

# Cytokine Profiling in Influenza A Virus and Staphylococcal (Co-)Infections

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## Abstract

Influenza A virus and *Staphylococcus aureus* are common causative agents of pneumonia. Co-infections with these two pathogens frequently occur and are characterized, among others, by higher morbidity and mortality due to hyper-inflammation of the lungs. Here, we aimed to profile systemic and local cytokine composition at early acute stages of pneumonia in a murine model. All mice recovered from single influenza A virus and/or staphylococcal infections. In contrast, co-infections led to a severe clinical outcome. While distinct cytokine patterns were detected in lungs of single-pathogen-infected animals, co-infections combined both virus- and bacteria-driven responses. However, analyses of infected human primary monocytic cells as well as bronchial epithelial cells did not reflect murine profiles. Based on infectious dose, mainly bacteria-driven responses were noted. The impact of single cells to cytokine composition of the lungs and translation of murine studies to humans remains uncertain and warrants further studies.

**Keywords:** influenza A virus; *Staphylococcus aureus*; co-infection; cytokines

## Introduction

In addition to the general annual seasonal influenza outbreaks, the last century witnessed several influenza pandemics.<sup>1</sup> During these outbreaks, the circulating influenza A virus (IAV) becomes the major cause of community-acquired pneumonia (CAP).<sup>2</sup> Co-infections with bacterial pathogens are frequent in influenza-related CAP.<sup>1</sup> Next to *Streptococcus pneumoniae*, *Staphylococcus aureus* is the second most common co-infecting gram-positive bacterial pathogen.<sup>3</sup> It is estimated that around 95% of all severe cases and deaths during the Spanish flu can be attributed to secondary bacterial infections.<sup>1</sup> In contrast, the novel IAV H1N1 identified in 2009 caused a rather mild self-limiting illness of the upper respiratory tract. However, up to 20% of infected humans

developed progressive severe pneumonia requiring admission to the intensive care unit (ICU).<sup>4–6</sup> Young age, pregnancy and obesity were identified as important risk factors.<sup>6–8</sup> Furthermore, co-infections were diagnosed in around 30% of cases within 72 hours of ICU admission, and *S. aureus* was the predominant bacterial species.<sup>9–11</sup>

Several studies have shown that IAV and bacterial co-infections are particularly associated with a more severe outcome.<sup>12–14</sup> Hypercytokinemia is partially dependent on pathogen etiology and plays a key role in the morbidity and mortality of CAP.<sup>15</sup> Lung epithelial cells, resident immune cells and recruited monocytes are the first responders against invading pathogens and the main cytokine producers during initial stages of infection.<sup>16</sup> It has been reported that IAV infection leads to a depletion of resident alveolar macrophages.<sup>17</sup> However, we have previously shown that the number of resident macrophages remains unaffected at early stages of single viral as well as viral and pneumococcal co-infections and that they display an activated phenotype. Furthermore, increased local cytokine production was noted in these co-infections.<sup>18</sup>

Here, we aimed to profile cytokine responses to single IAV, single staphylococcal and co-infections. Co-infected mice showed local hypercytokinemia combining IAV- and bacteria-driven responses. However, infections of human cells did not reflect the cytokine profiles observed in mice. Mainly bacteria-driven responses were noted.

## Results

Our aim was to profile a potential cytokine storm in response to viral and staphylococcal co-infections. Both pathogens had to meet the following criteria: (i) both, the chosen IAV as well as staphylococci, cause only mild symptoms in mice; and (ii) all animals can recover from the single-agent infections. Our hypothesis was that under the selected conditions, a fulminant cytokine response would be evident only in co-infections.

### *S. aureus* SA113 causes mild infection in mice

In order to select a staphylococcal strain of mild infectivity, mice were infected with *S. aureus* SA113 and two strains of the USA300 lineage (HGW\_USA300 and LUG2012; **Figure 1** and **Supplemental Figure 1**). Weight, clinical score, bacterial loads in the nasopharynx [nasal washes (NALs)] and bronchoalveolar lavage fluid (BALF) as

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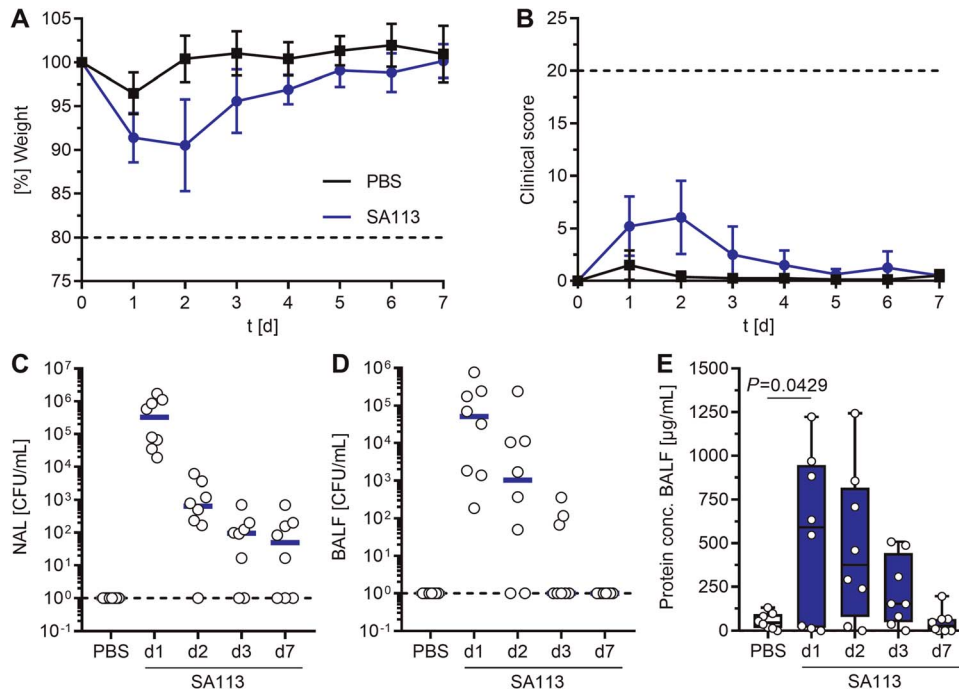
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**Figure 1. *S. aureus* SA113 causes mild infection in C57BL/6J mice.** Female C57BL/6J mice were intranasally infected with  $1 \times 10^8$  CFU of *S. aureus* SA113 or challenged with PBS and monitored over 7 consecutive days. A and B: Weight and clinical score were monitored daily and are displayed as mean values  $\pm$  SD. Horizontal dotted lines in A and B indicate termination criteria ( $\geq 20\%$  weight loss and/or a clinical score  $\geq 20$ ). C, D and E: At indicated time points, CFU counts in NALs, BALF and protein concentration in BALF as a general marker of inflammation were determined. Two independent experiments with four mice per group were performed (total:  $n = 8$ ). Each dot in C, D and E represents one mouse. The horizontal lines in C and D denote median values. The data in E are displayed as box plots. The levels of significance were determined using Kruskal-Wallis test with Dunn's multiple comparison post-test. CFU, colony forming units; PBS, phosphate-buffered saline; NALs, nasal washes; BALF, bronchoalveolar lavage fluid.

well as general lung inflammation were monitored over seven consecutive days. Single LUG2012 infections of mice resulted in severe clinical presentation within eight hours, and the experiment had to be terminated (Supplemental Figure 1A and 1B). High bacterial load and high inflammatory response were evident in the nasopharynx and lungs of the animals (Supplemental Figure 1C and 1D). In contrast, initial infections of mice with SA113 or HGW\_USA300 were characterized by a moderate weight drop and mild clinical symptoms peaking at Day 3 (Figure 1A and 1B and Supplemental Figure 1F and 1G). High bacterial loads in both respiratory compartments and protein concentrations in BALF were evident at Day 1 post bacterial application and decreased over the next six days (Figure 1C–1E and Supplemental Figure 1H–1J). No bacteria were detected in the lungs of the animals on Day 7. However, although the animals completely recovered from the infections, some contained staphylococci in the nasopharynx (Figure 1C and Supplemental Figure 1H). Since *S. aureus* SA113-infected animals showed a slightly better recovery, this strain was used for co-infection experiments.

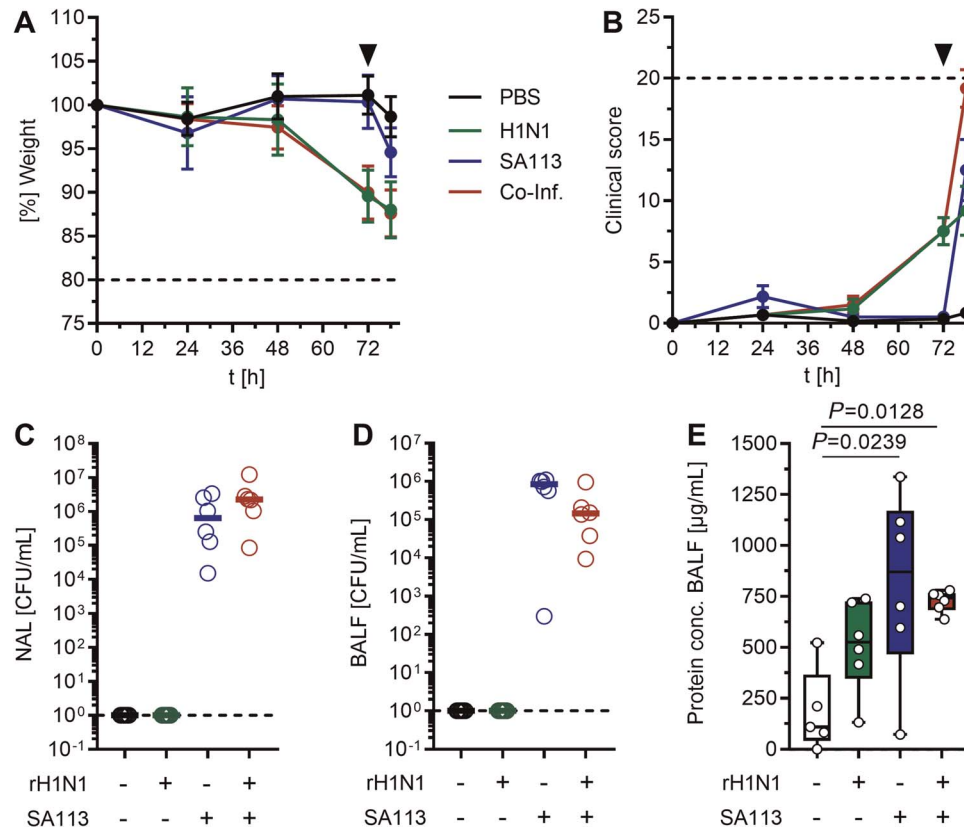
#### Co-infection leads to a severe clinical outcome and high local cytokine responses in mice

We have previously shown that the non-mouse-adapted influenza virus A/Bavaria/74/2009 (H1N1) causes only mild symptoms of viral pneumonia in mice. The mice showed a biphasic disease progression, with a short recovery phase on Day 4 to Day 7 post viral application.<sup>18</sup> This IAV strain was used for co-infection experiments.

Studies have shown that co-infections in humans occur within 72 hours.<sup>9–11</sup> Therefore, mice were infected with H1N1 for

72 hours followed by a bacterial infection with SA113. Phosphate-buffered saline (PBS) treatment as well as single viral and single bacterial infections served as controls. In accordance with the previous report,<sup>18</sup> infection of mice with H1N1 led to a continuous mild disease progression characterized by a minor weight loss and moderate clinical symptoms during the first three days (Figure 2A and 2B). On Day 3, mice were infected with bacteria, and six hours later, a minor weight loss was noted in all groups, most likely due to anesthesia effects (Figure 2A). In contrast, bacterial infections resulted in an elevated clinical score (reduced food intake, self-isolation and neurological symptoms), which particularly reached the termination criteria for the co-infected mice (Figure 2B). Based on the clinical score, all mice were sacrificed and bacterial burden in the respiratory tract (Figure 2C and 2D) as well as local and systemic inflammation were determined (Figures 2E and 3, and Supplemental Figure 2). Groups involving bacterial infections were characterized by a high bacterial burden in the nasopharynx and lungs (Figure 2C and 2D). In addition, the local inflammatory response was significantly increased in these groups, as shown by increased protein concentrations in BALF (Figure 2E).

Next, multiple local and systemic cytokine concentrations were determined in BALF and plasma, respectively (Figure 3 and Supplemental Figure 2). In general, mostly local inflammatory responses were noted due to the short period of bacterial infection. Furthermore, distinct cytokine patterns were observed based on the infectious agent. Single bacterial infections were characterized by elevated levels of tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\alpha$ , IL-1 $\beta$ , IL-6 and IL-17A in the lungs (Figure 3). In contrast, viral infections led to enhanced production of monocyte chemoattractant



**Figure 2. Severe clinical outcome in co-infections.** Female C57BL/6J mice were intranasally infected with influenza A virus H1N1 ( $1 \times 10^5$  PFU) for 3 days followed by a subsequent infection with  $1 \times 10^8$  CFU of *S. aureus* SA113 at  $t_{72h}$ . PBS challenge as well as single viral or bacterial infections served as controls. A and B: Weight and clinical score were monitored daily and are displayed as mean values  $\pm$  SD. Horizontal dotted lines in A and B indicate termination criteria ( $\geq 20\%$  weight loss and/or a clinical score  $\geq 20$ ). The black arrow indicates the time point of bacterial infection. C, D and E: Six hours (78 hours) post bacterial challenge, the animals were sacrificed and CFU counts in NALs, BALF and protein concentration in BALF as a general marker of inflammation were determined (total:  $n = 6$ ). Each dot in C, D and E represents one mouse. The horizontal lines in C and D denote median values. The data in E are displayed as box plots. The levels of significance were determined using Kruskal-Wallis test with Dunn's multiple comparison post-test. PFU, plaque forming units; CFU, colony forming units; PBS, phosphate-buffered saline; NALs, nasal washes; BALF, bronchoalveolar lavage fluid; Co-Inf., co-infection.

protein-1, interferon (IFN)- $\beta$  and IFN- $\gamma$  in the lungs (Figure 3). However, co-infections combined both bacterial and viral cytokine signatures locally (Figure 3). Overall, only a minor systemic response was noted. Elevated plasma levels of IL-6, IL-10 and IL-17A were seen in infections involving bacteria (Figure 3 and Supplemental Figure 2).

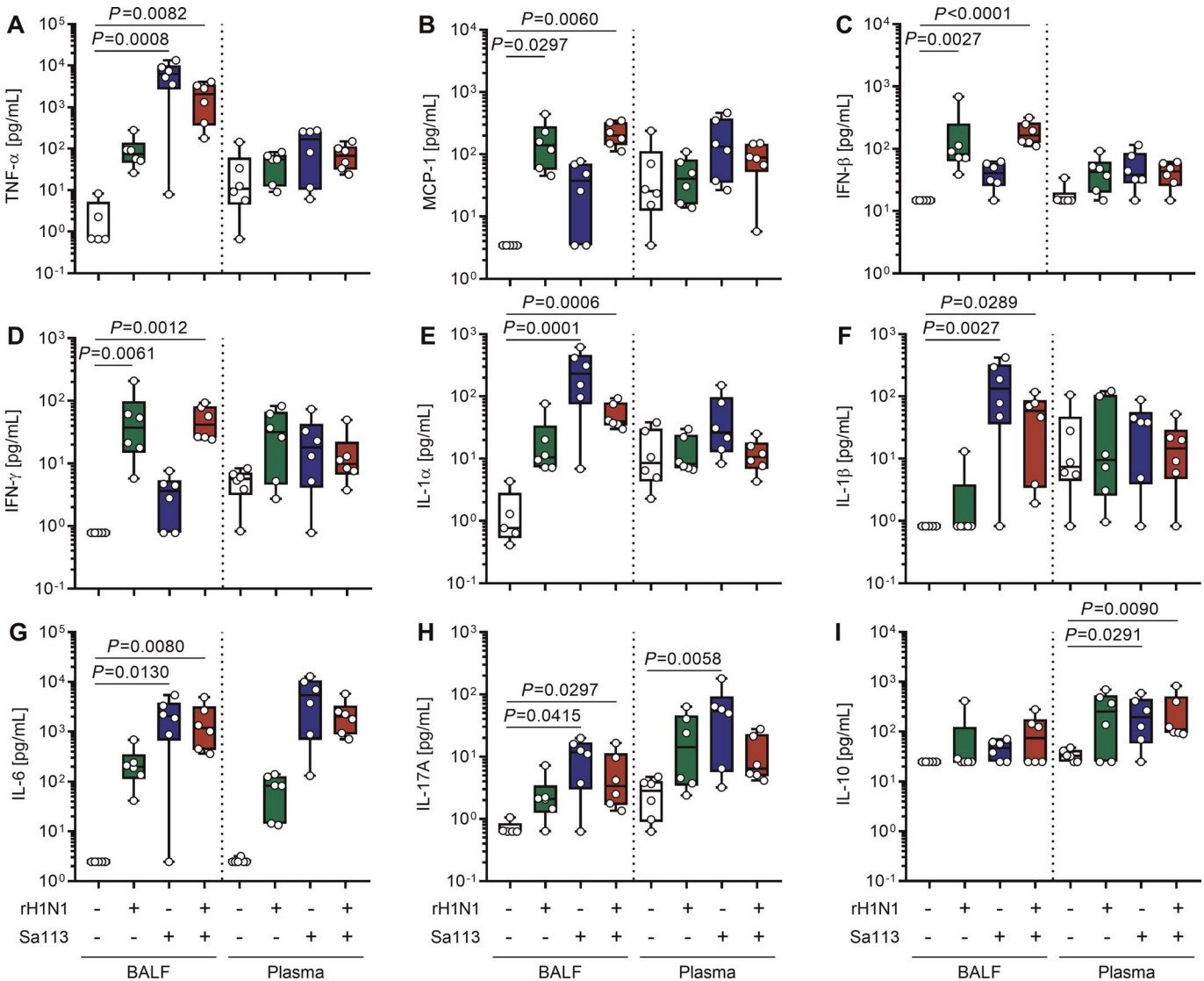
#### Human cells display mainly bacteria-driven cytokine response

Our previous analyses of single and co-infected mice revealed that all types of lung infections are associated with (i) an influx of classical monocytes into the vascular and alveolar/interstitial lung compartments, (ii) no major depletion of alveolar macrophages at early stages of viral infections, and (iii) increased levels of interstitial Type 3 macrophages in bacterial infections.<sup>18</sup> Therefore, we hypothesized that the high local cytokine release observed in staphylococcal and IAV (co-)infections might be attributed to the cells of myeloid lineage and/or epithelial cells, which are the first to encounter the pathogens. To determine whether the observed cytokine pattern would appear in human cells, human primary monocytes and monocyte-derived macrophages as well as 16HBE14o (16HBE) cells were (co-)infected with both pathogens and cytokine responses were assessed. Infections involving staphylococci resulted in a significant death of both monocytic cells, whereas H1N1 infections had a minor impact on cell viability (Supplemen-

tal Figure 3A and 3B). In contrast, irrespective of the infectious agent or combination, only minor bacteria-driven epithelial cell damage was seen (Supplemental Figure 3C). Assessment of cytokine release revealed mainly bacteria-driven responses (Figure 4 and Supplemental Figures 4–6). IFN- $\gamma$ , IL-6, IL-8 and IL-10 were exclusively elevated in single staphylococcal infections of macrophages, while enhanced levels of IL-1 $\beta$ , IFN- $\alpha$ 2, TNF- $\alpha$ , IL-12p70, IL-23 and IL-33 were detected in both single staphylococcal infections and co-infections (Figure 4 and Supplemental Figure 4). Monocyte infections mostly confirmed the observed bacteria-driven infection phenotype (Figure 4 and Supplemental Figure 5). IL-1 $\beta$ , TNF- $\alpha$ , IL-18 and IL-33 levels were increased in staphylococcal infections as well as co-infections, whereas IL-10, IL-12p70 and IL-23 were exclusively elevated in single bacterial infections. IFN- $\alpha$ 2 production was exclusively elevated in response to H1N1 infection of monocytes (Figure 4 and Supplemental Figure 5). In contrast to human primary monocytic cells, a uniform bacteria-driven cytokine release was noted in 16HBE (co-)infections (Figure 4 and Supplemental Figure 6).

#### Discussion

Cytokines are often used to evaluate severity of infections and to predict the clinical outcome. Progressive CAP is characterized by



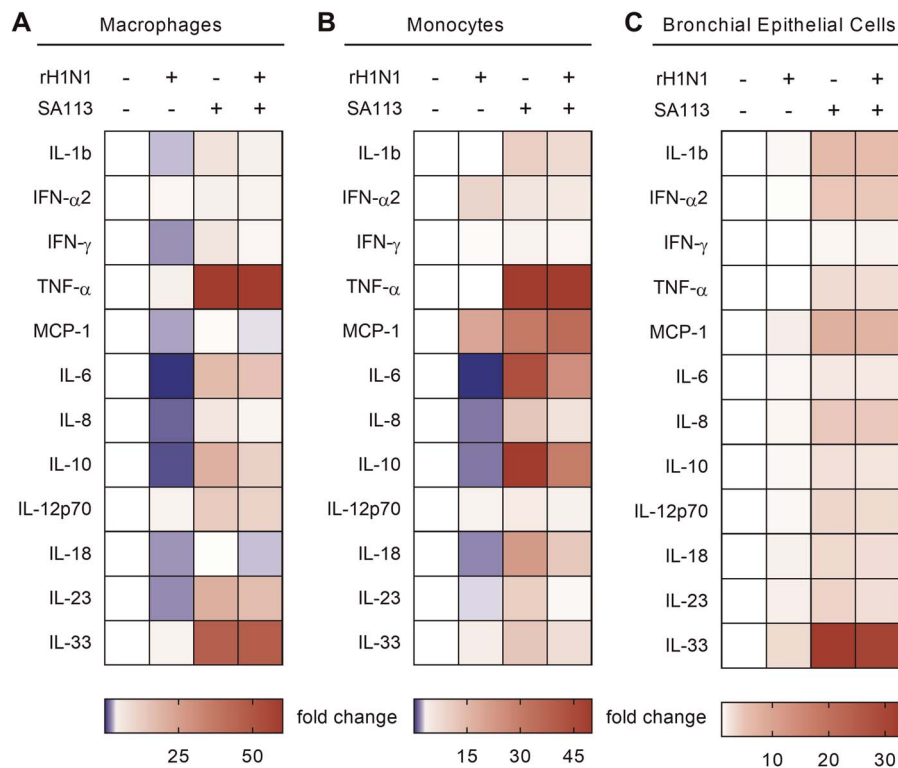
**Figure 3. High local cytokine response in influenza A virus H1N1 and staphylococcal co-infections.** Mice were sacrificed after 78 hours. A, B, C, D, E, F, G, H and I: TNF- $\alpha$ , MCP-1, IFN- $\beta$ , IFN- $\gamma$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-17A and IL-10 levels were determined in BALF and plasma ( $n \geq 5$ ). The data are displayed as box plots. Each dot represents one mouse. The level of significance was determined using Kruskal-Wallis test with Dunn's multiple comparison post-test. TNF, tumor necrosis factor; MCP, monocyte chemoattractant protein; IFN, interferon; IL, interleukin; BALF, bronchoalveolar lavage fluid.

excessive local and systemic inflammation, and therefore, systemic levels of IL-1 $\beta$ , IL-6, IL-8, IL-10 and TNF- $\alpha$  are commonly studied in relation to disease prognosis.<sup>19,20</sup> However, less is known about the early local cytokine response in CAP. Here, we show that infection of mice results in specific pathogen-mediated local cytokine profiles and co-infections combine both imprints. However, infection of human primary monocytic cells as well as epithelial cells did not mirror the murine cytokine profiles.

Several studies on pneumonia patients, who were admitted to the ICU, identified highly elevated levels of IL-6, IL-8,<sup>19,21,22</sup> IL-1 $\beta$ ,<sup>21,23</sup> IL-17 and IL-22<sup>19</sup> in BALF of these patients. These cytokines were found in all types of single infections and co-infections.<sup>24</sup> However, most of the studies were limited to a few cytokine profiles, and therefore, we aimed to analyze multiple markers. Our results obtained in mice are in line with several other analyses, which show the release of TNF- $\alpha$ ,<sup>25-27</sup> IL-1 $\alpha$ ,<sup>28</sup> IL-1 $\beta$ <sup>29</sup> and IL-6<sup>30</sup> in response to severe IAV and staphylococcal (co-)infections,

particularly at the late stages of infection. However, we show that these cytokines are already produced at early stages of infection and are mostly restricted to the lungs. In line with this, airway proteome analyses of *S. aureus* lung infections in mice revealed that IL-1 $\beta$ , IL-6 and TNF- $\alpha$  are elevated six hours post infection.<sup>31</sup>

Most of the aforementioned cytokines are usually linked to bacterial infections, although they are also found in viral infections. However, IAV-infected cells/organs potentially induce monocyte chemoattractant protein-1, type I and type II IFN production to restrict viral replication, among other functions.<sup>24,32</sup> Indeed, we observed production of these molecules in response to all infections involving IAV. Although many beneficial functions in viral infections are ascribed, type I IFNs are known to negatively affect monocytes in co-infections, resulting in compromised clearance of bacteria.<sup>33,34</sup> Furthermore, high levels of IFN- $\gamma$  were shown to contribute to local hyper-inflammation by inducing excessive TNF- $\alpha$  production in IAV-staphylococcal co-infections, resulting



**Figure 4. Cytokine release by human cells in response to IAV and staphylococcal (co-)infections.** Single and co-infections of human primary monocytes, monocyte-derived macrophages or 16HBE cells with IAV H1N1 (MOI of 0.1) and *S. aureus* SA113 (MOI of 10) were performed. Extracellular bacteria were killed by addition of antibiotics. A, B or C: The heat maps represent the fold change of cytokine concentrations in supernatants of infected macrophages, monocytes or 16HBE cells in relation to the uninfected control. IAV, influenza A virus; 16HBE, 16HBE14c; MOI, multiplicity of infection; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; MCP, monocyte chemoattractant protein.

in lethal outcomes.<sup>14</sup> Our results are in line with these observations. Co-infected mice reached high clinical scores within a short period of co-infection, hypercytokinemia was restricted to the lungs, and the experiment had to be terminated. Indeed, most of the mentioned cytokines are released at early stages of infections and are associated with the innate immune compartment. Previous studies investigating innate immune cell composition in early H1N1 infections have shown that H1N1 infection leads to an early influx of pro-inflammatory monocytes to the lungs and does not significantly deplete alveolar and interstitial macrophages at the beginning of infection. The high clinical score in co-infected animals might also be attributed to reactive oxygen species produced by infiltrating and resident phagocytes in response to *S. aureus* infection, which leads to subsequent inflammatory lung damage.<sup>35</sup> In addition, we hypothesized that the high local cytokine amounts in lung infections could be attributed to these cell types. Unfortunately, we observed an almost entirely bacteria-driven cytokine response in human monocytic and epithelial cells. Furthermore, even 24-hour infection of human dendritic cells with this viral strain resulted in negligible levels of cytokine production.<sup>36</sup> This low cytokine response might be caused by the mild viral strain or the low multiplicity of infection (MOI) that was used.

Although speculative, our results suggest that the severe outcome at early stages of co-infections is mainly mediated by hypercytokinemia and not, as suggested by several other studies, through increased bacterial burden in the lungs.<sup>26,37</sup> Further studies to determine detailed insights into the cytokine response during co-infections as well as the role of human cellular compartments in these infections are warranted.

## Material and methods

### Ethics statement

Buffy coats of blood, which were provided anonymously, were obtained from the blood bank at the University Medicine Greifswald. The ethics committee at the University Medicine Greifswald approved the study with number of BB 014/14 on January 24, 2014. All experiments were carried out in accordance with the approved guidelines.

Animal experiments were carried out in accordance with the regulations of the European Health Law of the Federation of Laboratory Animal Science Associations and the German Society for Laboratory Animal Science and are in compliance with the ARRIVE guidelines. Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern (LALLFV M-V, Rostock, Germany) approved the study with permit number of 7221.3-1.1-032/17.

### Bacterial and viral strains

*S. aureus* strains SA113, HGW\_USA300<sup>38</sup> and LUG2012 (USA300)<sup>39</sup> were cultivated overnight at 37 °C in casein hydrolysate and yeast extract medium. Influenza virus A/Bavaria/74/2009 (H1N1) was propagated as described previously.<sup>40</sup>

### Mice infections, monitoring and sampling

Two independent experiments with different infectious conditions were performed to ensure reproducibility of results. Groups of six to eight female C57BL/6J mice (eight weeks old; Janvier Labs) were intranasally infected under ketamine/xylazine anesthesia with H1N1

or *S. aureus*. For viral infections, 100,000 plaque forming units (PFU) in 42- $\mu$ L PBS were applied. For *S. aureus* infections, 20- $\mu$ L PBS containing  $1 \times 10^8$  colony forming units (CFU) (SA113),  $1 \times 10^8$  CFU (USA300\_HGW) and  $1 \times 10^7$  CFU (LUG2012) were administered. At indicated time points, mice were euthanized with isoflurane and blood; BALF and NALs were collected. BALFs and NALs were collected by rinsing the lungs and nasopharyngeal cavity with 1-mL PBS through the trachea, respectively. Cardiac puncture was used to collect blood. Plasma was obtained by centrifugation (10 minutes,  $1000 \times g$ ). Plasma was stored at  $-80^\circ\text{C}$  until further use. Daily monitoring of animals was performed, and weight and clinical score were documented (Supplemental Table 1).

### Co-infection

Mice were intranasally infected with 100,000 PFU H1N1 followed by an intranasal infection with  $1 \times 10^8$  CFU SA113 at  $t = 72$  hours. Single bacterial and viral infections were performed in parallel. Control mice were mock-treated with an equivalent volume of PBS. After 78 hours, mice were euthanized with isoflurane and sampled as described.

### Bacterial quantification in NAL and BALF

To quantify bacterial loads in mice, serial dilutions of NAL and BALF were prepared and plated on blood agar (Oxoid). The remaining cell-free BALF and NAL were stored at  $-80^\circ\text{C}$  for further analyses.

### Eukaryotic cells, culture conditions and infections

Human monocytes were isolated from buffy coats using CD14 S-pluriBead anti-human beads (PluriSelect) according to the manufacturer's instructions. CD14 and CD16 expression was validated via flow cytometry. The monocyte-derived macrophages were generated by culturing monocytes in RPMI1640 (Cytiva) medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) (Sigma-Aldrich) and 25-ng/mL granulocyte-macrophage colony-stimulating factor (Immunotools) for seven days. Medium was exchanged on Day 3 and Day 5. Infections of monocytes and macrophages were performed in RPMI1640 media supplemented with 10% FCS. Furthermore,  $1 \times 10^5$  monocytes or  $2 \times 10^5$  monocyte-derived macrophages were infected with H1N1 at an MOI of 0.1 for 24 hours.<sup>41</sup> The MOI was chosen based on previous results, showing equal viral loads for a range of tested MOIs.<sup>18,41</sup> Macrophages and monocytes were infected with SA113 at MOIs of 10 and 1, respectively. After one hour, the medium was removed, and extracellular bacteria were killed by addition of RPMI1640 containing 400- $\mu$ g/mL gentamicin or 2- $\mu$ g/mL lysostaphin (both Sigma-Aldrich). After a total of 24 hours of infection for macrophages and of six hours for monocytes, supernatants were collected and used for cytokine analysis. For co-infections, viral infection followed by subsequent bacterial infection was performed as described. Culture and infection of bronchial epithelial 16HBE cells were performed as described previously.<sup>42</sup> In brief,  $2.5 \times 10^5$  cells were seeded in 24-well tissue culture plates (Greiner) and maintained at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$  atmosphere for 24 hours. Next, cells were first infected with H1N1 at an MOI of 0.1 for 24 hours followed by a bacterial infection with SA113 at an MOI of 10. After 1 hour, the infection media were substituted with media containing antibiotics (400- $\mu$ g/mL gentamicin or 2- $\mu$ g/mL lysostaphin) to kill extracellular bacteria. After a total of 24 hours of bacterial infection, supernatants were collected and used for cytotoxicity measurement and cytokine analysis. Cytotoxicity was assayed using the CytoTox96

non-radioactive cytotoxicity assay (Promega) according to the manufacturer's instructions.

### Cytokine measurements

Cytokine concentrations in murine samples were measured via the LEGENDPlex mouse inflammation panel (13-plex) kit (BioLegend) according to the manufacturer's instructions. Cytokine concentrations in human cell supernatants were measured via the LEGENDPlex human inflammation panel (13-plex) kit (BioLegend) according to the manufacturer's instructions. Data were acquired with a FACSAria III flow cytometer using FACS DIVA Software (both BD Bioscience) and analyzed using LEGENDPlex software (BioLegend). Protein concentrations in BALF were determined using Bradford reagent (Sigma-Aldrich) according to the manufacturer's instructions.

### Flow cytometry

Dead cells were labeled using the Zombie Aqua Fixable Viability Kit (BioLegend). Unspecific binding of immunoglobulins was blocked using Human TruStain FcX (BioLegend) according to the manufacturer's instructions. Cells were incubated with titrated amounts of monoclonal antibodies for 30 minutes at  $4^\circ\text{C}$  in the dark. Cells were washed between each staining step and fixed using the Cyto-Fast Fix/Perm Buffer Set (BioLegend). Antibodies and clones directed against the following markers were used (target, clone and fluorochrome; all BioLegend): CD14 (63D3, APC) and CD16 (B73.1, PE). Data were acquired using a FACSAriaIII flow cytometer and FACS DIVA 8.0 Software (both BD Biosciences) and analyzed using FCS Express 7 Software (De Novo Software).

### Statistics

Kruskal-Wallis test with Dunn's multiple comparison post-test was used to determine statistical significance of differences. Statistics were performed using GraphPad Prism Version 7. A  $P$  value smaller than 0.05 was considered significant.

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### Supplemental digital content

Supplemental materials are available online at <http://links.lww.com/IMD/A26>, <http://links.lww.com/IMD/A27>, <http://links.lww.com/IMD/A28>, <http://links.lww.com/IMD/A29>, <http://links.lww.com/IMD/A30>, <http://links.lww.com/IMD/A31>, <http://links.lww.com/IMD/A32>.

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