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CD154+ CD4+ T cell dependence for effective memory influenza virus-specific CD8+ T cell responses.

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ABBREVIATIONS: DCs, dendritic cells; NP, nucleoprotein**;** PA, acid polymerase**;** WT, wild type.

Running title: CD154 dependent programming of flu memory T cell responses.

ABSTRACT

CD40-CD154 (CD40-ligand) interactions are essential for the efficient priming of CD8$^+$ cytotoxic T lymphocyte (CTL) responses. This is typically via CD4$^+$CD154$^+$ T cell dependent "licensing" of CD40$^+$ dendritic cells (DCs), however DCs infected with influenza A (IAV) virus upregulate CD154 expression thus enabling efficient CTL priming in the absence of CD4$^+$ T activation. Therefore, it is unclear whether CD4 T cells and DCs play redundant or unique roles in priming of primary and secondary CTL responses after infection. Here we determine the precise cellular interactions involved in CD40-CD154 regulation of both the primary and secondary IAV-specific CTL response. Infection of both CD40 KO and CD154 KO mice resulted in diminished quantitative and qualitative CTL responses after both primary and secondary infection. Adoptive transfer of CD154$^+$, but not CD154KO, CD4 T cells into CD154KO mice restored both primary and secondary IAV-specific CD8 T cell responses. These data show that although CD154 expression on CD4 T cells and other cell types (i.e. DCs) may be redundant for the priming of primary CTL responses, CD154 expression by CD4 T cells is required for the priming memory CD8 T cells that are capable of fully responding to secondary infection.

INTRODUCTION

Interactions between CD40 and its ligand, CD154, are central to ensuring both effective primary and secondary $CD8^+$ cytotoxic T lymphocyte (CTL) responses to infection. Activated $CD4^+$ T cells play a key role in the priming of optimal acute $CD8^+$ cytotoxic T lymphocyte (CTL) responses and establishment of CTL memory[1-4]. In the context of acute CTL responses, activated $CD4^+$ T cells that express CD154, "license" dendritic cells (DCs) via ligation of CD40[5] resulting in more efficient cross-presentation of antigen[1, 6, 7] and production of IL-12 production that can serve as a third signal[8], both of which ensure the development of highly differentiated CTL responses[9].

It has long been recognised that $CD4^+$ T cell help is key for initiating the developmental program that leads to the establishment of memory $CD8^+$ T cells. In particular, it has been proposed that IL-2 produced by activated CD4 T cells during an immune response acts to promote memory T cell programming[10]. However, a more recent study demonstrated that only when activated CD4 T cells could express CD154, needed to licence DCs via CD40 ligation, could $CD8^+$ memory T cells be generated[11]. What isn't clear is whether the CD40-CD154 interactions necessary for both primary and secondary CTL responses involve the same or distinct cell-cell interactions.

Several reports have now demonstrated that that acute CTL response to bacterial and viral infections, including influenza A virus (IAV), can be induced in the absence of $CD4^+$ T cell help[2, 4, 12, 13]. In the case of acute influenza A virus infection, the observed CD4 independence of primary virus-specific CTL responses has been explained by the ability of IAV infection to directly activate TLR7, and subsequently induce CD154 expression on DCs[14]. These activated DCs are then able to directly prime CD40-expressing virus-specific CD8 T cells[14]. Thus, a paradox is apparent with primary IAV-specific CTL responses readily primed via CD154-CD40 interactions imparted by DC-CTL contacts, rather than CD4-DC dependent licensing. However, while IAV infection is capable of inducing an

acute CTL response in the absence of $CD4^+$ T cell help, the establishment of effective memory CTL populations is deficient[13, 15].

In this study, we attempt to resolve this apparent paradox by determining the precise role of $CD154^+$ $CD4^+$ T cells in the establishment of optimal acute and memory $CD8^+$ T cell responses in the context of the IAV infection model. Mice deficient for both CD40- (CD40KO) and CD154 (CD154KO) displayed diminished acute CTL responses induced by influenza A virus infection. Importantly, adoptive transfer of virus-specific $CD154^+$ $CD4^+$ T cells into CD154KO, but not CD40KO mice, was able to restore both the magnitude and effector function of influenza A virus-specific CTL responses. The ability of $CD154^+$ $CD4^+$ T cells to restore effective CTL responses in CD154KO mice also correlated with improved viral clearance. Further, adoptive transfer of $CD154^+$ $CD4^+$ T cells into CD154KO mice also restored the ability of memory CTL to be effectively recalled. This suggests that $CD4^+$ T cells, activated in response to influenza A virus infection, are the major source of CD154, and hence ensure efficient programming of robust effector and memory virus-specific CTL responses.

RESULTS

Influenza-specific CD8 T cell responses are limited in CD40- and CD154-deficient mice.

Both CD40KO and CD154KO mice exhibit decreased virus-specific CTL responses and delayed viral clearance in a number of model systems[16-18]. To further investigate this phenomenon, wildtype (wt), CD40KO-CD154KO, and anti-CD154 (clone MR1)-treated mice were infected with the recombinant influenza A virus, A/HKx31-OVA_{323}[19] and the splenic D^bNP_{366}- and D^bPA_{224}-specific CTL responses enumerated 10 days after infection (**Fig 1A, B**). The magnitude of both the D^bNP_{366}- and D^bPA_{224}-specific CTL responses was decreased ($p<0.05$, ~4-fold) in CD40KO, CD154KO and nti-CD154-treated mice, when compared to wt controls (**Fig 1A, B)**. Thus, this supports earlier evidence that the CD40-CD154 nexus is key in determining optimal primary virus-specific CTL responses[18]. Interestingly, the ability of D^bPA_{224}-, but not D^bNP_{366}-specific, CTL to simultaneously produce IFN-γ and IL-2 was also diminished in the CD40KO, CD154KO and anti-CD154 treated mice when compared to wt controls (**Fig. 2A, B, p<0.05**).

WT CD4 T cell help rescues influenza-specific CD8 T cell responses in CD154-deficient mice.

To better define the cellular interactions that direct CD40-CD154 dependent priming of acute IAV-specific CTL responses, we adoptively transferred wt OTII TCR transgenic $CD4^+$ T cells into CD40KO, CD154KO or anti-CD154-treated mice, infected with HKx31-OVA_{323} and tissues harvested for analysis as described earlier. Adoptive transfer of wt OTII $CD4^+$ T cells restored D^bNP_{366}- and D^bPA_{224}-specific CTL responses in CD154KO mice, but not CD40KO or nti-CD154-treated mice (**Fig. 1A, B, *p*<0.05**). Moreover, adoptive transfer of wt OTII $CD4^+$ T cells restored the capacity of D^bPA_{224}-specific CTL in CD154-deficient mice to produce IL-2 (**Fig. 2B**), and rescued full IFN-γ production in both D^bNP_{366}- and D^bPA_{224}-specific CTL (**Fig. 2C, D, p<0.05**). These data suggest that in the absence of CD154 expression on APCs, CD154-expressing $CD4^+$ T cells are sufficient for

optimal primary IAV-specific CD8 T cell responses.

It is possible that molecules, other than CD154 on the transferred CD4 T cells, may be playing a role in rescuing CD8 T cell responses in CD154KO mice. However, adoptive transfer of CD154KO OTIIs into CD154KO recipients did not rescue either the magnitude or functional capacity of influenza A virus-specific CTL (**Fig. 1 and 2**). Thus, CD154 expression on activated $CD4^+$ T cells is both necessary and sufficient for promoting sustained CTL responses after IAV infection when CD154 expression is absent on other cells, such as DCs.

CD154-sufficient CD4 T cell help enhances viral clearance in CD154-/- recipients.

Given that CD40 and CD154 are required for optimal IAV-specific CD8 T cell responses in the spleen, we investigated if these interactions were also critical in generating robust pulmonary CTL responses that may impact viral clearance. In these studies, the lungs of naïve wt or CD154KO mice that received wt OTIIs or PBS were assayed for pulmonary influenza-specific CTL responses 6.5 days after infection with HKx31-OVA_{323}. While the magnitude of the D^bNP_{366}- and D^bPA_{224}-specific CTL responses in the lung were similar in wt mice, with or without adoptive transfer of OTII $CD4^+$ T cells (**Fig. 3A, B, *p*>0.05**), CD154KO mice exhibited significantly reduced numbers of pulmonary D^bNP_{366}- and D^bPA_{224}-specific CTL **(Fig. 3A, B, *p*<0.05**). Importantly, both the total number of tetramer and IFN-γ-producing D^bNP_{366}- and D^bPA_{224}-specific CTL was fully restored to wt levels in CD154KO mice that had received $CD154^+$ OTII CD4 T cells (**Fig. 3A-D**). Moreover, the functional capacity of D^bPA_{224}-specific CTL, both in terms of the amount of IFN-γ per cell (**Fig. 3E**) and proportion of TNF-α^+ of IFN-γ^+ CTL was restored in CD154KO mice that received $CD154^+$ OTII $CD4^+$ T cells.

To determine if adoptive transfer of wt OTII $CD4^+$ T cells enhanced clearance of virus from the lungs of CD154KO mice, we assessed viral titres in each group of mice after

infection. Firstly, both WT and CD154KO mice that received adoptive transfer of OTII CD4 T cells exhibited similar ($p>0.05$) numbers of OTII cells in the lungs at this time point, suggesting that these cells proliferated to a similar extent in both groups of mice (Fig 3G). Adoptive transfer of naive $CD4^+$ OTII T cells into wt mice reduced viral loads ~26-fold compared to PBS-treated mice (**Fig. 3H, *p*<0.05**), suggesting that virus-specific $CD4^+$ T cells may directly impact viral clearance. Further, PBS-treated CD154KO mice demonstrated delayed viral clearance compared to WT mice (**Fig. 3H, *p*<0.05**). CD154KO mice that received wt OTII $CD4^+$ T cells exhibited significantly lower viral titres (**Fig. 3H, *p*<0.05**) as compared to PBS-treated CD154KO mice. Importantly, the fold-decrease in viral load in CD154KO mice receiving wt OTII CD4 T cells (~100-fold) was greater than observed in wt mice that had also received OTII cells (~26-fold). These data suggest that the enhanced virus-specific CD8 T cell response observed in the $CD154^{-/-}$ mice that received wt $CD4^+$ T cell help further expedited viral clearance over what was observed with OTII cells alone. Thus, CD154 expression on activated CD4 T cells is sufficient for enhanced pulmonary virus-specific CTL responses after IAV infection and contributes to efficient clearance of virus from CD154KO lungs.

$CD154^+$ CD4 T cell help rescues robust secondary virus-specific CD8 T cell responses in CD154-deficient mice

While CD4 T cell help is not always necessary for the generation of primary influenza-specific CTL responses, it is required for robust recall responses after heterologous viral challenge[2, 4, 15]. While the above data suggest that $CD154^+$ CD4 T cell help is capable of rescuing a mature primary virus-specific CTL in an otherwise CD154-deficient environment, it remained unclear whether $CD154^+$ CD4 T cell help was also capable of rescuing secondary CTL responses after viral challenge in a CD154-deficient environment. To address this point, we adoptively transferred wt or CD154KO OTII CD4 T cells into either wt or CD154KO

hosts and infected mice as per Figure 1. Infected mice were rested for 45-60 days prior to i.n. challenge with A/PR8 influenza virus (lacking the OVA_{323} epitope) with CTL responses analysed 8 days after secondary infection. In these experiments, there were similar numbers of splenic D^bNP_{366}- and D^bPA_{224}-specific CTL in wt mice that had received OTII $CD4^+$ T cells as compared to PBS (**Fig 4A-C)**. Importantly, there was a marked decrease in the number of D^bNP_{366}- and D^bPA_{224}-specific CTL in the spleens of CD154KO mice that had received PBS as a control (**Fig. 4A-C, p<0.05**) and a small reduction in their capacity to produce IFN-γ, as measured by MFI, as compared to WT controls (Figure 4D, E, $p<0.05$) .

We next asked if transfer of virus-specific wt CD4 T cell help could rescue CD8 T cell responses after secondary infection. $CD154^{-/-}$ mice that had received wt OTII CD4 T cells exhibited a significant increase in the number of splenic D^bNP_{366}- and D^bPA_{224}-specific CTL as compared to CD154KO mice that had been given PBS (**Fig. 4A-C, p<0.05**). Furthermore, the numbers of D^bNP_{366}- and D^bPA_{224}-specific $CD8^+$ T cells and capacity of these cells to produce IFN-γ were virtually identical in wt and CD154KO mice that had received wt OTII $CD4^+$ T cells at the time of priming (**Fig. 4B, C)**, suggesting a full rescue of the secondary virus-specific CTL response. As with the primary response, adoptive transfer of CD154KO OTIIs into CD154KO recipients at the time of priming failed to rescue the magnitude of the D^bNP_{366}- and D^bPA_{224}-specific secondary CTL responses (**Fig. 4A-**C). Further, cells generated in these mice exhibited a distinct defect in their capacity to produce IFN-γ after re-stimulation as compared to controls (Fig 4D, E, $p<0.05$). These data strongly suggest that the upregulation of CD154 on activated $CD4^+$ T cells is necessary for the priming of both optimal primary and secondary $CD8^+$ T cell responses.

DISCUSSION

Generation of optimal primary CD8 T cell responses after infection depends on a number of shared factors that are largely independent of the pathogen. These factors include things such as ligation of the TCR (signal 1), co-stimulation (signal 2) and inflammatory signals (signal 3). Interestingly, the dependence for CD4 T cell help to promote effective primary CTL responses is highly dependent on the nature of the challenge. In particular, primary CTL responses to acute viral and bacterial infections are not dependent on CD4 help[4, 7, 10, 15]. As a potential explanation of this phenomenon, it has been suggested that primary CTL responses can be primed via CD154-CD40 interactions imparted by DC-CTL contacts, rather than CD4-DC dependent licensing[14]. Our data further supports the importance of CD40 and CD154 in generation of primary IAV-specific CD8 T cells responses, as mice with altered components of this system exhibit significantly impaired CTL responses (Fig 1). Further, adoptive transfer of wt $CD4^+$ T cell help into CD154-deficient hosts restored optimal IAV-specific CD8 T cell numbers after infection (Fig 1).

Interestingly, adoptive transfer of wt CD4 T cells improved the capacity of D^bPA_{224}-specific, but not D^bNP_{366}-specific, CTLs to produce IL-2 in CD154-deficient hosts (Fig. 2 and 3) Why CD4 help differentially affects these specificities is unclear. We have previously shown that D^bPA_{224}-specific CTL exhibit an increased capacity for simultaneous cytokine production, including increased IL-2 production, when compared to the D^bNP_{366}-specific set[20]. The greater functionality of D^bPA_{224}-specific, compared to D^bNP_{366}- specific, CTL has been correlated with higher T cell receptor avidity for peptide-MHC (pMHC)[20]. Thus, it is intriguing to speculate that CD40-CD154 interactions may preferentially contribute to the increased functionality of high avidity CTL populations.

A level of redundancy of CD154 expression is apparent after IAV infection with both activated $CD4^+$ and DCs capable of CD154 expression and the potential to contribute to sustained CTL responses[14]. These data beg the question, why does this level of redundancy

between CD4 T cells and DCs exist during primary infection? One possible explanation for this redundancy may involve generating CTL responses to pathogens with different cell or tissue tropisms. Viruses with restricted tropism for epithelial cells or specific tissues (ie RSV, HSV), would be unable to directly activate DCs by infection and allow for self-licencing. Further, a number of viruses (ie vaccinia virus) that do infect DCs also have mechanisms that directly inhibit *de novo* protein synthesis and MHC expression on the cell surface. Thus, it is likely that even if these infected cells can self-licence, they may not be able to induce proper CTL responses. In these scenarios, CD4 T cells would be required to licence cross-presenting DCs for optimal induction of anti-viral CD8 T cell responses. Previous work has demonstrated that IAV-infected DCs are potent inducers of virus-specific CD8 T cell responses[21]. However, IAV-infected DCs have a reduced capacity to cross-present non-IAV related antigens prime other specificities of CD8 T cells[22]. Based on these data, redundant expression of CD154 on DCs and CD4 T cells may ensure optimal CD8 T cells to *de novo* CTL responses in a situation where co-infection is prevalent.

The above show a redundancy between CD154-expressing CD4 T cells and other cell types that are capable of inducing primary IAV-specific CD8 T cell responses. However, there is no apparent redundancy in the recall of memory IAV-specific CD8 T cell responses after secondary infection. In virtually all situations, CD4 T cells are required for optimal secondary CD8 T cell responses[2, 3, 7, 15]. This is especially evident in IAV infection, where MHCII-deficient mice have a blunted virus-specific secondary CD8 T cell response[15]. We also show here that secondary virus-specific CD8 T cell responses are diminished in CD154-deficient mice (Figure 4). Like the primary response, secondary virus-specific CTL responses can also be rescued upon transfer of wt CD4 T cell help. These data indicate that CD154 expression on CD4 T cells is sufficient for this response. Interestingly, while CD154 expression on DCs is sufficient for primary IAV-specific CTL responses[14], our data suggest it is not sufficient for generation of memory CD8 T cells that can respond to secondary

infection. In fact, utilising mixed bone marrow chimeras, we have recently demonstrated that CD154 expression on activated CD4s, but not DCs, is necessary for programming memory IAV-specific CD8 responses (Olson et al., manuscript under revision). This is also supported by the presence of CD154-sufficient DCs in MHCII or CD4-depleted/deficient mice and the lack of pathogen-specific secondary CD8 T cell responses in these mice[3, 15]. Hence, programming of effective memory CTL populations likely occurs during the initial activation of naïve, virus-specific CTL and supports the notion that CD40-CD154 interactions are key for establishing memory CTL populations capable of robust recall responses[11].

We favour a model where activated CD4s licence DCs via a CD40L-CD40 interaction that promotes both robust primary and secondary CTL responses[6, 16, 19] (Figure 5). While CD154 signals from activated DCs can promote effector CTL differentiation, our data suggest they cannot program CTL memory formation. The implication is that CD40-CD154 interactions mediated by different cellular interactions that may result in different transcriptional programs within activated CTL. Thus, it will be of interest to define the transcriptional signatures of CTL that have received CD40-CD154 interactions from either $CD4^+$ T cells or DCs to delineate the precise molecular pathways that lead to full effector and memory CTL differentiation and we are currently following this line of investigation.

METHODS

Mice and viral infections.

Mice (C57BL/6J Ly5.2, CD40KO Ly5.2, CD154KO Ly5.2, OTII Ly5.1, and CD154KO OTII Ly5.1) mice were bred and housed in specific pathogen-free conditions at the Department of Microbiology and Immunology at the University of Melbourne (Parkville, VIC, Australia) or the Walter and Eliza Hall Institute (WEHI, Parkville, VIC, Australia). To examine primary CTL responses, mice were infected intranasally (i.n.) with 10^4 plaque forming units (PFU) of the H3N2 strain A/HKx31, engineered to expresses the chicken ovalbumin 323-339 peptide within the hemagglutinin head[23]. In some experiments, mice received $1x10^6$ WT OTII CD4 T cells or $1x10^6$ CD154KO OTII CD4 T cells, which recognize the $OVA_{323-339}$ peptide[24], isolated from LNs of OTII TCR transgenic mice one day prior to infection. Mice were treated with 200 µl of PBS or 50 µg of purified anti-CD154 antibody (MR1) one day prior to and one day after infection. In experiments where we examined the secondary response to influenza virus infection, wt or CD154KO mice received OTII CD4 T cells or PBS and were infected with A/HKx31-OVA_{323} as described above. These mice were rested for 45 days and subsequently infected with $2x10^3$ PFU of A/Puerto Rico/8/34 (PR8; H1N1).

Tissue Sampling and isolation of lymphocytes.

Spleens and LNs were harvested from mice and pressed between frosted glass slides to create a single cell suspension in Hank's Balanced Salt Solution (HBSS, University of Melbourne Media Preparation Unit). Mononuclear cells were isolated by digestion of the lungs from infected mice with 1 ug/ml collagenase in HBSS for 30 minutes at 37^oC. Following digestion, lungs were pressed through a 70 uM nylon mesh filter and washed and resuspended in HBSS containing 10% foetal calf serum (FCS). Red blood cells were lysed in spleen and lung samples by treatment with ammonium tris chloride buffer (0.14M NH_4Cl, 0.017M Tris) for 5 minutes at room temperature, followed by copious washing with HBSS.

MHC-I tetramer and antibody staining

Mononuclear cells (1-2 x10^6) from single cell suspensions were added to individual wells of a 96 well plate, pelleted by centrifugation (1600 rpm for 6 minutes at 4^oC) and washed once in FACS buffer (PBS containing 1% BSA and 0.02% sodium azide). Following washing, cells were resuspended in 50 ul of FACS buffer containing D^bNP_{366} and D^bPA_{224} MHC-I tetramers (ImmunoID) conjugated to phycoerythrin (PE) and incubated for 1 hour at room temperature. After this incubation, cells were washed once with FACS buffer and resuspended in an antibody cocktail containing anti-CD8 Pacific Blue (BD/Pharmingen) and CD44 phycoerythrin-Cy7 (eBioscience) for 30 minutes at 4^oC followed by washing twice in FACS buffer. Flow cytometric analysis was performed on a FACS Canto II (BD/Pharmingen) and data were analysed using FlowJo v8.8.5 (TreeStar) software.

Intracellular cytokine staining

Mononuclear cells (1-2x10^6) from single cell preparations from spleens or lungs were added to individual wells of a 96 well plate and incubated for 5-6 hours in the presence of 1 g/ml GolgiPlug (BD) and 10 U/ml of human IL-2 (Roche) in the presence or absence of 1μ $NP_{366-374}$ (ASNENMETM) or $PA_{224-223}$ (SSLENFRAYV) peptides (both from Auspep) at 37^oC and 5% CO_2. After incubation, cells were stained for surface expression of CD8α and intracellular IFN-γ (APC, eBioscience) and IL-2 (PE, eBioscience). Total numbers of IFN-γ^+ D^bNP_{366}- or D^bPA_{224}-specific CD8 T cells were determined by subtracting the frequency of IFN-γ^+ cells in unstimulated controls from those stimulated with peptide.

Determination of lung viral titers

Lungs from infected mice were harvested and washed immediately in cold HBSS. Lungs were placed in 2 ml of RPMI (Gibco) containing 100 U/ml of penicillin and 24 ug/ml of

gentamicin (both from Invitrogen) and homogenized using a tissue homogenizer (Polytron 1200 PT). Lung homogenates were centrifuged for 10 minutes at $4^{o}C$ to pellet debris and supernatants were aliquoted and frozen at $-80^{o}C$ for later viral titer determination on Madin-Darby canine kidney (MDCK) cells as previously described[25].

Data analysis and statistics

Data were analysed using Prism v4.0a software (GraphPad). Statistical analysis was done by Student's *t* test, or by ANOVA followed by a Tukey post-test where indicated. Differences were considered to be significant when $p<0.05$.

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Figure Legends

Figure 1. CD154-expression on CD4 T cells is sufficient and necessary for the generation of full magnitude primary virus-specific CD8 T cell responses in the absence of CD154 on other cell types. WT, CD40KO, anti-CD154-treated (MR1), and CD154KO mice were given PBS (filled bars) or $1x10^6$ OTII CD4 T cells (open bars) one day prior to i.n. infection with 10^4 PFU of A/HKx31-OVA_{323}. At 10 days post-infection, spleens were harvested from mice and the total number of D^bNP_{366}-specific (A) and D^bPA_{224}-specific (B) CD8 T cells were enumerated by IFN-γ ICS. These data represent at least 2 individual experiments with at least 7 mice per group. *, represents significantly different ($p<0.05$) as compared to WT mice that had received PBS. #, represents significantly different ($p<0.05$) as compared to WT mice that had received OTII CD4 T cells.

Figure 2. CD154 on CD4 T cells is necessary for optimal IFN-γ and IL-2 production by IAV-specific CD8 T cells. Mice were treated with PBS (left side of panels A, B) or OTII cells (right side of panels A, B), infected with A/HKX31-OVA_{323} and spleens were harvested at day 10 p.i. as per Figure 1. The capacity of D^bNP_{366}- (A) and D^bPA_{224}-specific (B) CD8 T cells to co-produce IFN-γ and IL-2 was determined by ICS in each group of mice. *, represents significantly different ($p<0.05$) as compared to WT mice that had received PBS. #, represents significantly different ($p<0.05$) as compared to WT mice that had received OTII CD4 T cells. The capacity of D^bNP_{366}- (C) and D^bPA_{224}-specific (D) CD8 T cells to produce IFN-γ, as measured by MFI was determined by ICS. *, represents significantly different ($p<0.05$) as indicated. These data represent at least 2 individual experiments with at least 7 mice per group.

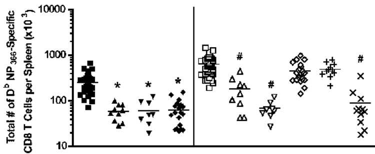
Figure 3. Early pulmonary virus-specific CD8 T cell responses and virus clearance in CD154KO mice are rescued following transfer of $CD154^+$ OTII CD4 T cell help. WT (filled

bars) or CD154KO mice (open bars) received PBS or $1x10^6$ OTII CD4 T cells and were infected as per Figure 1. Lungs were obtained and assessed for D^bNP_{366}- (A) and D^b PA_{224}- (B) specific CD8 T cell responses by MHC-I tetramer staining and by IFN-γ ICS (C, D). The capacity of D^b PA_{224}- specific CD8 T cells to produce IFN-γ (MFI, E) and to co-produce IFN-γ and TNF-α (%, F) was assessed by ICS. (G) The total number of OTII CD4 T cells were enumerated in the lung of each group of mice. (H) Lungs were also assessed for virus titers by plaque assay. These data represent 3 independent experiments with at least 9 mice per group. *, represents significantly different ($p<0.05$) as compared to wt mice that had received PBS. #, represents significantly different ($p<0.05$) as compared to CD154KO mice that had received PBS.

Figure 4. $CD154^+$ $CD4^+$ T cell help is sufficient for full virus-specific CD8 T cell responses after secondary infection. WT (filled bars) and CD154KO mice (open bars) received PBS or $2x10^6$ OTII CD4 T cells and were infected as per Figure 1. Mice were challenged i.n. with $2x10^3$ PFU of IAV/PR8 45 days after primary infection. Spleens were harvested 8 days later and assessed for the total number of D^bNP_{366}- (A, B) and D^bPA_{224}-specific (A, C) CD8 T cells by IFN-γ ICS. The relative capacity of D^bNP_{366}- (D) and D^bPA_{224}-specific (E) cells to produce IFN-γ was measured by ICS *, represents significantly different ($p<0.05$). These data represent at 2 individual experiments with at least 6 mice per group.

Figure 5. Proposed model of how $CD154^+$ T cell help restores both primary and secondary IAV CTL responses in $CD154^{-/-}$ mice. Primary infection with the A/HKx31 (H3N2) IAV virus results in a diminished primary CTL response when compared to wildtype mice. Moreover, a lack of CD154 signals at the time of initial infection also lead to poor memory CTL responses when challenged with a serologically distinct IAV (A/PR8, H1N1). Addition and subsequent activation after primary IAV infection of $CD154^+$ $CD4^+$ OTII T cells restores

the primary CTL response of CD154 deficient animals. Moreover, CD154-/- mice that received OTII help at the time of primary infection (OTII cells + infection with A/HKX31-OVA_{323}) are now able to mount robust secondary CTL responses after heterologous IAV challenge (A/PR8). The fact that this occurs in the absence of memory OTII activation suggests that CD4 dependent CD154-CD40 interactions at the time of primary infection are key for programming IAV-specific CTL memory responses.


A.
Total # of D^b NP 366-Specific CD8 T Cells per Spleen (x10^3)
10000
1000
100
10
*
*
*
#
#
#
B.
Total # of D^b PA224-Specific CD8 T Cells per Spleen (x10^3)
1000
100
10
*
*
*
#
#
#
WT
CD40KO
αCD154 (MR1)
CD154KO
OTII → WT
OTII → CD40KO
OTII → αCD154 (MR1)
OTII → CD154KO
CD154KO OTII → WT
CD154KO OTII → CD154KO

A.

% IL-2^{+} of NP_{366}-Specific CD8 T Cells

0 10 20 30 40 50

n.s. n.s. n.s. n.s. n.s. n.s. n.s. n.s. n.s.

B.

% IL-2^{+} of D^{b} PA_{224}-Specific CD8 T Cells

0 10 20 30 40 50 60

* * * # # #

WT
CD40KO
αCD154 (MR1)
CD154KO
OTII ➔ WT
OTII ➔ CD40KO
OTII ➔ αCD154 (MR1)
OTII ➔ CD154KO
CD154KO OTII ➔ WT
CD154KO OTII ➔ CD154KO

C.

$D^{b}NP_{366}$ Relative IFN-γ MFI

0.0 0.5 1.0 1.5 2.0

* *

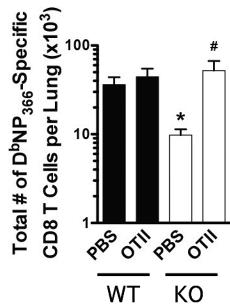
D.

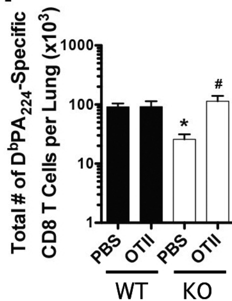
$D^{b}PA_{224}$ Relative IFN-γ MFI

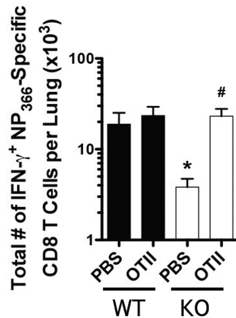
0.0 0.5 1.0 1.5 2.0

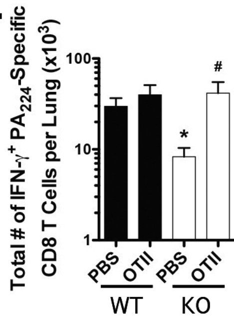
* *

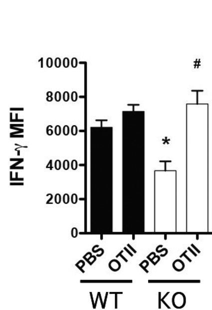
PBS ➔ WT
WT OTII ➔ WT
CD154KO OTI I➔ WT
PBS ➔ CD154KO
WT OTII ➔ CD154KO
CD154KO OTII ➔ CD154KO

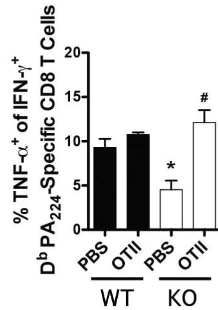

A.
Total # of D^bNP366-Specific CD8 T Cells per Lung (x10^3)
100
10
1
*
#
PBS OTII PBS OTII
WT KO

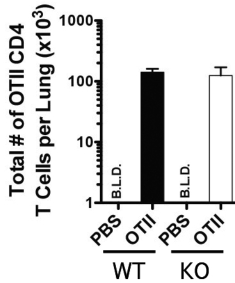

B.
Total # of D^bPA224-Specific CD8 T Cells per Lung (x10^3)
1000
100
10
1
*
#
PBS OTII PBS OTII
WT KO

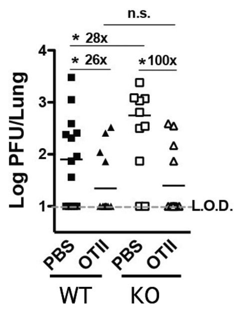

C.
Total # of IFN-γ+ NP366-Specific CD8 T Cells per Lung (x10^3)
100
10
1
*
#
PBS OTII PBS OTII
WT KO


D.
Total # of IFN-γ+ PA224-Specific CD8 T Cells per Lung (x10^3)
100
10
1
*
#
PBS OTII PBS OTII
WT KO


E.
IFN-γ MFI
10000
8000
6000
4000
2000
0
*
#
PBS OTII PBS OTII
WT KO


F.
% TNF-α+ of IFN-γ+ D^b PA224-Specific CD8 T Cells
20
15
10
5
0
*
#
PBS OTII PBS OTII
WT KO


G.
Total # of OTII CD4 T Cells per Lung (x10^3)
1000
100
10
1
B.L.D.
B.L.D.
PBS OTII PBS OTII
WT KO


H.
Log PFU/Lung
4
3
2
1
n.s.
* 28x
* 26x
*100x
L.O.D.
PBS OTII PBS OTII
WT KO

A.

No Peptide | $NP_{366-374}$ | $PA_{224-232}$

PBS → WT: 0.1 | 8.9 | 2

PBS → CD154KO: 0.2 | 2.6 | 0.6

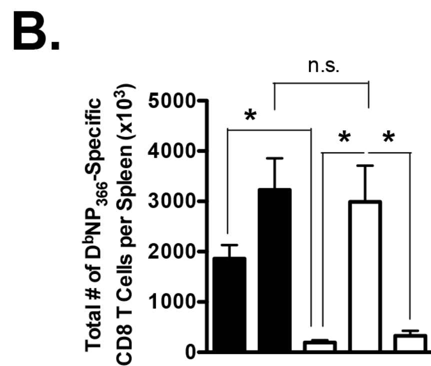
OTII → WT: 0.08 | 15 | 3.5

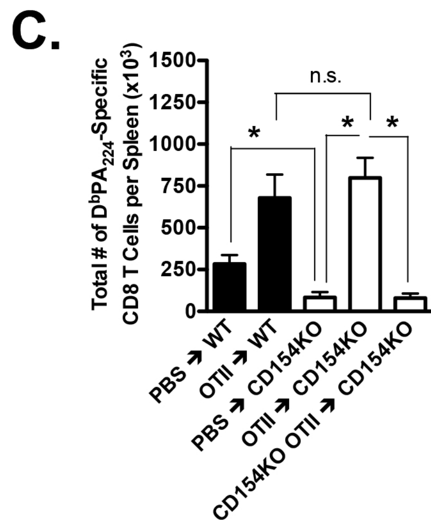
OTII → CD154KO: 0.1 | 20 | 4.1

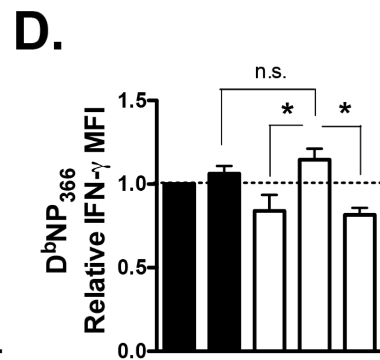
CD154KO OTII → CD154KO: 0.3 | 3.1 | 0.7

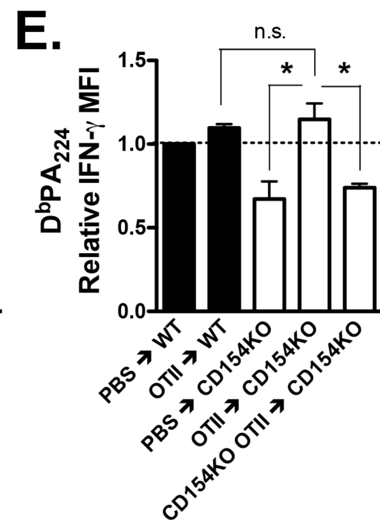
IFN-γ

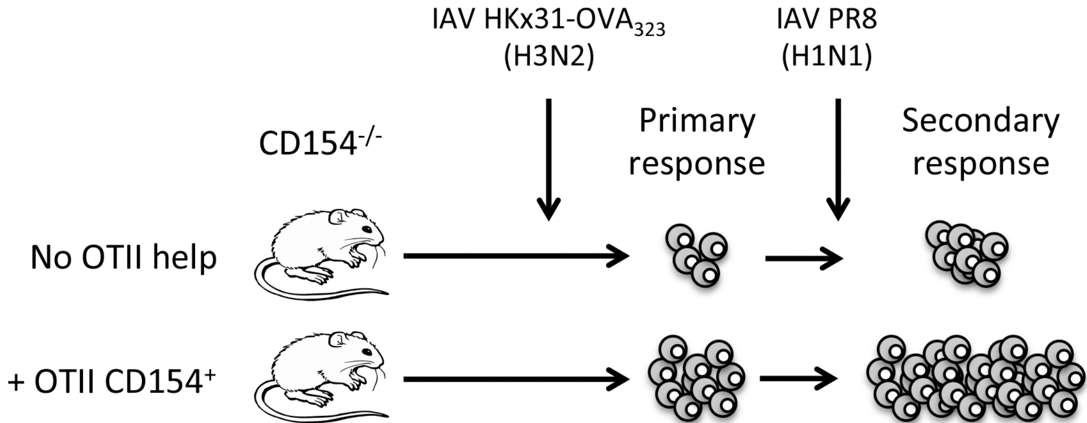
CD44










IAV HKx31-OVA323
(H3N2)
IAV PR8
(H1N1)
CD154-/-
Primary
response
Secondary
response
No OTII help
+ OTII CD154+