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# A hierarchical role of IL-25 in ILC development and function at the lung mucosae following viral-vector vaccination

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ABSTRACT

This study demonstrates that modulation of IL-25 and IL-33 cytokines responsible for innate lymphoid cell 2 (ILC2) activation/function can differentially regulate ILC profiles at the vaccination site, in a vaccine route-dependent manner. Specifically, recombinant fowlpox (rFPV) vector-based vaccine co-expressing an adjuvant that transiently sequestered IL-25 (FPV-HIV-IL-25 binding protein), delivered intramuscularly (i.m.) was able to induce significantly lower IL-25R$^+$ ILC2-deived IL-13 and ILC1/ILC3-derived IFN-γ expression with significantly elevated IL-17A in muscle. In contrast, intranasal (i.n.) delivery was able to induce all three known ILC2 subsets (ST2/IL-33R$^+$, IL-25R$^+$, and TSLPR$^+$ ILC2) to express varying amounts of IL-13 in lung, and also the TSLPR$^+$ ILC2 to express IL-4, unlike the unadjuvanted control, which only showed ST2/IL-33R$^+$ ILC2 to express IL-13. Interestingly, the sequestration of IL-25 in lung also induced a unique lineage$^-$ ST2/IL-33R$^-$ IL-25R$^-$ TSLPR$^-$ ILC2 population to express elevated IL-13 and IL-4. Moreover, both i.m. and, i.n. FPV-HIV-IL-25BP vaccination induced significantly elevated ILC1/ILC3-derived IL-17A in lung, indicating that ILC2 could directly impact ILC1/ILC3 activity. To our surprise, transient sequestration of IL-33 at the lung mucosae did not alter the lung ILC2 profiles or activity. These inhibitor studies showed that in the context of i.n. viral vector vaccination, IL-25 plays a predominant role in early ILC development/regulation than IL-33, and likely acts as a master regulator of ILC. Our previous findings have indicated that level of IL-4/IL-13 at the vaccination site impacts the quality/avidity of T cell immunity. Taken together data suggest that IL-25 binding protein could be used as an effective i.m. not an i.n. adjuvant to enhance quality of vaccine-specific T cell immunity. These findings evoke the notion that route-dependent manipulation of ILCs according to the target pathogen could be exploited to design more effective vaccines against chronic pathogens in the future.


## 1. Introduction

Innate lymphoid cells (ILCs), are currently categorized into three main subsets (ILC1, ILC2, and ILC3), and considered as the innate equivalent of T cells lacking antigen-specific receptors [1–3]. ILC1 have shown to act as the first line of defense against some intracellular pathogens by secreting IFN-γ and granulocyte-macrophage colony-stimulating factor (GM-CSF) [3-5]. Discovery of ILC2 materialized when in RAG-deficient mice (lacking B and T cells), a subset of cells were found to express IL-5 and IL-13 in response to IL-25 [6] and decade later were termed as ILC2 [7–9]. Currently, ILC2 are classified into three main subsets, depending on their expression of ST2/IL-33R, IL-25R, and TSLPR [10–13]. Interestingly, numerous studies have now shown that IL-33, IL-25, and TSLP differentially modulate ILC2 activity, specifically, in the context of tissue remodeling, allergy, and inflammation [14–17]. ILC3 were initially described in human tissues as mucosal-associated lymphoid cells that expressed NKp44 and produce IL-22 [18]. But currently, three ILC3 subsets; lymphoid tissue inducer (LTi) cells, NKp46$^-$ ILC3 and NKp46$^+$ ILC3 have been identified based on the expression of various surface markers and cytokines they express [19–21]. ILC3 are mainly known to express IL-17A and IL-22 cytokines, but due to high plasticity have also shown to express IFN-γ [3].

Recent studies indicate high plasticity between the different ILC subsets. The first observations of high plasticity of ILC were observed between ILC1/ILC3 and were shown to interchange under certain stimulatory conditions [22–24]. Specifically, under IL-12 and IL-23 stimulation RORγt$^+$ ILC3s have shown to down regulate RORγt and up-regulate the expression of T-bet and IFN-γ

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[21–23,25]. Moreover, Bernink *et al* have shown that CD127$^+$ ILC1 can differentiate to ILC3 in the presence of IL-2, IL-23, and IL-1β [24]. Similarly, high plasticity of ILC2 has also been reported and shown to differentiate to ILC1 or ILC3 [21,25,26]. For example, ILC2s isolated form human blood, when cultured in the presence of IL-1β were shown to express IL-12 receptor and when IL-12 was then added to culture, these ILC2 were shown to express IFN-γ, and down regulate IL-5 and IL-13 expression [26]. In another study following influenza virus infection, upon stimulation with IL-12 and IL-18, ILC2 were shown to down regulate GATA3 expression and convert to ILC1 and express IFN-γ [27]. Moreover, under IL-6 and transforming growth factor-β (TGF-β), ILC2 has also shown to become ILC3-like cells and produce IL-17A [28].

Interestingly, ILC function/regulation in the context of inflammation and vaccination/infection have also been vastly different [29,30]. Previous studies in our laboratory have established that intranasal vaccination with rFPV unadjuvanted vaccination induced ST2/IL-33R$^+$ ILC2 in the lung, whereas intramuscular vaccination induced IL-25R$^+$ ILC2 in muscle [29]. In naïve BALB/c lung only ST2/IL-33R$^+$ ILC2 were detected, however neither ST2/IL-33R$^+$ nor IL-25R$^+$ ILC2 subsets were detected in naïve muscle [29]. These results indicated that ST2/IL-33R$^+$ ILC2 were resident ILC in the lung, while IL-25R$^+$ ILC2 were most likely 'inflammatory' ILC2 that migrated to muscle from blood [28]. The inflammatory ILC2 have not only shown to play an important role in mediating anti-helminth immunity, but also shown to express RORγt and produce IL-17 in *Candida albicans* infection [28] which has indicated that there is high plasticity between ILC2 and ILC3. Interestingly, the ST2/IL-33R$^-$ IL-25R$^+$ inflammatory ILC2s have also shown to develop into ST2/IL-33R$^+$ ILC2s under certain conditions [28]. Therefore, to further understand the role of IL-33 and IL-25 in ILC2 development specifically in the context of intranasal and intramuscular viral vector-based vaccination, in this study two rFPV vaccines that transiently inhibited IL-33 or IL-25 activity at the vaccination site were constructed, and their impact on lung and muscle ILC development/cytokine expression were evaluated 24 h post vaccination. This time point was chosen as in our previous studies, data clearly established that ILC were significantly modulated 24 h post viral vector vaccination [29].

## 2. Results

### 2.1. Intramuscular FPV-IL-25BP vaccination induced reduced ILC2-derved IL-13 and elevated NKp46$^-$ ILC- derived IL-17A expression in the muscle

Knowing that lineage$^-$ ST2/IL-33R$^-$ IL-25R$^+$ ILC2s were detected in the muscle following i.m. vaccination [29] (Fig. S1), WT BALB/c mice were vaccinated intramuscularly (i.m.) with the unadjuvanted FPV-HIV vaccine and the FPV-HIV-IL-25BP adjuvanted vaccine, which sequestered IL-25 at the vaccination site muscle. Note that in this study, ILC cytokine expression was evaluated 24 h post vaccination, as in our previous studies peak ILCs activity was detected at this timepoint [29]. Firstly, when IL-13 expression by ILC2 was assessed, significantly lower expression of IL-13 was detected in FPV-HIV-IL-25BP vaccine compared to the control unadjuvanted group ($p < 0.01$) (Fig. 1a & b), and no IL-4 expression was detected in either groups.

Interestingly, in the context of ILC1/ILC3 subsets, sequestration of IL-25 in muscle significantly reduced the IFN-γ expression by lineage$^-$ ST2/IL-33R$^-$ IL-25R$^-$ NKp46$^+$ ILC subset compared to the unadjuvanted control vaccination (Fig. 1c & d), and no significant differences in IL-17A and IL-22 production were observed between the two groups (Fig. 1c & d). More interestingly, no IFN-γ expression was detected in the lineage$^-$ ST2/IL-33R$^-$ IL-25R$^-$ NKp46$^-$ ILC subset following i.m. FPV-HIV-IL-25BP adjuvanted vaccination, whilst significantly elevated IL-17A and IL-22 expression ($p < 0.0001$) were detected compared to the unadjuvanted control (Fig. 1e & f).

### 2.2. Intranasal FPV-HIV-IL-25BP vaccination induced unique ILC2 subsets expressing IL-13 in lung

Knowing that lung ILC2s were mainly ST2/IL-33R$^+$, next WT BALB/c mice were vaccinated intranasally (i.n.) with FPV-HIV-33BP, and unadjuvanted control and lung ILC subsets and their cytokine expression were evaluated at 24 h post vaccination. ILC subsets were gated as described previously [29] (Fig. S2). Interestingly, transient sequestration of IL-33 at the lung mucosae did not alter the ILC2 profiles compared to the control vaccination (Fig. 2c & f). Hence, next mice were vaccinated intranasally (i.n.) with FPV-HIV-25BP, which transiently sequestered IL-25 at the lung mucosae. Surprisingly, although no significant differences in lung lineage$^-$ ST2/IL-33R$^+$ ILC2s were observed between unadjuvanted control and IL-25BP adjuvanted vaccination groups (Fig. 2a & b), the IL-25BP adjuvanted vaccine induced significantly elevated lineage$^-$ ST2/IL-33R$^-$ IL-25R$^+$ ($p < 0.01$) and lineage$^-$ ST2/IL-33R$^-$ TSLPR$^+$ ILC2 ($p < 0.0001$) subsets in lung (Fig. 2a & b) compared to the unadjuvanted control.

When IL-13 expression was evaluated on ILC2 post intranasal FPV-HIV-IL-25BP vaccination, surprisingly, all three ILC2 subsets (ST2/IL-33R$^+$ ILC2, IL-25R$^+$ ILC2 and TSLPR$^+$ ILC2) were found to express IL-13 (Fig. 2d). Interestingly, although ST2/IL-33R$^+$ ILC2s showed significantly reduced IL-13 expression ($p < 0.0001$) (Fig. 2e), elevated expression was detected in ST2/IL-33R$^-$ IL-25R$^+$ and ST2/IL-33R$^-$ TSLPR$^+$ ILC2 subsets, compared to the unadjuvanted control (Fig. 2e). The IL-13 expression was significantly elevated in ST2/IL-33R$^-$ TSLPR$^+$ ILC2 subset compared to the ST2/IL-33R$^+$ subset ($p = 0.0308$) (Fig. 2e). Moreover, unlike our previous vaccine studies (specifically vaccine that transiently inhibited IL-4/IL-13) where no IL-4 expression was observed in ILC2s, following intranasal FPV-HIV-IL-25BP adjuvanted vaccination, elevated IL-4 expression was also detected in lineage$^-$ ST2/IL-33R$^-$ TSLPR$^+$ ILC2 (Fig. 2g & h). Interestingly, IL-4 subset did not express IL-13 (Fig. 2g).

### 2.3. Following FPV-HIV-IL-25BP vaccination elevated IL-17A expression detected in lung lineage$^-$ ST2/IL-33R$^-$ NKp46$^{+/-}$ ILC subsets

When lineage$^-$ ST2/IL-33R$^-$ NKp46$^+$ ILCs in lung were assessed 24 h post intranasal FPV-HIV-IL-25BP adjuvanted vaccination induced, significantly elevated numbers of lineage$^-$ ST2/IL-33R$^-$ NKp46$^+$ ILCs compared to the unadjuvanted vaccine (Fig. 3a & b), whereas the opposing was observed with FPV-HIV-IL-33BP vaccination (Fig. 3c). Next when IFN-γ expression was assessed, significantly elevated numbers of FPV-HIV-IL-25BP adjuvanted vaccinated ILC were found to express IFN-γ compared to the unadjuvanted control ($p < 0.0001$) (Fig. S3) although no such difference was observed when represented as a cell percentage (Fig. 3d & e) Interestingly, FPV-HIV-IL-25BP adjuvanted vaccine group also showed elevated IL-17A expression by ST2/IL-33R$^-$ NKp46$^+$ ILC (Fig. 3d & e), whilst IL-22 expression was significantly lower compared to the unadjuvanted control ($p < 0.001$) (Fig. 3d & e). Both IL-25BP adjuvanted and control groups, showed very low IFN-γ expression by lineage$^-$ ST2/IL-33R$^-$ NKp46$^-$ ILCs (in the context of both cell number/proportion and percentage) (Fig. 3g & h), whist significantly elevated IL-17A ($p < 0.0001$) and reduced IL-22 ($p < 0.001$) expression were detected following FPV-HIV-IL-25BP vaccination (Fig. 3g & h). Interestingly, FPV-HIV-IL-33BP vaccination only showed IFN-γ expression in the ST2/IL-33R$^-$ NKp46$^-$

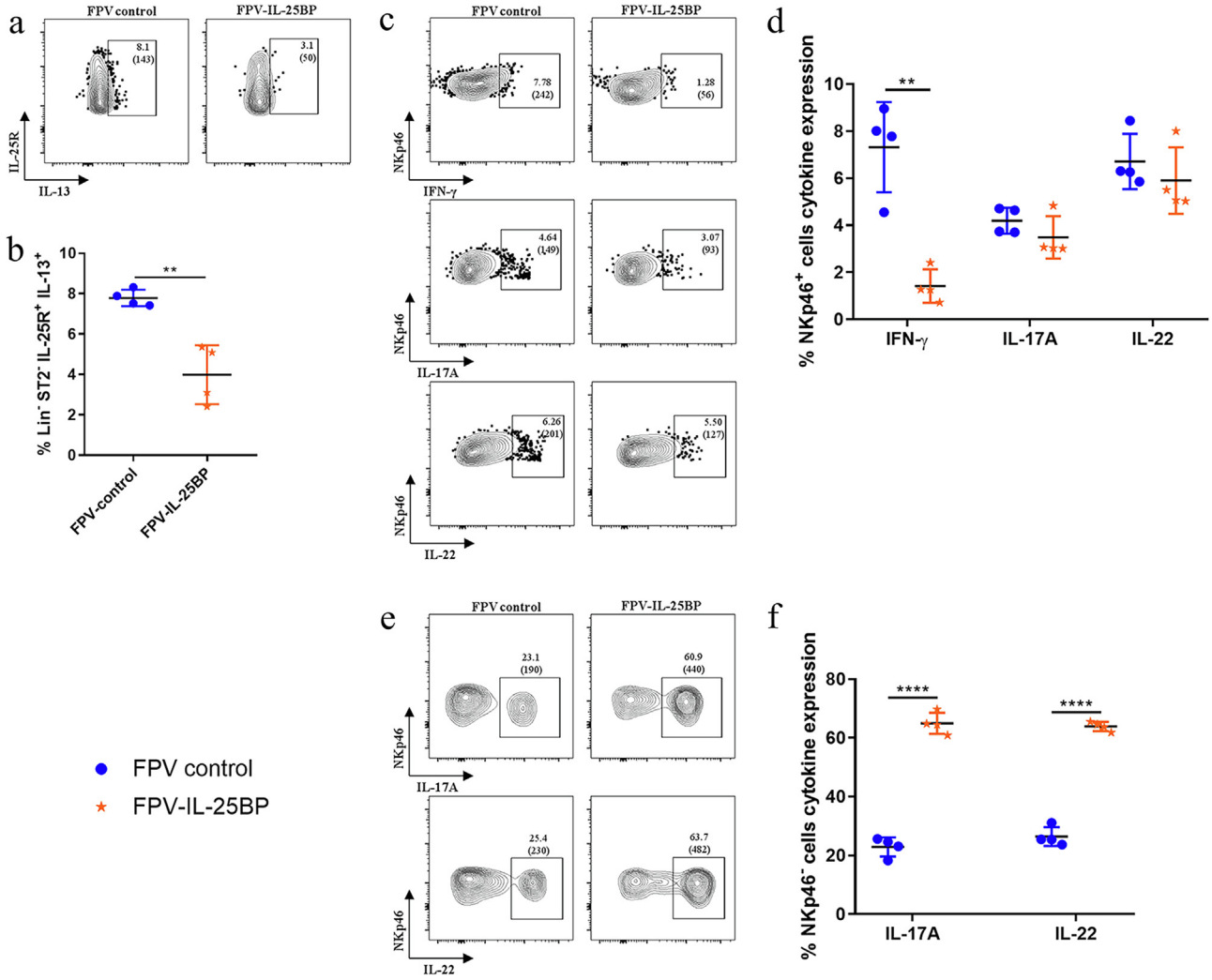


**Fig. 1. Evaluation of muscle ILC subsets in WT BALB/c mice following intramuscular IL-25BP adjuvanted vaccination.** WT BALB/c mice (n = 4) were immunized intramuscularly with unadjuvanted FPV-HIV or FPV-HIV-IL-25BP adjuvanted vaccine. ILC2s were identified as CD45$^+$ FSC$^{low}$ SSC$^{low}$ Lin$^-$ ST2/IL-33R$^-$ IL-25R$^+$ cells, and their IL-13 expression was evaluated. The FACS plots and graph indicated percentage of IL-13 producing ILC2 in muscle at 24 h post vaccination, the number of cells in each quadrant is indicated within brackets below the cell percentage (a & b). ILC1 and ILC3 were divided into CD45$^+$ FSC$^{low}$ SSC$^{low}$ Lin$^-$ ST2$^-$ NKp46$^{+/-}$ ILCs, and their IFN-γ, IL-17A, and IL-22 expression were evaluated using intracellular staining 24 h post vaccination. The FACS plots and graph indicate percentage of IFN-γ, IL-17A, and IL-22 producing NKp46$^{+/-}$ ILC in muscle (c–f). The error bars represent the mean and standard deviation (s.d.). The p-values were calculated using GraphPad Prism software (version 6.05 for Windows). * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001. For each group, experiments were repeated minimum three times.

ILC not NKp46$^+$ ILC and no expression of IL-17 or 22 were detected in either of the NKp46 subsets (Fig. 3f & i).

### *2.4. Intranasal FPV-HIV-IL-25BP vaccination induced a novel ST2/IL-33R$^-$ IL-25R$^-$ TSLPR$^-$ ILC2 subset expressing IL-13 and IL-4*

There was an unexpected discovery with FPV-HIV-IL-25BP adjuvanted vaccination where a lineage$^-$ IL-25R$^-$ ST2/IL-33R$^-$ TSLPR$^-$ cell population that not only to expressed IL-13 but also IL-4 were detected (Fig. 4a–c). This ILC2 subset, the IL-4 and IL-13 expression was much greater than the other three known ILC2 subsets ($p < 0.0001$) (Fig. 4d–f) and interestingly, a small subset of these cells were also found to be double positive for both IL-13 and IL-4 (Fig. 4d). These observations suggested that lineage$^-$ IL-25R$^-$ ST2/IL-33R$^-$ TSLPR$^-$ population could contain a novel ILC2 subset. As expected [3], all ILC2 subsets did not express IL-17A or IFN-γ (Fig. 4a–c). In this analysis the lineage$^-$ ST2/IL-33R$^-$ TSLPR$^-$ NKp46$^+$ and NKp46$^-$ cells that produced IL-17A and IFN-γ were most likely classic ILC1 and ILC3, not ILC2 as none of the known ILC2s expressed these two cytokines (Fig. 4d).

## 3. Discussion

Following intranasal FPV-HIV-IL-25BP vaccination, where IL-25 was temporarily sequestered from the vaccination site, significant impact on ILC development/function in lung mucosae were observed, similar to that of muscle following intramuscular vaccination. Specifically, in relation to lung ILC2, not only ST2/IL-33R$^+$ ILC2, but also IL-25R$^+$, TSLPR$^+$ ILC2 and also a unique lineage$^-$ IL-33R/ST2$^-$ IL-25R$^-$ TSLPR$^-$ ILC2 subsets that expressed IL-13 or IL-4 were detected 24 h post vaccination, unlike mice given the unadjuvanted FPV-HIV vaccination. Interestingly, knowing that sequestration of IL-33 (FPV-HIV-IL-33BP adjuvanted vaccine) had no impact on ILC2 development/function in lung, these unexpected finding suggested a hierarchical role of IL-25 in ILC2 development compared to IL-33, specifically in the context of viral-vector vaccination.

Different ILC2 subsets arise from a common progenitor cell and under different cytokine conditions/anatomical location differentiate into ILC2 that are IL-33R$^+$, IL-25R$^+$ or TSLPR$^+$ [31,32]. Previous studies in our laboratory have shown that in naïve mice,

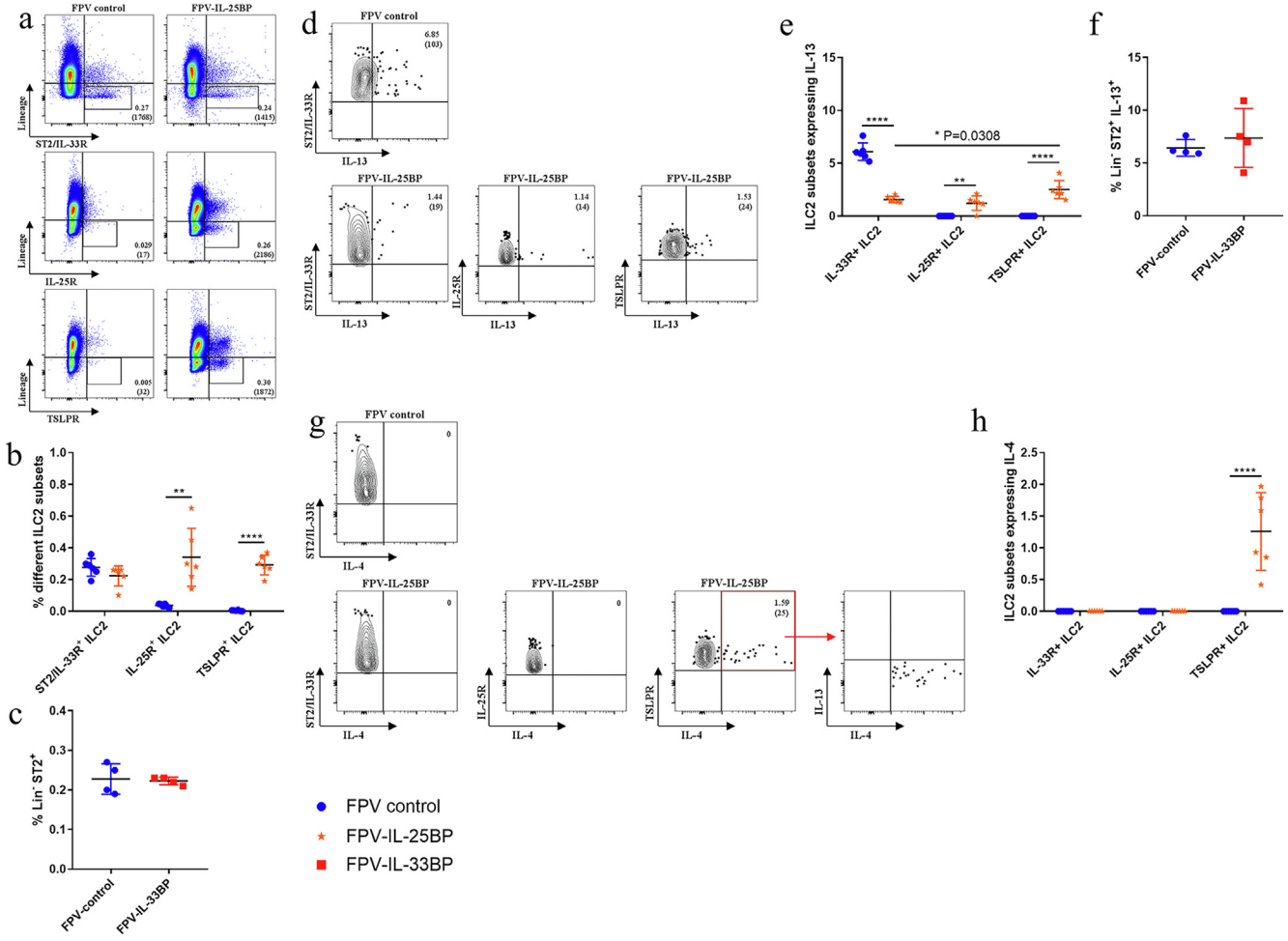


**Fig. 2. Evaluation of lung lineage$^-$ ILC2 subsets and their IL-13 and IL-4 expression following intranasal IL-25BP and IL-33BP adjuvanted vaccination.** WT BALB/c mice (n = 4–6) were immunized intranasally with unadjuvanted FPV-HIV, IL-25BP or IL-33BP adjuvanted vaccines. ILC2s were defined as CD45$^+$ FSC$^{low}$ SSC$^{low}$ Lin$^-$ ST2/IL-33R$^+$, CD45$^+$ FSC$^{low}$ SSC$^{low}$ Lin$^-$ IL-25R$^+$, or CD45$^+$ FSC$^{low}$ SSC$^{low}$ Lin$^-$ TSLPR$^+$ cells. The FACS plots indicate the percentage of different lung ILC2 subsets (lineage$^-$ ST2/IL-33R$^+$, lineage$^-$ IL-25R$^+$, and lineage$^-$ TSLPR$^+$) 24 h post vaccination, the number of cells in each quadrant is indicated within brackets below the cell percentage (a). Graph represents percentages of the different ILC2 subsets following unadjuvanted FPV-HIV, IL-25BP and IL-33BP adjuvanted vaccinations (b & c). The representative FACS plots and graphs indicate the three different ILC2 subsets expressing IL-13 and/or IL-4 (d–h). The error bars represent the mean and standard deviation (s.d.). The p-values were calculated using GraphPad Prism software (version 6.05 for Windows). * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001. For each group experiments were repeated minimum three times.

ST2/IL-33R$^+$ ILC2 are resident in lung unlike IL-25R$^+$ ILC2, which are known to be "inflammatory" ILC [28] and are recruited to the muscle following i.m. vaccination [29]. The current study demonstrated that transient inhibition of IL-25 at the vaccination site can promote some ILC2s at the lung mucosae to express both IL-13 as well as IL-4. Interestingly, the IL-4 expressing ILC2s induced by FPV-HIV-IL-25BP adjuvanted vaccination did not express IL-13, suggesting that these were two distinct ILC2 populations. Recent studies have also shown that addition of IL-25 and IL-33 can promote differential cytokine expression by lung ST2/IL-33R$^+$ ILC2. For example, when lung ST2/IL-33R$^+$ ILC2 were cultured in the presence of IL-25 in vitro, these cells were shown to produce elevated IL-13 but reduced IL-5 whereas the inverse was reported when cells were cultured in the presence of IL-33 [33,34]. Furthermore, *Chen et al* using IL-13-GFP reporter mice have also shown that compared to IL-33, intranasal administration of recombinant IL-25 can induce elevated IL-13 expression by lung ILC2 [33]. Interestingly, the current study demonstrated that removal of IL-25 from the cell milieu can have profound impact on ILC2 differentiation (induction of numerous ILC2 subsets) including IL-13 and IL-4 expression, unlike overexpression of IL-25. Thus, these observations further supported the notion that IL-25 plays a dominant role in ILC2 development in lung compared to IL-33.

Our previous studies have shown that mucosal vaccination induced high avidity CD8$^+$ T cells and this was associated with low level of IL-13 expressed by T cells [35]. In contrast, systemic vaccination induced low avidity CD8$^+$ T cells associated with elevated level of IL-13 [35]. Transient inhibition of IL-4 and IL-13 at the vaccination site has shown to induce (i) T cells of high avidity [36] and (ii) unlike IL-13Rα2 adjuvanted vaccination IL-4R antagonist vaccination has also shown to induce excellent antibody differentiation [36,37], suggesting IL-13 plays an important role in modulating both T and B cell immunity [38,39]. These studies also showed that level of IL-4/IL-13 at the vaccination site can significantly alter the activity of antigen presenting cells [40]. When trying to dissect which cells at the vaccination site produced IL-13, our previous studies have clearly established that ILC2 were the major source of IL-13 at the vaccination site 24 h post viral vector vaccination [29]. Interestingly, low IL-4/IL-13 levels have been associated with recruitment of CD11b$^+$ CD103$^-$ conventional DCs, and induction of high avidity HIV-specific CD8$^+$ T cells [40]. Intranasal FPV-HIV-IL-25BP adjuvanted vaccination induced significantly elevated IL-4 and IL-13 expression by ILC2 at the lung mucosae 24 h post vaccination (Table 1). Taken together our previous findings, the current data suggest that sequestration of IL-25 at the lung mucosae may be detrimental for the induction of high

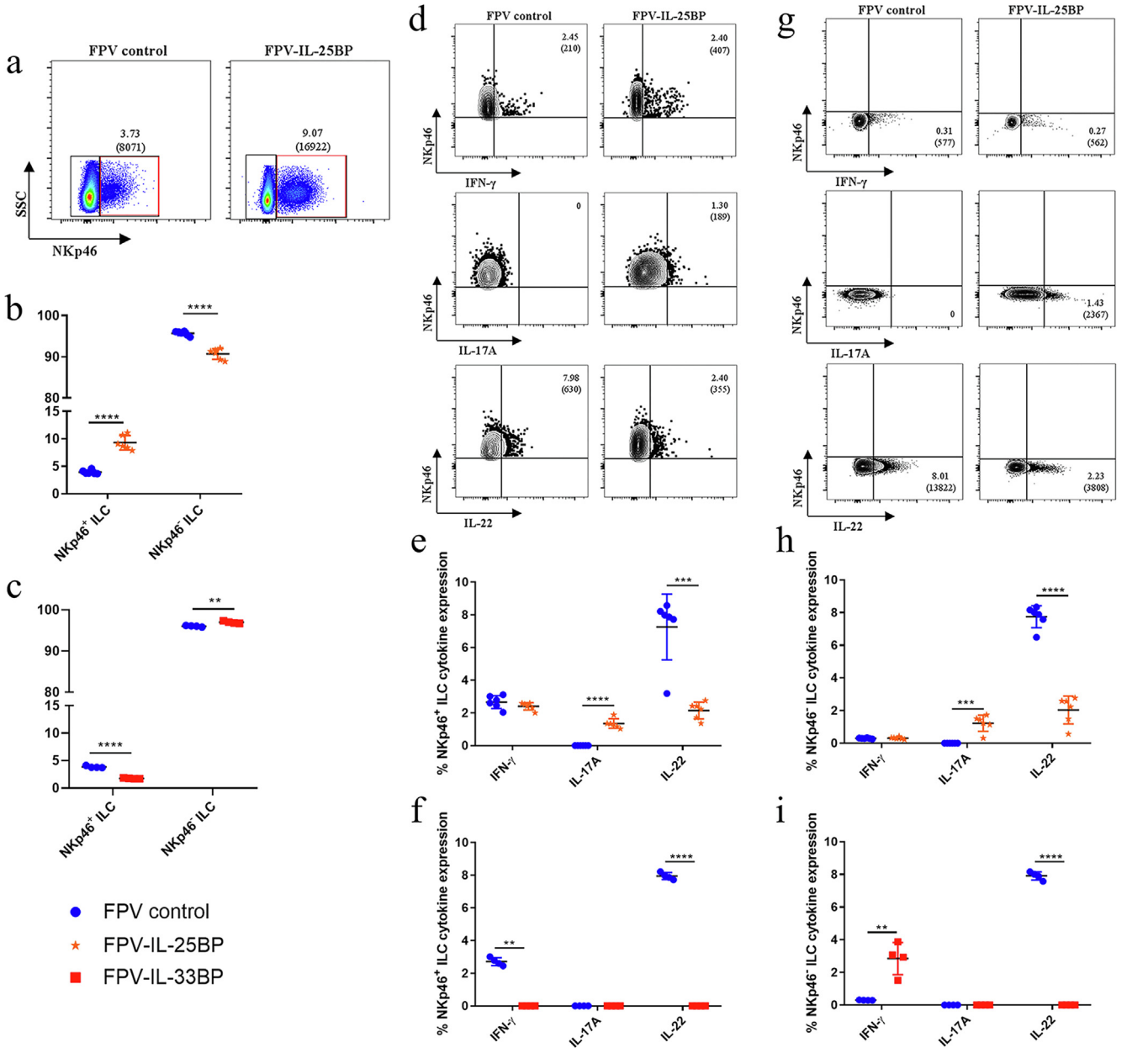


**Fig. 3. Evaluation of IFN-γ, IL-22 and IL-17A expression by IILC1/ILC3 following intranasal IL-25BP and IL-33BP adjuvanted vaccination.** WT BALB/c mice (n = 4–6) were immunized intranasally with unadjuvanted FPV-HIV vaccine, IL-25BP or IL-33BP adjuvanted FPV vaccine. ILC1/ILC3 were gated as CD45$^+$ FSC$^{low}$ SSC$^{low}$ Lin$^-$ ST2$^-$ NKp46$^{+/-}$. The representative FACS plots indicate the lineage$^-$ ST2/IL-33R$^-$ NKp46$^{+/-}$ ILC for each vaccine group 24 h post vaccination (a). The graph represents percentage of lineage$^-$ ST2/IL-33R$^-$ NKp46$^+$ and ST2/IL-33R$^-$ NKp46$^-$ ILC1/ILC3 following FPV-HIV, FPV-HIV-IL-25BP, or FPV-HIV-IL-33BP vaccination (b & c) Representative FACS plots and the graphs indicate the expression of IFN-γ, IL-22 and IL-17A by the above cell subsets (d–i). The error bars represent the mean and standard deviation (s.d.). The p-values were calculated using GraphPad Prism software (version 6.05 for Windows). * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001. For each group experiments were repeated minimum three times.

avidity T cells. However, intramuscular FPV-HIV-IL-25BP vaccination significantly inhibited both IL-4 and IL-13 expression by ILC2 in muscle (Table 1), suggesting that IL-25BP could be a highly efficacious intramuscular adjuvant than an intranasal adjuvant.

Interestingly, FPV-HIV-IL-25BP adjuvanted vaccination not only manipulated the ILC2 differentiation/function but also ILC1 and ILC3 at the vaccination site. This was highly unexpected as cytokine IL-25 was thought to be an activator of ILC2 but not ILC1/ILC3 [31]. Surprisingly, compared to the unadjuvanted rFPV vaccination, transient removal of IL-25 at the muscle and lung mucosae induced elevated expression of IL-17A by lineage$^-$ ST2/IL-33R$^-$ NKp46$^{+/-}$ ILC1 and ILC3 (Table 1). Ravichandran *et al.* have previously demonstrated that IL-4 and IL-13 can differentially regulate IL-17A in antigen-specific CD8$^+$ T cells [41]. As FPV-HIV-IL-25BP vaccination also significantly manipulated IL-4 and IL-13 expression by the different ILC2 subsets (ST2/IL-33R$^+$, IL-25R$^+$, TSLPR$^+$, and the unique lineage$^-$ ST2/IL-33R$^-$ IL-25R$^-$ TSLPR$^-$ ILC2 subsets), we postulate that the significant changes in IL-17A production in ILC1/ILC3 could be associated with the high level of IL-4/IL-13 expressed by the different ILC2 subsets, including the novel lineage$^-$ ST2/IL-33R$^-$ IL-25R$^-$ TSLPR$^-$ ILC2 subset (Table 1). Moreover, IL-25 (IL-17E), signals via the IL-25 receptor complex IL-17RA/IL-17RB, and IL-17RA/ IL-17RC is the major receptor complex associated with IL-17A signaling [42,43]. Therefore, following IL-25BP

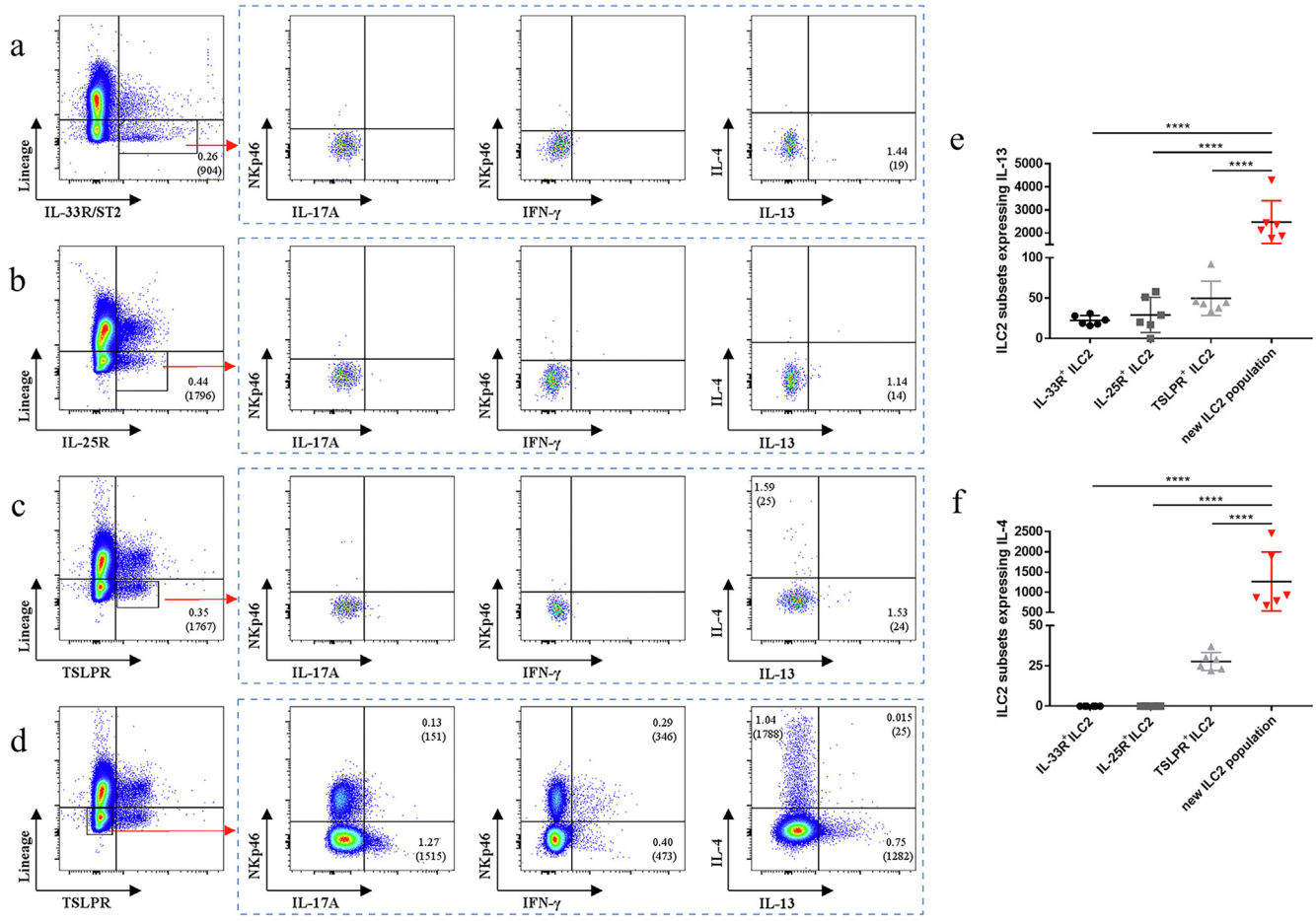


**Fig. 4. Evaluation IL-13 and IL-4 expression in the novel ST2/IL-33R⁻ IL-25⁻ TSLPR⁻ population following intranasal IL-25BP adjuvanted vaccination.** WT BALB/c mice (n = 6) were immunized intranasally with IL-25BP adjuvanted FPV vaccine. Lineage⁻ ST2/IL-33R⁺, lineage⁻ ST2/IL-33R⁻ IL-25R⁺, lineage⁻ ST2/IL-33R⁻ TSLPR⁺ and lineage⁻ ST2/IL-33R⁻ TSLPR⁻ cells were gated as per described in Fig. S2. The IL-13, IL-4 and also IL-17A and IFN-γ expression in these cells were evaluated using intracellular cytokine staining. The representative FACS plots indicate the expression of the different cytokines by lineage⁻ ST2/IL-33R⁺ (a), lineage⁻ ST2/IL-33R⁻ IL-25R⁺ (b), lineage⁻ ST2/IL-33R⁻ TSLPR⁺ (c), and lineage⁻ ST2/IL-33R⁻ TSLPR⁻ cells (d). The graph represents the number of cells expressing IL-13 (e) and IL-4 (f) by the different ILC2 subsets, (note that these cell numbers were back calculated to CD45⁺ population as described in materials and methods). The error bars represent the mean and standard deviation (s.d.). The p-values were calculated using GraphPad Prism software (version 6.05 for Windows). * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001. For each group experiments were repeated minimum three times.

**Table 1**
Summary of ILC-derived cytokine expression profiles 24 h post rFPV vaccination.

| | | ILC2 | | | | | | ILC1/ILC3 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | IL-33R/ST2⁺ IL-13 | IL-25R⁺ IL-13 | TSLPR⁺ IL-13 | TSLPR⁺ IL-4 | New subset IL-13 | New subset IL-4 | NKp46⁺ | | | NKp46⁻ | | |
| | | | | | | | | IFN-γ | IL-17A | IL-22 | IFN-γ | IL-17A | IL-22 |
| IM | Control | – | ** | – | – | – | – | ** | * | ** | – | *** | *** |
| | IL-25BP | – | * | – | – | – | – | * | * | ** | – | **** | **** |
| IN | Control | ** | – | – | – | – | – | * | – | ** | * | – | ** |
| | IL-25BP | * | * | * | * | **** | **** | * | * | * | * | * | * |
| | IL-33BP | ** | – | – | – | – | – | – | – | – | * | – | – |

(–) Not expressing, * < 5%, ** 5% – 10%, *** 10% – 60%, **** > 60% or > 1000 cells calculated as indicated in Materials and Methods.
Control = FPV-HIV (unadjuvanted); IL-25BP = FPV-HIV-IL-25BP, IL-33BP = FPV-HIV-IL-33BP (adjuvanted).

vaccination the elevated IL-17A expressed by ILC1/ILC3 could also be related to the balance of IL-17A and IL-25 signaling. For example: sequestration of IL-25 initiating the IL-17A signaling via NF-κB, Activator Protein 1 (AP-1) and CCAAT-enhancer-binding-protein (C/EBP) pathways. This could be similar to what has been observed during transient inhibition of IL-13, in which IL-4 signaling via the STAT6 pathway was initiated [29,36,37]. The level of IL-13 expressed by ILC2 was also shown to alter the expression of IFN-γ by ILC1 and ILC3 [29]. Specifically, transient blockage of IL-4/IL-13 signaling via STAT6 pathway, compared to transient inhibition of IL-13 at the vaccination site differentially regulated IL-13 expression by ILC2 and IFN-γ expression by ILC1/ILC3. These findings support the notion that in the context of viral vector vaccination, IL-13 produced by ILC2 could be the master regulator of ILC1 and ILC3 activity, specifically the IFN-γ, IL-17 and also IL-22 expression by these cells 24 h post vaccination (Table 1).

Although following intranasal FPV-HIV-IL-33BP vaccination, no significant differences in the lung ILC2 subsets or their IL-13

production were observed, surprisingly, sequestration of IL-33 impacted the IFN-γ expression by NKp46$^-$ ILC1/ILC3, but not IL-17A or IL-22 (Table 1). These observations indicated that apart from maintaining a balance between IL and 13 and IFN-γ activity, cytokines such as IL-33 which is critical for ILC2 development may also have an significant impact on the downstream ILC1/ILC3 differentiation/function [44,45]. Recent studies have also shown that under IL-1 family cytokine stimulation, ILC2 can become more like ILC1, that produce IFN-γ [26,27]. Knowing that IL-33 is an IL-1 family cytokine, current data suggest that sequestration of IL-33 could have impacted the IL-1 cytokine balance resulting in the high IFN-γ expression by ST2/IL-33R$^-$ NKp46$^-$ ILC.

This study clearly established that manipulating IL-25 at the lung mucosae can not only have dramatic impact on ILC2 and their IL-13/IL-4 expression, but also have significant effects on ILC1/ILC3 and their IFN-γ/IL-22/IL-17A production (Table 1). Consistent with our findings, several studies, have shown that IL-25 and IL-33 may have different impacts on ILC development and function [14,15,46]. Stier et al. have shown that IL-33 plays a crucial role in promoting ILC2 egress from the bone marrow [47]. Hence, we propose that IL-33 may be critical for ILC homing and trafficking to tissue, whereas IL-25 may be important for initial ILC development and function. Following IL-25 sequestration, knowing the high plasticity of ILC, the lineage$^-$ ST2/IL-33R$^-$ IL-25R$^-$ TSLPR$^-$ ILC2 population observed, could be an undifferentiated ILC2 subset (i.e. similar to CD4$^-$ CD8$^-$ T cell development) or a not yet defined, novel ILC2 subset [48]. In conclusion, current findings further substantiate that the adjuvants used and the route of delivery play an important role in modulating ILC activity and ILC-driven cytokine expression at the vaccinations site, and these early events need to be seriously taken into consideration when designing effective vaccines against chronic pathogens.

## 4. Methods

### 4.1. Mice

5–7-week-old pathogen free female wild type (WT) BALB/c mice were obtained from the Australian Phenomics Facility, the Australian National University. All animals were maintained, and experiments were performed in accordance with 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 Australian National University Animal Experimentation and Ethics Committee (AEEC). This study was approved by the AEEC and listed under ANU ethics protocol numbers A2014/14 and A2017/15.

### 4.2. Immunization and preparation of lung and muscle lymphocytes

In this study, $1 \times 10^7$ PFU unadjuvanted FPV-HIV, FPV-HIV-IL-33BP or FPV-HIV-IL-25BP adjuvanted vaccines were administered to WT BALB/c mice (n = 6 per group) i.n. or i.m. under mild isoflurane anaesthesia. FPV-HIV-IL-33BP vaccine co-expressed HIV antigens together with an IL-33 binding protein (the extracellular binding domain of IL-33R/IL-1RL1) that can sequester IL-33 temporarily at the vaccination site, and FPV-HIV-IL-25BP vaccine co-expressed HIV antigens together with an IL-25 binding protein (the extracellular binding domain of IL-17RB) that can sequester IL-25 temporarily at the vaccination site. Both IL-33BP and IL-25BP DNA sequences were custom synthesized by Genscript. The i.n. vaccines were given 10–15 μl per nostril (total 25–30 μl volume) and i.m. vaccines, 50 μl per quadriceps muscle as per described previously [29,36,37]. Vaccines were diluted in sterile PBS and sonicated 3 times for 15 s at 50 output using a Branson Sonifier 450 on ice just prior to administration. Lungs or quadriceps muscle tissues were harvested in 2 ml of complete RPMI 24 h post immunization, and single cell suspensions were prepared as per described previously [29]. Briefly, lungs 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 mashed and passed through a falcon cell strainer and resulting lung cell suspensions were then lysed with RBCs, washed and once again passed through gauze to remove debris. Quadriceps muscle tissues were also cut into small pieces and digested with 0.5 mg/ml collagenase, 2.4 mg/ml Dispase, 5 Units/ml DNase and complete RPMI for 30 min at 37 °C, passed through a falcon cell strainer (without mashing to avoid creating smaller debris) and gauze to remove debris similar to lung. The cells were then suspended in complete RPMI and rested overnight at 37 °C with 5% $CO_2$ as per our previous studies [29,36]. All cells were treated with 1% Brefeldin A for 5 h prior to staining and analysing using multi-colour flow cytometry.

### 4.3. Flow cytometry

ST2/IL-33R$^+$ and IL-25R$^+$ ILC2 staining: APC/Cy7-conjugated anti-mouse CD45 (clone 30-F11), and FITC-conjugated anti-mouse CD3 (clone 17A2), CD19 (clone 6D5), CD11b (clone M1/70), CD11c (clone N418), CD49b (clone HMα2), FcεRIα (clone MAR-1) (all linage positive markers were selected as FITC) were used to identify the lineage$^-$ cells. PE-conjugated anti-mouse ST2/IL-33R (clone DIH9), and APC-conjugated anti-mouse IL-25R (clone 9B10) were used to identify the different ILC2 subsets. Brilliant Violet 421-conjugated anti-mouse IL-4 (clone 11B11) and PE-eFlour 610-conjugated anti-mouse IL-13 (clone eBio13A) were used to evaluate intracellular expression of these cytokines in ILC2. The gating strategy indicated in Fig. S1, S2 & S4 was used to identify the different ILC2 subsets and their cytokine expression. Similar to our previous studies, single color and FMO controls were used to set up the respective gates [29].

ST2/IL-33R$^+$ & TSLPR$^+$ ILC2 staining: APC/Cy7-conjugated anti-mouse CD45, and FITC-conjugated lineage cocktail were used to identify the lineage$^-$ cells as per indicated above. PerCP/Cy5.5-conjugated anti-mouse ST2/IL-33R (clone DIH9), and APC-conjugated anti-mouse TSLPR (clone FAB5461A) were used to identify different ILC2 subsets. (In this staining cocktail, since the TSLPR antibody was found to interact with PE-conjugated anti-mouse ST2/IL-33R antibody, PerCP/Cy5.5-conjugated anti-mouse ST2/IL-33R was used). To avoid spectral overlap, Brilliant Violet 421-conjugated anti-mouse IL-4 and PE-conjugated anti-mouse IL-13 (clone eBio13A) were used to evaluate the intracellular cytokine expression in ILC2, as per indicated in Fig. S1, S2 & S4.

ILC1 and ILC3 staining: APC/Cy7-conjugated anti-mouse CD45, and FITC-conjugated lineage cocktail were used to identify lineage$^-$ cells. PE-conjugated anti-mouse ST2/IL-33R, and Brilliant Violet 421-conjugated anti-mouse CD335 (NKp46) (clone 29A1.4) were used to identify the ILC1 and ILC3 populations. Brilliant Violet 510-conjugated anti-mouse IFN-γ (clone XMG1.2), APC-conjugated anti-mouse IL-22 (clone Poly5164), and Alexa Fluor 700-conjugated anti-mouse IL-17A (clone TC11-18H10.1) were used to evaluate the intracellular cytokine expression in ILC1 and ILC3 subsets as per indicated in Fig. S1, S2 & S4.

### 4.4. Statistical analysis

In this study, cell numbers were calculated using the formula (cytokine expressing cells/number of CD45$^+$ cells) $\times 10^6$. The graphs represent the mean and standard deviation (s.d.). The p-values were calculated using GraphPad Prism software (version

6.05 for Windows). One-way ANOVA or two-way ANOVA using Tukey's multiple comparisons test were used to calculate statistical significance. The P-values were denoted as follows: ns – $p \geq 0.05$, * – $p < 0.05$, ** – $p < 0.01$. *** – $p < 0.001$, **** – $p < 0.0001$. In this study, n = 4–6 mice per group were used and all experiments were repeated at least three times.

## 5. Data availability statement

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

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## Author contributions

Z.L. conducted all the i.n. and i.m. experiments, data analysis and prepared the manuscript, R.J.J. designed/constructed the IL-25BP and IL-33BP adjuvanted vaccines and, C.R. helped design all the experiments and critical evaluation and preparation of the manuscript.

## Declaration of Competing Interest

The authors have no conflicts of interests.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jvacx.2019.100035.

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