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

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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-17A. 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]. ILC3s have shown to down regulate 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 (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].
[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 mouse 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 ILC2 have also shown to develop into ST2/IL-33R+ ILC2 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-derived 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-HPV vaccine and the FPV-HPV-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-HPV-IL-25BP vaccinated 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-HPV-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-HPV-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-HPV-33BP, and unadjuvanted control and lung ILC subsets and their cytokine expression were evaluated at 24 h post vaccination. ILC2 subsets were gated as described previously [29] (Fig. S2). Interestingly, transient sequestration of IL-33 at the lung mucosa did not alter the ILC2 profiles compared to the control vaccination (Fig. 2c & f). Hence, next mice were vaccinated intranasally (i.n.) with FPV-HPV-25BP, which transiently sequestered IL-25 at the lung mucosa. 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-HPV-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-HPV-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-HPV-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-HPV-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-HPV-33BP vaccination (Fig. 3c). Next when IFN-γ expression was assessed, significantly elevated numbers of FPV-HPV-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-HPV-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 < 0.0001) (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), whilst significantly elevated IL-17A (p < 0.0001) and reduced IL-22 (p < 0.001) expression were detected following FPV-HPV-IL-25BP vaccination (Fig. 3g & h). Interestingly, FPV-HPV-IL-33BP vaccination only showed IFN-γ expression in the ST2/IL-33R– Nkp46-
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 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$^+$/IL-25R$^+$/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 mucosa was 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,
ST2/IL-33R+ ILC2 are resident in lung unlike IL-25R+ ILC2, which are known to be "inflammatory" ILC2 and are recruited to the muscle following i.m. vaccination. 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. Furthermore, Chen et al using IL-13-GFP reporter mice have also shown that compared to IL-25, intranasal administration of recombinant IL-25 can induce elevated IL-13 expression by lung 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.

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. In contrast, systemic vaccination induced low avidity CD8+ T cells associated with elevated level of IL-13. Transient inhibition of IL-4 and IL-13 at the vaccination site has shown to induce (i) T cells of high avidity and (ii) unlike IL-13Rα antagonist vaccination has also shown to induce excellent antibody differentiation, suggesting IL-13 plays an important role in modulating both T and B cell immunity. These studies also showed that level of IL-4/IL-13 at the vaccination site can significantly alter the activity of antigen presenting cells. 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. 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. Intra-nasal 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

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**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+ FSClow SSClow Lin−ST2/IL-33R−, CD45+ FSClow SSClow Lin−IL-25R−, or CD45+ FSClow SSClow 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. 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.
avidity T cells. However, intramuscular FPV-HIV-IL-25BP vaccina-
tion significantly inhibited both IL-4 and IL-13 expression by ILC2 in muscle (Table 1), suggesting that IL-25BP could be a highly effi-
cacious 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 cyto-
kine IL-25 was thought to be an activator of ILC2 but not ILC1/
ILC3 [31]. Ravichandran et al. have previ-
ously demonstrated that IL-4 and IL-13 can differentially regulate
IL-17A in antigen-specific CD8+ T cells [41]. As FPV-HIV-IL-25BP vaccina-
tion 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 lin-
eage ST2/IL-33R+ IL-25R+ TSLPR+ ILC2 subset (Table 1).

Fig. 3. Evaluation of IFN-γ, IL-22 and IL-17A expression by ILC1/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$^{+/-}$.
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 NFκB, 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

Table 1

| IL-25R/ST2 | IL-25R | TSLPR | TSLPR | New subset | New subset |
|-----------|--------|-------|-------|------------|------------|
| ILC2      | IL-13  | IL-13 | IL-13 | IL-13      | IL-13      |
| IM Control | ** - | - | - | - | - |
| IL-25BP   | - | - | - | - | - |
| IN Control | - | - | - | - | - |
| IL-25BP   | * | * | * | * | * |
| IL-33BP   | * | * | * | * | * |

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).
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 a 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 ILC 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 × 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 temporally 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-17R) that can sequester IL-25 temporally 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 washed 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 rinsing 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% CO2 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), CD40b (clone HM52), FcRγI (clone MAR-1) (all lineage positive markers were selected as FITC) were used to identify the lineage− cells. PE-conjugated anti-mouse ST2/IL-33R−/IL-25R− (clone D1H9), 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 D1H9), 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 TC11-18H110.1) 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) × 10^6. The p-values were calculated using GraphPad Prism software (version
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.jvacc.2019.100035.

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