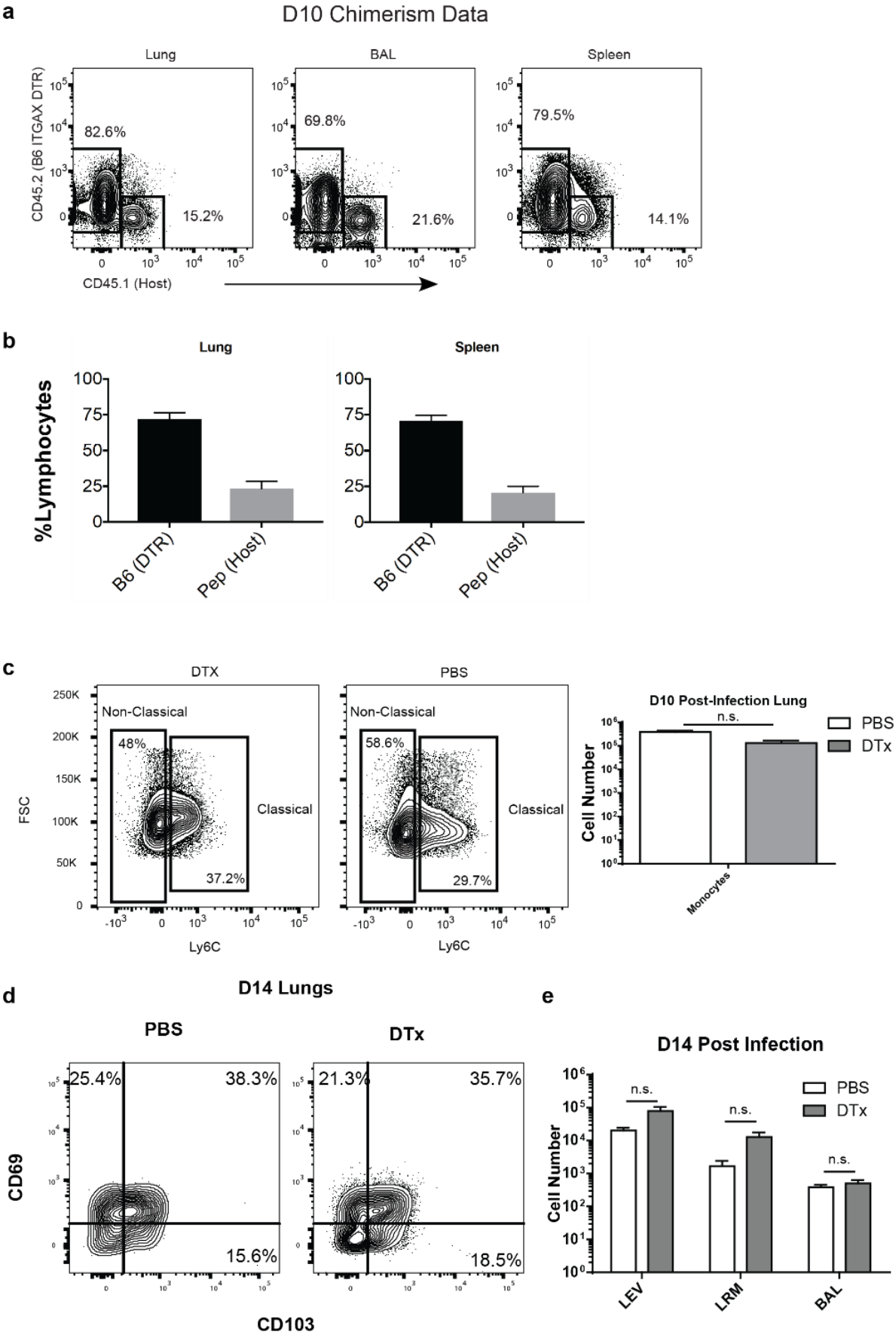
a
D10 Chimerism Data
Lung
BAL
Spleen
CD45.2 (B6 ITGAX DTR)
CD45.1 (Host)
82.6%
15.2%
69.8%
21.6%
79.5%
14.1%
b
Lung
Spleen
%Lymphocytes
B6 (DTR)
Pep (Host)
c
DTX
PBS
Non-Classical
Classical
48%
37.2%
58.6%
29.7%
FSC
Ly6C
D10 Post-Infection Lung
n.s.
PBS
DTx
Cell Number
Monocytes
d
D14 Lungs
PBS
DTx
25.4%
38.3%
15.6%
21.3%
35.7%
18.5%
CD69
CD103
e
D14 Post Infection
n.s.
PBS
DTx
Cell Number
LEV
LRM
BAL

Figure 3. Validation of CD11c Itgax-DTR chimeras. (A) Representative flow plots of chimerism with Itgax DTR and host cells in uninfected, untreated chimeras. (B) Quantification of chimerism with Itgax DTR and host cells in uninfected, untreated chimeras. (C) Representative flow plots and quantification of total monocytes and monocyte subsets in the lung 10 days post infection showing lack of depletion of monocytes in DTx treated animals. (D) Example staining of D14 post-infection DTx- and PBS-treated chimeras, showing CD69 and CD103 staining on flu-specific CD8 T cells in the lung. (E) Number of FluNP-specific CD8 T cells in the extra-vascular compartment of the lung (LEV), CD69+ CD103+ cells of the extra-vascular compartment (LRM) and airways (BAL) on day 14 post infection.

Lung-resident dendritic cells and monocytes are among the most numerous cell types presenting influenza antigens during the initial appearance of lung T_{RM}

This finding led us to broaden our approach to identify additional APC subsets potentially involved in lung T_{RM} differentiation. We investigated which APC subsets in the lung were displaying antigen to flu-specific T cells, particularly at the conclusion of the effector T cell response, when resident memory is established. CD8 T cells in the lung make direct contact with many different APC subsets, including dendritic cells, macrophages, and monocytes (Fig. 4A). To determine which APC subsets were capable

of presenting influenza antigens in the lung, we performed intravital labeling to identify different lung extra-vascular APC subsets, (Fig. 5) and assessed intracellular influenza nucleoprotein (FluNP) content (Zaynagetdinov et al., 2013). This staining approach was specific for the influenza nucleoprotein, as isotype staining, as well as staining of lung APCs following Sendai virus infection, failed to give a positive signal (Fig. 4B). Intracellular FluNP staining was detected in pulmonary monocytes, monocytic respiratory dendritic cells (MoRDCs), $CD11b^{hi}$ DCs, and $CD103^{+}$ DCs, and this staining was restricted to the lung-resident ($CD45.2^{-}$) population of each subset (Fig. 4C). The number of cells containing FluNP in each subset was highest at day 6 post-infection, and substantial numbers of pulmonary APCs containing FluNP were detected through day 15 post-infection (Fig. 4D) (Zaynagetdinov et al., 2013). Although, as expected, substantial numbers of $FluNP^{+}$ DCs were present in the lung, it was surprising to observe that pulmonary monocytes were also among the most numerous $FluNP^{+}$ APC subset from days 10-15 post-infection. Thus, we began to assess what role these monocytes may play in the differentiation of lung $T_{RM,}$ as well as any differences in the roles of the classical and non-classical monocyte subsets.

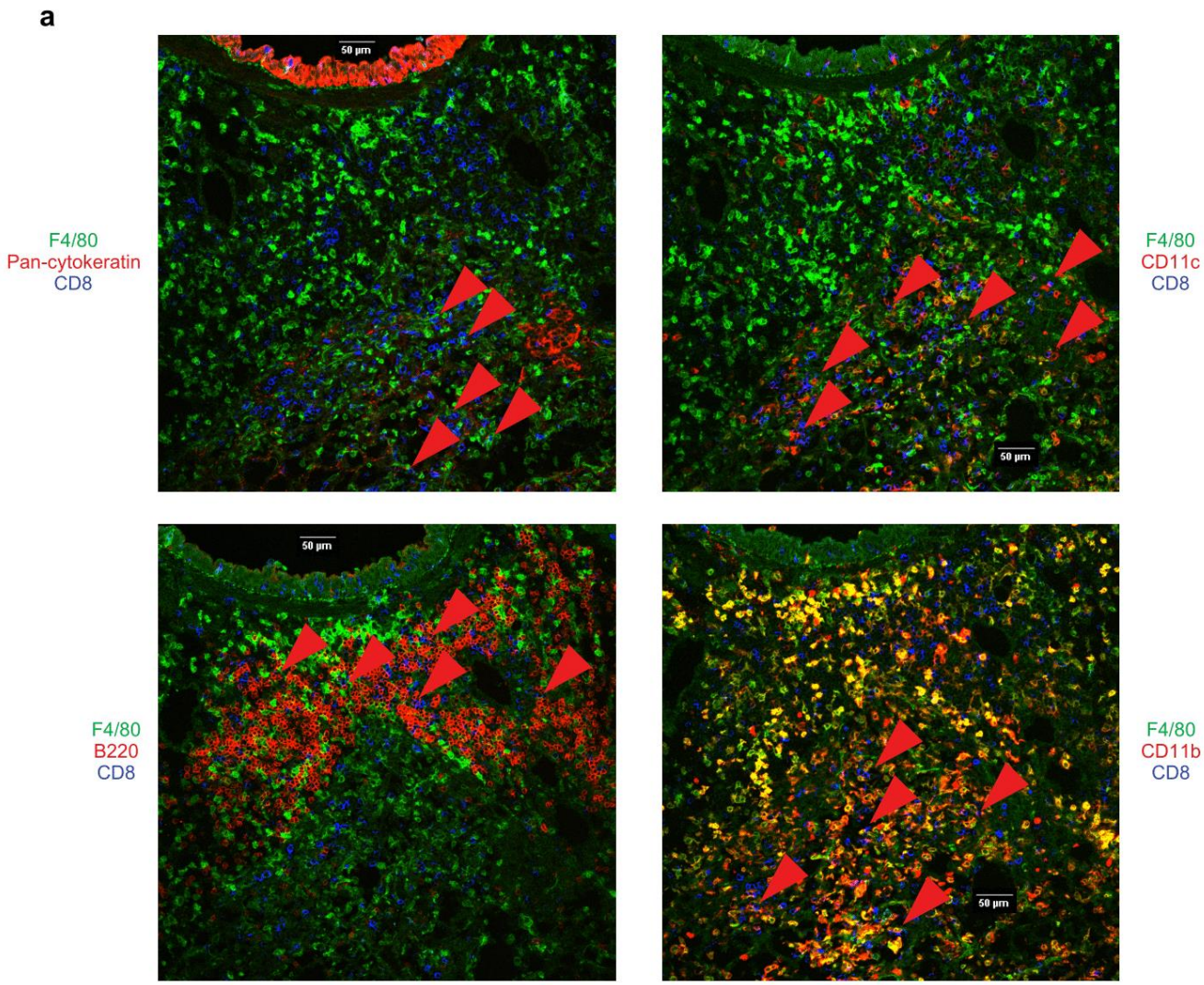
a
F4/80
Pan-cytokeratin
CD8
F4/80
CD11c
CD8
F4/80
B220
CD8
F4/80
CD11b
CD8
50 µm
b
Normalized To Mode
FluNP-FITC
x31, isotype
Sendai, anti-FluNP
x31, anti-FluNP
c
Total-Monocytes
MoRDCs
CD11b^hi DCs
CD103+ DCs
CD45.2-PE(IV)
FluNP Protein-FITC
17.2%
19.1%
25.1%
23.3%
d
FluNP Protein Bearing APCs
Cell number
Day post-infection
Total-Monocytes
MoRDCs
CD11b^hi DCs
CD103+ DCs

Figure 4. Large numbers of pulmonary DCs and monocytes contain influenza antigens following viral clearance and during the initial appearance of lung T_{RM}. (A) Representative confocal microscopy images showing the localization of CD8 T cells and various APC subsets in the lung of influenza infected mice. Red arrows highlight areas of T cell and APC co-localization. (B) Representative staining showing the specificity of intracellular FluNP staining in pulmonary monocytes following infection with influenza x31 (black histogram) or Sendai virus (gray histogram). Isotype control staining is shown by the dashed line. (C) Representative staining of intracellular FluNP in extra-vascular (CD45.2 IV-) lung APC subsets on day 7 post-infection. (D) The number of FluNP-containing total monocytes (black), MoRDCs (blue), $CD11b^{hi}$ DCs (red), and $CD103^{+}$ DCs (green) in the lung over the course of influenza infection. Data are representative of 3 independent experiments with 5 mice per time point. All graphs error bars are S.E.M.

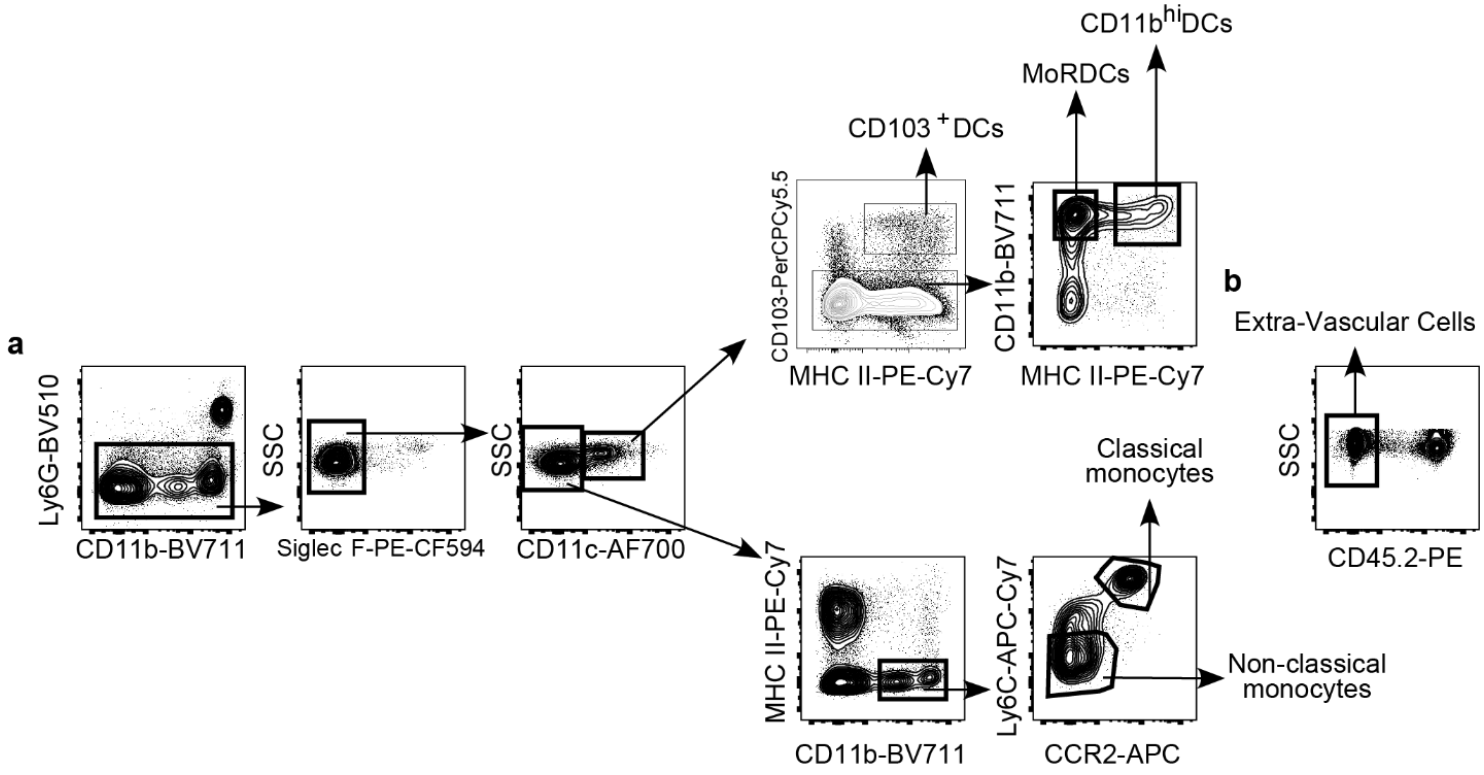

Figure 5. Gating strategy for pulmonary APCs. (A)Representative flow plots and gating strategy for the identification of extravascular pulmonary APC subsets of C57BL/6J mice intranasally infected with x31 influenza virus. Discrimination of vascular versus extra vascular cells via i.v. staining with anti-CD45.2 was performed in each experiment as shown. plots are representative of 3 independent experiments with 5 mice used in each experiment.

Inhibiting monocyte recruitment to the lung significantly decreases lung CD8 T_{RM} development

To investigate the role pulmonary monocytes may be playing in the development of lung T_{RM} *in vivo,* we used mice deficient in C-C chemokine receptor type 2, or CCR2, which lack the ability to efficiently traffic monocytes from the circulation into sites of mucosal inflammation. Previous studies characterizing influenza infection in $CCR2^{-/-}$ mice observed no defect in the flu-specific effector CD8 T cell response or viral clearance [31, 32], but the mice do show decreased monocyte-driven immunopathology (Aldridge et al., 2009). To test this, we seeded WT and $CCR2^{-/-}$ mice with naïve OT-I T cells, infected the mice with x31-OVA, and tracked the OVA-specific as well as endogenous fluNP-specific T cell response (Fig. 7A). As expected, we observed a significant decrease in the number of monocytes recruited to the lung in $CCR2^{-/-}$ mice following influenza infection, but no difference in the numbers of other lung APC subsets, including MoRDC, $CD103^{+}$, and $CD11b^{hi}$ DC subsets (Fig. 6). Similar to previous reports, at day 10 post-infection there were no differences in the number of OT-I effector T cells in the BAL, lung interstitium (lung extra-vascular, LEV), or spleen between WT and $CCR2^{-/-}$ mice (Fig. 7B and 7C) (Desai et al., 2018). In addition, there was no difference in the number of $CD69^{+}$ $CD103^{+}$ lung-resident OT-I cells at this peak of the acute CD8 T cell response (Fig. 7C). To determine whether $CCR2^{-/-}$ mice showed a defect in overall memory T cell development, we assessed the number of memory precursor cells (MPECs) in the lung and spleen (Fig. 7D). Similar to our observations of the overall effector T cell pool, there was no difference in the number of $CD127^{hi}$ $KLRG1^{lo}$ MPECs in the lung or spleen. Thus, $CCR2^{-/-}$ mice showed no defect in the flu-specific effector CD8 T cell response, even within the lung tissue and airways (BAL).

In contrast to the effector T cell response, CCR2$^{-/-}$ mice showed a significant decrease in the number of OT-I lung T_{RM} in both the airway (BAL) and interstitium (LEV) at memory (Fig. 7E and 7F). Importantly, there was no difference in the number of OT-I memory T cells in the spleen, indicating that the role of pulmonary monocytes in the generation of CD8 T cell memory was restricted to the lung-resident pool. In addition to a significant decrease in the total number of lung extra-vascular T cells, there was a significant decrease in the number of CD69^{+} CD103^{+} T_{RM} OT-I cells in both the airways and interstitium in CCR2$^{-/-}$ mice (Fig. 7F). Although there is some overlap between the number of lung T_{RM} observed in WT and CCR2-/- mice, the compiled data show a significant defect in the average number of flu-specific lung T_{RM} in the CCR2-deficient mice.

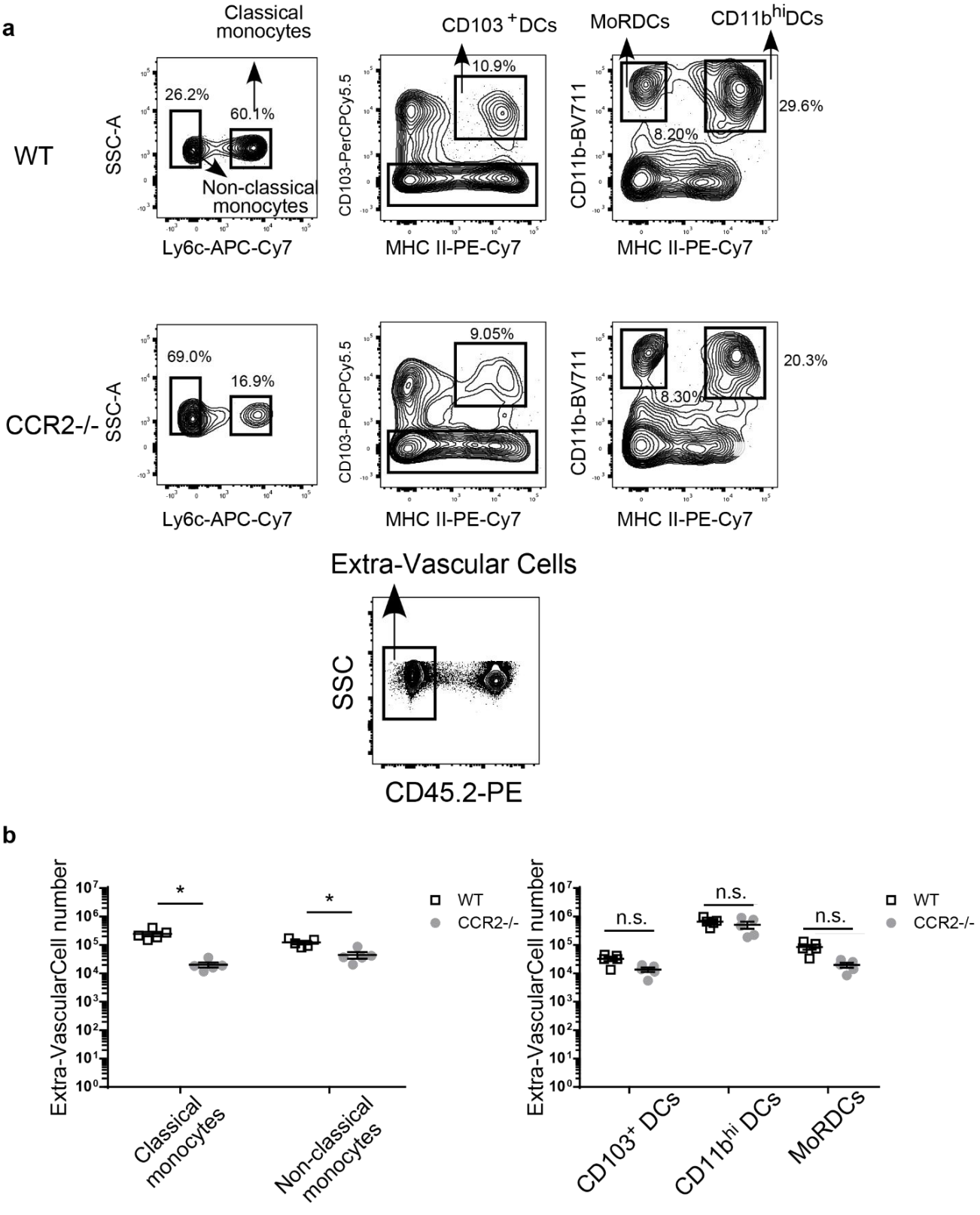
a
Classical monocytes
CD103 +DCs
MoRDCs
CD11b^hiDCs
WT
SSC-A
26.2%
60.1%
Non-classical monocytes
Ly6c-APC-Cy7
CD103-PerCPCy5.5
10.9%
MHC II-PE-Cy7
CD11b-BV711
29.6%
8.20%
MHC II-PE-Cy7
CCR2-/-
SSC-A
69.0%
16.9%
Ly6c-APC-Cy7
CD103-PerCPCy5.5
9.05%
MHC II-PE-Cy7
CD11b-BV711
20.3%
8.30%
MHC II-PE-Cy7
Extra-Vascular Cells
SSC
CD45.2-PE
b
Extra-VascularCell number
*
*
WT
CCR2-/-
Classical monocytes
Non-classical monocytes
Extra-VascularCell number
n.s.
n.s.
n.s.
WT
CCR2-/-
CD103+ DCs
CD11bhi DCs
MoRDCs

Figure 6. Number of APCs in the lung of WT and CCR2-/- mice 10 days post infection with x31 influenza virus. (A)Representative flow plots of APCs present in WT and CCR2-/- mouse lungs on day 10 post-infection. (B) Number of extra-vascular classical and non-classical monocytes (left graph) or DC subsets (right graph) in the lungs of WT and CCR2-/- mice on day 10 post-infection. All graphs error bars are S.E.M. Data are representative of 3 independent experiments with 5 mice per time point. * $p<0.05$ n.s. $p>0.05$(two-tailed Student's *t*-test).

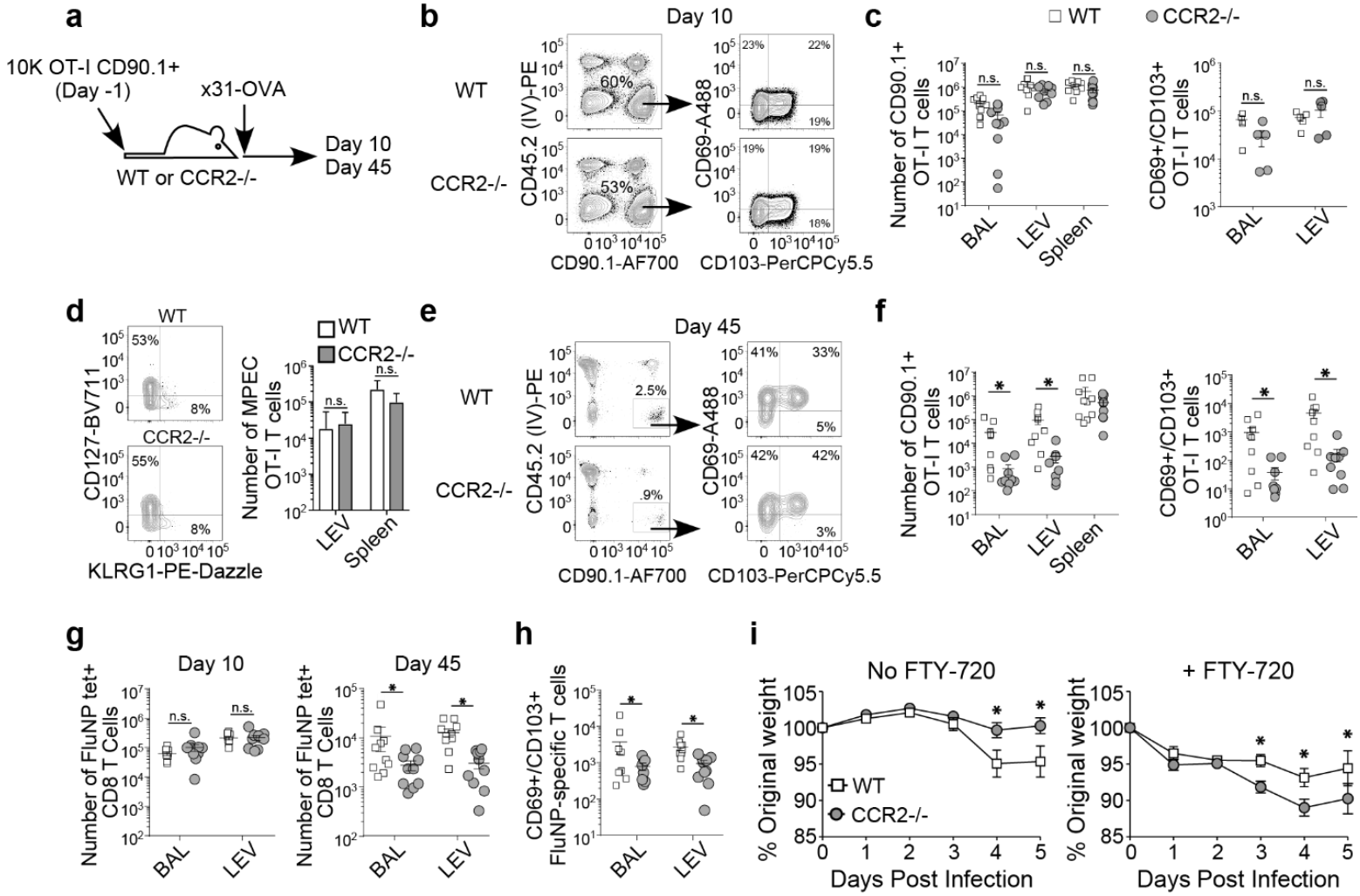

Figure 7. Inhibiting monocyte recruitment to the lung significantly reduces the number virus-specific lung extra-vascular and lung T_{RM} following influenza infection. (A) Experimental design for investigating the role of pulmonary monocytes in lung T_{RM} establishment. (B) Representative staining and (C) numbers of total and $CD69^+$ $CD103^+$ OT-I CD8 T cells in the airways (BAL), lung extra-vascular (LEV), and spleen on day 10 post-infection in WT and $CCR2^{-/-}$ mice. (D)Representative staining and numbers of $CD127^+$ $KLRG1^-$ MPEC OT-I T cells in the lung and spleen on day 10 post-infection. (E) Representative staining and (F) numbers of total and $CD69^+$ $CD103^+$ OT-I CD8 T cells in the airways (BAL), lung extra-vascular (LEV), and spleen on

day 45 post-infection in WT and CCR2$^{-/-}$ mice. (G) Number of FluNP-specific CD8 T cells in the airways (BAL) and extra-vascular in the lung (LEV) on days 10 and 45 post-infection in WT and CCR2$^{-/-}$ mice. (H) Number of CD69^{+} CD103^{+} FluNP-specific CD8 T cells resident in the airways (BAL) and lung (LEV) on days 10 and 45 post-infection in WT and CCR2$^{-/-}$ mice. (I) Weight loss of WT and CCR2$^{-/-}$ influenza x31-OVA-immune mice challenged with PR8-OVA in the presence (right graph) or absence (left graph) of FTY-720. Data represent 3 independent experiments with 5 mice per group (B-H), or 3 independent experiments with 6 mice per group (I). All graphs error bars are S.E.M. * $p<0.05$(two-tailed Student's *t*-test).

To differentiate between the potential role classical and non-classical monocytes may be playing in the CCR2-/- model, we generated CX3CR1-DTR bone marrow chimeras to allow for the selective depletion of CX3CR1+ non-classical monocytes (Fig 8A) (Diehl et al., 2013). However this model showed no difference at memory between the PBS and DTx treated mice, with similar numbers of memory OT-Is being generated in the lung extra-vascular compartment, the airways, and the spleen (Fig 8B and 8C). This indicated that the classical monocytes, but not non-classical monocytes, had a prominent role in driving the differentiation of lung T_{RM} following influenza infection.

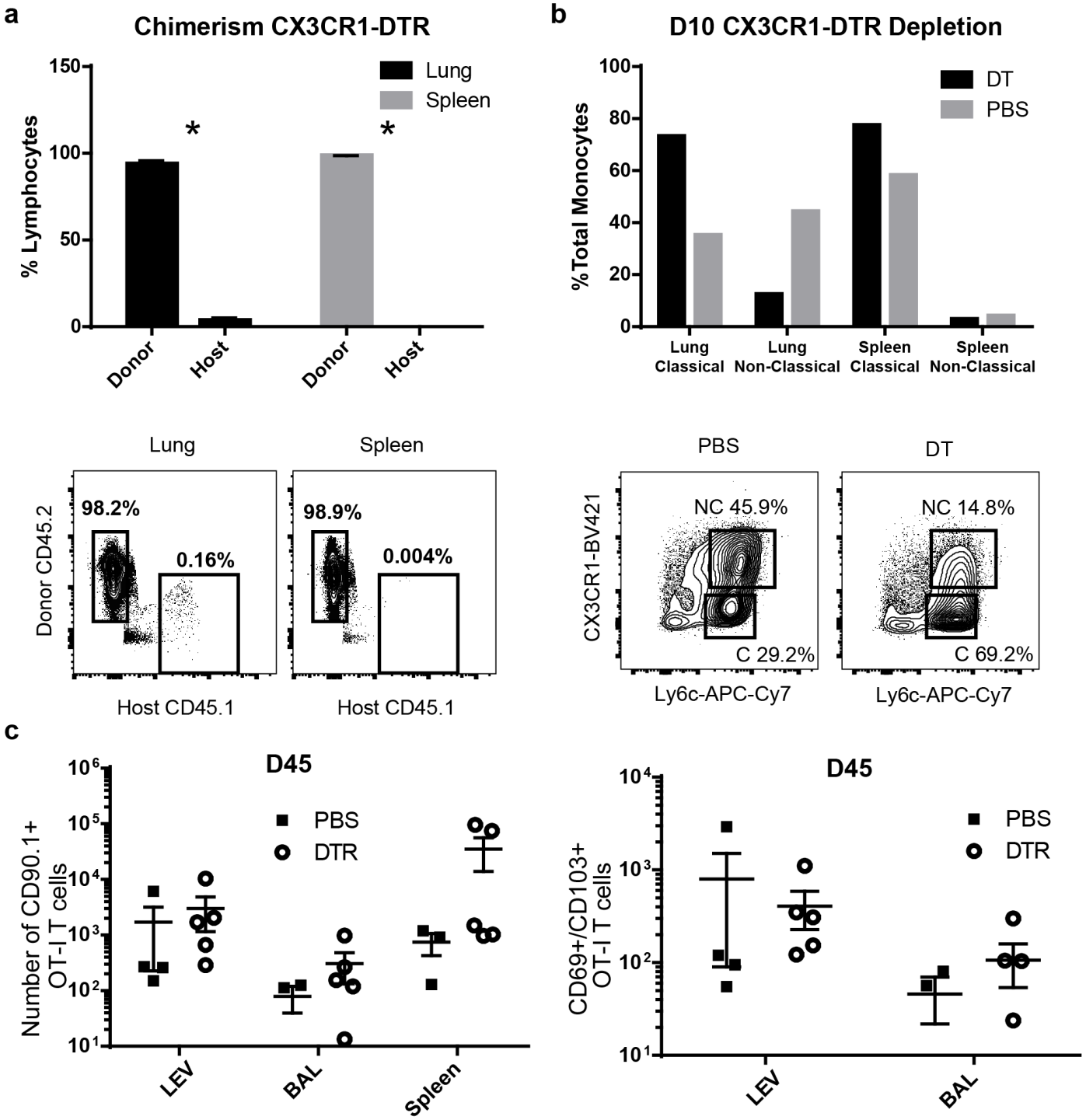
a
Chimerism CX3CR1-DTR
% Lymphocytes
Lung
Spleen
Donor
Host
Donor
Host
Lung
Spleen
Donor CD45.2
98.2%
0.16%
98.9%
0.004%
Host CD45.1
Host CD45.1
b
D10 CX3CR1-DTR Depletion
%Total Monocytes
DT
PBS
Lung Classical
Lung Non-Classical
Spleen Classical
Spleen Non-Classical
PBS
DT
CX3CR1-BV421
NC 45.9%
C 29.2%
NC 14.8%
C 69.2%
Ly6c-APC-Cy7
Ly6c-APC-Cy7
c
D45
Number of CD90.1+ OT-I T cells
PBS
DTR
LEV
BAL
Spleen
D45
CD69+/CD103+ OT-I T cells
PBS
DTR
LEV
BAL

Figure 8. Depletion of non-classical monocytes in CX3CR1-DTR mice has no effect upon lung T_{RM} generation following influenza infection. (A) Graph and representative flow plots of frequency of total lymphocytes in the lung and spleen derived from donor bone marrow versus host lymphocytes 6 weeks after chimera production. Gated to exclude CD45- singlet lymphocytes. (B) Graph and representative flow plots of the frequency of classical and non-classical monocytes in the lung and spleen of chimeras treated with DTx or PBS 10 days post infection. Plots are gated on MHC-II lo, Ly6g-, CD11b+, CD11c- singlet lymphocytes.(C) Graph of number of OT-I CD8 T cells day 45 post infection in PBS and DTx treated mice in the lung extra-vascular compartment (LEV), bronchoalveolar lavage (BAL), and spleen, and Graph of number of CD69+ CD103+ OT-I CD8 T cells day 45 post infection in PBS and DTx treated mice in the lung-extra vascular compartment and bronchoalveolar lavage. All graphs error bars are S.E.M. * $p<0.05$ n.s. $p>0.05$(two-tailed Student's *t*-test) Data are representative of one experiment.

To confirm the results of the transferred OT-I cells in the CCR2-/- model, we also investigated the endogenous polyclonal flu-specific T cell response. Similar to the OT-I response, there was no difference in the number of effector fluNP-specific CD8 T cells in the airways and lung on day 10 post-infection, but a significant decrease in the number of fluNP-specific lung extra-vascular T cells in the airways and interstitium in $CCR2^{-/-}$ mice at memory (Fig. 6G). Furthermore, in $CCR2^{-/-}$ mice, the overall decrease in fluNP-specific lung extra-vascular T cells was paralleled by a significant decrease in the number of $CD69^{+}$ $CD103^{+}$ T_{RM} in the airway and interstitium (Fig. 6H).

Assessing the impact of reduced numbers of lung T_{RM} on cellular immune protection in CCR2$^{-/-}$ mice is complicated due to the reduced monocyte recruitment, which results in decreased immunopathology and weight loss (Aldridge et al., 2009; Lin et al., 2008). To overcome this issue, we treated (H3N2) influenza x31-immune WT and CCR2$^{-/-}$ mice with FTY-720 to sequester circulating immune cells in secondary lymphoid organs and limit the response to the virus to lung T_{RM} and challenged the mice with heterologous (H1N1) influenza PR8 to assess the protective efficacy of the lung T_{RM} population. Similar to previous reports, mock FTY-720-treated CCR2$^{-/-}$ mice showed decreased weight loss compared to WT mice following PR8 challenge (Fig 6I). In contrast, treatment with FTY-720 resulted in significantly greater weight loss in CCR2$^{-/-}$ mice compared to WT mice, indicating that the decreased numbers of lung T_{RM} in CCR2$^{-/-}$ mice are associated with impaired immune protection.

Pulmonary monocytes interact with virus-specific CD8 T cells *in vivo* and are sufficient to drive CD8 T cell activation and differentiation *in vitro*

The effect of defective monocyte trafficking on lung T_{RM} establishment suggested two possibilities: that the inflammatory milieu of the lung was altered in a manner detrimental to T_{RM} differentiation, or that T_{RM} development is driven by direct interactions between antigen-specific T cells and monocytes in the lung tissue. Given that antigen re-encounter in the tissue is necessary to establish lung T_{RM} following an influenza infection (McMaster et al., 2018), we focused on the potential role of pulmonary monocytes presenting antigens directly to virus-specific CD8 T cells. The lung is a large organ, making the precise localization of individual cells crucial if they are to directly engage one another. As monocytes are among the most abundant APC

subsets containing influenza antigens when lung T_{RM} first appear, we examined whether different fluorescently-marked monocyte subsets were interacting with virus-specific CD8 T cells in the lung (Fig. 9A). Influenza antigen-bearing monocytes were observed in close contact with virus-specific CD8 T cells in the lung on day 12 post-infection (Fig. 8B, red arrows). To ensure that the monocytes not only contained but were presenting antigen on MHC-I as described by Jakubzick et al. (Larson et al., 2016), we stained lung monocytes for H-2K^b bound to SIINFEKL, finding that both classical and non-classical monocytes were capable of presenting virus-derived peptide on MHC-I (Fig. 9C). Furthermore, we found that a subset of these H-2K^b-SIINFEKL positive cells also contained intracellular FluNP protein, thus demonstrating both flu antigen uptake as well as presentation on MHC-I (Fig. 9D). Though the cells were in contact, it was still possible they did not contribute to T cell activation and differentiation. To test this, we sorted classical and non-classical monocytes from the lungs of mice infected with x31, pulsed the monocytes with SIINFEKL peptide, and cultured the monocytes with naïve OT-I T cells. Both monocytes subsets were able to induce substantial proliferation of OT-I cells as measured by CTV dilution (Fig. 9D). However, in highly divided cells, only classical monocytes were able to generate a population of OT-I T cells that co-expressed the T_{RM} markers CD69 and CD103 (Fig. 9E and 9F). Surprisingly, dendritic cell subsets, while able to induce CD103 upregulation, did so to a lesser degree than classical monocytes (Fig. 8H). Both subsets of monocytes were able to induce the expression of CD127 on highly divided cells, indicating that they were capable of generating OT-I cells with the potential to become memory T cells (Fig. 10). Together, these data indicate that lung monocytes, particularly classical monocytes, are present in the lung micro-environment, co-localize with CD8 T cells, and are sufficient to drive T cell

differentiation, including the expression of CD103 on a subset of highly-divided cells. Overall, these data demonstrate a novel role for antigen presentation by pulmonary monocytes in the establishment of virus-specific lung T_{RM}, but not systemic T cell memory, following influenza infection.

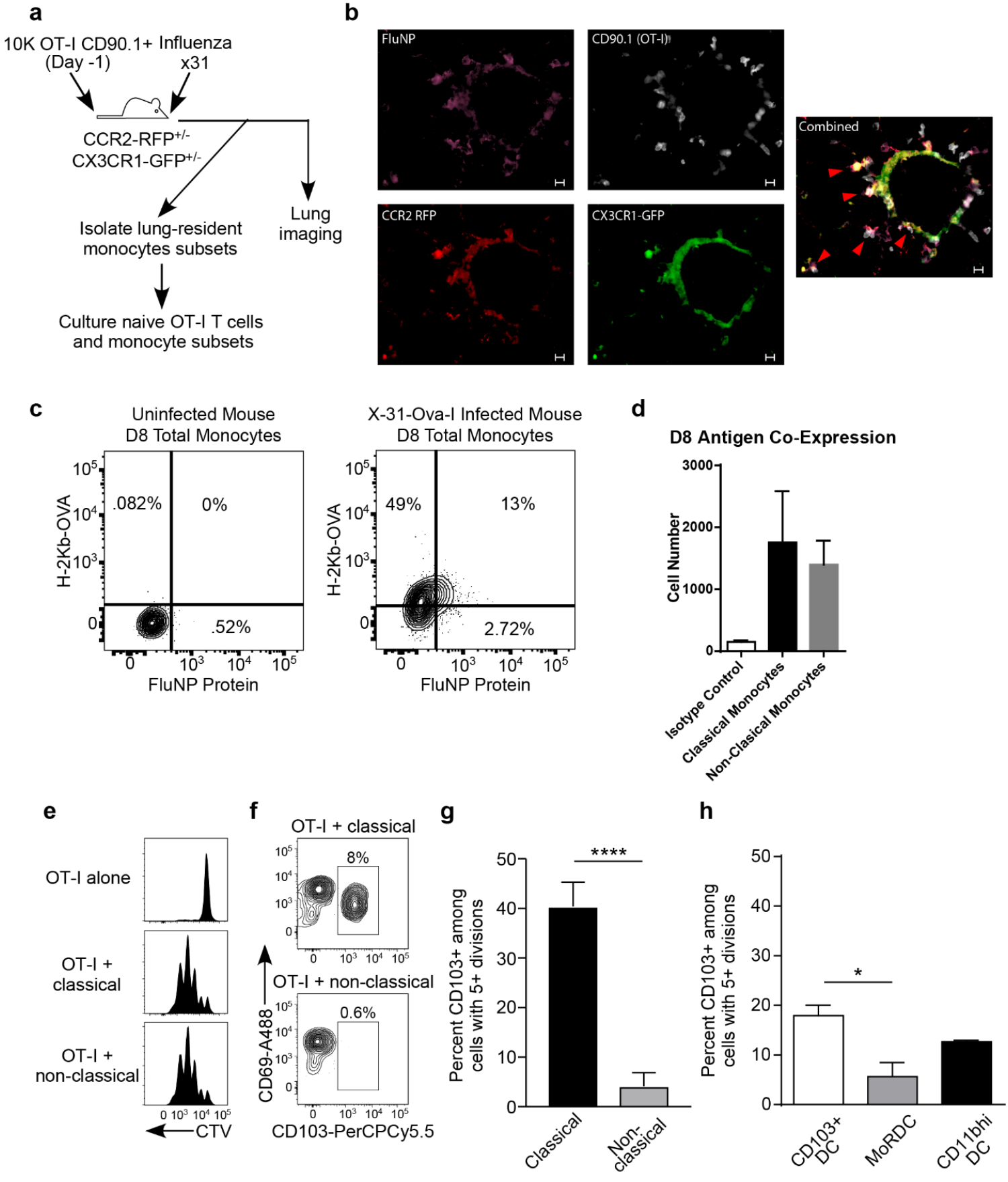
a
10K OT-I CD90.1+ Influenza
(Day -1) x31
CCR2-RFP+/-
CX3CR1-GFP+/-
Isolate lung-resident monocytes subsets
Lung imaging
Culture naive OT-I T cells and monocyte subsets
b
FluNP
CD90.1 (OT-I)
Combined
CCR2 RFP
CX3CR1-GFP
c
Uninfected Mouse
D8 Total Monocytes
X-31-Ova-I Infected Mouse
D8 Total Monocytes
H-2Kb-OVA
FluNP Protein
.082%
0%
.52%
49%
13%
2.72%
d
D8 Antigen Co-Expression
Cell Number
Isotype Control
Classical Monocytes
Non-Clasical Monocytes
e
OT-I alone
OT-I + classical
OT-I + non-classical
CTV
f
OT-I + classical
8%
OT-I + non-classical
0.6%
CD69-A488
CD103-PerCPCy5.5
g
Percent CD103+ among cells with 5+ divisions
****
Classical
Non-classical
h
Percent CD103+ among cells with 5+ divisions
*
CD103+ DC
MoRDC
CD11bhi DC

Figure 9. Pulmonary monocytes interact with T cells in the lung during infection, present influenza-derived antigen, and are sufficient to drive the activation and differentiation of T_{RM}-like CD8 T cells *in vitro*. (A) Experimental design for using CX3CR1$^{+/GFP}$ CCR2$^{+/RFP}$ dual reporter mice infected with x31-OVA for microscopy and monocyte isolation for in vitro culture. (B) Representative fluorescent microscopy images from the lung at day 12 post-infection. OT-I T cells interacting with FluNP-containing monocytes are indicated with red arrows. Scale bar is 20 μm. (C) Representative co-staining of uninfected and x31-OVA-I infected mice with surface H-2Kb-OVA and intracellular FluNP protein, showing specificity of staining (left plot) as well as monocytes with both surface H-2Kb-OVA and intracellular FluNP protein (right plot). (D) Number of lung extra-vascular monocytes co-expressing FluNP protein and H-2Kb-OVA staining day 8 post infection. (E) Cell trace violet dilution of OT-I T cells cultured in the presence or absence of classical or non-classical monocytes for three days. (F) Representative staining of CD69 and CD103 on OT-I CD8 T cells with 5+ cell divisions as indicated by CTV dilution. (G) Frequency of CD103^{+} OT-I CD8 T cells stimulated by classical or non-classical monocytes with more than 5 cell divisions. **** $p<0.0001$ (two-tailed Student's *t*-test) (H) Frequency of CD103^{+} OT-I CD8 T cells stimulated by CD103+ DC, MoRDC, or CD11bhi DC subsets with more than 5 cell divisions. *$p<.05$ (two-tailed Student's *t*-test) Data are representative of 3 independent experiments, with each *in vitro* culture (D-I) run in triplicate. All graphs error bars are S.E.M.

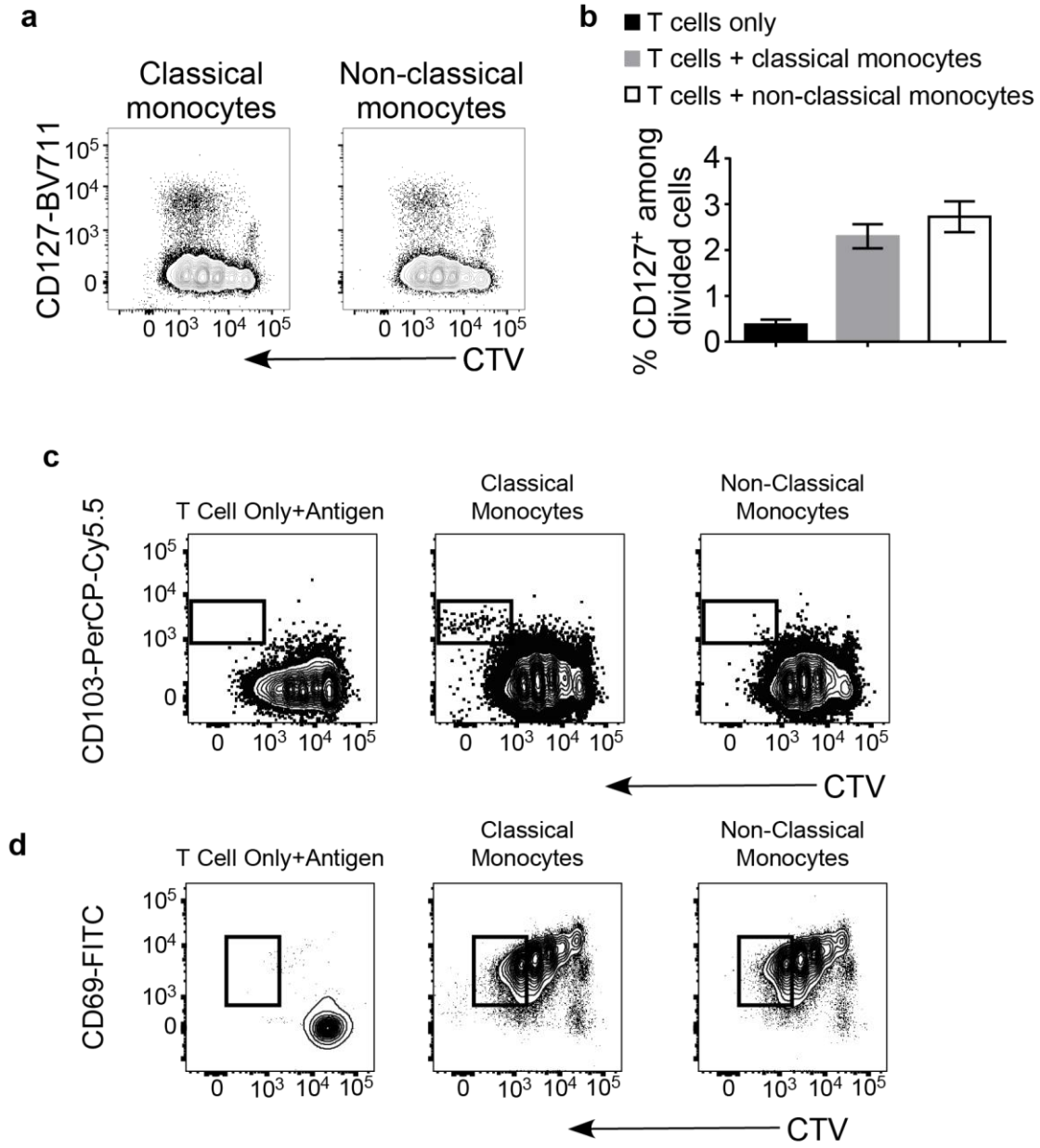
a
Classical monocytes
Non-classical monocytes
CD127-BV711
CTV
b
T cells only
T cells + classical monocytes
T cells + non-classical monocytes
% CD127+ among divided cells
c
T Cell Only+Antigen
Classical Monocytes
Non-Classical Monocytes
CD103-PerCP-Cy5.5
CTV
d
T Cell Only+Antigen
Classical Monocytes
Non-Classical Monocytes
CD69-FITC
CTV
e
% Parent CD62L+
% Parent CD49a+
T only + ag
Classical +ag
NC+Ag
DC 103+ ag
MoRDC +ag
11b+ag

Figure 10. Phenotype of OT-I T cells after three days of in vitro stimulation with lung APC subsets. (A) Representative flow plots of CD127 staining on CD8 OT-I T cells following three days of *in vitro* culture with OVA peptide pulsed monocytes of indicated subtypes. (B) Frequencies of CD127 positivity among highly divided CD8 OT-I T cells following three days of *in vitro* culture with OVA peptide pulsed monocytes of indicated subtypes. Error bars SEM, data represents 3 experiments using 3 technical replicates *in vitro* each. (C) Representative flow plots of CD103+ staining on CD8 OT-I T cells following three days of *in vitro* culture with OVA peptide pulsed monocytes of indicated subtypes. (D) Representative flow plots of CD69+ staining on CD8 OT-I T cells following three days of *in vitro* culture with OVA peptide pulsed monocytes of indicated subtypes.

DISCUSSION

Many studies have shown the importance of dendritic cells for the initiation of antiviral T cell responses following influenza infection, with particular subsets such as $CD8\alpha^+$ and $CD103^+$ DCs playing specific roles in naïve T cell activation and differentiation (Hao et al., 2008; Infusini et al., 2015; Kim and Braciale, 2009; Kim et al., 2014). Given the requirement for antigen re-encounter in the tissue for establishing lung T_{RM}, it was surprising that depletion of $CD11c^+$ cells after initial T cell activation showed that DCs were dispensable for lung T_{RM} formation. In contrast, inhibiting monocyte recruitment to the lung had a dramatic impact on the establishment of lung T_{RM}, despite having no effect on the magnitude of the effector T cell response. Thus, the ability of monocytes to promote T cell responses against influenza is not through the initial priming and expansion of antiviral T cells, but through their ability to present viral antigens to effector T cells in the infected lung tissue and drive T cell differentiation.

Classical monocytes have been characterized as innate inflammatory mediators that produce large amounts of IL-1, IL-6, and TNFα, and promote tissue damage (van Leeuwen-Kerkhoff et al., 2017), but their ability to drive adaptive immune responses through antigen presentation has been understudied (Jakubzick et al., 2017). Monocytes have been shown to promote T_H1 responses during viral infection through direct priming of naïve T cells in the lymph node (Kim and Braciale, 2009; Nakano et al., 2009; Zigmond et al., 2012). However, as we observed no defect in the flu-specific effector CD8 T cell response or the systemic flu-specific memory CD8 T cell pool in $CCR2^{-/-}$ mice, it is unlikely that the decreased numbers of lung T_{RM} in these mice are due to a defect in the initial priming of the flu-specific T cell response. Rather, our data show

that antigen presentation by pulmonary monocytes to effector CD8 T cells in the lung tissue is important for lung T_{RM} establishment. Consistent with these findings, Ly6C$^+$ inflammatory monocytes are efficient at cross-presentation to CD8 T cells in the presence of TLR agonists, especially TLR7 (Desch et al., 2014; Kastenmuller et al., 2011; Larson et al., 2016; Oh et al., 2011; Qu et al., 2009). Together, these findings support a model where monocytes in the infected lung are activated by viral TLR agonists, promoting efferocytosis of dying cells and enhancing cross-presentation of influenza antigens to flu-specific CD8 T cells, which ultimately drives the establishment of lung T_{RM}.

Several studies have identified roles for specific APC subsets in the establishment or maintenance of T_{RM}. Cross-presentation by DNGR-1$^+$ dendritic cells was shown to be required for optimal generation of T_{RM}, but not circulating memory T cells, in a model of cutaneous Vaccinia virus infection(Iborra et al., 2016). In the lung, targeting vaccines to respiratory dendritic cells or alveolar macrophages was shown to induce local T_{RM} that could protect mice against respiratory challenge(Macdonald et al., 2014; Vega-Ramos and Villadangos, 2013). Recently, it was reported that inflammatory monocytes were important for the maintenance of both lung T_{RM} and circulating memory T cell subsets following Vaccinia virus infection, but the mechanism by which monocytes were promoting memory maintenance was unknown(Desai et al., 2018). We have extended these findings to demonstrate a critical role for antigen presentation by monocytes in establishing lung T_{RM}. Although we did not observe a defect in the circulating memory T cell pool in our study, this discrepancy is likely due to the differences in tissue tropism between influenza and Vaccinia viruses. As influenza replication and inflammation is localized to the respiratory tract, the impact of monocyte antigen-presentation on

memory T cell development during a respiratory virus infection would be limited to the lung.

Although our data support a direct role for antigen presentation by pulmonary monocytes in driving lung T_{RM} establishment, there may be additional monocyte-derived factors contributing to this process, such as cytokines or the propagation of tissue repair. For example, monocytes can produce IL-15, which has been implicated in the initial lodgment of T_{RM}(Mackay et al., 2015). Monocytes are also prevalent in areas of tissue repair following viral clearance in the lung, and these sites have been identified as anatomical niches, termed repair-associated memory depots, that promote the maintenance of lung T_{RM}(Takamura et al., 2016). Future studies investigating antigen-independent functions of monocytes in T_{RM} differentiation and maintenance, and the interplay between monocytes and other APCs in these processes, will be required to fully understand the contributions of monocytes for the development of resident T cell memory.

Monocytes and effector CD8 T cells are recruited to discrete sites of inflammation in the lung through CCR2- and CXCR3-dependent mechanisms, respectively(Henri et al., 2001; Tsou et al., 2007). Given the large size of the lung, having a mechanism to recruit both APCs and virus-specific CD8 T cells to the same inflamed local microenvironment where virus is present is an efficient means to ensure T cells have access to antigen and accessory signals such as co-stimulation and cytokines that will promote their differentiation. The high number of antigen-bearing monocytes in the lung, their co-localization with antigen specific T cells, and their sufficiency to drive T cell differentiation *in vitro* all point to monocytes being critical mediators of T_{RM} differentiation.

It should be noted that monocytes did not induce robust expression of T_{RM} markers such as CD103 on all OT-I cells in our *in vitro* culture system, and we did not examine the transcriptional profile of these cells to assess the complete T_{RM} program(Kumar et al., 2017; Mackay et al., 2016) (MacKay Science, Farber Cell Reports). As the *in vitro* culture system cannot recapitulate all the complex interactions that guide CD8 T cell differentiation *in vivo*, we believe it is unlikely that monocytes alone are sufficient to program lung T_{RM} development following an influenza inflection. It seems likely that multiple T cell – APC interactions, separated by time (initial T cell priming and antigen re-encounter) and location (lymph node and infected lung) are required. One potential developmental pathway suggested by our data is that dendritic cells may induce the initial T_{RM} program, and that pulmonary monocytes may provide additional signals that lead to further progression or enforcement of this program. We are currently investigating these possibilities.

While significant technical hurdles remain, if strategies can be devised by which vaccine-derived antigens are presented by monocytes to activated T cells in the lung, this may enhance vaccine efficacy through the establishment of greater numbers of lung T_{RM}. These findings also provide a rationale for antibody-targeted mucosal vaccines, such as used by Villadangos et al(Wakim et al., 2015), to directly target vaccine antigens to lung monocytes in order to produce more robust T_{RM} responses. Combined with the previously demonstrated efficacy of dendritic cell targeting vaccines, this could offer an avenue towards a combination vaccine capable of generating robust cellular immunity in the lung. In summary, we have identified a novel role for antigen presentation by pulmonary monocytes in driving the establishment of lung T_{RM} following influenza virus infection. Further exploration of the mechanisms by which monocytes promote T_{RM}

differentiation may aid in the development of new strategies for vaccination against respiratory pathogens.

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AUTHOR CONTRIBUTIONS

P.R. Dunbar, S. Takamura, and J.E. Kohlmeier designed the experiments. P.R. Dunbar, E.K. Cartwright, A. N. Wein, T. Tsukamoto, Z.-R.T. Li, N. Kumar, I.E. Uddbäck, S.L. Hayward, S. Ueha, and S. Takamura performed experiments and analyzed data. I.E. Uddbäck and S. Takamura edited the manuscript. P.R. Dunbar and J.E. Kohlmeier wrote the manuscript.

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CD8+ T Cell Intrinsic IL-27R signaling is Required for the Development of a Robust CD8 Effector Response Following Influenza Infection

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Abstract:

IL-27 has pleiotropic effects on a range of immune cells, but its roles in shaping T cell responses during infection are not fully understood. Previous studies have shown IL-27 can influence both CD4 T cell differentiation and suppression, and recent studies have revealed a role for IL-27 in reducing immunopathology following viral respiratory infection. However, the impact of IL-27 signaling on antigen-specific CD8 T cells responding to a respiratory viral infection has not been studied. We utilized a direct competition model to compare antiviral CD8 T cell response in the presence and absence of IL-27 signaling following influenza infection. We find that while CD8 T cells lacking the IL-27 receptor (IL-27R) are competent to expand following stimulation *in vitro*, they exhibit a severe accumulation defect in both peripheral and lymphoid tissues during acute infection. Although this defect was supported by decreased proliferation in the lung and the development of fewer terminally-differentiated effector cells in the absence of IL-27 signaling, these observations could not fully account for the impaired expansion of virus-specific IL-27R$^{-/-}$ CD8 T cells. However, RNA-sequencing analysis of WT and IL27R$^{-/-}$ CD8 T cells revealed differential expression of genes involved in apoptosis. Furthermore, we observed that IL-27R$^{-/-}$ CD8 T cells apoptose at a greater

rate than their WT counterparts in both peripheral and lymphoid tissues. Surprisingly, the secondary effector response was similarly affected following heterologous influenza challenge, as IL-27R$^{-/-}$ memory CD8 T cells failed to accumulate to the degree of their WT counterparts. Together, these data show a cell-intrinsic role for IL-27 signaling in promoting the differentiation and survival of effector CD8 T cells during respiratory virus infection.

Introduction:

Respiratory viruses such as influenza require a robust cellular immune response for the propagation of an antiviral state within the infected tissue and viral clearance. (Braciale, 1977) While both CD4 and CD8 T cells are important for this control, virus-specific effector CD8 T cells play a critical role via direct killing of influenza-infected epithelial cells. (Slutter et al., 2013) Following initial TCR engagement in naïve CD8 T cells, the development of an antiviral T cell response is dependent on co-stimulatory cues, including the cytokine milieu, that guides the continued differentiation and programming of effector CD8 T cells. For example, IL-12 and type I interferons promote the development of highly cytolytic short-lived effector CD8 T cells through the induction of T-bet.(Wilson et al., 2008) The numerous cytokines that activated T cells encounter during an infection, either while expanding in the lymph nodes or upon entry into the infected tissue, will continually shape the virus-specific effector CD8 T cell response and the eventual establishment of CD8 T cell memory. Although the impact of many cytokines on effector CD8 T cell differentiation have been previously studied, we still lack a complete understanding of how different inflammatory cytokines that are

produced during an infection can influence the development of antiviral T cell responses. In this study, we focused on the role of IL-27 signaling in the generation and differentiation of effector CD8 T cells following influenza virus infection.

IL-27 is a pleiotropic IL-12 family member cytokine that has been shown to play diverse roles in immune responses. Produced primarily by antigen presenting cells, including monocytes and dendritic cells, it's receptor expression and effect on DCs, NK cells and CD4s are all fairly well described.(Gafa et al., 2006; Gwyer Findlay et al., 2013; Kumar et al., 2019; Laroni et al., 2011; Ruckerl et al., 2006) In CD4 T cells, IL-27R signaling promotes Th1 differentiation via STAT1-dependent expression of transcription factor T-bet, while suppressing Th17 differentiation. (Kumar et al., 2015; Takeda et al., 2003) However, the overall impact of IL-27 signaling on immunity is context dependent, and can have either a deleterious or protective role. In murine models of *Mycobacterium tuberculosis* and Cytomegalovirus infection, IL-27 signaling drives IL-10 production by CD4 T cells, thereby preventing effective control of the bacteria.(Batten et al., 2008; Clement et al., 2016; Kumar et al., 2015) In contrast, during parainfluenza virus infection, rapid IL-27 production in the lung is important for reducing immunopathology driven by neutrophil influx . (Sun et al., 2009) These contradictory roles highlight the context sensitive appropriateness of IL-27R signaling during an immune response. Less well understood, however, is the potential importance of cell-intrinsic IL-27R signaling for the development of antiviral CD8 T cell responses following respiratory virus infection.

While it is known that CD8 T cells express the IL-27 receptor, the importance of IL-27 signaling on the development of effector CD8 T cell responses during respiratory

infection has not been well defined. Previous studies have shown that IL-27R-deficient effector CD8 cells in the airways fail to produce IL-10, leading to increased immunopathology.(Jiang et al., 2016; Perona-Wright et al., 2012) However, these studies did not examine the impact of IL-27 signaling on the early development of the effector CD8 T cell response. Furthermore, due to the wide range of effects IL-27 can mediate in other immune cell types, a more direct experimental system to investigate CD8 T cell-intrinsic effects of IL-27 signaling is required. In this study we used a dual transfer OT-I system to directly compare WT and IL-27R-deficient CD8 T cells during influenza infection. We found that, while CD8 T cells lacking IL-27R are competent to activate and divide *in vitro*, the absence of IL-27R signaling *in vivo* resulted in a severe impairment of the effector CD8 T cell response. Compared to WT CD8 T cells in the same host, IL-27R-deficient effector CD8 T cells failed to accumulate during the early stage of infection, showing decreased proliferation, increased apoptosis, and a skewing from a short-lived effector phenotype to a memory precursor phenotype. RNA sequencing of WT and IL-27R-deficient CD8 T cells showed a defect in the type I interferon signature in the absence of IL-27 signaling, and decreased expression of ISGs associated with cell survival during viral infection. Together, these findings highlight the importance of IL-27 signaling for the development of a robust effector CD8 T cell response following respiratory virus infection and identify a novel role for IL-27 in promoting the survival of virus-specific effector CD8 T cells.

Results

Competition model and Intrinsic signaling

We established a competition model to directly compare wild type versus IL-27R$^{-/-}$ CD8s of the same antigen specificity in an otherwise normal host. To this end we generated congenically mismatched wild type OT-I transgenic mice on the Pep Boy (CD45.1+, CD45.2-, CD90.1-, CD90.2+) as well as IL-27R$^{-/-}$ OT-I transgenic mice on the B6 background (CD45.1-, CD45.2+, CD90.1-, CD90.2+). From these naive OT-I CD8s were isolated from the spleens, mixed 1:1 WT and IL-27R$^{-/-}$ and 10,000 total cells adoptively transferred intravenously into Pep Boy X B6 mice (CD45.1+, CD45.2+, CD90.1-, CD90.2+) one day prior to intra-nasal infection with x31 (H3N2) influenza expressing OVA protein (Fig. 1A). As seen in Fig. 1B these populations of OT-I CD8 T cells both respond to the x31-OVA-I and are easily distinguished from one another, as well as host CD8s, by flow cytometry. This system allows us to preform pairwise comparisons between OT-I T cell populations in the same mouse.

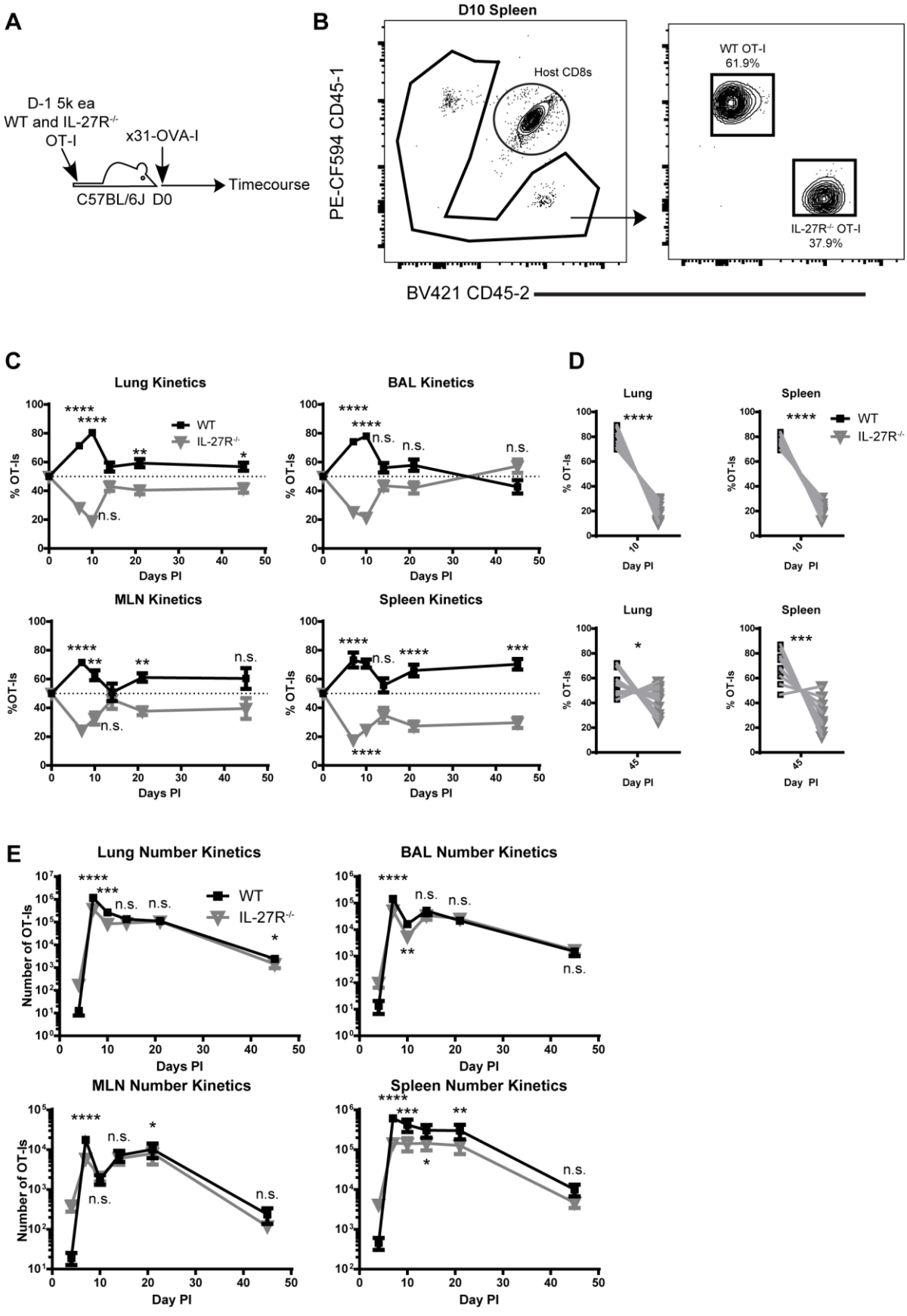

A
D-1 5k ea
WT and IL-27R-/-
OT-I
x31-OVA-I
C57BL/6J D0
Timecourse
B
D10 Spleen
Host CD8s
WT OT-I
61.9%
IL-27R-/- OT-I
37.9%
PE-CF594 CD45-1
BV421 CD45-2
C
Lung Kinetics
BAL Kinetics
MLN Kinetics
Spleen Kinetics
WT
IL-27R-/-
% OT-Is
%OT-Is
Days PI
D
Lung
Spleen
Day PI
E
Lung Number Kinetics
BAL Number Kinetics
MLN Number Kinetics
Spleen Number Kinetics
Number of OT-Is
Days PI
Day PI

Figure 1. IL-27R$^{-/-}$ OT-Is are identifiable in competition model, and fail to accumulate to normal numbers during influenza infection. A. Experimental Diagram of competition model. B. Representative flow plots and gating strategy for identification of WT and IL-27R-/- OT-Is, data shown is from D10 post infection spleen total CD8s. C. Frequency of total OT-I's of each genotype in indicated tissue. D. Frequency of total OT-Is presented as paired data from D10, D45 post infection. E. Number of OT-Is of indicated genotype in indicated tissue during course of infection. **** p<0.0001, *** p 0.0001 to .001, ** p 0-.001 to 0.01, * p ≤0.05, ns p >0.05(two-tailed paired Student's *t*-test) All graphs error bars are S.E.M. Data are representative of 3 independent experiments with 5 mice per time point.

Lack of receptor leads to defect in acute response as seen in terms of frequency as well as number

Over the course of x31 influenza infection in mice we see a robust response by 7 days post infection, the peak of the flu specific CD8 T cell response at day 10, and the collapse of this populations numbers into memory days 14-21 We consider day 45 post infection to be well into memory as it is long after any detectible virus was present in the lung, and after the flu specific CD8 population has largely stabilized in number. Thus, in our competition model we ran a time-course, sampling mice at 7,10,14,21, and 45 days post infection. During acute infection days 7 and 10 the WT OT-I T cells show a distinct and significant advantage in both frequency and number over the CD8 OT-Is lacking the IL-27 receptor. This is observed both in primary lymphoid tissues where the CD8s are initially activated such as the mediastinal lung draining lymph node (MLN) and the

spleen, but also in the infected tissue of the lung as well as in the lung airways as sampled via bronchiolar lavage (BAL) (Fig. 1 A,B). This difference across infected tissues, primary response generation sites, as well as in the periphery indicates that this is a systemic defect in the CD8s that is independent of the environment present in the infected lung or airway. During the collapse of the short lived effector population days 14 and 21 post infection the defect in accumulation of IL-27R$^{-/-}$ OT-I T cells persists, with the WT OT-Is maintaining a significant advantage, though it is notable that this advantage is reduced from that seen during the acute response, going from 80% vs 20% in favor of the WTs to 60% 40% in most tissues (Fig 1A,B). This may indicate a selective reduction of a particular T cell subset; this possibility is explored later in this paper. This significant but lessened difference persists through memory. Looking at the paired data at days 10 and 45 post infection, it is apparent that there is a clear defect in the IL-27R$^{-/-}$ OT-Is during the acute infection, but this relationship is less clear at the memory time-points (Fig 1D). So while on a population level there is certainly a trend towards less IL-27R$^{-/-}$ CD8s than WT CD8s in memory, there appears to be a larger role for stochastic variance in each individual host, indicating the potential for either the WT OT-Is to decline proportionally more than the IL-27R$^{-/-}$ OT-Is, or for the IL-27R$^{-/-}$ OT-Is to proliferate to similar numbers as the WTs. Looking at the dynamics of the T cell response in terms of cell number, we find that both the WT and IL-27R$^{-/-}$ OT-Is exhibit similar kinetics over the course of infection, but that the IL-27R$^{-/-}$ OT-Is accumulate significantly less than the WT OT-Is (Fig 1E).

IL-27R$^{-/-}$ OT-Is are biased towards MPEC generation

As our data indicated that the defect seen in the OT-Is lacking IL-27R was severe during acute infection, but lessened in severity into memory, we suspected that there may be differential establishment of memory precursor (MPEC) versus short lived effector (SLEC) T cells. Here we define MPECs as CD127+, KLRG-1- and SLECs as CD127-, KLRG-1+ OT-I T cells (Fig2 A). At D7 post infection there is a significant difference in the frequency of MPECs and SLECs. We see that the IL-27R$^{-/-}$ T cells, while reduced in number (Fig 1E) have a significant bias towards the generation of MPECs above that of the WT OT-I T cells (Fig 2B). Likewise, the WT T cells have a significant bias towards the generation of SLECs. Thus it may be the case that the WT OT-Is are generating many short lived effector cells as well as a normal number of memory precursors while the IL-27R$^{-/-}$ OT-Is are producing fewer short lived effectors but a nearly normal number of longer lived memory precursors. This would explain the dramatic difference during acute infection but the smaller difference seen after population collapse and into memory time-points.

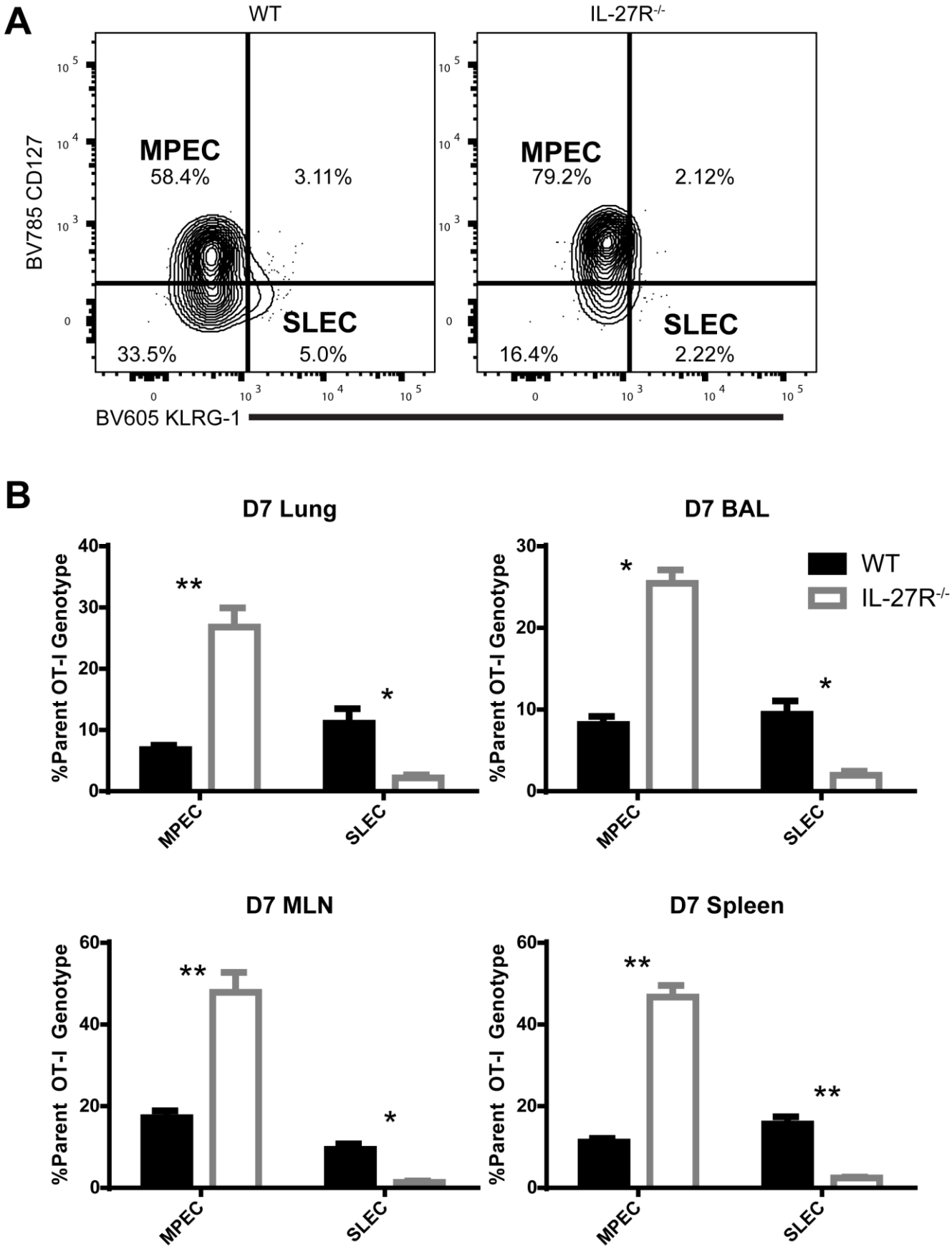

A
WT
IL-27R-/-
BV785 CD127
BV605 KLRG-1
MPEC
58.4%
3.11%
SLEC
33.5%
5.0%
MPEC
79.2%
2.12%
SLEC
16.4%
2.22%
B
D7 Lung
D7 BAL
D7 MLN
D7 Spleen
%Parent OT-I Genotype
WT
IL-27R-/-
MPEC
SLEC

Figure 2. IL-27R-/- OT-Is are biased towards MPEC, not SLEC generation following influenza infection. A. Representative flow diagram from D7 post infection MLN indicating data and gating strategy. B. Frequency of MPEC/SLEC populations as a proportion of parent OT-I genotype. MPEC defined as CD127+,KLRG-1-, SLEC defined as CD127-, KLRG-1+. **** p<0.0001, *** p 0.0001 to .001, ** p 0-.001 to 0.01, * p ≤0.05, ns p >0.05(two-tailed paired Student's *t*-test) All graphs error bars are S.E.M. Data are representative of 3 independent experiments with 5 mice per time point.

Polyclonal CD8 T cell response re-capitulates findings from OT-I model

To confirm that this phenotype was generalizable to CD8s of various specificities and affinities, and not an artifact high affinity transgenic OT-I T cells we established a competition system using mixed bone marrow chimeras, mixing WT and IL-27R$^{-/-}$ bone marrow 50:50 in a congenic host (Fig 3A). This allowed for clear identification of our target WT and IL-27R$^{-/-}$ CD8s, and CD44 and *FluNP366*-374D^{b} tetramer staining allowed for identification of the influenza specific cells generated (Fig 3B). We find that the general phenotype is the same, that WT CD8s accumulate to a greater degree than their IL-27R$^{-/-}$ counterparts during acute infection, but interestingly the dynamics/kinetics of this phenotype are slightly different (Fig 3C). In the mixed bone marrow chimera, IL-27R$^{-/-}$ CD8s have an advantage in frequency as well as number day 7 post infection, before the peak of the T cell response at day 10 post infection, by which

time the WT CD8s have overtaken the IL-27R$^{-/-}$ CD8s in both number and frequency (Fig 3 C,D). This indicates that in a polyclonal response IL-27R may have a differential role to play early on depending on the TCR affinity of the CD8 T cell, but this variance is insufficient to eliminate the general phenotype of dependence on IL-27R for accumulation during acute infection. Similar to the results of the direct competition model, the dynamics of the response as seen via T cell number are also similar between the WT and IL-27R$^{-/-}$ OT-Is, though again there is a failure of the IL-27R$^{-/-}$ OT-Is to accumulate to normal numbers (Fig. 3D).

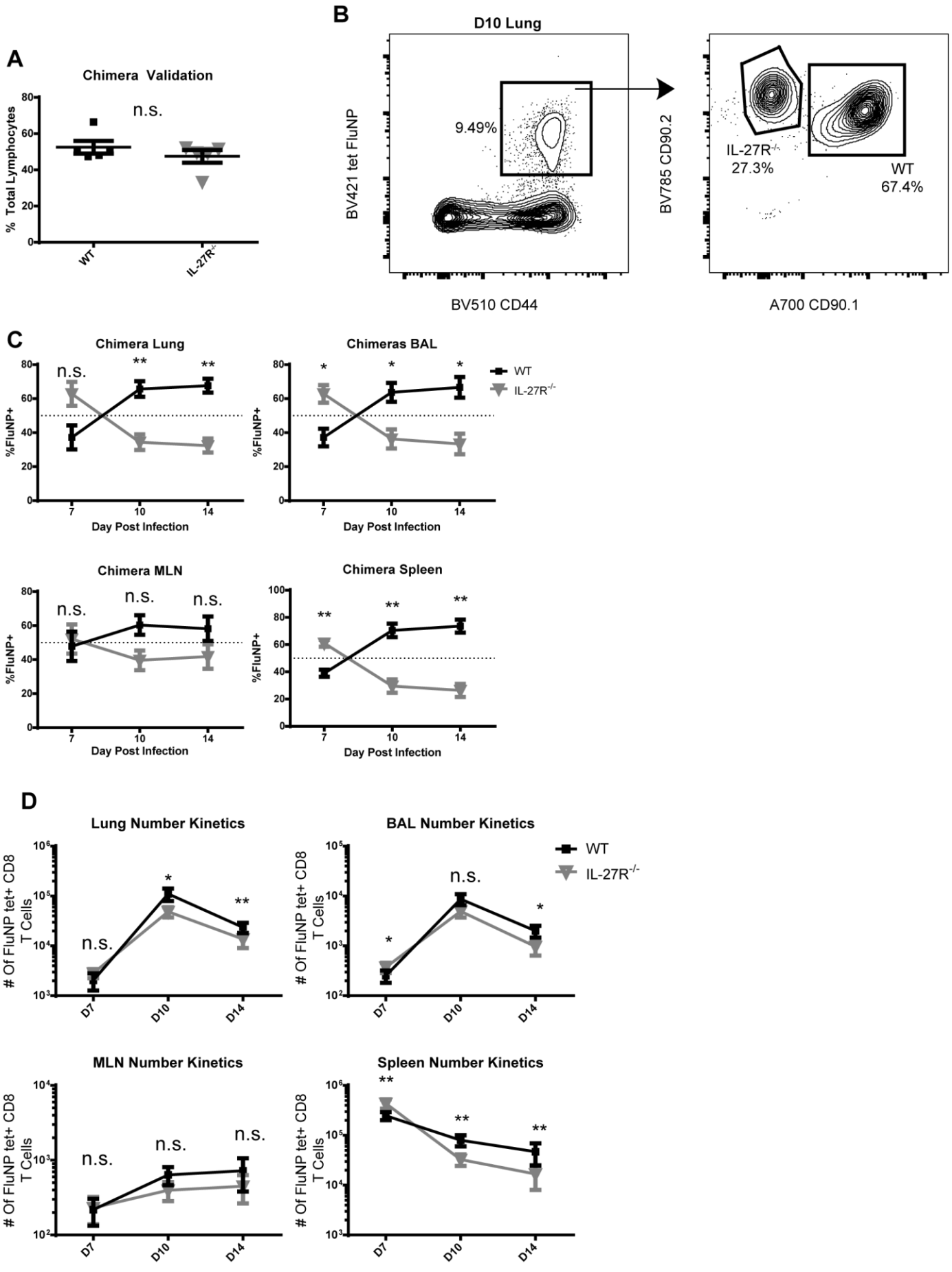

A
Chimera Validation
% Total Lymphocytes
n.s.
WT
IL-27R-/-
B
D10 Lung
BV421 tet FluNP
9.49%
BV510 CD44
BV785 CD90.2
IL-27R-/-
27.3%
WT
67.4%
A700 CD90.1
C
Chimera Lung
Chimeras BAL
Chimera MLN
Chimera Spleen
%FluNP+
Day Post Infection
WT
IL-27R-/-
D
Lung Number Kinetics
BAL Number Kinetics
MLN Number Kinetics
Spleen Number Kinetics
Of FluNP tet+ CD8 T Cells
D7
D10
D14

Figure 3. Polyclonal response to influenza infection in mixed chimeras show similar defect in IL-27R-/- CD8s. A. Validation of 50/50 mixed bone marrow chimeras with frequency of each genotype as a percentage of total lymphocytes. B. Representative flow plots and gating strategy for the identification of WT and IL-27R-/- FluNP specific CD8 T cells. C. Frequency of total FluNP specific CD8 Response of each genotype in indicated tissue. D. Number of WT and IL-27R-/- FluNP specific CD8 T cells in indicated tissue during course of infection. **** p<0.0001, *** p 0.0001 to .001, ** p 0-.001 to 0.01, * p ≤0.05, ns p >0.05(two-tailed paired Student's *t*-test) All graphs error bars are S.E.M. Data are representative of 3 independent experiments with 5 mice per time point.

IL-27R$^{-/-}$ OT-Is are competent to activate *in vitro*

To ensure the basic function of our IL-27R OT-Is they and WT OT-Is were isolated as naiive CD8s from spleens, stained with cell trace violet (CTV) and cultured for 3 days in the presence of CD3/CD28 stimulating beads to test if the cells were competent to activate and divide in vitro. After 3 days in culture, the IL-27R$^{-/-}$ OT-Is accumulated to significantly higher cell counts (Fig. 4B), and CTV staining indicated significantly more IL-27R$^{-/-}$ OT-Is divided (Fig. 4A,C). Further, significantly more IL-27R$^{-/-}$ OT-Is underwent three cell divisions, while almost no WT OT-Is did (Fig. 4C). Thus we may conclude that not only are IL-27R$^{-/-}$ OT-Is competent to activate and divide, they do so more robustly than OT-Is with the IL-27 receptor.

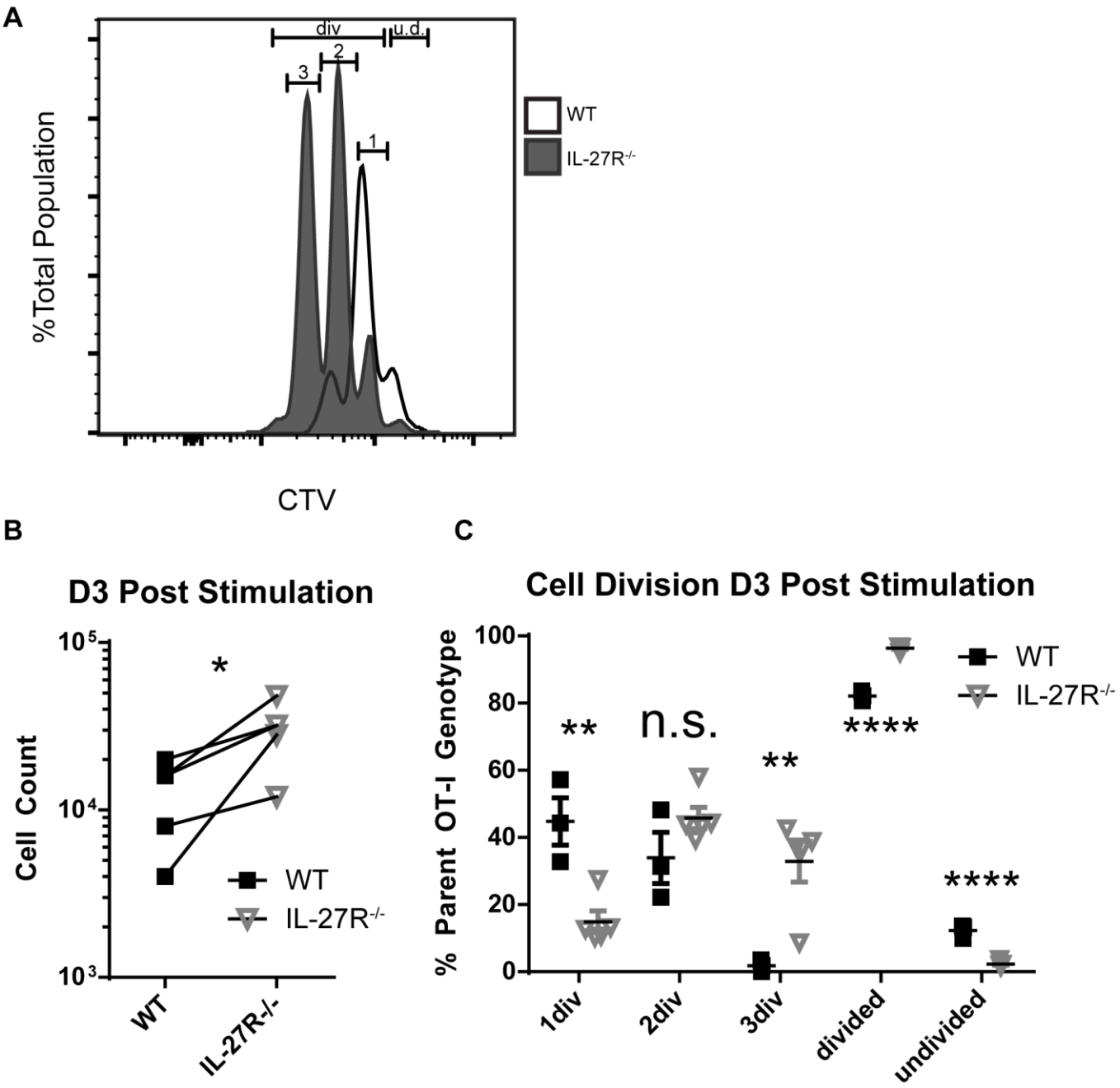


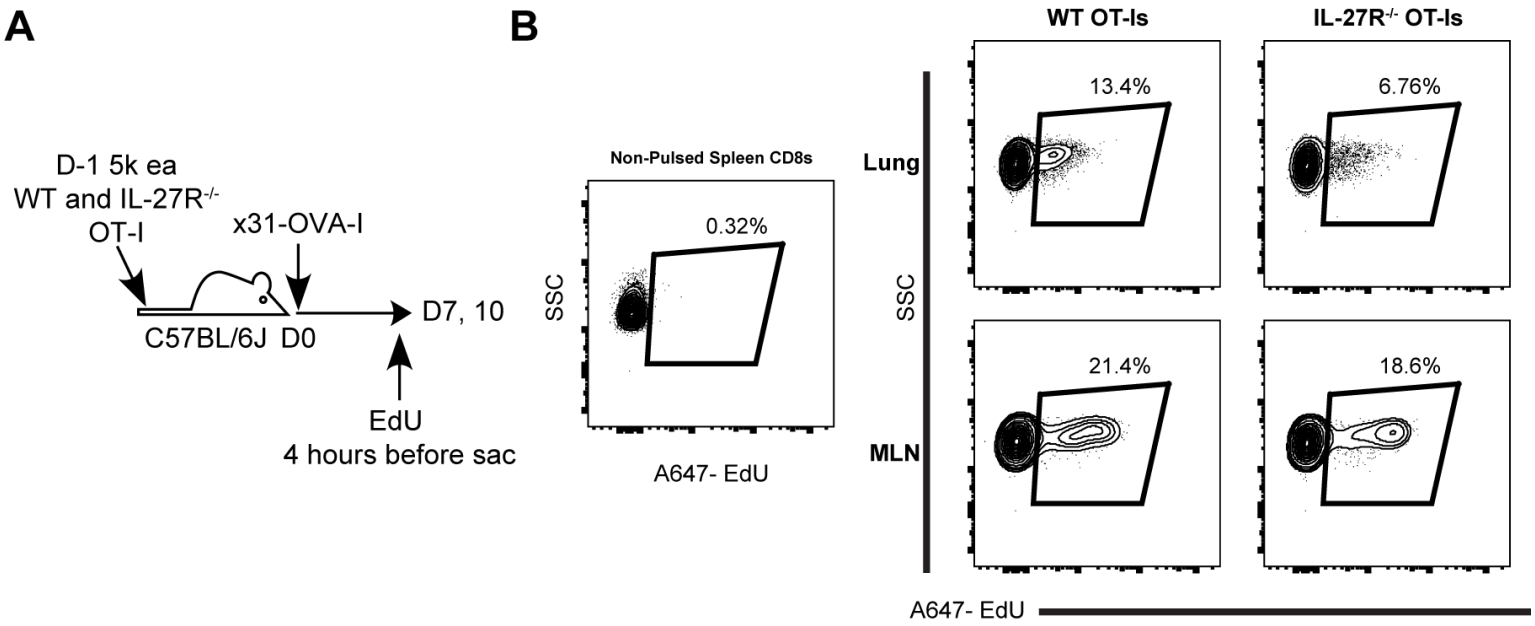
Figure 4. In vitro stimulation indicates IL-27R-/- are competent to activate and divide following TCR stimulation. A. Representative histogram showing data and gating strategy for cells after 3 days of in vitro stimulation with CD3/CD28 beads. B. Cell counts of each genotype in each well after 3 days of stimulation. C. Frequency of

division status of each genotype following 3 days of stimulation. **** p<0.0001, *** p 0.0001 to .001, ** p 0-.001 to 0.01, * p ≤0.05, ns p >0.05(two-tailed paired Student's *t*-test) All graphs error bars are S.E.M. Data are representative of 2 independent experiments with 3 wells of technical replicates for each sample.

IL-27R$^{-/-}$ OT-Is fail to continue dividing in infected tissues

IL-27 receptor engagement can lead to STAT-I signaling on CD4 T cells, and STAT-I signaling is implicated in cell proliferation in CD8 T cells. Thus, if similar signaling is occurring on IL-27R engagement in CD8s it is reasonable that the lack of the receptor could lead to less cell proliferation. Further, other groups have found that a key aspect of the CD8 effector response against influenza is the continued division of the effector T cells in the infected tissue.(Lawrence and Braciale, 2004; McGill and Legge, 2009) To verify the proliferation of WT and IL-27R$^{-/-}$ OT-I CD8s in vivo we used our competition model and dosed the mice with the nucleotide analogue 5-ethynyl-2'-deoxyuridine (EdU) 4 hours prior to sacrifice (Fig. 5A). During staining we then used a Click-iT EdU cell proliferation kit to identify the cells that proliferated during that 4-hour window, as indicated by their incorporation of EdU (Fig. 5B). This indicated that both WT and IL-27R$^{-/-}$ OT-Is proliferated to a similar degree day 7 post infection in the secondary lymphoid organs (MLN and spleen), indicating that the generation of both WT and IL-27R$^{-/-}$ OT-I T cell responses and the proliferation of WT and IL-27R$^{-/-}$ OT-I T cells in the periphery was not defective (Fig. 5C). However, at the sites of infection in the lung as well as in the lung airways the IL-27R$^{-/-}$ OT-Is proliferated significantly less (Fig. 5C). Further, 10 days post infection cell division in the infected tissues has largely ceased for both populations (Fig. 5C). However, in the spleen a significantly larger proportion of

IL-27R$^{-/-}$ OT-Is are continuing to divide compared to WT. This may further explain the total dynamics seen of an early, dramatic advantage for the WT OT-Is shifting to a more moderate advantage into memory, the 27R$^{-/-}$ OT-Is may be proliferating longer, allowing some limited catching up that contributes to the lessened defect at memory timepoints.


A
D-1 5k ea
WT and IL-27R-/-
OT-I
x31-OVA-I
C57BL/6J D0
D7, 10
EdU
4 hours before sac
B
Non-Pulsed Spleen CD8s
0.32%
SSC
A647- EdU
WT OT-Is
IL-27R-/- OT-Is
Lung
MLN
13.4%
6.76%
21.4%
18.6%
SSC
A647- EdU

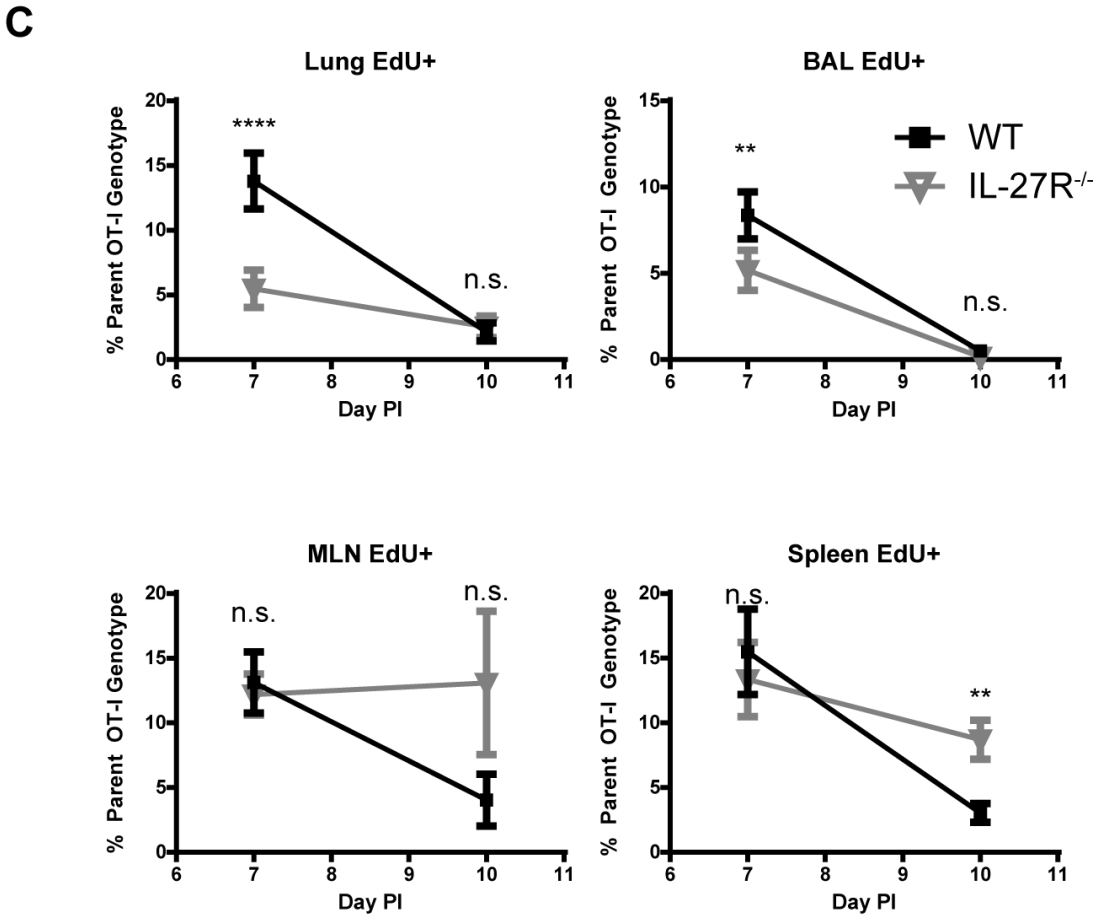
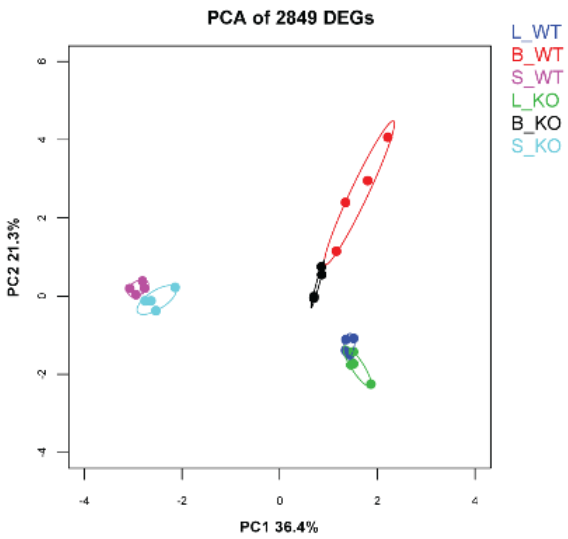

C
Lung EdU+
BAL EdU+
MLN EdU+
Spleen EdU+
% Parent OT-I Genotype
Day PI
WT
IL-27R-/-
****
**
n.s.

Figure 5. In vivo EdU staining indicates IL-27R-/- OT-Is fail to divide in influenza infected tissues. A. Experimental Diagram of competition model with EdU pulse. B. Representative flow diagrams indicating gating strategy and data from D7 post infection. C. Frequency of parent genotype that was EdU+. **** p<0.0001, *** p 0.0001 to .001, ** p 0-.001 to 0.01, * p ≤0.05, ns p >0.05(two-tailed paired Student's *t*-test) All graphs error bars are S.E.M. Data are representative of 3 independent experiments with 5 mice per time point.

RNA Sequence Analysis Implicates Type One Interferon Signaling

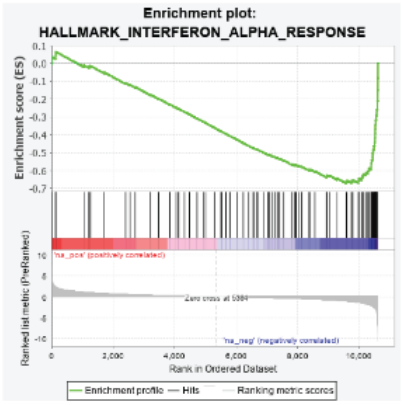
To investigate the potential phenotypic differences in our WT and IL-27R$^{-/-}$ populations we preformed RNA-seq analysis on samples sorted from the lung spleen and BAL ten days post infection from our competition model. Via PC analysis we found that the populations sort together based on tissue of origin more so than genotype, indicating that the local environment has a larger impact on these cells than their IL-27 receptor status (Fig. 6A). Gene Set Enrichment Analysis indicated that the interferon alpha response pathways failed to upregulate in the IL-27R$^{-/-}$ OT-I population (Fig. 6 B). Further analysis of genes of interest indicated that this was the case across tissues, and may point to a reduced anti-viral response of IL-27R$^{-/-}$ CD8s (Fig 6C). This lack of type 1 interferon signaling may lead to early apoptosis of activated cells, which would potentially explain in part the accumulation defect observed.

A

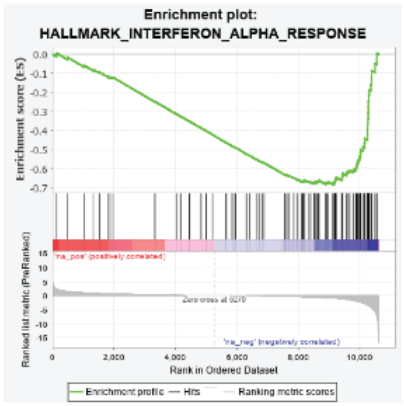


B

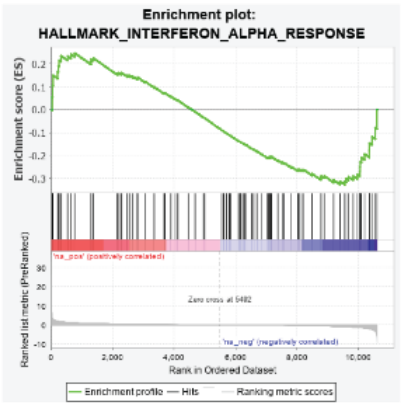
Lung



BAL



Spleen



C

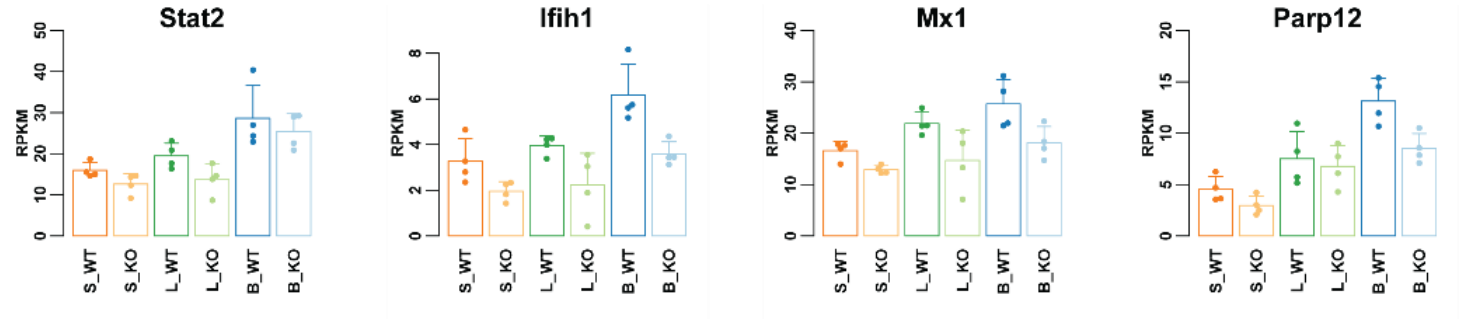
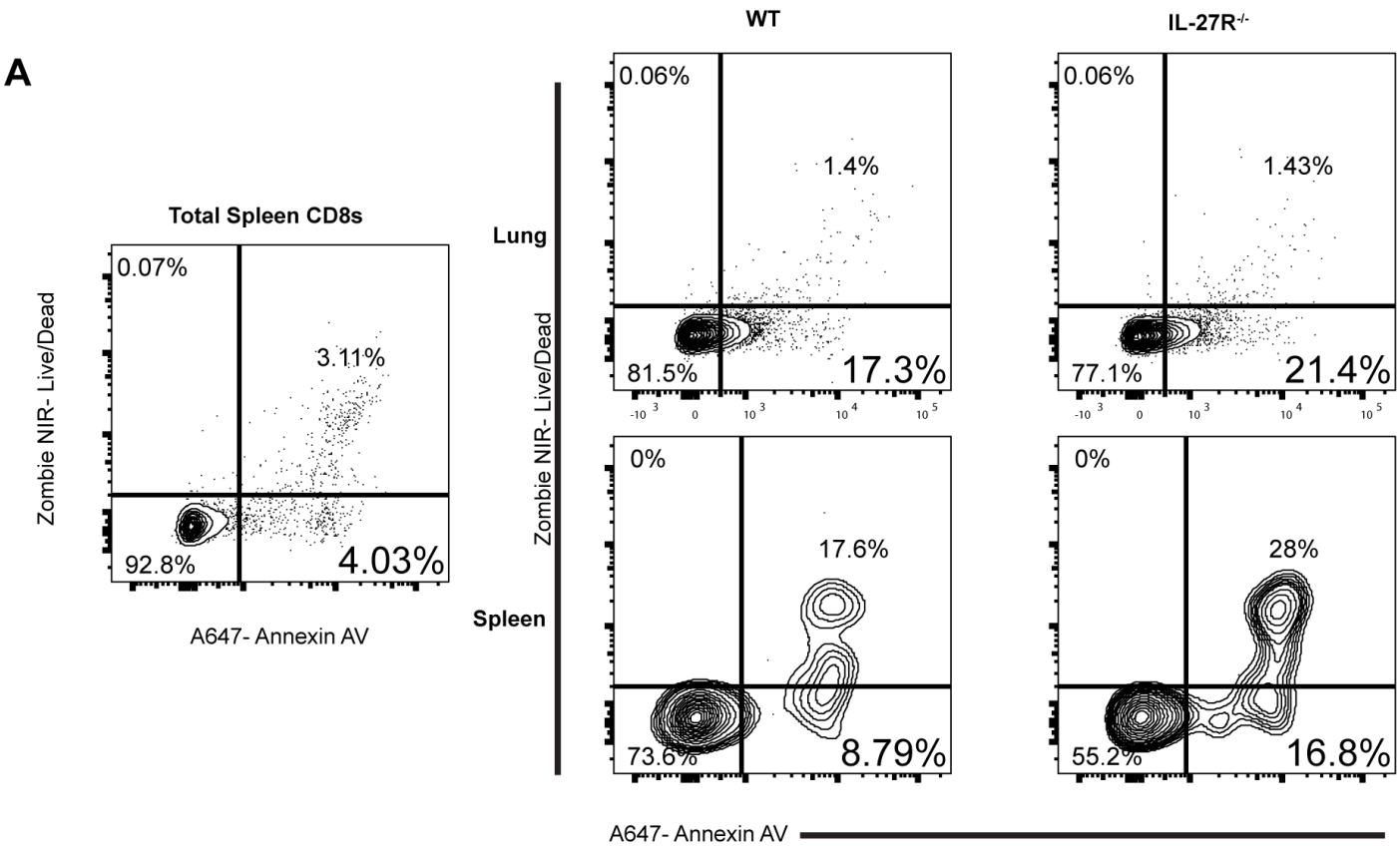


Figure 6. RNA Sequence analysis indicates a lack of Type 1 Interferon Signaling. A. Principle component analysis validating populations of cells sorted from indicated organs 10 days post infection. B. Gene Set Enrichment Analysis of interferon alpha responses. C. Differential expression of individual genes indicated in interferon alpha responses.

IL-27R$^{-/-}$ OT-Is apoptose at a significantly greater rate across tissues

Thus, given the results of our RNA seq experiments we hypothesized that the IL-27R deficient cells are activating and dividing, but dying off before they can accumulate to similar numbers as the WT OT-Is. To test this, we combined zombie live/dead staining that reacts with the primary amine groups only accessible once a cells membrane integrity has failed with Annexin AV staining which binds the phosphatidyl serine groups that are exposed on the outer-leaflet of the plasma membrane as a cell begins to apoptose. This allows us to functionally detect and discriminate between live, early apoptotic and dead cells, based on their staining with neither marker, Annexin 5, or both Annexin 5 and zombie stain respectively (Fig. 7A). At 7 days post infection we find that in all tissues the IL-27R$^{-/-}$ OT-Is are apoptosing at a greater rate, which would explain the severe systemic defect we see in their accumulation (Fig. 7B). This leads us to a model where both WT and IL-27R$^{-/-}$ OT-Is are activating and dividing normally in response to viral insult, but the IL-27R$^{-/-}$ OT-Is are losing more cells to early apoptosis in addition to failing to continue to proliferate after trafficking to the infected tissues.


A
WT
IL-27R-/-
Total Spleen CD8s
Lung
Spleen
Zombie NIR- Live/Dead
A647- Annexin AV
0.07%
3.11%
92.8%
4.03%
0.06%
1.4%
81.5%
17.3%
0.06%
1.43%
77.1%
21.4%
0%
17.6%
73.6%
8.79%
0%
28%
55.2%
16.8%

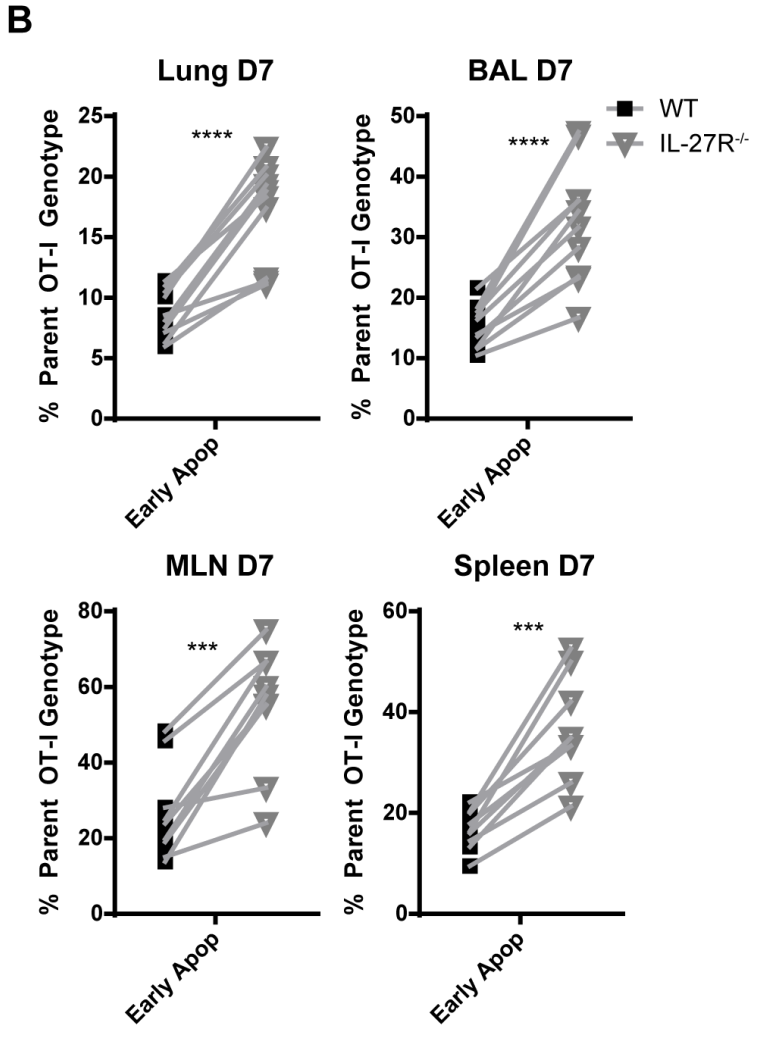

B
Lung D7
BAL D7
MLN D7
Spleen D7
WT
IL-27R-/-
% Parent OT-I Genotype
Early Apop
****
****
***
***

Figure 7. IL-27R-/- OT-Is apoptose at a greater frequency than WT OT-I T cells following influenza infection. A. Representative flow diagrams and gating strategy for early apoptotic cells defined as Annexin AV+, Zombie NIR-. B. Frequency of early apoptotic cells as a fraction of total OT-I population of indicated genotype. **** p<0.0001, *** p 0.0001 to .001, ** p 0-.001 to 0.01, * p ≤0.05, ns p >0.05(two-tailed paired Student's *t*-test) All graphs error bars are S.E.M. Data are representative of 3 independent experiments with 5 mice per time point.

IL-27R$^{-/-}$ OT-Is exhibit defective memory response to heterologous challenge

It is possible that while there is a defect in the accumulation of IL-27R$^{-/-}$ OT-Is following acute infection, the receptor may be dispensable for a recall/memory response. To test this we established our competition model as before, infecting the mice with H3N2 X31-OVA-I (Fig. 8A). Once in memory, 35 days post infection, we sampled the mice blood to establish baseline OT-I frequency in the periphery, revealing that in the periphery there was no significant difference between the WT and IL-27R$^{-/-}$ OT-Is at memory (Fig. 8B, C). We then infected the mice with H1N1 PR-8-OVA-I to provide a heterologous challenge, avoiding the previously generated antibody response against H3N2 X31 virus. 7 days post heterologous challenge we find that the defect in accumulation of IL-27R$^{-/-}$ OT-Is not only persists, but has returned to the same severity observed during acute infection (Fig. 8D, E). This indicates that not only is IL-27R signaling important in the establishment of a normal primary response to acute viral infection, but is also important in the generation of a robust response upon secondary challenge as well.

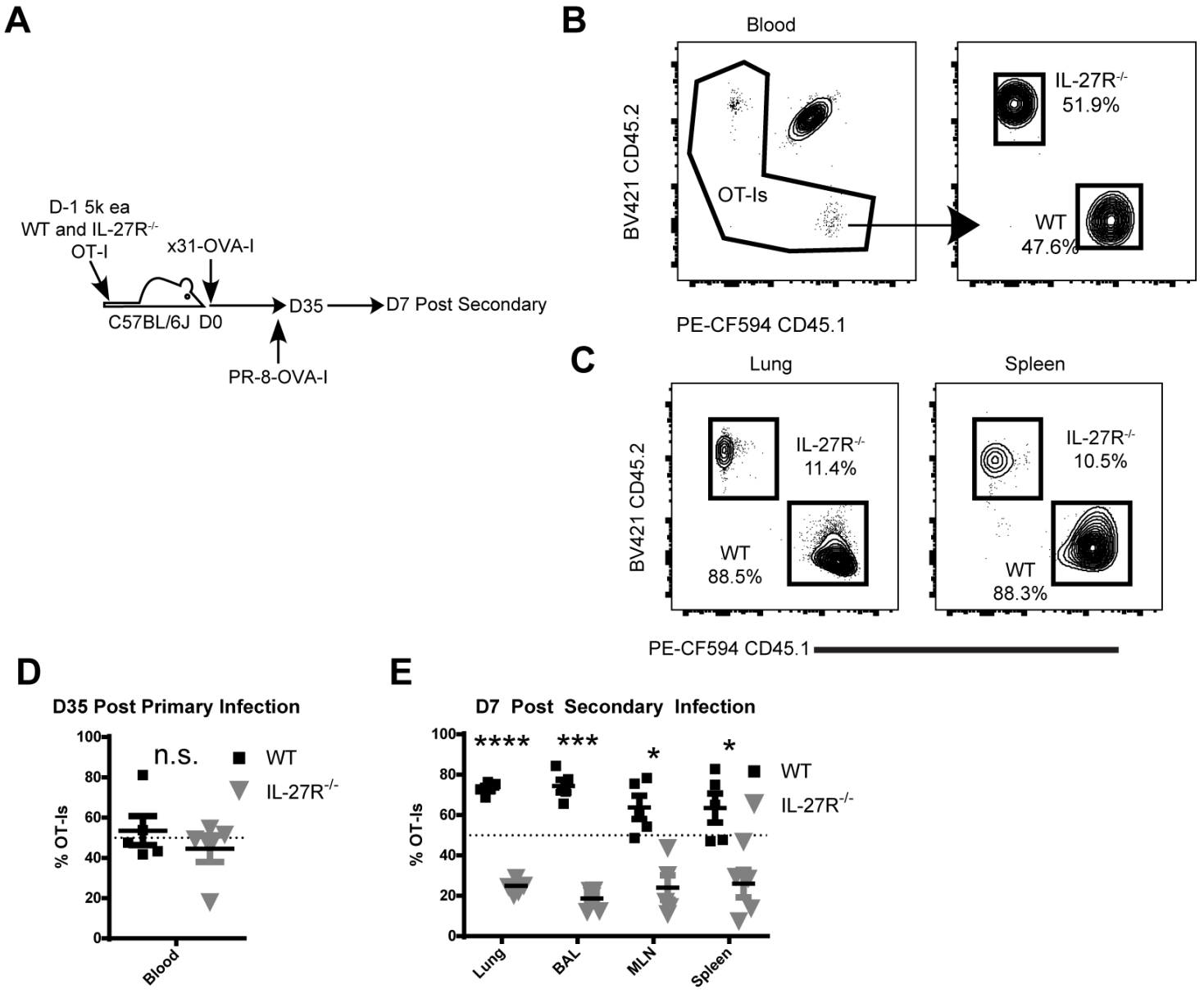


Figure 8. IL-27R-/- OT-Is fail to divide in response to secondary viral challenge. A. Experimental diagram of competition model for secondary memory response examination. B. Representative flow plots and gating strategy for identification of WT and IL-27R-/- genotype OT-Is in peripheral blood D35 post primary infection C. Representative flow plots and gating strategy for identification of WT and IL-27R-/-

genotype OT-Is in lung and spleen 7 days post-secondary infection. D. Frequency of total OT-I's of each genotype in peripheral blood D35 post primary infection E. Frequency of total OT-I's of each genotype in each indicated tissue 7 days post-secondary infection. **** p<0.0001, *** p 0.0001 to .001, ** p 0-.001 to 0.01, * p ≤0.05, ns p >0.05(two-tailed paired Student's *t*-test) All graphs error bars are S.E.M. Data are representative of 3 independent experiments with 5 mice per time point.

Discussion:

While the role of IL-27R is increasingly appreciated for its effects on CD4 T cell differentiation in the context of cancer, as well as viral and bacterial infections, much less is known about the role of the receptor on CD8 T cells. Previous studies have indicated a strong potential for a cell intrinsic role for the IL-27 receptor on CD8s, but have not tested this directly. In this study we established a sensitive and specific system for testing the role of the receptor on CD8 T cells in an in vivo model accounting for the host environment as well as T cell specificity and affinity. Our data indicates that CD8 T cells lacking the IL-27 receptor suffer a severe defect in accumulation during the acute phase of an anti-influenza virus response. However, in vitro testing indicates that these cells are capable of responding to TCR engagement and co-stimulation to a degree at least equivalent to wild type T cells. Thus this difference must be driven by the use of IL-27 receptor engagement as a "signal-3" in T cell activation. This defect is persistent but reduced through collapse and memory following clearance of virus, which may be

partially explained by a bias shift towards the production of MPECs as opposed to SLEC CD8s. In this model the IL-27R$^{-/-}$ OT-Is may be proliferating but not forming the rapidly dividing SLECs, while producing almost normal numbers of MPECs. Further this defect may be contributed to by the fact that CD8s lacking the IL-27 receptor proliferate normally in secondary lymphatic tissues but fail to continue to proliferate in the infected tissues as their wild type counterparts do. However, this tissue specific proliferation defect would not explain the systemic phenotype exhibited. A more complete explanation may be seen in the fact that the IL-27R$^{-/-}$ OT-I CD8s apoptose at a greater rate across all tissues tested, offering an explanation for the dramatic systemic defect seen, when taken along with the SLEC/MPEC polarization differences as well as the lack of proliferation in the infected tissue. In a polyclonal mixed bone marrow system, we see similar results, though with slightly altered initial kinetics. In the polyclonal system the IL-27R$^{-/-}$ CD8s appear to have an early advantage at 7 days post infection, but by day 10 the WT CD8s have significantly overtaken them, and this relationship persists through memory, largely recapitulating the results of our OT-I competition model and confirming this is not a defect limited to OT-I T cells. Shockingly the defect reasserts itself in the heterologous memory response, indicating that the IL-27R$^{-/-}$ memory cells established are less able to respond to viral re-challenge, and indicating a probable role for IL-27 in the mediation of a recall response. This evidences a further role for IL-27R signaling not just in the generation of the acute response, but in the response of memory cells. This, along with the apoptosis data indicates that IL-27 may be a previously unappreciated survival factor for CD8 T cells responding to acute viral infection. Signal 3 is critical for the ultimate fate of generated T cells, and increasing appreciation of IL-27 as a key signal 3 required for the mediation of an effective acute response may offer

insights into interventions in acute viral settings. IL-27 is an apparently important signal to consider in the generation of a robust and efficacious vaccine response, and attention should be paid to vaccination strategies and adjuvants that elicit IL-27 and IL-27 competent cells.

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Summary

Chapter 1 gave a review of the innate and adaptive immune systems, and in particular highlighted the role of CD8 T cells in mediating effective anti-viral responses. In this summary, a primary focus was on CD8 T cell activation and differentiation. Using the three-signal hypothesis as our framework, we explored current canonical ideas of T cell activation in the generation of an acute response. In addition, we surveyed distinguishing T cells by their anatomic compartment, giving an overview of effector, central, and resident memory T cells. Of special note were pulmonary tissue resident memory T cells, which are well positioned to provide a rapid and broad response. Pulmonary antigen presenting cells as drivers of CD8 T cell differentiation were also discussed as a part of this focus. The later chapters built on this information to provide new data indicating both novel roles for pulmonary monocytes in CD8 T_{RM} differentiation as well as a newly described role for cytokine IL-27 in mediating an anti-viral CD8 T cell response following influenza infection. This summary also identified multiple deficiencies in the knowledge of the field, as well as how they can be addressed. In particular, prior to this work little was known about the individual role of pulmonary antigen presenting cell subsets in the generation of pulmonary tissue resident memory T cells. While much was known about the role of these antigen presenting cells in the generation of a T cell response to influenza in general, the particular contributions of each subset to the generation of tissue resident memory T cells was poorly understood. Further, much of the previous work had focused on the role of pulmonary dendritic cells, with much less focus on pulmonary monocytes. While monocytes have been studied for their role mediating immune pathology during acute infection, these studies

did not focus on T cell memory responses formed with the help of monocytes. Concerning the role of cytokine IL-27 in effector CD8 T cell responses, much attention had been paid to the cytokines effect upon CD4 T cells, but much less was known about its role for CD8 responses. In particular, a system to directly compare CD8 T cells with and without the IL-27 receptor had not previously been developed.

Chapter 2 described the ability and sufficiency of pulmonary inflammatory monocytes to drive CD8 T cell activation, division, and differentiation to a tissue resident phenotype following influenza infection. Further, these cells were demonstrated to be drivers of protection against heterologous challenge mediated by influenza specific lung tissue resident memory CD8s. This identification of monocytes as a key driver of lung tissue resident memory CD8 T cell differentiation outlines some potential difficulties as well as opportunities moving forward to a vaccine to elicit lung T_{RM} based protection. As inflammatory monocytes are drivers of immunopathology, we cannot merely draw them into the lung for a vaccine response, as such inflammation in the lung would be not just unpleasant but potentially dangerous for a large part of the population (Aldridge et al., 2009). However, with the causative cell type identified, further refined studies may be performed to investigate first the exact signals that pulmonary monocytes are making. Further testing of these factors using the in vitro system established in Chapter 2 to explore T cell activation and differentiation into a T_{RM} like phenotype may prove a fertile testing bed for rapid exploration of a variety of factors in isolation and combination. This information may lead to finding novel signals to drive T_{RM} phenotypes while avoiding excessive inflammation. The import of a vaccine to elicit a protective pulmonary T_{RM} response to influenza was outlined in detail in

Chapter 1, but it is worth repeating that such a vaccine would provide not just effective protection against a disease that debilitates millions and kills tens of thousands of people a year, but would provide broader protection against a rapidly mutating virus, allowing easier vaccine development versus the current strategy of selecting probable B cell epitopes from predicted strains. This strategy would also further reduce the need for yearly vaccination, making vaccination campaigns easier, cheaper, and presumably higher compliance as a consequence. Currently, given vaccination efficacy and vaccination rates, effective herd immunity is a pipe dream, but a vaccine that provides a wider baseline of protection, in combination with current vaccine strategies may be able to get us to a threshold where herd immunity reduces overall influenza transmission and burden.

Current issues with furthering the development of monocyte signals include the difficulty of culturing the monocytes. In vivo, monocytes are a short-lived cell once they activate unless they differentiate into longer lived macrophages. Even this developmental pathway is relatively rare, with monocytes developing into macrophages generally only in environments that have been depleted of their resident macrophage populations (Geissmann et al., 2010). However, as monocytes, they are pulled into an inflamed tissue, serve their purpose and die off rapidly. Some may live slightly longer if they differentiate into non-inflammatory monocytes and assist with the resolution of inflammation. Even in this scenario. monocytes are short lived and difficult to work with outside of a tissue. Despite this, Chapter 2 lays out a method for the isolation and culture of monocytes isolated from virally infected tissues. This method provides a way to culture ex-vivo monocytes either in isolation or with other immune cells such as T

cells. This provides a platform to study factors such as T cell activation and differentiation as in Chapter 2. This platform may also be broadened to study the individual signals being produced by monocytes isolated from different tissues or different infection conditions. Thus, there is much promise in adoptive cell therapies where we engineer cells to produce the desirable products of a monocyte without needing the traditional monocyte itself.

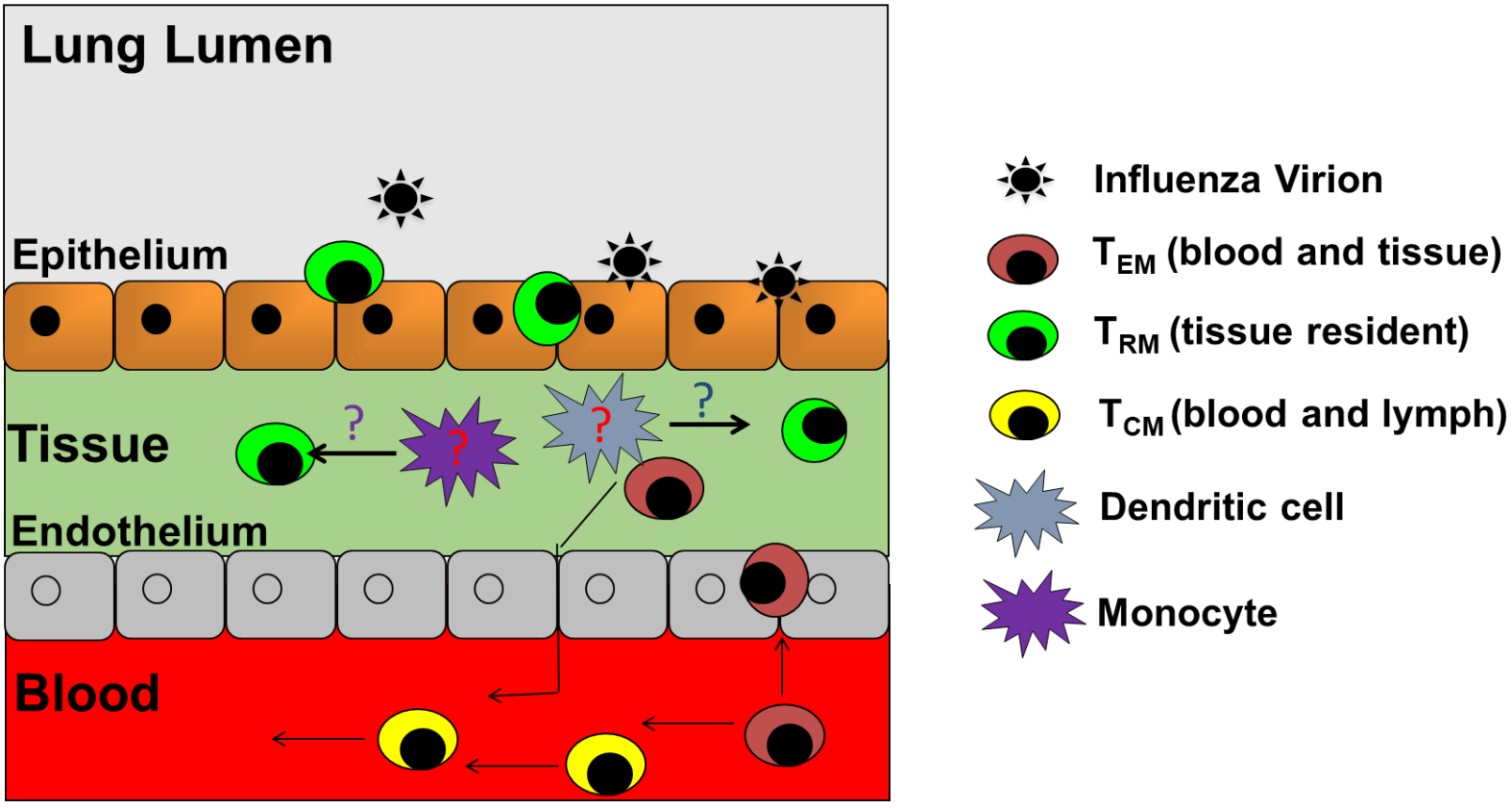

Figure 1. Working model of pulmonary T cell-APC interactions, indicating potential roles for pulmonary antigen presenting cells explored in Chapter 2. Figure adapted from slides provided by Jacob Kohlmeier PhD.

Chapter 3 laid out the multiple roles IL-27 plays in infectious disease. It explained that IL-27 is produced by a variety of antigen presenting cells in response to a

variety of TLR stimulation. As a consequence, IL-27 is present following a variety of bacterial as well as viral infections. The IL-27 receptor is also a widely expressed receptor, appearing on lymphocytes including CD4 T cells, CD8 T cells, natural killer cells as well as antigen presenting cells including dendritic cells and monocytes. Despite this omnipresence, its role is not simple, varying between immune-stimulatory and immune inhibition depending on context. Likewise, the desirability of the cytokine is also highly context dependent, varying from being a key inhibitor of an effective response against chronic disease to being a desirable component to prevent unnecessary immunopathology. This makes the study of the cytokine in specific contexts such as influenza infection particularly important. Despite this wealth of information about the cytokine, very little had been studied about its potential role on CD8 T cells. We felt this represented an obvious hole in the field. Given the receptor expression on CD8 T cells, and IL-27's role in preventing immunopathology during pulmonary viral challenges, we felt it likely that IL-27 may also be suppressing CD8 T cells during influenza infection. To test this, we developed a new competition model exploiting the adoptive transfer of wild type and IL-27R$^{-/-}$ OT-I T cells to develop a system to compare CD8 T cells of the same specificity, in the same host, and thus in the same micro-environments, with and without the receptor of interest. This provided a much cleaner systems than those previously used in the field, which relied on mixed bone marrow chimeras. These previous models had the caveat that there were multiple aberrant cell types acting in them, which could skew results. Our direct competition system on the other hand, allows for direct comparison of CD8 T cells with and without the IL-27 receptor in the same host. To our surprise we found that CD8 T cells lacking the IL-27 receptor, rather than increasing in number after being released from an immune-suppressive signal,

accumulated to much lower numbers than CD8 T cells with the IL-27 receptor present. Further analysis revealed this defect to be due to a combination of factors including them not proliferating in infected tissues, as well as the activation of a pro-apoptotic program across all tissues. This indicates a surprising role for IL-27 as an unappreciated survival factor for CD8 T cells during an anti-viral response. While we saw no tissue specific response, we saw that a lack of IL-27 receptor signaling led to an accumulation and apoptotic defect across tissues. This opens the way for potential therapies to boost anti-viral responses exploiting the IL-27 receptor signaling pathway, either through the use of recombinant IL-27 or more likely through the use of small molecule agonists that signal through the IL-27 receptor. Modulating the IL-27 pathway has already been described in cancer models to increase CD8 killing in models of solid tumors engineered to overexpress IL-27 (Gonin et al., 2013). As IL-27 has already been described to reduce immunopathology, it is much more likely to be a well-tolerated therapy in comparison to the ideas of harnessing monocytes to direct CD8 activity described above. IL-27 may have a role to play not just as a therapeutic option but also as a vaccine adjuvant if it's supplementation or stimulation of increased production can lead to the establishment of a larger viral pathogen specific CD8 pool following vaccination. Viral specific CD8 T cell numbers are predictive of protection in many systems, so non-inflammatory methods of increasing them are crucial to the future of effective T cell eliciting vaccination strategies. Thus IL-27 therapy shows some potential as either a vaccine adjuvant or therapeutic treatment targeted to generating an increased CD8 T cell effector response during influenza infection.

Combining Ideas

Thus, this thesis outlines methods of increasing the viability and efficacy of T cell-based vaccination strategies, as well as the future potential of T cell-based therapies. Methods explored include both manipulation of T cell differentiation and localization, as well as identification of factors important to the size of the initial T cell response. The factors described outline two contrasting themes present in immune responses; the necessity of inflammation for an effective response, and the immunopathology driven damage that can cause. It also demonstrates the need for survival factors that are themselves often immuno-suppressive, and the balance that must be struck between causing enough damage to elicit an effective response, versus providing sufficient survival and memory factors to mediate the transition from acute effector response to the establishment of long-lived memory CD8 T cells. All of this must also occur without excessively suppressing the acute response itself. Some of the ideas going forward in balancing these ideas include developing vaccines around timed release of different signals, which presents interesting problems of packaging, and dosing (Veiseh et al., 2015). Packaging the vaccine presents challenges and opportunities for delivery of stimulus to not just specific locations in the lung, but also the delivery of specific stimulus at specific times. Dosing challenges include maximizing efficacy while minimizing both immunological pathogenicity and cost. Of particular interest is the development of materials that could allow a timed-release schedule of various factors(Vegas et al., 2016). This could enable temporal isolation of various signals that are individually appropriate for the initiation and later differentiation of a response. This is important to balance the factors described above, as many memory promoting

cytokines such as IL-10 also have immune suppressive effects if present early in an immune response. Temporal isolation of signals important in the initial generation of the response which may include IL-27 and factors from inflammatory monocytes, from memory T cell promoting factors will likely be key to developing effective T cell vaccines. A hypothetical system for influenza may be the delivery of an intra-nasal vaccine using some combination of materials including antigen and adjuvant that are initially available to cause the minor inflammation needed to activate APCs and draw T cells back into the tissue for antigen re-encounter. This goes along with what was discussed in Chapter 2 with early activation of T cells in response to influenza needing DCs but them being dispensable for later T_{RM} differentiation. On a delayed release could be factors specific for the development of T_{RM} including TGF-β and other factors that may be discovered in further studies of monocytes. This delayed release could serve a twofold purpose. As TGF-β can be immunosuppressive (Yoshimura and Muto, 2011), it is important that it not be present in the generation of the initial response, and delaying its release until activated T cells are actually present in the tissue can also reduce the amount required, making the vaccine cheaper and easier to produce. Further delay for the release of immune suppressing factors such as IL-10 and possibly IL-27 that are important in mediating the differentiation of long- lived memory T cells may serve a similar dual purpose as well. Fundamentally the generation of long-lived memory CD8 T cells is the goal, and any push in that direction may increase the overall efficacy of the vaccines. Possibly this could also enable the use of harsher, more inflammatory adjuvants in the initial stages, to mediate a large and rapid response that is then quickly and efficiently differentiated and tamped back down by the release of immune-suppressive signals. While less ideal than a system not requiring inflammation, at

present it seems unlikely that we will develop effective vaccines that don't require an inflammatory response, and thus careful modulation and control of this response may be the most appropriate intermediate goal. Use of both a minimum of specific inflammatory signals, as well as the delivery of those and other factors in the proper controlled order will be key to recapitulating the natural responses that lead to durable T cell memory formation.

As for the far future of T cell vaccines, the increasing interest in creating engineered T cells for therapeutic use against cancer may provide some illustrative ideas of what could be next for T cells against infectious disease. Chimeric-antigen-receptor or CAR T cells are an engineered cell type that may be made specific to any conceivable antigen. This can be done regardless of the hosts MHC alleles that would normally limit antigen presentation. Further, recent developments in the generation of these cells allow them to be programmed with basic logic loops, allowing them to be tailored for particular environments, or otherwise made "smarter" to prevent unnecessary immunopathology (Chakravarti et al., 2019). As an example, a CAR could be engineered with a system to detect its presence in the gut mucosa via binding to integrin $\alpha4\beta7$, and using that engagement to drive a residency program to establish itself as a long-lived tissue resident T cell in the gut. Thus, an engineered a cell could avoid the need for local inflammation in development of a residency phenotype, greatly increasing the safety of the therapeutic strategy. This could avoid factors such as the inflammatory signals being provided by pro-inflammatory monocytes described above. Such a system could thereby avoid undue immunopathology by using alternate means to drive desirable differentiation paths in the CD8 T cells. While no such single factor has been found for

the lung, it is likely that there are factors unique to the lung that could be exploited, and even in the unlikely absence of those, factors limited to a few tissues like the lung and liver like CXCL16 may provide sufficient specificity of activation to allow the cell to establish itself largely in the tissue of interest. Combining the ideas above, this work outlines the importance of individual signals in the development of effector and tissue specific responses to influenza virus infection. These signals however need to be delivered in a controlled manner to limit undesirable effects, and this control may be provided by future developments in both material engineering as well as cell therapy discoveries.

This may even be amenable to a more generalizable strategy. As mentioned in Chapter 1, there are many transcription factors that appear to underlie common tissue residency programs for CD8 T cells. Instead of designing logic circuits to turn on a variety of individual genes, we may be able to exploit the transcription factors as chokepoints where induction of just the transcription factor can lead to the development of a tissue resident memory cell. Thus, the necessary complexity of the system can be reduced. This could provide a further avenue to avoid immunopathology as discussed above. By exploiting otherwise benign signals to induce the programs normally instigated by signals with deleterious side effects we can deliver safer, but still efficacious, treatment. At present there are many difficulties of such ideas, as they are largely limited to autologous treatments that would require a lot of specialized labor as well as time, leading to very high individual patient cost. However as presented at AAI2019 there are a variety of ideas coming up in the field looking into the development of “off-the-shelf” allogenic cell therapies using T cells engineered to be themselves

immunologically stealthy. These ideas may provide methods to create products that could be delivered to the public at large, rather than having to be produced for individual patients. Such products would also avoid potential pitfalls that would be expected in products relying on patients to have particular HLA alleles or other specific variable genetic factors that would be required for a successful vaccination.

Thus, the work contained in this thesis has advanced the field. It has done this by identifying gaps in current knowledge about the role of individual pulmonary antigen presenting cell subsets in generating lung tissue resident memory CD8 T cells protective against influenza virus. This gives new insight into the roles of monocytes as antigen presenting cells important in the differentiation of lung tissue resident memory CD8 T cells. It also provides a methodology for the ex-vivo culture of pulmonary monocytes, providing techniques for the exploration of a difficult to culture cell type. Further, it identifies a paucity of information about the potential action of cytokine IL-27 on CD8 T cells during influenza infection. By developing a new experimental system, it finds that IL-27 is an important mediator of CD8 T cell accumulation during the response to influenza. as well as discovering a novel role for IL-27 receptor signaling on CD8 T cells in the effective generation of an effector response. In summation, this work provides new insight into the development of both lung tissue resident, as well as general effector CD8 T cell responses following influenza infection, and this information may be carried forward into future studies geared towards producing effective T cell-based influenza vaccines.

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