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Interleukin 17 (IL-17) manipulates mouse bone marrow- derived neutrophils in response to acute lung inflammation

Phongsakorn Chuammitri[a,c,*], Kanruethai Wongsawan[a], Kidsadagon Pringproa[a,c], Roongroje Thanawongnuwech[b]

[a] Department of Veterinary Biosciences and Public Health, Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai, Thailand
[b] Department of Pathology, Faculty of Veterinary Sciences, Chulalongkorn University, Bangkok, Thailand
[c] Excellent Center in Veterinary Biosciences (ECVB), Chiang Mai University, Chiang Mai, Thailand

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ABSTRACT

Interleukin 17 (IL-17) mediates neutrophil migration to the lungs during acute inflammation, potentially leading to lung tissue damage. In the present study, we evaluated whether IL-17 could facilitate certain neutrophil functions in a mouse model. Mice were divided into four groups and intranasally challenged with PBS (1 = Control), Influenza A (H1N1) and *Klebsiella pneumoniae* (2 = Mix), Influenza A alone (3 = Flu), or *K. pneumoniae* (4 = KP) alone. Bone marrow, BAL cells, and lung specimens were collected seven days post-challenge for analysis. Mice in the Flu group showed the highest mortality rate. Neutrophils were the prominent cell type in BAL from Mix and KP, whereas lymphocytes were most numerous in Flu. Lesions in the lungs revealed considerably damage in the Mix, Flu, and KP groups. Isolated bone marrow-derived neutrophils were *in vitro* primed with mouse recombinant IL-17A protein (*r*IL-17A) followed by various functional assays. The reactive oxygen species (ROS) levels in *r*IL-17A primed cells showed significant elevations in all groups. Phagocytosis and bacterial destruction showed no significant difference between (+) or (−) *r*IL-17A groups. The formation of neutrophil extracellular traps (NETs) in *r*IL-17A-primed neutrophils showed elevated NET production. We next monitored expressions of genes in neutrophils. IL-17A mRNA expression was significantly increased in Mix and Flu; IL-1β mRNA only significantly increased in Flu, and IL-17RA showed constitutive expressions in all groups. In summary, neutrophils may cause tissue damage during lung inflammation through specific functions influenced by IL-17.

1. Introduction

The outbreak and transmission of influenza viruses among humans and animals have recently drawn a great deal of public concern. Acute Respiratory Distress Syndrome (ARDS) and Acute Lung Injury (ALI) are regularly observed in highly pathogenic influenza infections with severe secondary bacterial sepsis, such as in *Streptococcus pneumoniae* and *Klebsiella pneumoniae* [1,2]. Animal models have suggested a correlation between neutrophils, ALI, and neutrophil-dependent lung injury.

The influenza A virus (IAV) infection, with or without secondary complications, stimulates cellular and humoral immune responses in the lungs. Presence of the virus triggers either robust innate immunity or complex adaptive immune responses in pursuit of virus clearance [3,4]. The recruitment and activation of circulating neutrophils to infected lungs are influenced by a cytokine of paramount importance, IL-17, produced by a slate of IL-17-producing immune cells [5–7].

Interleukin-17 (IL-17) is an endogenous cytokine that attracts the accumulation of neutrophils in bronchoalveolar tissues and the lung parenchyma [8,9]; the mechanisms that underpin this process, however, are not well-established. Data concerning whether IL-17 is primarily implicated in neutrophil function remains elusive. Th17 lineage cells are critical to fighting extracellular infections *via* immune effector Module 3 [10] wherein the production of IL-17 cytokines is associated with inflammation in the fight against bacteria and fungi [5,8,11]. Many immune cells are capable of producing IL-17, including neutrophils [6,7,12]. The peripheral blood neutrophil lineage is thought to be one of the primary IL-17-secreting subsets of cells, although this idea is not without controversy [7,13].

IL-17 molecules are synthesized *de novo* upon Toll-like receptor 2 and 4 (TLR2 and TLR4) stimulation [5,9]. IL-17 acts on cell types such

* Corresponding author at: Department of Veterinary Biosciences and Public Health, Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai 50100, Thailand.
E-mail addresses: phongsakorn.c@cmu.ac.th, phongsakorn@gmail.com (P. Chuammitri).

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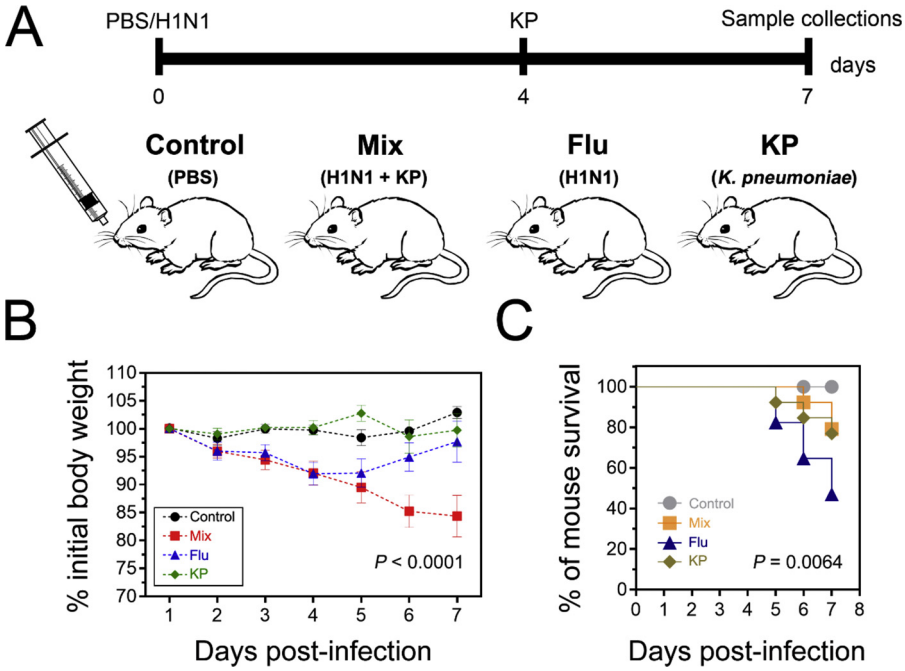


Fig. 1. Model of acute lung inflammation in mouse-adapted swine H1N1 influenza virus. **(A)** Schematic of mouse challenge experiments. Mice were divided into 4 groups and intranasally (*i.n.*) challenged with PBS (Control), swine influenza H1N1 virus plus *K. pneumoniae* (Mix), swine influenza H1N1virus (Flu), or *K. pneumoniae* (KP) at days 1 and 4 of the experiments (amount of virus or bacteria used detailed in *Materials and Methods*). **(B)** Percentage daily changes in body weight, data are mean ± SE of 3 independent experiments (n = 9–15 mice/group), two-factor ANOVA. (C) Survival curves from infection; results are inclusive of three experiments (n = 9–15 mice/group) by Log-Rank test.

as airway epithelial cells and myeloid cells including neutrophils. These cells likely possess IL-17 receptors (IL-17R) [7,14]. The fact that neutrophil activation may positively correlate with the presence of IL-17, however, has been proposed [15], as well as that activated neutrophils could perform effector functions, such as ROS generation, phagocytosis, and degranulation. A recently discovered extracellular mechanism, the production of neutrophil extracellular traps (NETs), has also been implicated by a release of DNA, histones, granule enzymes, and other toxic substances into the extracellular milieu. This extracellular process could contribute to the severity of the damage incurred by IAV-infected lungs [1,16].

To our knowledge, information regarding *ex vivo* stimulation of neutrophils by IL-17 and tests of specific neutrophil effector functions have yet to be comprehensively reported. We aimed first to establish and validate viral titer endpoints in the influenza virus (IAV-H1N1). We then determined the ability of recombinant mouse IL-17A protein (rIL-17A) as an exogenous IL-17 source to activate neutrophils in mice challenged with IAV, *K. pneumoniae*, or combinations thereof by examining *in vitro* neutrophil functions.

2. Materials and methods

2.1. Mice

Eight to ten weeks old, pathogen-free BALB/cMlac mice were obtained from the National Laboratory Animal Center, Mahidol University, Nakhon Prathom, Thailand. Mice were housed in specific pathogen-free animal facilities. All experiments were approved by the Institutional Animal Care and Use Committee, Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai, Thailand (FVM-ACUC Approval # S06/2557). All animals received standard husbandry and *ad libitum* food and water. Mice used in the IAV challenge were housed in a BSL-2 laboratory facility at the Faculty of Veterinary Medicine, CMU.

2.2. Virus

The pandemic (H1N1) Influenza virus (A/SW/Thailand/CU-RA29/2009) was used throughout the study [17]. The GenBank accession no. of the virus is CY062297–CY062304. The original virus was provided courtesy of the Veterinary Diagnostic Center, Chulalongkorn University, Thailand.

2.3. Cell culture

Madin–Darby canine kidney (MDCK) cells were propagated and maintained with Dulbecco's Modified Eagle Medium (DMEM, Gibco, Thermo Fisher Scientific, Waltham, MA, U.S.A.) supplemented with 10% v/v fetal bovine serum, 200 IU/ml penicillin G, and 200 µg/ml streptomycin (Gibco).

2.4. Klebsiella pneumoniae and fluorescent staining of Klebsiella pneumoniae

K. pneumoniae (ATCC 27736) were freshly prepared (1×10^8 CFU/ml) in Luria-Bertani broth (LB broth, Caisson Laboratories, North Logan, UT, U.S.A.) before use in the assay. Live bacteria (1×10^8 CFU/ml) were heat-killed and fluorescently stained as previously described [18]. Fluorescently stained *K. pneumoniae* was adjusted to 1×10^7 CFU/ml, and stored at 4 °C until use.

2.5. Titration of pandemic (H1N1) Influenza virus

The $TCID_{50}$, EID_{50}, Viral plaque assays (PFU), and MID_{50} were performed as described in the *Supplemental Materials and Methods section*.

2.6. In vivo challenge studies

Mice were randomly divided into 4 groups with 3 to 5 mice in each group as illustrated in Fig. 1A. Experiments were repeated 3 times with the same settings. At the beginning of the experiment (day 0), mice in group 1 (Control) and group 4 (KP) received 50 microliters (µl) PBS intranasally (*i.n.*); groups 2 (Mix) and 3 (Flu) received 50 µl of 5 × MID_{50} pandemic H1N1 Influenza virus as indicated previously. On day 4 of the experiment, mice from groups 1 and 3 received intranasal inoculation of PBS; mice from groups 2 and 4 were intranasally infected with 50 µl of 10^5 CFU/ml KP. Mice were observed daily for clinical signs of respiratory disease or flu-like signs for 7 days. At day 7 post-initial challenge, all mice were sedated and euthanized by cervical

dislocation. Sample collections of bone marrow cells, bronchoalveolar lavage (BAL), and lung tissues were performed immediately after euthanasia.

2.7. Bone marrow neutrophil isolation

After euthanasia, both ends of the femurs were sterile cut, and bone marrow cells (BM) were flushed as earlier described [19,20]. BM cells were placed on a discontinuous density gradient, at a specific gravity of 1.077 over 1.119 g/ml (Histopaque; Sigma-Aldrich, St. Louis, MO, U.S.A.) and centrifuged. In the final step, cell density was adjusted to approximately one million (1×10^6) cells per ml in RPMI-1640 supplemented with 1% FBS.

2.8. Flow cytometric analysis of surface molecules of neutrophils

After cell isolation, BM-neutrophils (1×10^5 cells in RPMI-1640) were pelleted and stained with 0.25 µg Alexa Fluor 647 anti-mouse Ly-6 G/Ly-6C (Gr-1) Clone RB6-8C5 (BioLegend, San Diego, CA, U.S.A.). Sample analysis and data acquisition were performed on a CyAn ADP Flow Cytometer, and analysis was conducted with Summit Software (Beckman-Coulter) and FlowJo (Tree Star, Ashland, OR, U.S.A.). Mean Fluorescence Intensity (MFI) was then determined.

2.9. Bronchoalveolar lavage (BAL)

Bronchoalveolar lavage fluids were collected from the lungs of euthanized mice by protocols previously described [1]. BAL samples recovered from one mouse were pooled. For differential cell counts, an aliquot of BAL was spun onto microscopic slides and subjected to Dip-Quick staining. Four-hundred cells per mouse were differentially counted at a magnification of 400×.

2.10. Stimulation of the neutrophils with rIL-17A and neutrophil functional assays

At day 7 post-initial challenge, neutrophils (1×10^5 cells) were seeded onto a 96-well flat plate in duplicate [14]. Cells were pre-stimulated with recombinant mouse IL-17A protein (*r*IL-17A, eBiosciences, Thermo Fisher Scientific) at 100 ng/ml (final concentration) or PBS (Control), for 30 min at 37 °C with 5% CO_2 [21]. After pre-stimulation, neutrophils were subjected to functional assays.

2.10.1. Reactive oxygen species (ROS) assay

Pre-stimulated neutrophils were induced to produce ROS with 100 nM PMA (MilliporeSigma, Burlington, MA, U.S.A.) in HBSS with Ca^{2+} and Mg^{2+} (HBSS-CM) for 15 min at 37 °C at 5% CO_2. Subsequently, 20 µM/ml carboxyl-H_2DCF-DA (Life Technologies) was added. Samples were analyzed with a CyAn ADP flow cytometer [18,22].

2.10.2. Phagocytosis

Fluorescently-stained bacteria were opsonized with 10% heat-inactivated normal mouse serum at 37 °C for 20 min. Phagocytosis was assessed *via* flow cytometry by protocols previously described [18,23].

2.10.3. Bacterial killing assay

Mouse neutrophil bacterial killing was evaluated using a formazan metabolic conversion in the MTT colorimetric assay as previously described [18].

2.10.4. Mouse Neutrophil Extracellular Trap (NET) induction, fluorescence measurement of mouse NET release by plate reader, and fluorescence imaging of mouse NETs

*r*IL-17A-stimulated neutrophils (1×10^5 cells) were induced to form NETs with 100 nM PMA in HBSS-CM for 150 min at 37 °C, 5% CO_2. The supernatant (200 µl) was transferred to new plates. Fifty microliter (µl) of fluorescent dye (Hoechst 33342, Life Technologies) at 5 mg/ml was used to stain DNA in the NET structures [24]. Fluorescent measurement of stained NETs-DNA was measured with Synergy™ HT Multi-Detection Microplate Reader (BioTek, Winooski, VT, U.S.A.) using an excitation wavelength of 360 nm and an emission wavelength of 470 nm [19].

Pre-stimulated neutrophils (2.5×10^4 cells) were seeded on circular coverslips with 0.001% poly-L-lysine coating, followed by activation with 100 nM PMA in HBSS-CM for 150 min at 37 °C, 5% CO_2 to allow NET formation and release [25]. The cells were fixed with 4% PFA, and NET structures were fluorescently stained and mounted with ProLong Gold with DAPI (Life Technologies). The NET structures were recorded using Zeiss Axio Scope A1 with Axio Vision Image System (Carl Zeiss, Oberkochen, Germany) at 5× and 10× objectives [26]. DAPI-stained NETs were counted by three blinded observers (five random fields at 5× objective). Cytospin slides were prepared and stained with Dip Quick to confirm the presence of NET structures.

2.11. Histological examination

Lung samples of euthanized mice were aseptically collected at day 7. Tissue sections (5 µm thickness) were used for routine hematoxylin and eosin (H&E) histopathology. The severity of lung damage was scored by three well-trained pathologists in a semi-quantitative blinded manner. Histology scores (0–4) to rate severity of inflammation infiltration were as follows: 0, no inflammation; 1, perivascular cuff, peribronchiolar inflammation, or hemorrhage, accounting for 5–10% of tissue; 2, mild inflammation extending less than 25% of the lung; 3, moderate inflammation covering 25–50% of the lung; 4, severe inflammation involving over one-half of the lungs [27].

2.12. Mouse neutrophil gene expressions by real-time RT-PCR

Bone marrow-isolated neutrophils (2×10^5 cells) from challenge studies were preserved in RNA*later* (Life Technologies) [22]. Total RNA was extracted and purified by NucleoSpin RNA (Macherey-Nagel, Düren Germany). To determine the expression levels of mRNA by real-time RT-PCR of IL-1β (*Il1b*), IL-17A (*Il17a*), IL-17RA (*Il17ra*), and GAPDH (*Gapdh*) as reference gene [19], a Tetro cDNA Synthesis Kit (Bioline) and SensiFAST SYBR Hi-ROX Kit (Bioline, Taunton, MA, U.S.A.) were used as previously reported [19,22].

Primer pairs were designed by Primer3plus using information from GenBank. The sequences were as follows: *Il1b* (NM_008361), forward, 5′– GTGGCAGCTACCTGTGTCTT –3′, reverse, 5′– GGAGCCTGTAGTGC AGTTGT –3′; *Il17a* (NM_010552), forward, 5′–TCTCCACCGCAATGAA GACC–3′, reverse, 5′– CACACCCACCAGCATCTTCT –3′; *Il17ra* (NM_008359), forward, 5′–GTGGATCTGTTGCCCTACGG–3′, reverse, 5′–CCAGCACTTGAGAGAGCACA–3′; *Gapdh* (NM_008084), forward, 5′–TGATGGGTGTGAACCACGAG–3′, reverse 5′– AGTGATGGCATGGACT GTGG–3′. The primers were synthesized by Macrogen, Seoul, Korea.

The real-time RT-PCR assays were performed on an ABI 7300 Real-Time PCR (Applied Biosystems, Thermo Fisher Scientific) with the following cycling conditions: an initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 5 s, and annealing/extension at 60 °C for 30 s. Subsequently, specificity was confirmed by dissociation curve analysis (T_m). The relative expressions of target genes were calculated using the formula $2_T^{-\Delta\Delta C}$ after normalization to *Gapdh* [28]. Amplicon sizes 164, 202, 158, and 152 bp were considered specific products of *Il1b*, *Il17a*, *Il17ra*, and *Gapdh*, respectively.

2.13. Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 (GraphPad Software, San Diego, CA, U.S.A.). A P value < 0.05 was considered statistically significant. Data from three independent experiments were subjected to D'Agostino-Pearson normality tests prior to

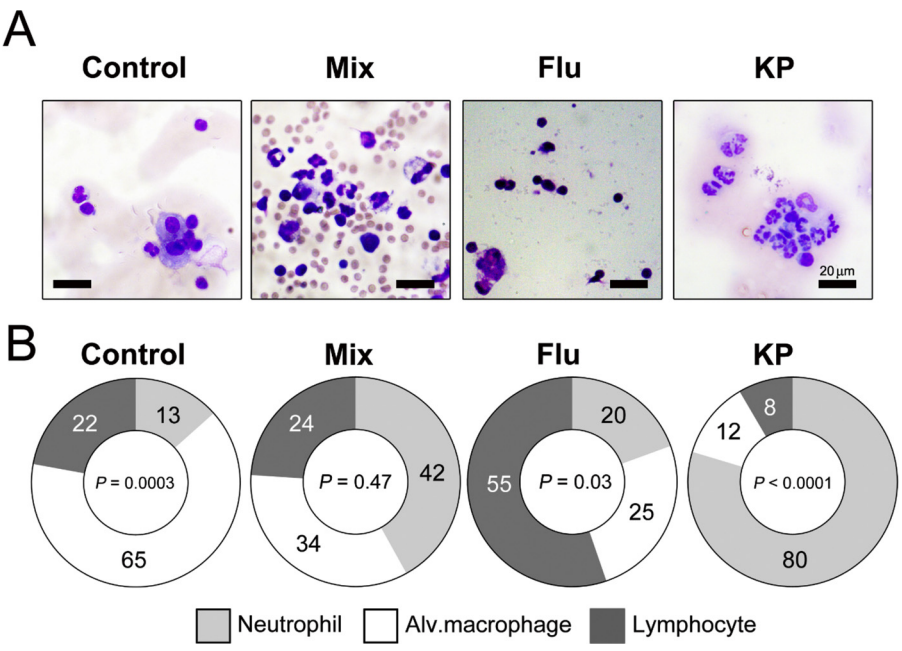


Fig. 2. BAL cell population in response to influenza infection.
(A) Representative cytospin preparations illustrate cell compositions found in BAL from challenged mice at 7 days post-infection, 200× magnification. **(B)** Donut graphs represent differential cell counts of BAL cells. The numbers on the graphs indicate the mean percentage of neutrophils, alveolar macrophages, and lymphocytes from three experiments (n = 9–12 mice/group), one-way ANOVA.

performing statistical analyses. Unpaired t test or Mann-Whitney tests (nonparametric test), one-way ANOVA or Kruskal-Wallis tests (nonparametric test), and two-factor ANOVAs were used followed by multiple comparisons using the Tukey test where indicated. For survival analysis, Log-rank (Mantel-Cox test) for trend was used when comparing survival curves. Most data are presented as mean/median with interquartile range (IQR), or percentage.

To correct for inter-experimental variability in flow cytometry settings and the fluorescent plate reader, MFIs or RFUs were normalized to corresponding samples not treated with *r*IL-17A cells from the same experiment. Absolute MFI or RFU values of corresponding samples in the same experiment were shown. Information obtained from statistical analyses was represented as graphs generated by GraphPad Prism 7.

3. Results

3.1. The A/SW/Thailand /CU-RA29/2009 is a pandemic influenza virus in mice

In order to explore if the pandemic H1N1 influenza virus (A/SW/Thailand /CU-RA29/2009) possesses mouse infectivity, we intranasally infected healthy mice with H1N1 influenza virus and monitored them daily for 10 days. Mice infected with viruses did show flu-like symptoms and also showed clinical signs related to respiratory distress, such as rapid breathing and wheezing, beginning at 5 days post-infection (d.p.i.). Certain mice also manifested various degrees of depression, anorexia, and other non-respiratory signs with lower virus dilutions. Mice that received PBS (Control) showed no clinical signs throughout the challenge studies. The Log_{10} MID_{50} per ml of this specific H1N1 influenza virus was 3.8 as determined by Reed and Muench method. Initial data suggested that the virulence of this virus made it amenable for the *in vivo* challenge experiment.

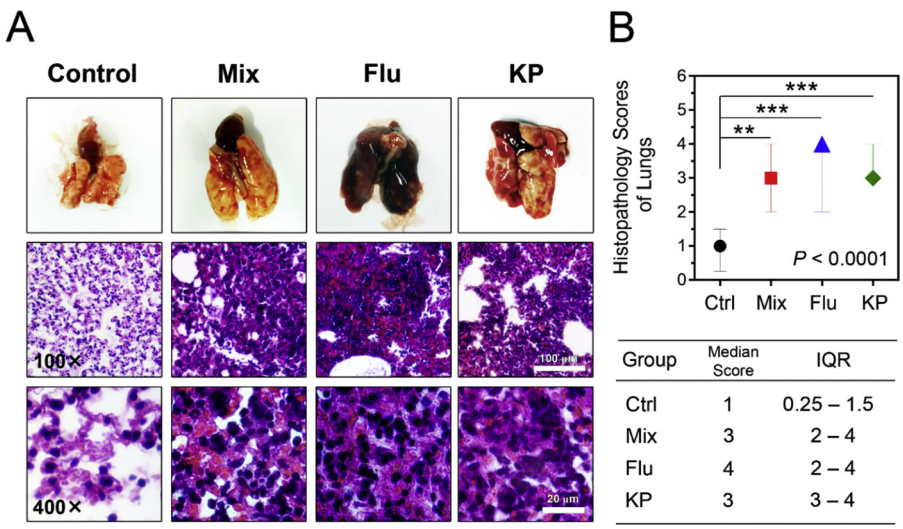
3.2. In vivo establishment of influenza A virus (H1N1) and bacterial infection

The influenza A/SW/Thailand/CU-RA29/2009 was further utilized in mouse experiments. Experimental infection of pandemic H1N1 Influenza A alone (Flu) or in combination with *Klebsiella pneumoniae* (Mix) was associated with decreased average daily body weights, whereas those of mice in the Control and KP groups remained unchanged. Body weight in Flu declined at first, but had recovered almost to starting weight by the end of the experiment (Fig. 1B). Mice in Mix (influenza co-infected with KP) showed significantly decreased body weight, as seen in Fig. 1B (P < 0.0001). We also monitored challenged mice daily throughout the experiment. We found that mice in the Control group were explicitly healthy, in contrast with the mice in the Mix, Flu, and KP groups, which showed flu-like symptoms and clinical signs related to respiratory problems. Certain mice in those groups also had anorexia, inactivity, ruffled fur, and huddling behavior. Percentages of mice survival at the end of the experiment were as follows; 100% (Control), 79.12% (Mix), 47.06% (Flu), and 76.92% (KP) (Fig. 1C, P = 0.0064).

The presence of circulating neutrophils to infected lungs is proposed to play a role during IAV and bacterial infection. The function of IL-17 family cytokine to mediate the clearance of extracellular bacteria, such as *Klebsiella pneumoniae* is one of many important roles of this cytokine. The function of IL-17 derived from innate and adaptive sources (Th17 and other IL-17-producing cells) may attract and stimulate specific neutrophil populations through incorporation of many equipped functions within these cells. We next asked whether the infiltration of neutrophils into the lung during experimental lung infection is an indicator of lung inflammation. Lung histopathology and bronchial lavage (BAL) were further investigated.

3.3. BAL cell populations in response to influenza infection

Apart from the clinical signs, cytology from BAL and histopathologic examination could be carried out for all those who suffered or clinically healthy to reveal of lung lesions. As a consequence of influenza or co-infection of bacteria, microscopic examination provided evidence of the involvement of neutrophils during acute lung inflammation. Bronchial lavage samples (BAL) in all mice from challenge experiments were obtained and used to analyze the cell composition in a clinically meaningful way. Differential cell counts of white blood cells in the BAL of mice varied across experimental groups (Fig. 2A–B). In the Control group, the majority of BAL cells were alveolar macrophages (P = 0.0003). Neutrophils, as well as alveolar macrophages, were equally observed in the Mix group. Lymphocytes were the majority in the Flu challenge group (P = 0.03). In the KP group, a considerable number of neutrophils were found in the BAL (P < 0.0001). Our findings of the numerous neutrophils accumulation in BAL of Mix, Flu, and KP groups



Group	Median Score	IQR
Ctrl	1	0.25 – 1.5
Mix	3	2 – 4
Flu	4	2 – 4
KP	3	3 – 4

Fig. 3. Lung pathology of mice infected with swine pandemic H1N1 influenza virus. Mice were intranasally challenged as described in Fig. 1A. Lung samples of euthanized mice were aseptically collected at day 7 of experiments. **(A)** Representative gross lesions (*top panels*) and H&E stained lung histopathology slides (*middle and bottom panels*) at 7 dpi. Lung lesions of mice in the Mix, Flu, and KP groups showed peribronchiolar inflammation, alveolitis, emphysema, hemorrhage, and infiltration of inflammatory cells depending on the type of infection. **(B)** Median with IQR of histopathology scores of lungs from three independent experiments (n = 9–15 mice/group). $^{**}P < 0.01$, $^{***}P < 0.001$ by Kruskal-Wallis test, followed by Tukey test.

suggest the role of IL-17 in the attraction of neutrophils to bronchoalveolar tissues during IAV infection.

3.4. Mice infected with pandemic H1N1 influenza virus showed severe lung pathology

Histopathological examinations of lung tissues collected from challenged mice showed lesions concomitant with either primary viral infection or complications from secondary bacterial infection (Fig. 3A). In the Control group, lung tissues revealed a clear alveolar space without the infiltration of inflammatory cells. The lungs of mice in the Mix, Flu, and KP groups varied in severity depending upon the type of initiating infection (Fig. 3A). On the basis of the lung scores, many lesions were consistent with perivascular cuffs, peribronchiolar inflammation of inflammatory cells, or hemorrhages (Fig. 3A). When evaluating the lung scores, we noticed that vast amounts of streaming neutrophils were evident in all challenge groups except for Control. The median lung scores in challenged groups were 3 to 4 with 4 being the highest possible score. Statistical analysis confirmed that the lung lesions were significantly different from Control, $P < 0.0001$, (Fig. 3B).

In light of IAV infection results described above, the findings in Mix, Flu, and KP groups of lung histology and BAL altogether imply the role of IL-17 in the attraction of neutrophils to bronchoalveolar tissues during IAV infection. The fact that IL-17 can facilitate and promote cooperative functions of neutrophils is largely unknown. We asked the question whether supplementation of *r*IL-17A could enhance neutrophil functions. Next, we have explored the *in vitro* function of neutrophils supplemented with *r*IL-17 using freshly isolated neutrophils. The following functions: reactive oxygen species (ROS), phagocytosis, bacterial killing, and Neutrophil Extracellular Trap (NET) were reported in detail.

3.5. Neutrophil functional assays

As seen from the previous section, we have shown the successful establishment of IAV infection in mice. We next aimed to evaluate isolated neutrophils from mice with different infected groups may elicit different responses when supplemented with *r*IL-17. The features of *r*IL-17 will draw general conclusions about its performance. The surface Gr-1 antigen of bone marrow-isolated cells indicated that the majority of the positively stained cell population was comprised of neutrophils, as shown in Suppl. Fig. S1. This finding supported the use of this neutrophil population in subsequent assays. The morphology of isolated cells was also depicted in Suppl. Fig. S1 by light and fluorescent microscopy. Mouse neutrophils from each experimental group were divided into two assay groups; one group received recombinant mouse IL-17A protein (with *r*IL-17A) whereas another group was stimulated with PBS (wo/ *r*IL-17A). All neutrophils were subjected (where indicated) to one or several effector functional assays. The effect of the *r*IL-17 was profound and currently unknown to directly primed neutrophils *in vitro* setting. We have shown that isolated mouse bone marrow neutrophils were relatively functional, capable of performing particular functions through an application of *r*IL-17A (Figs. 4–6).

3.5.1. Neutrophils increased reactive oxygen species (ROS) production in response to rIL-17A supplementation

We found significantly higher levels of intracellular ROS staining in neutrophils after *in vitro* stimulation with *r*IL-17A across all groups compared with PBS (Fig. 4C, $P < 0.05$). Neutrophils treated with *r*IL-17A were compared to corresponding cells without *r*IL-17A to derive a normalized value of ROS MFIs. From the data, normalized ROS MFIs in Mix had the highest average value (Fig. 4C). Overall, supplementation of *r*IL-17A significantly enhanced ROS production in mouse neutrophils, especially in the Mix infection group (Fig. 4C). The increased *Il17a* gene expression (Mix and Flu) and *Il17ra* gene expression in all treatment groups (Mix, Flu, and KP) compared with control in neutrophils were noted (Fig. 7B, C). These findings suggested that the receptor for IL-17 is readily prepared to function as a cognate receptor of IL-17A, which is necessary for signal transduction and to generate further function of this cytokine in cells. The gene expression results may indicate the potentiate and active signaling, ready to produce ROS when properly stimulating threshold signals are reached.

3.6. Neutrophil phagocytosis was unaltered by supplementation of rIL-17A

In phagocytosis assays, isolated cells were treated with *r*IL-17A or PBS before adding fluorescent labeling *Klebsiella pneumoniae*. Normalized MFIs of phagocytosis in cells treated with *r*IL-17A were slightly lower than those receiving PBS (Control) in all assay groups except in Mix. (Fig. 5B). Comparison of normalized phagocytosis MFIs from Control ($P = 0.22$), Mix ($P = 0.94$), Flu ($P = 0.69$), and KP ($P > 0.99$) showed no difference between challenge groups (Fig. 5B).

3.7. Decreased intracellular bacterial killing ability was observed in neutrophils stimulated with rIL-17A

Our assay setting used the MTT method to examine neutrophil bactericidal ability. Bacterial killing ability in neutrophils (average percentage of killing) without *r*IL-17A stimulation exceeded that of those with *r*IL-17A stimulation in Control, Mix, and KP groups;

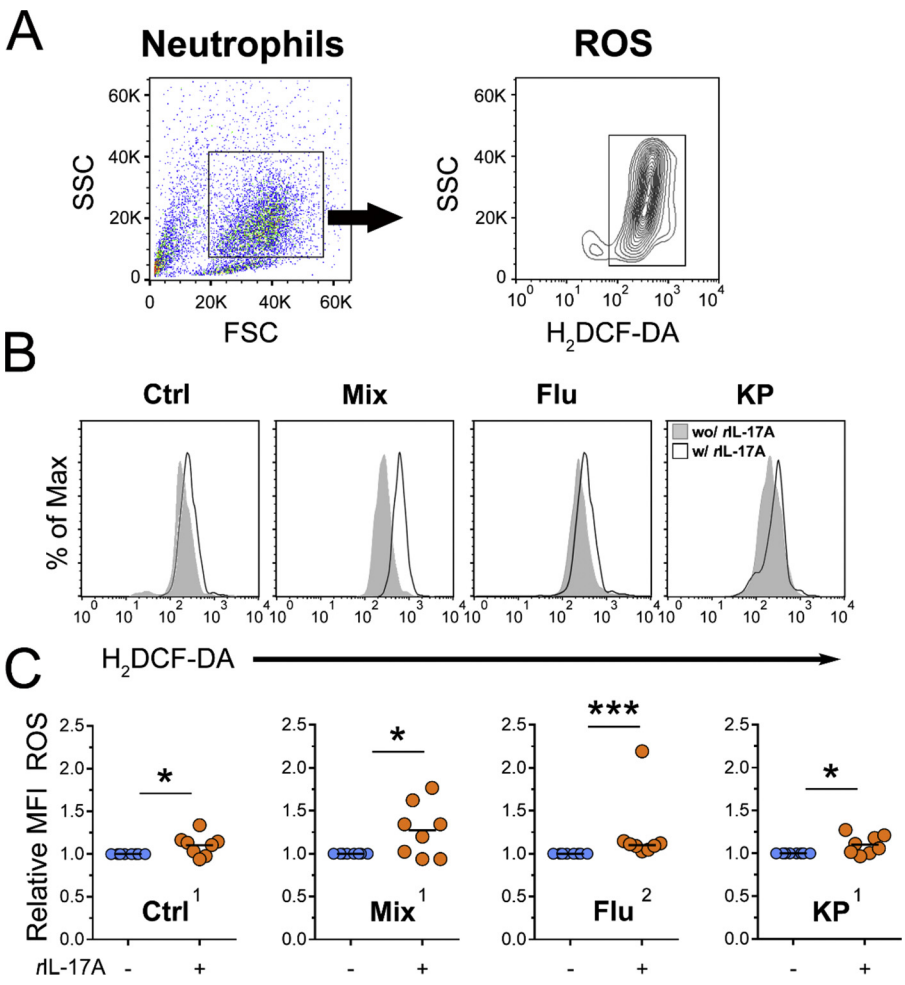


Fig. 4. Neutrophils increased ROS production in response to *r*IL-17A supplementation. **(A)** Representative dot plots and gating strategies of ROS positive (H_2DCF-DA$^+$) neutrophils **(B)** Representative histograms show positive H_2DCF-DA fluorescence of *r*IL-17A stimulated neutrophils (open histogram) and unstimulated cells (filled histogram). Data are representative of three independent experiments. **(C)** Inclusive flow cytometry data of relative MFI ROS as described in (B). Each data point in the scatter plot represents an individual mouse. Horizontal bars indicate mean/median values of three independent experiments (n = 8 mice each group). *$P < 0.05$, *** $P < 0.001$ by unpaired t test ([1]) or Mann-Whitney test ([2]).

however, bacterial killing properties of neutrophils were enhanced by *r*IL-17A in Flu. Differences in bacterial killing among all challenge groups were not significant (Fig. 6A). Even though the results of *Il17ra* gene expression (Fig. 7C) suggested the trend in sensing of *r*IL-17A, the functional assay regarding phagocytosis, and subsequent bacterial killing showed unaltered functions in these two assays.

3.8. Quantification of NET release revealed significant effects of rIL-17A on neutrophils

The induction of NET production by IL-17 has not been demonstrated up to this point. Here we assayed and quantified the release of NETs using fluorescently-labeled DNA. Data showed that mouse neutrophils initially stimulated with *r*IL-17A released more NETs than unstimulated neutrophils in all challenge groups (Fig. 6B). The co-

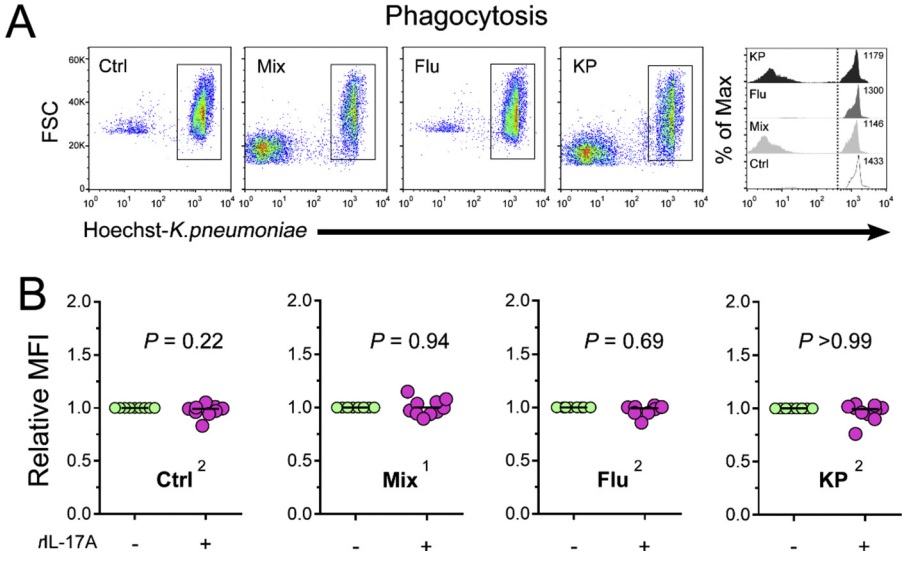


Fig. 5. Neutrophil phagocytosis of Hoechst-labeled *K.pneumoniae* was unaltered by supplementation with *r*IL-17A. **(A)** Representative pseudocolor dot plots show phagocytosis in *r*IL-17A-stimulated neutrophils (gating area) in each challenged group. Overlay histograms from data of gating area. The number in the histograms indicates MFI of gated cell populations. **(B)** Inclusive flow cytometry data of relative MFI of phagocytosis as described in (A). Each data point in the scatter plot represents an individual mouse. Horizontal bars indicate mean/median values of three independent experiments (n = 8–10 mice/group) analyzed by the unpaired t test ([1]) or Mann-Whitney test ([2]).

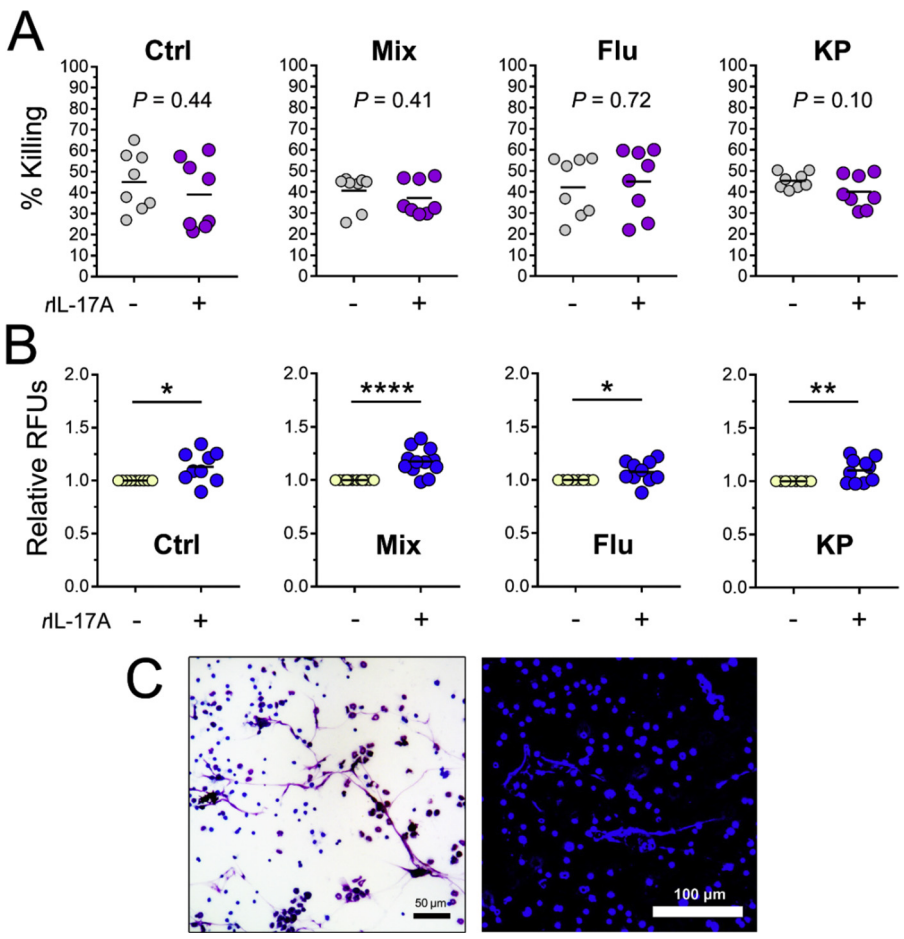


Fig. 6. Bacterial killing (MTT) assays (A) and neutrophil extracellular traps (NETs) release in mouse neutrophils (B–C). **(A)** Inclusive data on killing percentages of live *K. pneumoniae* from two independent experiments with $n = 8$ mice each group. Horizontal lines in dot plots indicate mean values, unpaired *t* test. **(B)** Inclusive scatter plot showing relative RFUs of NETs from *r*IL-17A-stimulated neutrophils in each challenge group. Horizontal bars indicate mean values of three independent experiments ($n = 9$–12 mice/group). *$P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ by unpaired *t* test. **(C)** Representative images of stained NET structures, Dip Quick, 200× magnification (*left*) or DAPI-stained structures, 50× magnification (*right*).

infection of bacteria and virus (Mix) was associated with the most significant increase in NET release ($P < 0.0001$). The published literature indicated that neutrophils expressing RORγt and IL-17 [36] were able to form and accumulate extracellular traps. Our results were in agreement on the fact that neutrophils received *r*IL-17 were able to produce more NETs in a combination with increased *Il17a* gene expression (Fig. 7B).

3.9. Relative mRNA expression of Il1β, Il17a, and Il17ra gene in neutrophils from challenged mice by qRT-PCR

Total RNAs were extracted from isolated mouse BM neutrophils as stated in the methods section. The gene expression levels of *Il1b*, *Il17a*, and *Il17ra* from mRNA transcripts, with *Gapdh* as a reference gene, were examined. We first determined the specificity of primers used in qRT-PCR. Dissociation curves confirmed that our primers generated the expected products as depicted by a single peak dissociation curve and accurate melting temperature (T_m) while amplifying target PCR products earlier than 35 cycles ($C_T < 35$) (data not shown). The T_m values of *Il1b*, *Il17a*, *Il17ra*, and *Gapdh* were 83.12, 82.27, 76.52, and 80.26 °C, respectively.

We expected that neutrophils in the presence of influenza virus or a combination of bacteria in secondary infection would express IL-17 gene family cytokines. Relative expression levels of IL-17A and its receptor (IL-17RA) were quantified by qRT-PCR. The data from mouse neutrophils from Mix and Flu showed increases in expression of the *Il17a* and *Il17ra* genes (Fig. 7B–C). The expression of IL-17RA was upregulated across all challenge groups (Fig. 7C). The expression of proinflammatory cytokine *Il1b* was only increased in Flu (Fig. 7A). Overall, the increase in gene expression of *Il17a* in Mix and Flu groups, and *Il17ra* in Mix, Flu, and KP is therefore only the indicative of starting point in protein is being synthesized. The detection of internally expressed gene encoding for IL-17A cytokine, and receptor (IL-17RA) may not necessarily mean the actual protein in being produced. The association between the neutrophil functions (namely; ROS, phagocytosis, NETs) and IL-17, through the signaling pathways involved in those

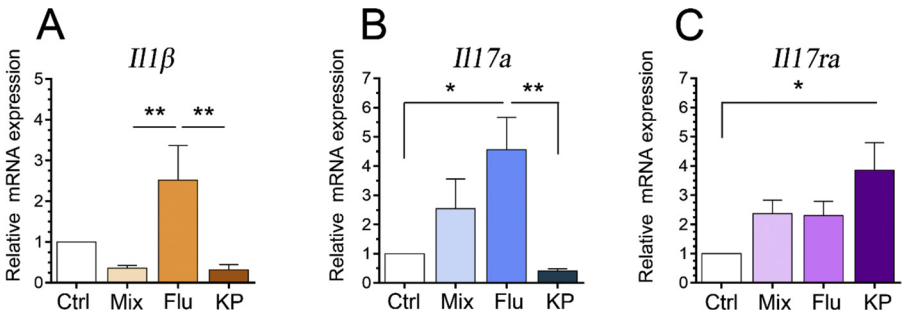


Fig. 7. Relative mRNA expressions of *Il1β*, *Il17a*, and *Il17ra* genes in neutrophils from challenged mice by qRT-PCR. The bar graphs show relative expression of *Il1β* **(A)**, *Il17a* **(B)**, and *Il17ra* **(C)** in neutrophils from two independent experiments ($n = 8$–10 mice/group). Data are means ± SE, *$P < 0.05$, ** $P < 0.01$ by one-way ANOVA, followed by Tukey test.

functions has to be further unveiled.

4. Discussion

Global outbreaks and major flu casualties from past to present are derived from the influenza A virus. Patients who suffered from a pandemic outbreak of influenza H1N1 (swine flu) in 2009 showed lung lesions that may have been the result of secondary bacterial lung infection or bacterial pneumonia [29]. In this study, we created a model of influenza virus infection by utilizing virus isolates from swine origin pandemic H1N1 Influenza A/SW/Thailand/CU-RA29/2009 [17,30]. This virus infects a range of hosts, from pigs to humans and other mammals. We also initiated a co-infection between influenza A virus and *Klebsiella pneumoniae*, the latter of which is commonly found to be a cause of secondary bacterial infection in the respiratory tracts of patients [31].

The pandemic swine H1N1 influenza virus (CU-RA29/2009) maintained its infectivity in other mammalian hosts similar to previously published research pertaining to the mouse adapted H1N1 A/PR/8/34 and other strains [29,32,33]. Many studies have been conducted on the H1N1 viral infection alone or in co-infection with bacteria (*e.g.*, *Streptococcus pneumoniae*), resulting in high morbidity and mortality in challenged mice [4,16,33]. Survival curve data showed that challenged mice were dying from day 5 of the experiments. This finding may correlate to the peak of viral replication in the lungs [34]. Histopathology of lung lesions revealed hemorrhage as well as a vast neutrophil infiltration, which corresponds to ARDS-like and ALI pathologies in mice [35].

Mouse neutrophils are also a producer of the proinflammatory cytokines IL-1α, IL-1β, IL-6, IL-17, IL-18, and MIF [7]. The IL-17 producing neutrophil has been previously described [14,36]. Relatively recent studies state that IL-17A and IL-17F are the most two potent cytokines and play a key role in the augmentation of neutrophil migration to inflammation sites [32,37,38]. IL-17A in concert with IL-1β is thought to be a key mediator facilitating neutrophilia in influenza-induced lung inflammation [34]. We have described in this study that an indirect effect of IL-17 persists in inflamed lung tissues by observing that neutrophils are the major cell population in BAL and lung histology, a finding consistent with previous reports [16]. IL-17 when synergized with IL-1, IL-6, and TNF-α promotes neutrophil infiltration into tissues, thus aiding in the elimination of bacteria or fungi [14]. Gr-1^+ mature neutrophils, as well as BM-neutrophils, can produce IL-17A; as a result, IL-17A signaling is a requirement of IFN-γ signaling [14,39]. This current study recapitulated these results, wherein Gr-1^+ BM neutrophils specifically expressed the *Il17a* mRNA transcript after exposure to the influenza virus alone or co-infection with virus and bacteria. In addition, mouse neutrophil *Il17ra* mRNA levels were upregulated by both viral and bacterial challenges.

Our results demonstrated intracellular and extracellular effector functions of mouse neutrophils following *in vitro* stimulation with *r*IL-17A. We showed this cytokine affects mouse neutrophil functionality beyond its well-established role as a chemoattractant. Results pointed toward selected neutrophil functions that may be enhanced by this cytokine, such as enhancement of ROS generation as well as NET release in challenged mice, in a similar manner as previously reported [14]. In our ROS study, mouse BM neutrophils produced robust ROS molecules after stimulation with *r*IL-17A; this observation is consistent with other published work [14,40]. A correlation among IL-17, IL-17 receptors and ROS production has previously been proposed [14].

The considerable accumulation of ROS molecules in neutrophils in the infected lung areas promoted neutrophil cell death, from an apoptosis pathway stimulation or NETosis [19,41]. As a result of NET release, cellular and nuclear portions, as well as granule proteins from neutrophils, may diffuse into the surroundings and cause uncontrolled tissue damage. Ermert et al. [25] and Röhm et al. [42] suggested that ROS was required for NET production. Our ROS assay results also showed an increase in ROS molecules in *r*IL-17-primed cells. Altogether, the increase in ROS level may somehow assist in NET formation, although a mechanism remains unclear [19,26].

In this study, we provide insights into the release of NETs in influenza or *K. pneumoniae*-challenged mice in that it is partially regulated by *r*IL-17A. The data are, in part, consistent with other reports that found multiple NET structures, predominantly in areas of tissue damage within the lungs, in mice challenged with influenza, *Streptococcus pneumoniae*, or dual infection [1,16]. The production and release of NETs by activated neutrophils may have caused the more detrimental effects in those reports and in this study. As previously reported, bacterial clearance within the lung tissues has been impaired in experimental influenza virus infections [29,43]. Findings from the current study regarding phagocytosis and bacteria killing are consistent with previous findings of phagocytosis in *Streptococcus pneumoniae* [43].

The IL-17 axis is highly organized and can be linked to IL-1, IL-6, IL-15, IL-21, and IL-23 [4,14,44,45]. To further elucidate the necessity of *Il17a* and shared receptor *Il17ra* gene expression in host defense mechanisms, our qRT-PCR results identified the upregulation of IL-17A and IL-17RA in challenged mouse neutrophils. The increase in *Il17a* mRNA levels in influenza or co-infection may result from signals transmitted *via* TLR, NF-κB-, PI3K-AKT-, or MAPK (ERK, p38, and JNK)-dependent signaling pathways or other undetermined pathways [5].

In conclusion, we have successfully produced a mouse model of co-infection between swine influenza and the commonly found respiratory bacteria *Klebsiella pneumoniae*. Neutrophil functional activities were explored after cells were stimulated *in vitro* with *r*IL-17A protein. We found that IL-17 was able to promote ROS and NET production in mouse neutrophils. Results from this study highlight that IL-17 may be key to the enhancement of ROS and NET production in neutrophils. Furthermore, IL-17 by itself is likely to augment neutrophil migration into virus- or bacteria-infected areas and contribute to the innate immune effector module 3 in the lungs. Future experiments could be build based on this study, perhaps to find a way to mitigate or prevent the lung lesions from occurring as of a significance of IL-17 on neutrophils.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.cimid.2019.101356.

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