

Viral vector and route of administration determine the ILC and DC profiles responsible for downstream vaccine-specific immune outcomes



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ABSTRACT

This study demonstrates that route and viral vector can significantly influence the innate lymphoid cells (ILC) and dendritic cells (DC) recruited to the vaccination site, 24 h post delivery. Intranasal (i.n.) vaccination induced ST2/IL-33R⁺ ILC2, whilst intramuscular (i.m.) induced IL-25R⁺ and TSLPR⁺ (Thymic stromal lymphopoietin protein receptor) ILC2 subsets. However, in muscle a novel ILC subset devoid of the known ILC2 markers (IL-25R⁺ IL-33R⁺ TSLPR⁺) were found to express IL-13, unlike in lung. Different viral vectors also influenced the ILC-derived cytokines and the DC profiles at the respective vaccination sites. Both i.n. and i.m. recombinant fowlpox virus (rFPV) priming, which has been associated with induction of high avidity T cells and effective antibody differentiation exhibited low ILC2-derived IL-13, high NKp46⁺ ILC1/ILC3 derived IFN- γ and low IL-17A, together with enhanced CD11b⁺ CD103⁺ conventional DCs (cDC). In contrast, recombinant Modified Vaccinia Ankara (rMVA) and Influenza A vector priming, which has been linked to low avidity T cells, induced opposing ILC derived-cytokine profiles and enhanced cross-presenting DCs. These observations suggested that the former ILC/DC profiles could be a predictor of a balanced cellular and humoral immune outcome. In addition, following i.n. delivery Rhinovirus (RV) and Adenovirus type 5 (Ad5) vectors that induced elevated ILC2-derived IL-13, NKp46⁺ ILC1/ILC3-derived-IFN- γ and no IL-17A, predominantly recruited CD11b⁺ B220⁺ plasmacytoid DCs (pDC). Knowing that pDC are involved in antibody differentiation, we postulate that i.n. priming with these vectors may favour induction of effective humoral immunity. Our data also revealed that vector-specific replication status and/or presence or absence of immune evasive genes can significantly alter the ILC and DC activity. Collectively, our findings suggest that understanding the route- and vector-specific ILC and DC profiles at the vaccination site may help tailor/design more efficacious viral vector-based vaccines, according to the pathogen of interest.

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1. Introduction

In the last two decades, inactivated, live attenuated, replication-competent or -defective viruses have been extensively tested as viral vector-based vaccines. Interestingly, poxviruses such as Modified Vaccinia Ankara (MVA), New York strain of vaccinia virus (NYVAC), which are attenuated versions of vaccinia virus (VV), and Avipoxvirus; canarypox and fowlpox (FPV) viruses, used in prime-boost modalities have yielded uniquely different immune outcomes, dependent upon the route of delivery and/or the vaccine

vector combination [1–4]. For example, heterologous rFPV/rVV compared to rVV/rFPV vaccination has shown to induce highly poly-functional/ high avidity T cells [3,5–7], moreover, rMVA used as a booster, as opposed to a prime has shown to induce more effective T cell immunity [8–10]. Similarly, both replication-competent and -defective recombinant Adenovirus-based vaccines have also shown to induce T cell responses associated with immune protection in animal models [11–13]. Moreover, viruses such as, Influenza A, Human RV, Cytomegalovirus, and Vesicular stomatitis virus, have also been assessed as promising vaccine delivery vehicles [10,14–16]. In a recent prime-boost vaccination study, mucosal RV prime vaccination was shown to induce HIV-specific T cell responses associated with protection in mice [17]. To improve vaccine-specific immunity, variants of viral vectors,

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such as IL-1 β R and/or IL18 binding protein (IL-18bp) deletion mutants of MVA and Adenoviral vectors have also been recently tested [18–20]. Despite the knowledge of different viral vector-based vaccines conferring different adaptive immune outcomes, the underlying innate immune mechanisms governing these processes at the vaccination site still remains elusive, specifically the role of innate lymphoid cells (ILCs) and dendritic cells (DCs).

ILCs, although derived from a common progenitor, are lineage negative in nature and according to the transcription factors, receptors and cytokines they express, are broadly classified into three main categories (ILC1, ILC2 and ILC3) [21]. ILC2, due to their ability to express IL-13, have been heavily studied under chronic inflammation, allergic asthma and helminth infections [22]. During intracellular pathogen infection, ILC1 have shown to express IFN- γ and tumour necrosis factor (TNF)- α [23], whilst during extracellular bacterial and fungal infections, ILC3 have been associated with interleukin (IL)-17A and IL-22 expression [24,25]. Although ILCs have three distinct phenotypes, studies have shown that they have the ability to interconvert between the phenotypes, according to the external stimuli, and thus thought to be highly plastic [26,27]. It is postulated that ILCs can polarize the immune response, according to the immune cell milieu or pathogen encountered, towards Th1, Th2 or Th17 immunity. However, the role of ILCs in viral vector-based vaccination is not well characterised.

DCs sample antigens at various body surfaces; skin, gastrointestinal tract and lungs, and are among the first line of defence against many pathogens. Based on the anatomical location and the invading pathogen, distinct DC subsets carry out differential functions [28]. For example; lung DCs have been extensively studied under respiratory infections. Lung conventional CD11b⁺ CD103[−] DCs (cDCs) and cross-presenting CD11b[−] CD103⁺ DCs have been associated with CD8 T cell priming [29,30]. Although conflicting evidence suggest that cDCs are functionally more important in mounting an effective antiviral response [31,32], there is growing evidence to support the notion that the activity of a particular DC subset is determined by the specific infection. For example: control of acute influenza virus infection is associated with CD11b[−] CD103⁺ DCs cross presentation to CD8 T cells [33], whilst, CD11b[−] CD8⁺ DCs, which share a common developmental origin with CD11b[−] CD103⁺ DCs, have been associated with activation of cytotoxic CD8 T cells against non-respiratory pathogens such as West Nile Virus [34]. In the context of respiratory syncytial virus (RSV) infection, CD11b⁺ and CD103⁺ DC subsets have been involved in antigen presentation to both CD4 and CD8 T cells [35]. In addition, during Influenza A infection, CD11b⁺ DCs have also been associated with humoral immunity [36]. Furthermore, plasmacytoid DCs (pDCs) also have been associated with distinct functions during viral infections [37,38].

It is now well established that the route of delivery, cytokine milieu, viral vectors and the order in which they are administered can yield vastly different adaptive immune outcomes [3,5,7,39,40]. We have previously shown that (i) IL-13, although detrimental for high avidity/poly-functional CD8 T cell immunity, was necessary for effective antibody differentiation [41–43]. (ii) Using rFPV adjuvanted vaccines that transiently inhibited IL-13 activity at the vaccination site, we have recently established that ILC2 (not other lineage⁺ cells) were the major source of IL-13 at the vaccination site 24 h post vaccination [44]. (iii) Furthermore, using the same vaccines we have also shown that elevated IL-13 in the milieu recruited CD11b[−] CD103⁺ cross-presenting DCs, associated with low avidity CD8 T cells [42,45]. Therefore, in this study to further understand which specific innate immune cell subsets play a predominant role in shaping the downstream adaptive immune outcomes, replicating and non-replicating viral vectors were delivered intranasally and intramuscularly and subsequent ILC-

derived cytokine profiles and DCs subsets were assessed 24 h post vaccination.

2. Results

2.1. Different viral vector-based vaccines can induce uniquely different ILC2-derived IL13 profiles following intranasal and intramuscular vaccination

BALB/c mice were vaccinated intranasally or intramuscularly with four different poxviral vectors rFPV, rMVA, rVV and rMVA Δ IL-1 β R and three non-poxviral vectors Influenza A, Human rhinovirus (RV) and Adenovirus type 5 (Ad5). Percentage of lung and muscle ILC2 and their corresponding IL-13 expression were assessed 24 h post vaccination. ILC2 were gated as CD45⁺ FSC^{low}, SSC^{low}, lineage[−] ST2/IL-33R⁺ cells for lung (Fig. S1) or lineage[−] IL-25R⁺, TSLPR⁺ and ST2/IL-33R⁺ for muscle (Fig. S2), as indicated in Materials and Methods and Li et al 2018 [44]. Among all the vectors tested, following i.n. delivery, Influenza A vector recruited the highest percentage of Lin[−] ST2/IL-33R⁺ ILC2 to the vaccination site (lung mucosae). In contrast, RV and Ad5 recruited the lowest percentage of ILC2, which was much lower than unimmunized control ($p = 0.0014$ and $p = 0.0011$ respectively) (Fig. 1A and S3). However, despite this, RV and Ad5 expressed elevated IL-13 levels, which were similar to rMVA and Influenza A (Fig. 1B). Among the three poxviral vectors tested, the highest IL-13 level was detected in rMVA (rFPV vs rMVA $p < 0.0001$, rMVA Δ IL-1 β R vs rMVA $p < 0.0001$), whilst rMVA Δ IL-1 β R showed the lowest (rFPV vs rMVA Δ IL-1 β R $p = 0.4159$) (Fig. 1B). It is also noteworthy that, all the vectors showed significantly elevated IL-13 expression by Lin[−] ST2/IL-33R⁺ ILC2 compared to the unimmunized control (rFPV $p = 0.0028$; rMVA $p < 0.0001$; rMVA Δ IL-1 β R $p = 0.0412$; Influenza A $p < 0.0001$; RV $p < 0.0001$; Ad5 $p < 0.0001$) (Fig. 1A and B).

Following i.m. vaccination, mainly IL-25R⁺ ILC2s and TSLPR⁺ ILC2, ranging from 0.25% to 2% were detected. In the context of IL-25R⁺ ILC2, rMVA and Ad5 vector vaccination showed significantly elevated numbers compared to unimmunised control ($p = 0.0183$ and $p = 0.0178$ respectively). Furthermore, Ad5 vaccination also showed higher proportion of IL-25R⁺ ILC2s compared to influenza A ($p = 0.0004$) (Fig. 1G, H and I). Interestingly, rMVA Δ IL-1 β R (1.8% average) showed a significantly elevated proportion of TSLPR⁺ ILC2 compared to rFPV and rMVA vaccination ($p < 0.0001$ and $p = 0.0240$ respectively) (Fig. 1G, H and I). Ad5 also showed elevated TSLPR⁺ ILC2s compared to rFPV and influenza A vaccination ($p = 0.0103$ and $p = 0.0006$ respectively) (Fig. 1G, H and I). Following i.m. vaccination, similar to our previous studies extremely low or no ST2/IL-33R⁺ ILC2 were detected with all vaccine groups tested (Fig. 1G, H and I).

Surprisingly, following i.m. delivery, canonical ILC2 subsets (IL-25R⁺, TSLPR⁺) were found to express marginal IL-13. In contrast, compared to the unimmunised control, a not yet defined ILC2 subset that lacked IL-25R, ST2/IL-33R and TSLPR were found to express IL-13 (Fig. 1E and F). Out of the vectors tested, Ad5 showed remarkably higher proportion (2 to 3-fold) of IL-25R[−] IL-33R[−] TSLPR[−] cells expressing IL-13 ($p < 0.0001$) (Fig. 1E and F), which was comparatively lower than i.n. Ad5 vaccination (Fig. 1B). It is noteworthy that, the ILC2-derived IL-13 expression by each vector was significantly higher following i.m. delivery compared to i.n. delivery. (Note that: The parent ILC2 population in the i.m. groups were much greater than the i.n. ST2⁺/IL-33R⁺ ILC2s. Thus, the difference in IL-13 expression by these two ILC subsets were also represented normalised to the CD45⁺ subset, described in materials and methods (Fig. 1J).

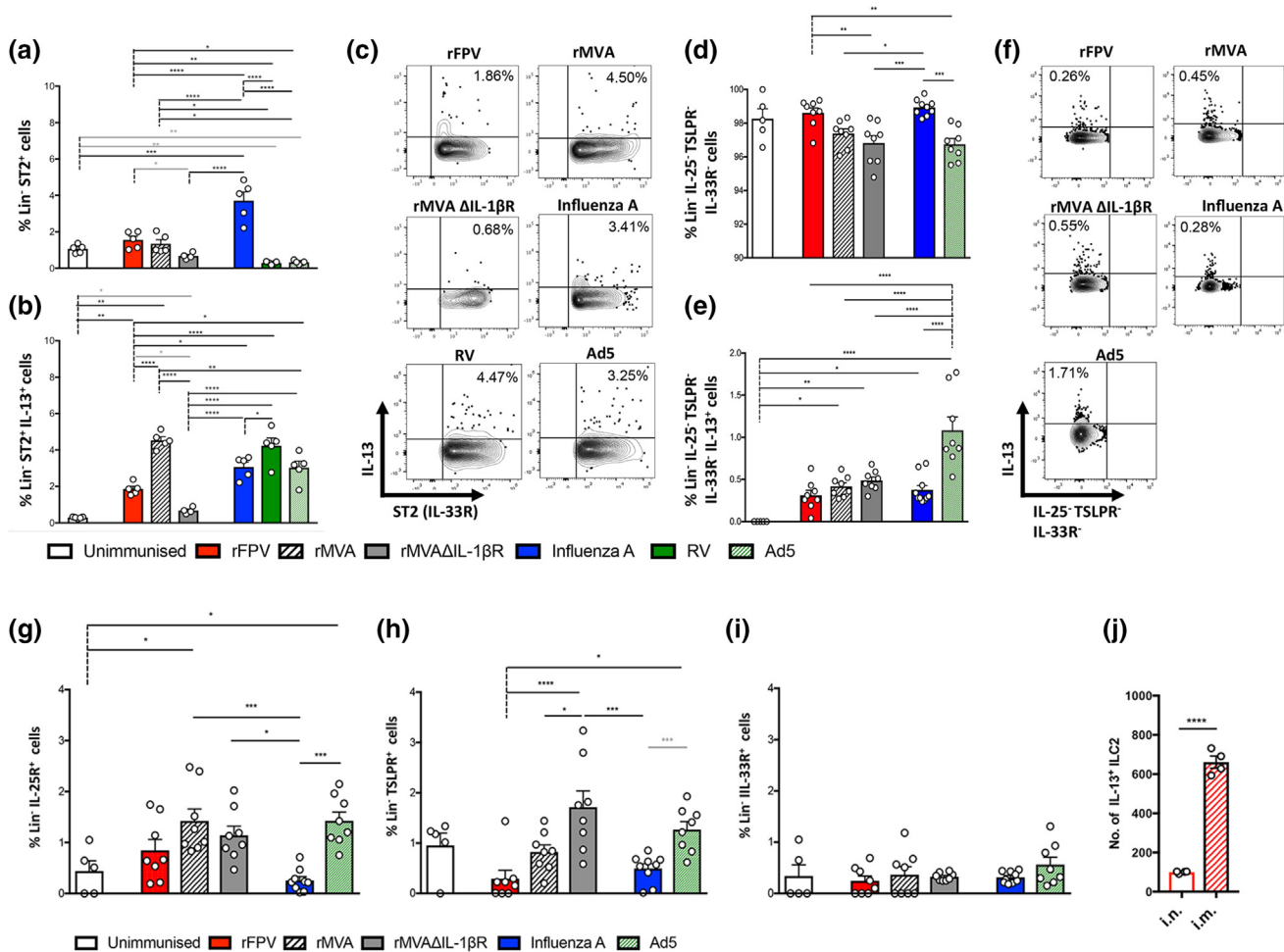


Fig. 1. Evaluation of lung and muscle ILC2 and corresponding IL-13 expression following intranasal and intramuscular viral vaccination. BALB/c mice ($n = 5-9$ per group) were i.n. or i.m. immunised with rFPV, rMVA, rMVA- Δ IL-1 β R, Influenza A, RV or Ad5. 24 h post vaccination lungs were harvested and single cell suspensions were stained for ILC2s and their IL-13 expression and analysed using flow cytometry. Cells were pre-gated on CD45 $^{+}$ FSC low SSC low cells using FlowJo software as described in Materials and Methods and **Figs. S1 and S2.** Lung ILC2 graphs show (A) the percentage of Lin $^{-}$ ST2 $^{+}$ /IL-33R $^{+}$ ILC2 and (B) IL-13 expression by Lin $^{-}$ ST2 $^{+}$ /IL-33R $^{+}$ ILC2. (C) Representative FACS plots show percentage of Lin $^{-}$ ST2 $^{+}$ /IL-33R $^{+}$ ILC2 expressing IL-13. Muscle ILC2 graphs show percentage of (D) Lin $^{-}$ IL-25 $^{+}$ TSLPR $^{-}$ ST2 $^{-}$ /IL-33R $^{+}$ ILC2 and (E) IL-13 expression by this novel ILC2 subset. (F) Representative FACS plots show percentage of Lin $^{-}$ IL-25 $^{+}$ TSLPR $^{-}$ ST2 $^{-}$ /IL-33R $^{+}$ ILC2 expressing IL-13, and graphs show muscle (G) IL-25 $^{+}$ ILC2s, (H) TSLPR $^{+}$ ILC2s and (I) ST2 $^{+}$ /IL-33R $^{+}$ ILC2s. To compare and contrast lung and muscle ILC2-derived IL-13, (J) bar graph represents IL-13 expression by Lin $^{-}$ ST2 $^{+}$ /IL-33R $^{+}$ ILC2 (i.n.) and Lin $^{-}$ IL-25 $^{+}$ TSLPR $^{-}$ ST2 $^{-}$ /IL-33R $^{+}$ ILC2 (i.m.) following rFPV vaccination. Error bars represent Standard Error of mean (SEM) and p values were calculated using One-way ANOVA followed by Tukey's multiple comparison test (black lines) and paired student's *t* test (grey lines). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Experiments with each vector were repeated minimum 2–3 times.

2.2. Poxviral and non-poxviral vectors showed significantly different ILC1/ILC3- derived IFN- γ and IL-17A expression profiles

Our recent intranasal rFPV vaccination studies have shown that the transient inhibition of ILC2-derived IL-13 at the vaccination site can directly impact the level of IFN- γ and IL-17A expression by NKp46 $^{+}$ and NKp46 $^{-}$ ILC1/ILC3s at the vaccination site 24 h post vaccination [44]. Hence, we next investigated the induction of IFN- γ and IL-17A expression by ILC1/ILC3s by different viral vaccine vectors as per indicated in Materials and Methods using flow cytometry gating strategies described in Fig. S1. Following i.n. vaccination, although no significant differences in the percentages of NKp46 $^{+}$ ILC1/ILC3s were detected compared to the unimmunized control (Fig. 2A), compared to Influenza A, Ad5 showed significantly reduced numbers of NKp46 $^{+}$ ILC1/ILC3s ($p = 0.042$). Whilst rMVA Δ IL-1 β R recruited NKp46 $^{+}$ ILC1/ILC3s similar to rFPV, rMVA recruited significantly lower numbers of NKp46 $^{+}$ ILC1/ILC3s compared to rFPV ($p = 0.036$). In the context of IFN- γ expression by NKp46 $^{+}$ ILC1/ILC3s, RV induced the highest (average 14.5%), followed by Ad5 (average 5%) and rFPV (average 2.9%) (Fig. 2B).

Unlike rFPV, the deletion mutant rMVA Δ IL-1 β R and rMVA showed significantly lower IFN- γ expression ($p = 0.0187$, and 0.0011 respectively), which was also lower than the unimmunized control ($p = 0.0086$ respectively) (Fig. 2B). Expression of IFN- γ by Influenza A was similar to that of the unimmunized control.

Interestingly, following i.n. delivery 95–98% ILC1/ILC3s were found to be NKp46 $^{-}$ (Fig. 2A and D). Although there were no differences observed between the numbers of NKp46 $^{-}$ ILC1/ILC3s recruited by any of poxvirus vectors (Fig. 2D), IFN- γ expression was vastly different. rFPV was amongst the highest inducers of IFN- γ expression by NKp46 $^{-}$ ILCs (Fig. 2E), whilst showing modest IFN- γ expression also by NKp46 $^{+}$ ILCs (Fig. 2B). Out of all the vaccine vectors tested, rMVA Δ IL-1 β R showed the lowest IFN- γ expression by NKp46 $^{-}$ ILC1/ILC3s (Fig. 2E). Although Influenza A recruited significantly lower numbers of NKp46 $^{-}$ ILC1/ILC3s compared to RV and Ad5 ($p = 0.0004$, $p < 0.0001$ respectively), it induced the highest IFN- γ expression among the non-poxviral vectors (Fig. 2E). Interestingly, the IFN- γ expression by NKp46 $^{-}$ ILC1/ILC3s was very similar between Influenza A and rFPV vaccinated groups (Fig. 2E). It is noteworthy that, although the unimmunized

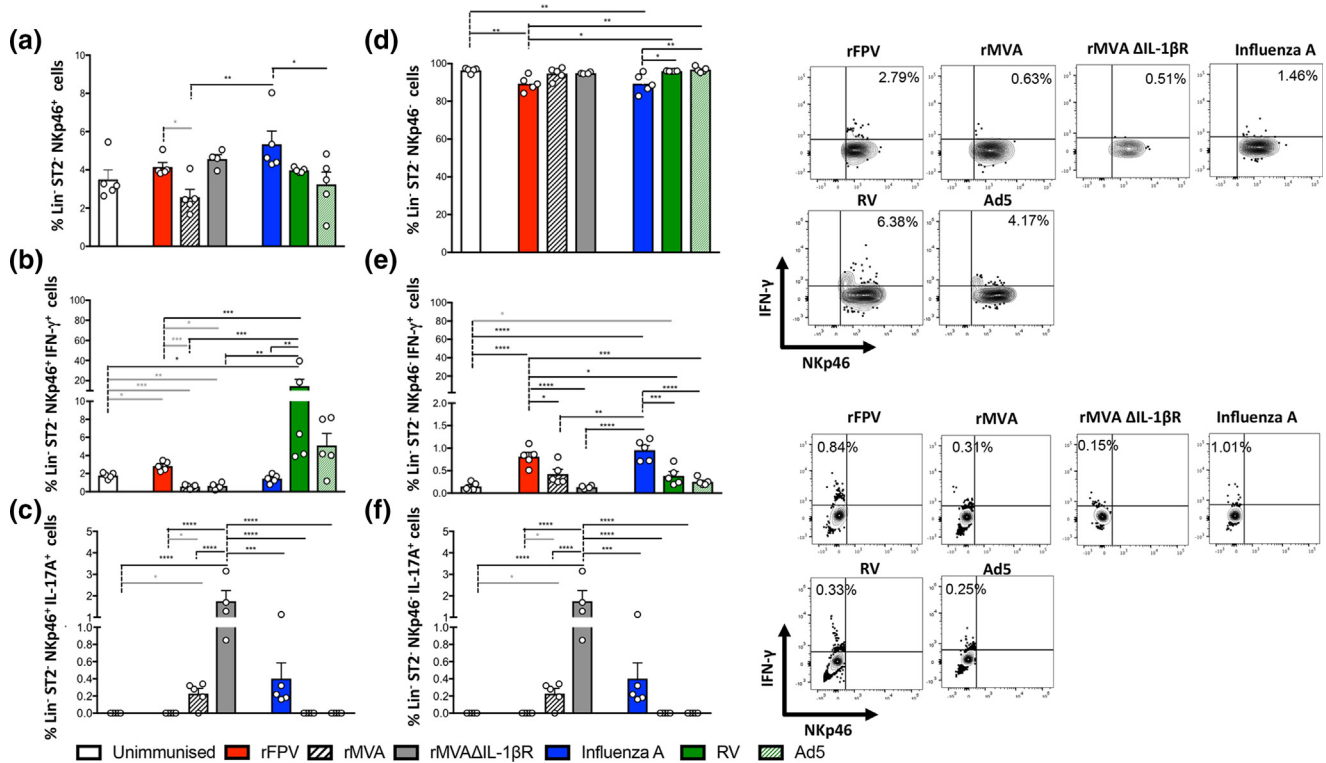


Fig. 2. Evaluation of lung Lin⁺ ST2⁺ NKp46⁺ and NKp46⁺ ILC-derived IFN- γ and IL-17A expression following intranasal viral vector vaccination. BALB/c mice (n = 5) were i.n. immunised with same vectors as per in Fig. 1, and stained for Lin⁺ ST2⁺/IL-33⁺ NKp46⁺ and NKp46⁺ ILC and their cytokine expression. Cells were pre-gated on CD45⁺ FSC^{low} SSC^{low} cells as described in Materials and Methods and Fig. S1. Graphs show percentage of (A) Lin⁺ ST2⁺/IL-33⁺ NKp46⁺ ILC and (B) corresponding IFN- γ and (C) IL-17A expression by these cells, (D) percentages of Lin⁺ ST2⁺/IL-33⁺ NKp46⁺ ILC and (E) corresponding IFN- γ and (F) IL-17A expression. Error bars represent SEM and p values were calculated using One-way ANOVA followed by Tukey's multiple comparison test (black lines) and paired student's t test (grey lines). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Representative FACS plots show IFN- γ expression by Lin⁺ ST2⁺/IL-33⁺ NKp46⁺ (top) and NKp46⁺ (bottom) ILC. Experiments for each group was repeated minimum 2–3 times.

control showed elevated NKp46⁺ ILC1/ILC3 numbers, low or no expression of IFN- γ was observed (Fig. 2D, E and S3). Remarkably, rMVA Δ IL-1 β R induced the highest IL-17A expression by both NKp46⁺ (Fig. 2C) and NKp46⁺ ILC1/ILC3 subsets (Fig. 2F). rMVA and Influenza A vectors induced modest IL-17A expression by both these subsets, whilst rFPV, Ad5 and RV showed no IL-17A expression, similar to the unimmunized control (Fig. 2C and F).

Unlike i.n., following i.m. delivery, the proportion of NKp46⁺ ILC1/ILC3 in the muscle was very minimal (0–0.8%) across all vaccine vectors (Fig. 2A and Fig. 3A), with significant differences observed between rMVA compared to rFPV, rMVA Δ IL-1 β R and Ad5 ($p = 0.0087$, $p = 0.0049$, and $p = 0.0397$ respectively). Additionally, only rFPV and Influenza A vaccinated groups showed any expression of IFN- γ by NKp46⁺ ILC1/ILC3 (Fig. 3B). Interestingly, IFN- γ expression by these subsets was much greater following i.m. versus i.n. vaccination (rFPV i.m. ~12.06% i.n. 2.5% and influenza A i.m. ~4.67% i.n. ~1.5%) (Fig. 2B and Fig. 3B). In the context of IL-17A expression by NKp46⁺ ILC1/ILC3, only Influenza A vaccinated animals showed any significant expression (average 8.39%, $p < 0.0001$ influenza A vs. all vaccine vectors) (Fig. 3C). Of the poxviral vectors tested, rMVA Δ IL-1 β R vaccinated group also showed an increase in the proportion of NKp46⁺ ILC1/ILC3 expressing IL-17A (average 0.89%) although not significant and was similar to what was observed with i.n. delivery (average 1.0%).

Moreover, following i.m. delivery, different IFN- γ and IL-17A expression profiles were detected by NKp46⁺ ILC1/ILC3. Unlike i.n. delivery, very low IFN- γ expression was detected following i.m. vaccination, and only influenza A (~0.01%) and Ad5 (~0.03%) showed any IFN- γ expression (Fig. 2E and Fig. 3E). All vectors showed different NKp46⁺ ILC1/ILC3-derived IL-17A expression

profiles. Specifically, out of the vectors tested, Ad5 and rMVA Δ IL-1 β R showed the highest expression (~0.58% and ~0.84% respectively) (Fig. 3F). Interestingly, the NKp46⁺ ILC1/ILC3-derived IL-17A expression by the rMVA Δ IL-1 β R group was significantly elevated compared to unimmunised, rFPV, rMVA and influenza A ($p < 0.0001$, $p = 0.0064$, $p < 0.0001$, and $p < 0.0001$ respectively) (Fig. S4). Whilst, Ad5 showed significant differences compared to unimmunised, rMVA, and influenza A vaccinated groups ($p = 0.0048$, $p = 0.0172$ and $p = 0.0219$ respectively) (Fig. S4).

2.3. rFPV and rMVA Δ IL-1 β R lead to preferential recruitment of CD11b⁺ CD103⁺ conventional DCs to the lung mucosae, 24 h post intranasal vaccination

Our previous studies have shown that transient inhibition of IL-13 at the vaccination site can significantly modulate DC recruitment and resulting avidity of CD8⁺ T cells, including B cell immunity [41,42,45]. Since we have shown that ILC2 are the major source of IL-13 at the vaccination site and this is also viral vector-dependent [44], in this study we have also assessed the influence of viral vector on lung DC recruitment 24 h post i.n. vaccination (as per indicated in Figs. S5). In this study, four different lung DC subsets was assessed (CD11b⁺ CD103⁺ cDC, CD11b⁺ CD103⁺ cross-presenting DC, CD11b⁺ CD8⁺ cross-presenting DC and CD11b⁺ B220⁺ pDC (not other immune cell infiltrates)). Percentage of each DC subset, for a given viral vector was calculated as a proportion of total MHC-II⁺ CD11c⁺ DCs, as described in Materials and Methods.

In agreement with Trivedi et al 2014, these studies also showed that rFPV recruited significantly elevated proportions of CD11b⁺

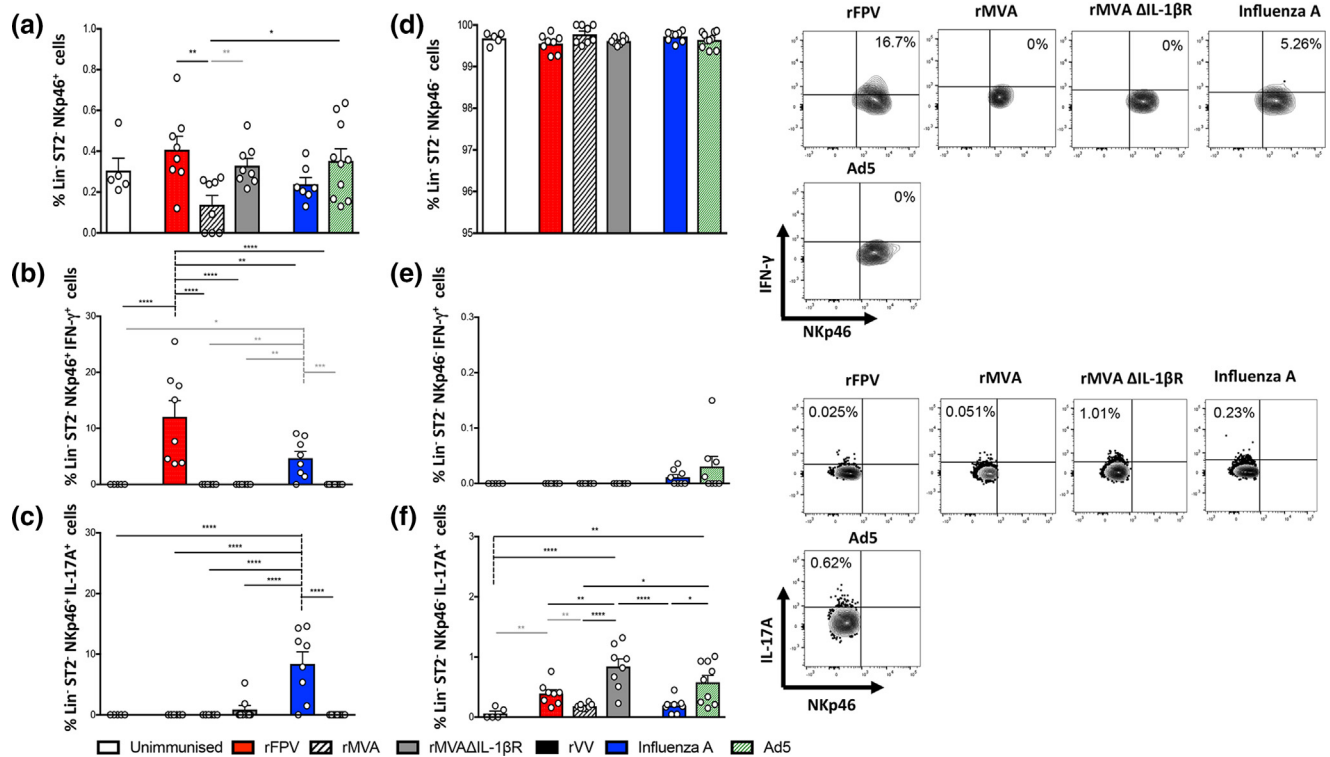


Fig. 3. Evaluation of muscle $\text{Lin}^- \text{ST2}^- \text{Nkp46}^+$ and Nkp46^- ILC-derived IFN- γ and IL-17A profiles post intramuscular viral vector vaccination. BALB/c mice ($n = 5-9$) were i.m. immunised with rFPV, rMVA, rMVA- $\Delta\text{IL-1}\beta\text{R}$, Influenza A or Ad5. 24 h post vaccination muscles were harvested and cell suspensions were stained for $\text{Lin}^- \text{ST2/IL-33R}^- \text{Nkp46}^+$ and Nkp46^- ILC and their cytokine expression. Cells were pre-gated on $\text{CD45}^+ \text{FSC}^{\text{low}} \text{SSC}^{\text{low}}$ cells as described in Materials and Methods and Fig. S2. (A) Graphs show percentage of $\text{Lin}^- \text{ST2/IL-33R}^- \text{Nkp46}^+$ ILCs and (B) corresponding IFN- γ and (C) IL-17A expression by these cells, (D) percentage of $\text{Lin}^- \text{ST2}^- \text{Nkp46}^-$ ILCs and (E) their corresponding IFN- γ and (F) IL-17A expression. Error bars represent SEM and p values were calculated using One-way ANOVA followed by Tukey's multiple comparison test (black lines) and paired student's t test (grey lines). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Representative FACS plots show IFN- γ and IL-17A expression by $\text{Lin}^- \text{ST2/IL-33R}^- \text{Nkp46}^+$ (top) and Nkp46^- (bottom) ILC. Experiments for each group was repeated minimum 2–3 times.

$\text{CD103}^- \text{cDCs}$ compared to rMVA and rVV ($p = 0.0062$, $p = 0.0322$ respectively). Additionally, the deletion mutant rMVA $\Delta\text{IL-1}\beta\text{R}$ recruited the highest percentage of $\text{CD11b}^+ \text{CD103}^- \text{cDCs}$, whilst Ad5 recruited the lowest (Fig. 4A and B). Furthermore, $\text{CD11b}^+ \text{CD103}^- \text{cDC}$ recruitment by Influenza A was similar to that of rFPV, rMVA, rVV and RV (Fig. 4A and B). Compared to the unimmunized control, rFPV, rMVA $\Delta\text{IL-1}\beta\text{R}$ and Influenza A showed significant elevated $\text{CD11b}^+ \text{CD103}^- \text{cDC}$ recruitment ($p = 0.0069$, $p < 0.0001$ and $p = 0.0077$ respectively).

2.4. Intranasal rVV vaccination recruited elevated numbers of $\text{CD11b}^- \text{CD103}^+$ and $\text{CD11b}^- \text{CD8}^+$ cross-presenting DCs to the lung mucosae 24 h post vaccination

Unlike $\text{CD11b}^+ \text{CD103}^- \text{cDC}$ recruitment, rFPV induced significantly lower $\text{CD11b}^- \text{CD103}^+$ cross-presenting DCs compared to that of the unimmunized control ($p = 0.0224$), and these values were significantly lower than that of rVV, Influenza A and RV vectors ($p < 0.0001$, $p = 0.0065$ and $p < 0.0001$ respectively) (Fig. 4A and C). Interestingly, compared to all viral vectors tested, rVV recruited the highest percentage of $\text{CD11b}^- \text{CD103}^+$ cross-presenting DCs to the lung mucosae 24 h post vaccination. Whilst, rFPV recruited the lowest number similar to rMVA, rMVA $\Delta\text{IL-1}\beta\text{R}$ and Ad5 (Fig. 4C). Furthermore, the proportion of $\text{CD11b}^- \text{CD8}^+$ cross-presenting DCs recruited by all the vaccine vectors showed a comparable profile to that of the $\text{CD11b}^- \text{CD103}^+$ cross-presenting DCs, where rVV showed the highest proportion of $\text{CD11b}^- \text{CD8}^+$ cross-presenting DCs (Fig. 5A and C). It is noteworthy that the cross-presenting $\text{CD11b}^- \text{CD103}^+$ DCs recruited by rVV, Influenza A and RV were significantly higher than unimmunized control ($p < 0.0001$, $p = 0.0067$ and $p = 0.0113$ respectively) (Fig. 4A and C). Whereas, cross-presenting $\text{CD11b}^- \text{CD8}^+$ DCs recruited by rVV and Influenza A although were significantly higher than unimmunized control ($p < 0.0001$, $p = 0.0498$ respectively), Ad5 recruitment was significantly lower ($p = 0.0164$) (Fig. 5A and C).

2.5. Compared to the other viral vectors, RV and Ad5 recruited elevated $\text{CD11b}^- \text{B220}^+$ plasmacytoid DCs to the lung mucosae 24 h post intranasal vaccination

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Next when the $\text{CD11b}^- \text{B220}^+$ pDC recruitment profile was assessed, these DCs showed a unique profile compared to the other three DC subsets examined. At 24 h post vaccination, RV and Ad5 recruited the highest percentage of $\text{CD11b}^- \text{B220}^+$ pDCs to the lung mucosae, whilst Influenza A, rFPV and rMVA $\Delta\text{IL-1}\beta\text{R}$ showed the lowest (Fig. 5B and D). Among the poxviral vectors, rVV recruited the highest proportion of $\text{CD11b}^- \text{B220}^+$ pDCs whilst rFPV recruited the lowest, and rMVA and rMVA $\Delta\text{IL-1}\beta\text{R}$ showed a similar pDC profile. Compared to the unimmunized control, rVV, RV and Ad5 vectors showed significant differences in pDC recruitment 24 h post vaccination ($p = 0.0025$, $p < 0.0001$ and $p < 0.0001$ respectively) (Fig. 5B and D).

2.6. Following intranasal vaccination different viral vectors showed different kinetic profiles 0 to 48 h post vaccination

Next, we also evaluated the DC recruitment kinetics 0 to 48 h post vaccination. Distinct DC kinetic profiles for each of the vectors were detected over time. rFPV showed significant regulation of

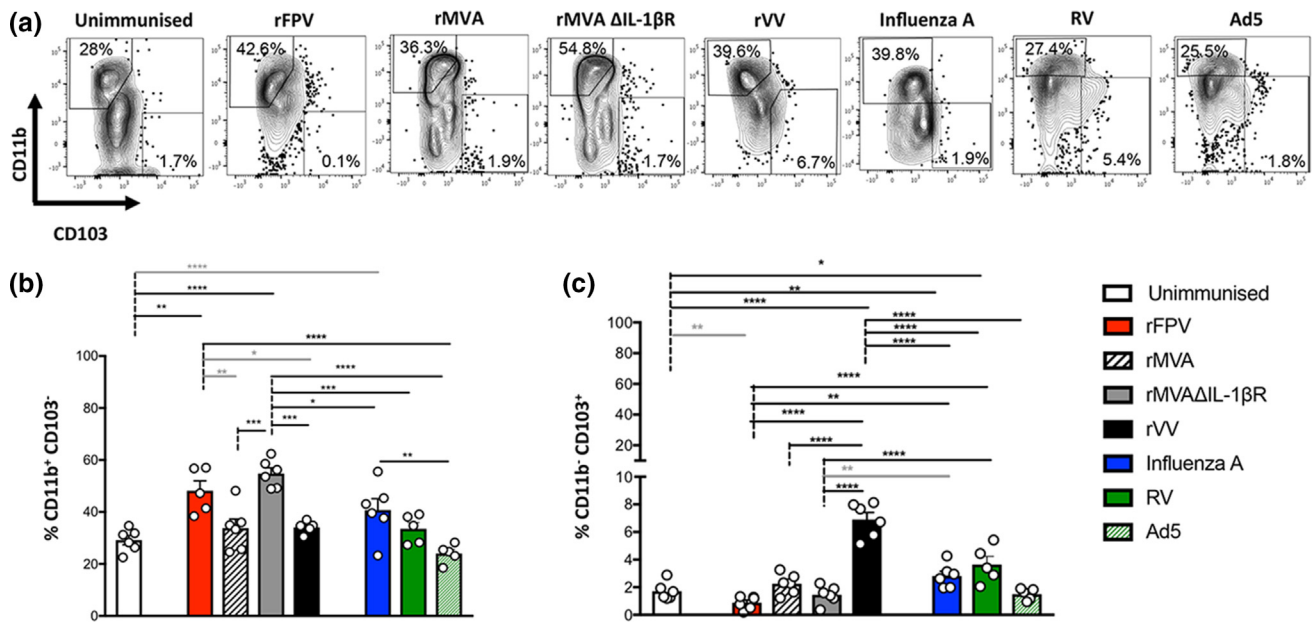


Fig. 4. Evaluation of CD11b⁺ CD103⁻ cDCs and CD11b⁻ CD103⁺ cross-presenting DCs following intranasal viral vector vaccination. BALB/c mice (n = 5) were i.n. immunised with rFPV, rMVA, rMVA-ΔIL-1βR, rVV, Influenza A, RV or Ad5. 24 h post vaccination lungs were harvested single cell suspensions were prepared and stained for different DC subsets and analysed using flow cytometry as described in Materials and Methods. Cells were pre-gated on MHC-II⁺ CD11c⁺ cells using fluorescence minus one (FMO) controls for each virus as described in Materials and Methods and Fig. S5. (A) Representative FACS plots show percentage of CD11b⁺ CD103⁻ DCs (gated top left) and CD11b⁺ CD103⁺ DCs (gated bottom right) recruited to lung mucosae. (B) Percentage of CD11b⁺ CD103⁻ DCs and (C) CD11b⁻ CD103⁺ DCs are shown as bar graphs, recruited by each vaccine vector. Percentages were calculated as a proportion of total MHC-II⁺ CD11c⁺ DCs generated by each vector as indicated in Materials and Methods. Error bars represent SEM and p values were calculated using One-way ANOVA followed by Tukey's multiple comparison test (black lines) and paired student's t test (grey lines). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Experiments with each vector were repeated minimum 2–3 times.

CD11b⁺ CD103⁻ cDCs, which was similar to the cDC profile induced by the rMVA deletion variant (rMVAΔIL-1βR), unlike the parental rMVA (Fig. 6A and S6). The replication competent rVV showed regulation of all DC subsets, with significant modulation of cross-presenting DCs. Interestingly, cDC recruitment kinetics between rVV, rMVA and Influenza were very similar (Fig. 6B and S6). Ad5 recruited a pDC profile similar to RV and a CD11b⁻ CD8⁺ profile similar to rVV (Fig. 6B, C and S6).

3. Discussion

This study has clearly demonstrated that not only the route of vaccination, but also different viral vector-based vaccines can induce significantly different ILC subsets at the respective vaccination sites 24 h post delivery. In the context of ILC2, Lin⁻ ST2/IL-33R⁺ ILC2 were predominant in lung, whilst Lin⁻ IL-25R⁺ or/and Lin⁻ TSLPR⁺ ILC2 were found in muscle 24 h post viral vector vaccination. This was not entirely surprising as Lin⁻ IL-25R⁺ ILC2 has been associated with circulation [46,47], whilst Lin⁻ TSLPR⁺ ILC2 is known to be skin-resident [48]. Although, Lin⁻ ST2/IL-33R⁺ ILC2 was the major source of IL-13 in lung, Lin⁻ IL-25R⁻ TSLPR⁻ ST2/IL-33R⁻ ILC2s were the predominant source of IL-13 in muscle. Interestingly, recently we have also found that following viral vector vaccination IL-5 expression was specific to lung ILC2, not muscle (Jaeson *et al.* submitted), reaffirming that ILCs can be highly plastic under different conditions (specifically chronic inflammatory conditions versus vaccination or infection) [26,49], and why different routes of delivery may yield uniquely different innate and adaptive immune outcomes.

In addition to ILC2, i.n. versus i.m. vaccinations induced different proportions of NKp46⁺ ILC1/ILC3s unlike NKp46⁻ ILC1/ILC3s. Specifically, significantly lower numbers of NKp46⁺ ILC1/ILC3s were detected in muscle compared to the lung (~1% vs 4–8%), confirming that circulatory ILC1/ILC3s are scarce as opposed to tissue

resident ILCs [50]. Both NKp46⁺ and NKp46⁻ ILC1/ILC3s were able to express different levels of IFN-γ, that were vaccine route- and vector-dependent. Specifically, whilst both NKp46^{+/+} ILC1/ILC3 subsets were able to express IFN-γ in lung, only the NKp46⁺ ILCs in muscle expressed IFN-γ, albeit by two vaccination groups, where the expression was in the order of rFPV > Influenza A. Moreover, muscle NKp46⁻ cells expressed extremely low IFN-γ following Influenza and Ad5 vaccination. Majority of i.m. delivered vectors induced elevated ILC2-driven IL-13 and minimal ILC1/ILC3-driven IFN-γ expression compared to i.n. delivery. Additionally, our previous studies with pox-viral vectors have shown that, compared to i.m., i.n. delivery can induce T cells of higher avidity, associated with low IL-13 at the vaccination site [5,6,44]. Furthermore, i.n. rFPV priming has shown to induce high avidity T cells compared to i.n. rVV and Influenza priming vaccination [3,7,51], (Tan, Derosé *et al.* personal communication). In agreement with our current study, i.n. Ad5 vaccination has also shown comparable ILC2 gene expression profiles to i.n. rFPV, unlike i.m. Ad5 delivery (Jaeson *et al.* submitted). Taken together, these findings may explain why systemic vaccination with some viral vectors may lead to suboptimal antiviral immunity, compared to mucosal vaccination [6,7,52,53].

Besides the route of delivery, each viral vector also induced a uniquely different ILC2-driven IL-13 and ILC1/ILC3-driven IFN-γ expression profiles. Specifically, both i.n. and i.m. rFPV vaccination induced low ILC2-derived IL-13, and high NKp46⁺ or NKp46⁻ ILC1/ILC3-derived IFN-γ. In contrast, i.m. rMVA vaccination induced lower ILC2-derived IL-13 compared to i.n. delivery. Knowing that, low IL-13 is associated with improved T cell immunity, our current data may explain why previously rMVA has been found to be more efficacious as an i.m. delivery vector than a mucosal delivery vector [8,10]. Moreover, whilst i.n. delivery of rMVA, Influenza A, RV and Ad5 induced elevated ILC2-derived IL-13, the expression of IFN-γ was lower in NKp46⁺ ILC1/ILC3s following rMVA, Influenza A;

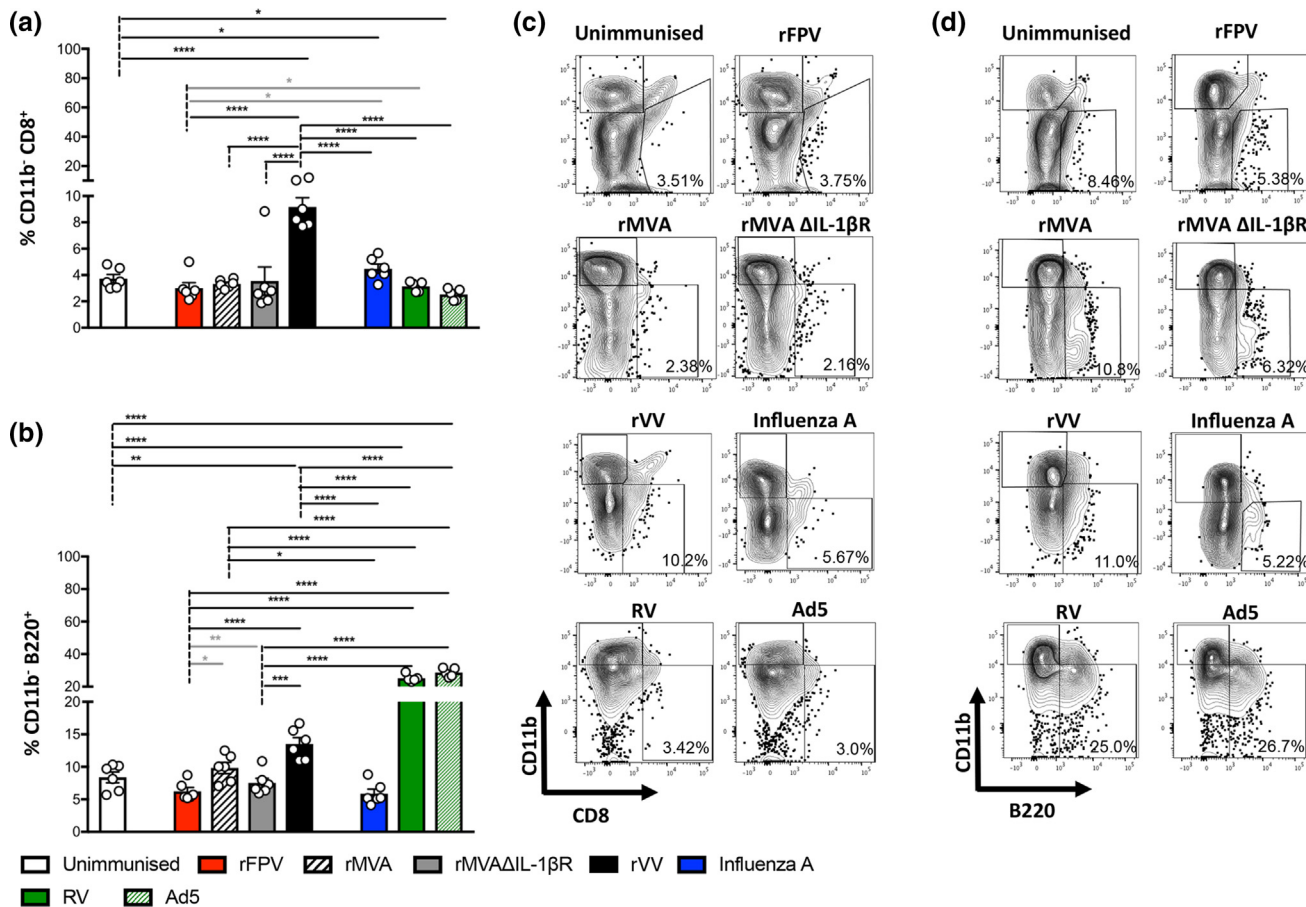


Fig. 5. Evaluation of CD11b⁻ CD8⁺ cross-presenting and CD11b⁻ B220⁺ pDC subsets following intranasal viral vector vaccination. BALB/c mice (n = 5) were i.n. immunised with same vectors as in Fig. 4 and lung cells were analysed using flow cytometry for DC subsets as per described in Materials and Methods. Cells were pre-gated on MHC-II⁺ CD11c⁺ cells using fluorescence minus one (FMO) controls as described in Fig. S5. Left panel bar graphs show (A) percentage of CD11b⁻ CD8⁺ DCs and (B) CD11b⁻ B220⁺ DCs recruited to the lung mucosae compared to the unimmunised control. Right panel representative FACS plots show percentage of (C) CD11b⁻ CD8⁺ DCs and (D) CD11b⁻ B220⁺ DCs recruited to the vaccination site 24 h post vaccination by each vector. Percentages were calculated as a proportion of total MHC-II⁺CD11c⁺ DCs generated by each vector as indicated in Materials and Methods. Error bars represent SEM and p values were calculated using One-way ANOVA followed by Tukey's multiple comparison test (black lines) and paired student's t test (grey lines). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Experiments with each vector were repeated minimum 2–3 times.

and NKp46⁺ ILC1/ILC3s following RV and Ad5 vaccinations. Interestingly, we have previously shown that IL-4R antagonist adjuvanted vaccination that transiently inhibited IL-13 signalling via STAT6, induced low ILC2-derived IL-13 expression associated with elevated expression of NKp46⁺ ILC1/ILC3-derived IFN-γ [44]. Additionally, enhanced *IfngR* gene expression on ILC2 was also recently associated with low ILC2-derived IL-13 (Jaeson et al. submitted). Taken together, these observations suggest that enhanced ILC1/ILC3-derived IFN-γ expression regulates ILC2-derived IL-13 at the vaccination site, similar to the Th1/Th2 paradigm. Hence, we propose that ILC-derived IL-13 and IFN-γ balance at the vaccination site crucially impacts the downstream vaccine-specific immunity.

Different vectors also lead to differential expression of IL-17A by NKp46⁺ and NKp46⁻ ILC1/ILC3. Specifically, i.n. rMVA, rMVAΔIL-1βR and Influenza A vectors induced elevated IL-17A by both ILC1/ILC3 subsets at the lung mucosae 24 h post vaccination. However, majority of the vectors induced different levels of IL-17A by NKp46⁻ ILC1/ILC3 subsets in the muscle. In asthma studies the importance of maintaining IL-13 and IL-17 balance has been well documented [54]. Similarly, our vaccination studies have also shown that IL-13 can regulate IL-17A expression on T cells at the transcriptional and translational level, and IL-17A is associated with T cell mediated protective immunity [55]. Knowing that (i) rVV and its derivatives (rMVA) perform better as a booster vaccine than a prime [7,8] (ii) Influenza A prime yield poor adaptive

immune outcomes (Tan, Derosé et al. personal communication) [51] and (iii) systemic Ad5 immunization have shown to induce less effective antiviral T cell responses [12,56–58], collectively our data suggest that the early onset of high ILC1/ILC3-derived IL-17A together with low IFN-γ and high ILC2-derived IL-13 could be detrimental for inducing effective cellular immunity.

Our study demonstrated that in addition to different ILC profiles, mucosal vaccination with different viral vectors yielded uniquely different lung DC profiles at the vaccination site 24 h post vaccination. We have previously shown that IL-13 levels at the vaccination site can significantly alter DC phenotype, specifically, inhibition of IL-13 can recruit elevated CD11b⁺ CD103⁻ cDCs associated with high avidity T cells [45]. This study further substantiated our previous findings of enhanced recruitment of CD11b⁺ CD103⁻ cDCs as opposed to CD11b⁻ CD103⁺ cross-presenting DCs following i.n. rFPV vaccination. Moreover, moderate proportions of CD11b⁻ B220⁺ pDCs were also observed with rFPV vaccination. pDCs are known to induce antibody differentiation via IFN-γ production [40] and their clustering with cDCs have shown to induce efficient T cell mediated antiviral immunity [38]. We have already established that rFPV priming can induce robust high avidity T cells and differentiated antibodies, involved in protective immunity against viral pathogens such as HIV [7,41]. Thus, our current findings suggest that although in the context of certain viral vectors, the cDC/pDC balance may govern the quality of T and

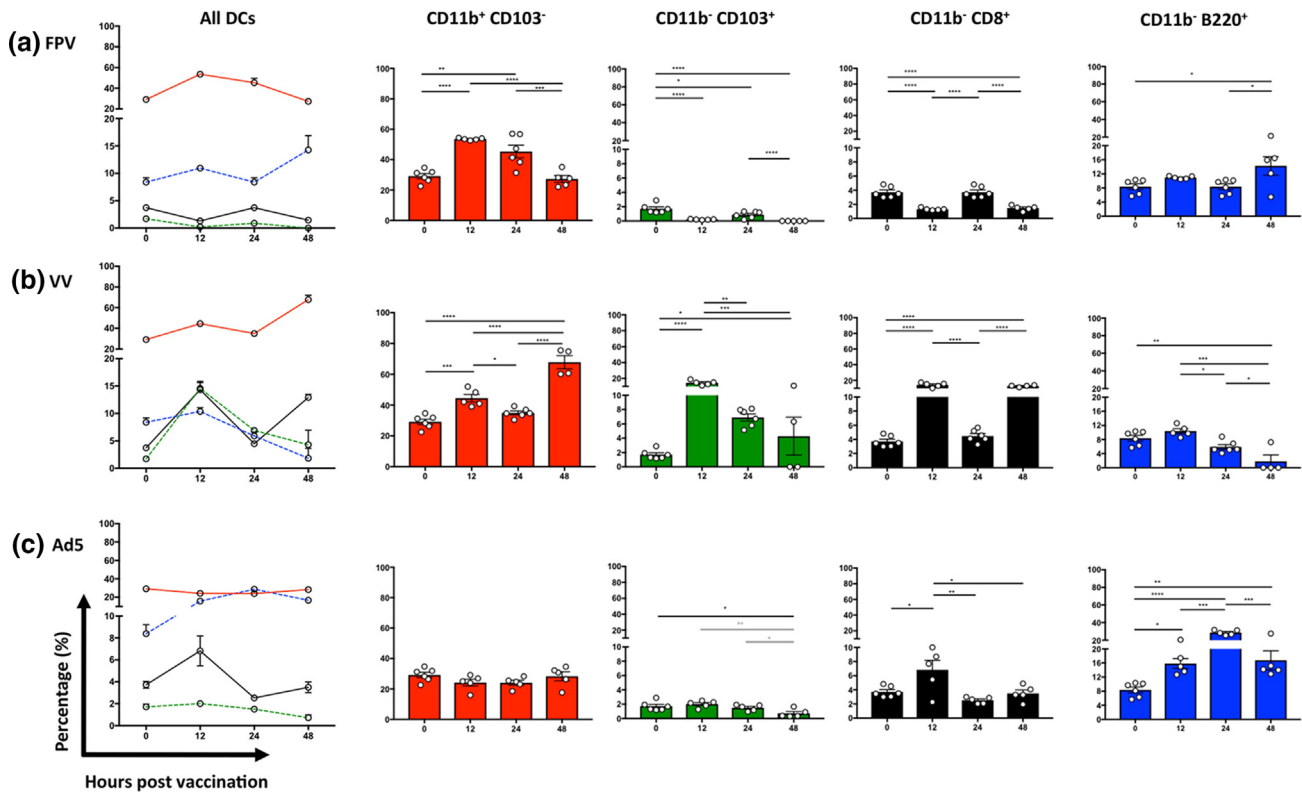


Fig. 6. DC kinetics following intranasal viral vector based vaccination 0–48 h post vaccination. BALB/c mice ($n = 5$) were i.n. immunised with rFPV, rVV and Ad5. Lungs were harvested at 12, 24 and 48 h post vaccination and lung DC subsets and analysed using flow cytometry as described in Materials and Methods. Cells were pre-dated on MHC-II⁺ CD11c⁺ cells using fluorescence minus one (FMO) controls as described in Fig. S5. Line graphs (left panel) and bar graphs (right four panels) show percentage of CD11b⁺ CD103⁻ DCs (red), CD11b⁻ CD103⁺ DCs (green), CD11b⁻ CD8⁺ DCs (black) and CD11b⁻ B220⁺ DCs (blue) recruited by (A) rFPV, (B) rVV and (C) Ad5 to the lung mucosae 0 to 48 h post vaccination. Error bars represent SEM and p values were calculated using One-way ANOVA followed by Tukey's multiple comparison test (black lines) and paired student's t test (grey lines). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Experiments with each vector were repeated minimum 2–3 times. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

B cell immunity, replicating vectors such as Influenza A may employ other mechanisms (as Influenza A showed similar cDC/pDC profile to rFPV associated with poor quality T cells).

In contrast to rFPV vaccination, rMVA lead to elevated ILC2-derived IL-13, similar to rVV (data not shown), and both vectors significantly enhanced recruitment of CD11b⁻ CD103⁺ cross-presenting DCs to the lung mucosae, as shown previously [45]. This may explain why rMVA and rVV priming lead to low avidity T cells following recombinant HIV vaccination [3,7]. Moreover, intranasal Influenza A, RV and Ad5 vaccination which also lead to high ILC2-derived IL-13, preferentially induced CD11b⁻ CD103⁺ cross-presenting DCs as opposed to cDCs. In a prime-boost vaccine modality, recombinant Influenza A priming has shown to induce enhanced magnitude of vaccine-specific T cells, however, are of low avidity unlike rFPV priming (Tan, Derosé et al. personal communication). Similarly, recombinant Ad5 vaccination has also shown to induce high magnitude of vaccine-specific CD8 T cells [13]. Therefore, these observations suggest that these vectors although lead to enhanced magnitude of vaccine-specific T cell immunity (IFN- γ production by T cells), may lead to low avidity T cells against chronic infections such as HIV-1. Despite low cDCs, Ad5 and RV exhibited a bias towards pDC recruitment. Knowing that pDC-driven IFN- γ can induce effective antibody responses, we postulate that Ad5- and RV-based vaccines could be more efficacious in inducing humoral immunity. Similar to CD11b⁻ CD103⁺ cross-presenting DCs, rVV additionally induced elevated CD11b⁻ CD8⁺ cross-presenting DCs to the lung mucosa. These observations suggested that, early induction of CD11b⁻ CD8⁺ cross-presenting DCs, could also be associated with induction of low avidity T cells.

However, in the context of some pathogens, (for example, *Leishmania*, and also some viruses, Influenza and HSV-1 infections), induction of cross-presenting DCs have been associated with protective immunity [30,59,60]. Thus, when designing recombinant viral vector-based vaccines, careful selection of the vector, according to the pathogen of interest may be of great importance.

rMVA Δ IL-1 β R is known to induce effective memory T cell responses compared to parental rMVA vaccination [61]. Unlike rMVA, rMVA Δ IL-1 β R induced low ILC2-derived IL-13 and elevated cDCs similar to rFPV, which has shown to induce high avidity T cells with better protective immunity. These findings indicated that removal of a single immune evasive gene from the viral vector can significantly alter the innate immune outcomes, specifically the ILCs and DCs, associated with effective protective immunity. However, compared to rFPV (which showed elevated IFN- γ and no IL-17A expression), rMVA Δ IL-1 β R vaccination induced suboptimal ILC1/ILC3-derived IFN- γ and high IL-17A expression at the vaccination site. It is well established that IFN- γ is crucial for antiviral immunity, and overexpression of IL-17A can lead to immune imbalance [62]. It is also known that viral IL-18 bp neutralize host IL-18 and prevent IFN- γ production [63]. Thus, the residual IL-18 bp in the rMVA Δ IL-1 β R vector could be responsible for the observed ILC1/ILC3-derived IFN- γ profile. Thus, we postulate that rMVA vector lacking both IL-1 β R and IL-18 bp genes may lead to ILC/DC profiles similar to rFPV and balanced T and B cell outcomes.

Furthermore, rVV, rMVA and rMVA Δ IL-1 β R data clearly demonstrated that the attenuation status of a viral vector and the presence or absence of virokinases significantly modulated the ILC cytokine expression and DC profile. The rFPV and rMVA Δ IL-1 β R

data indicated that viral vectors that do not interfere with the host immune system could be more efficacious at inducing vaccine-specific immunity in humans (e.g.- Avipoxvirus compared to Orthopoxvirus). These observations strongly highlight the notion that when designing viral vector-based vaccines, in addition to the safety and genetic stability, inherent properties of the viruses themselves need serious consideration (in this case, its replicative ability within the mammalian host).

We have previously shown that ILC2s are the only source of IL-13 at the vaccination site, 24 h post vaccination and IL-13 level in the milieu can crucially impact the DC recruitment at the lung mucosae [42,44,45]. Hence, collectively our findings suggest that, early ILC2-derived IL-13, together with ILC1/ILC3-derived IFN- γ and IL-17A, differentially impact DC recruitment/regulation at the vaccination site, associated with adaptive immune outcomes and this warrants further investigation. Therefore, we postulate that (i) following vaccination, ILC and DC profiles may act as predictors of downstream vaccine-specific immunity and (ii) selection of viral vector according to the pathogen of interest (eg: virus, bacteria or parasites) may help tailor/design effective viral-vector based vaccines against chronic pathogens.

4. Materials and Methods

Mice. Pathogen-free 6–8 weeks old female BALB/c mice were purchased from the Australian Phenomics Facility, The Australian National University. All animals were maintained, monitored daily and cervically dislocated at the endpoint according to the Australian NHMRC guidelines within the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and in accordance with guidelines approved by the ANU Animal Experimentation and Ethics Committee (AEEC), protocol number A2014/14 and A2017/15.

Viral vector-based Vaccines. Recombinant FPV, VV and MVA expressing HIV antigens described previously were used in this study [44,64]. The rMVA- Δ IL-1 β R was constructed and kindly provided by Dr. Jackson. Influenza A and Adenovirus (Ad5) vectors were kindly provided by Prof. Arno Mullbacher, JCSMR, ANU. Recombinant Human Rhinovirus serotype 1A (RV) was kindly provided by Prof. Gowans and Dr. Wijesundara, Basil Hetzel Institute, University of Adelaide [16].

Immunisation. BALB/c mice were intranasally or intramuscularly immunised with 1×10^7 plaque forming units (pfu) of each of the poxviruses rFPV, rVV, rMVA, rMVA- Δ IL-1 β R; 2×10^7 pfu (i. n.) or 2.5×10^7 pfu (i.m.) of Ad5, 5×10^6 TCID₅₀ of RV or 500 pfu of Influenza A. Note that, doses used were comparable to those used in previous studies, optimal to induce adaptive immune outcomes. Mice were vaccinated with 10 μ L per nostril (i.n.) or 50 μ L per leg (i.m.) under mild isoflurane anaesthetic. rFPV, rVV, rMVA, rMVA- Δ IL-1 β R were sonicated three times for 15 s on ice at 50% capacity using Branson Sonifier 450 immediately prior to vaccination.

Preparation of lung lymphocytes. Lung tissues were collected 24 h post vaccination in complete RPMI for ILC studies as described previously [44]. For DC studies, lungs were harvested at 12, 24 and 48 h post vaccination. Lung tissues were prepared as described previously [44]. Briefly, tissues were cut into small pieces, and enzymatically digested for 45 min at 37 °C in digestion buffer containing 1 mg/ml collagenase (Sigma-Aldrich, St Louis, MO), 1.2 mg/ml Dispase (Gibco, Auckland, NZ), 5 Units/ml DNase (Calbiochem, La Jolla, CA) in complete RPMI. Samples were crushed and passed through a 100 μ m falcon cell strainer and resulting lung cell suspensions were then treated with red cell lysis buffer followed by extensive washing to remove the lysis buffer. Samples were then passed through gauze to remove debris, cells were re-suspended

in complete RPMI, rested overnight at 37 °C under 5% CO₂ as per our previous studies prior to staining [5,42].

Preparation of muscle lymphocytes. Muscle tissues were harvested 24 h post vaccination in complete RPMI and prepared as previously indicated [44]. Briefly, tissues were, homogenised and enzymatically digested for 45 min at 37 °C in a digestion buffer containing 2 mg/mL collagenase, 2.4 mg/mL dispase and 5 Units/mL of DNase in complete RPMI. Subsequently, samples were very gently pushed through a 70 μ m Falcon cell strainer, to avoid debris. Resulting cell suspension was then washed, resuspended in complete RPMI and rested overnight as per lung prior to staining [5,42].

Evaluation of lung and muscle ILCs using flow cytometry. Monoclonal antibodies FITC-conjugated anti-mouse CD3 (T cells) clone 17A2, CD19 (B cells) clone 6D5, CD11b (macrophages and dendritic cells) clone M1/70, CD11c (dendritic cells) clone N418, CD49b (NK, NKT, T cells) clone HM α 2, Fc ϵ R1 α (Mast cells and Basophils) clone MAR-1 (all lineage positive markers were selected as FITC), PE-conjugated anti-mouse ST2/IL-33R (clone DIH9), APC-conjugated IL-25R (clone 9B10), APC/Cy7-conjugated anti-mouse CD45 (clone 30-F11), Brilliant Violet 421-conjugated anti-mouse CD335 (NKP46) (clone 29A1.4), Brilliant Violet 510-conjugated anti-mouse IFN- γ (clone XMG1.2), Alexa Fluor 700-conjugated IL-17A (clone TC11-18H10.1) were obtained from BioLegend. PE-eFluor 610-conjugated anti-mouse IL-13 (clone eBio13A) was purchased from eBioscience and APC-conjugated anti-mouse TSLPR R&D systems. ILC2 and ILC1/3s were stained separately to avoid fluorochrome overlap. Specifically, FITC-conjugated lineage cocktail antibodies and APC/Cy7-conjugated anti-mouse CD45 were used in both ILC2 and ILC1/ILC3 staining. For lung and muscle ILC2 staining, PE-conjugated anti-mouse ST2/IL-33R, and PE-eFluor 610-conjugated anti-mouse IL-13 were used and for muscle ILC2 staining, additionally APC-conjugated IL-25R and APC-conjugated anti-mouse TSLPR were used. Brilliant Violet 421-conjugated anti-mouse NKP46, Brilliant Violet 510-conjugated anti-mouse IFN- γ , Alexa Fluor 700-conjugated IL-17A were only used in ILC1/3s staining. Briefly, for intracellular staining, samples were treated with Brefeldin A for 5 h, washed, cell surface staining was performed followed by and intracellular staining after fixing and permeabilising the cells as per our previous protocols [42]. Once the staining was completed all samples were fixed with 0.5% paraformaldehyde, 1.4×10^6 events from each lung sample were acquired and 3.0×10^6 events were acquired for muscle on a BD LSR Fortessa. Data were analysed using Tree Star FlowJo software (version 10.0.7) using gating strategies indicated in **Figs. S1 and S2**.

Evaluation of lung DCs using Flow cytometry. 2×10^6 cells were blocked with anti-mouse CD16/CD32 Fc Block antibody (BD Biosciences, USA) for 20 min at 4 °C and cells were surface stained with APC conjugated MHCII I-Ad (e-Biosciences, USA), biotin conjugated CD11c (N418 clone, Biolegend, USA), followed by streptavidin Brilliant violet 421 (Biolegend, USA) and other DC markers CD8 APC-eFluor780 (53–6.7 clone, ebiosciences, USA), B220 PercpCy5.5 (RA3-6B2 clone, e-Biosciences, USA), CD11b AlexaFluor 700 (M1170 clone, Biolegend, USA) and CD103 FITC (2E7 clone, e-Biosciences, USA) for 30 min on ice. Cells were resuspended in PBS and analysed using BD LSRII flow cytometer Becton Dickinson, San Diego, CA). 5×10^5 events per sample were collected and results were analyzed using FlowJo software version 10.0.7, as described in **Fig. S2**. Note that, live/dead staining was also performed using viability dye 7-amino-actinomycin D (7-AAD Biolegend, USA) (**Figs. S2A and S2B**).

Statistical analysis. Cytokine expression by ILCs was calculated as a percentage of the parent ILC subset. To depict the differences in IL-13 expression, following i.n. vs i.m. vaccinations, number of ILC2 expressing IL-13 were also back calculated to CD45⁺ population and normalized to 1×10^6 . The muscle ILC2 subset percent-

ages were calculated as (subset of interest/Lin⁺ population × 100%). The DC subsets were represented as a percentage of total MHC-II⁺ CD11c⁺ DCs. The *p*-values were calculated using two-tailed paired parametric student's *t*-test, unpaired parametric student's *t*-test or Ordinary One-way ANOVA with Tukey's multiple comparison post-test. All experiments were repeated minimum 2–3 times.

5. Data availability

The authors declare that all data supporting the findings of this study are available within the paper and [supplementary files](#).

Acknowledgements

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Author Contribution

SR designed and performed the DC studies, preparation of some vaccine stocks, analysis of the DC data and preparation of figures and the manuscript, SM performed most of the ILC studies and data analysis, ZL performed the RV and Ad5 ILC studies and data analysis, MJ contributed to the ILC studies and manuscript preparation. RJ constructed the rMVAΔIL-1βR. DKW, BG and EJG provided RV vaccine and critical evaluation of the manuscript. CR conceived the idea and helped design the experiments, data analysis and critical evaluation and preparation of the manuscript.

Disclosure

The authors declare no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.vaccine.2019.01.045>.

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