The interaction between influenza and pneumococcus

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1 INTRODUCTION

Since the last two decades, the importance of knowledge about possible dangers of the influenza virus has been emphasized. The interest was especially caused by the outbreak of the avian influenza A H5N1 virus among poultry and its transmission to humans in 1997 [1]. When this virus emerged again in 2003, the fear raised that an influenza strain which is transferable between humans could develop [2]. As the H5N1 virus has a mortality rate of about 60 percent, a scenario like 1918, when the Spanish Flu killed over 40 million people, might reappear [3]. For this reason intensive research was performed in order to investigate factors that affect virulence of different influenza types.

The influenza virus belongs to the Orthomyxoviridae family. Orthomyxoviruses are enveloped and have a negative-sense RNA genome, consisting of 8 segments. The membrane envelope of an influenza virus contains two glycoproteins, hemagglutinin (HA) and neuraminidase (NA). HA is responsible for the binding of the virus to sialic acid on epithelial cell surface receptors. Also, HA is the main target of the neutralizing antibody response initiated by the immune system. NA facilitates the release of new viruses from infected cells. The NA-molecule is the target of most antiviral drugs like zanamivir and oseltamivir. Frequent mutations of the viral RNA leads to gradual changes in the HA protein (antigenic drift). Because HA is the main antigen for detection by the immune system, these mutations cause the annual epidemics. When a more abrupt change causes a new HA to emerge (antigenic shift), there is no pre-existing immunity against the uncommon protein. A worldwide pandemic might follow due to the lack of an effective reaction by the immune system of most people. Antigenic shift is caused by reassortment or zoonosis. Reassortment occurs when a human or animal cell is coinfected with different strains of influenza. Thereby, a hybrid virus can be created with a new combination of HA and NA. Zoonosis is the direct transmission of an animal influenza strain to humans. When this strain gradually adapts to human properties, a human to human transmissible virus can develop.

The clinical symptoms of an influenza infection are not uncommon. Many people suffer every year of a fever, malaise, myalgia, sore throat and coughing. However, for those who belong to the risk group, like children or elderly, the disease can be worse. The infection can cause gastrointestinal tract symptoms, otitis media and myositis. In this group the risk of developing complications is significant. Complications like viral or bacterial pneumonia, cardiac involvement or neurologic syndromes can cause life-threatening dangers. Especially secondary bacterial pneumonia is known to be a major cause of death in periods of influenza epidemics or pandemics [3]. The most common pathogen inducing bacterial pneumonia is Streptococcus *pneumoniae*.

S. *pneumoniae* is a gram-positive bacterium, covered with a polysaccharide capsule. It colonizes the oropharynx through binding to epithelial cells by means of surface protein adhesins. The pneumococcus counteracts envelopment into the mucus by producing secretory IgA protease and pneumolysin. In specific situations, like during an influenza infection, the bacterium is able to spread to the lungs, paranasal sinuses, or middle ear. Here, it activates leukocytes by its teichoic acid and peptidoglycan fragments. This will lead to release of cytokines such as IL-1 and TNFα by leukocytes causing migration of inflammatory cells. Especially the inflammation process, but also release of hydrogen peroxide by S. *pneumoniae* can induce tissue damage. Besides, the bacterium can bind to receptors for platelet-activating factor (PAFr) present on endothelial cells, leukocytes, platelets and tissue cells. By binding this receptor the pathogen can enter the cell and protect itself from immune cells. An infection of S. *pneumoniae* can cause middle ear infection, meningitis, bacteremia and pneumonia.

During the Spanish Flu pandemic it was already observed that death usually occurred when the patient suffered of a secondary Pneumococcal infection [19]. With the risk of the development of a pandemic influenza strain, like H5N1, it is of major importance to unravel the relationship between the two pathogens. This can help us to know how to handle if a potential high virulent virus strikes. At the moment, many country's are preparing for this threat by development of a vaccine and stocking of antiviral drugs. However, the exact features of a possible new virus can never be predicted. During last-years flu-epidemic a oseltamivir-resistant influenza strain was observed [1]. This raises the concerns about how to prepare against a virus that does not yet exists. Even in annual influenza epidemics, flu followed by bacterial pneumonia can be lethal for elderly, children or patients with an impaired immune system.

The last decades it was proposed that epithelial damage due to influenza leads to stimulation of bacterial adherence. This fitted into clinical observations like dry cough, indicating loss of mucous production. Although this is a reasonable assumption, recent studies have shown that the interaction between pneumococcus and influenza is more complicated [23]. Still, the exact mechanisms remain unclear. The goal of this paper is to elucidate the possible mechanisms that contribute to the interaction between the Influenza virus and bacterial pneumonia. This information can give insights into development of methods to prevent death by a secondary bacterial infection. Besides, the knowledge discussed can help us to find new ways to target influenza.

Different recently investigated subjects which are shown to be important in this problem will be reviewed. At first, the specific mechanism causing enhanced pneumococcal adhesion will be discussed. Secondly, the role of the immune system is shown to be involved in the stimulation of secondary bacterial infection and inflammatory damage. The last subject of interest is PB1-F2, this is a recently discovered influenza protein of which it was proved that it has several effects on influenza virulence and pneumococcal infection.

2 PNEUMOCOCCAL ADHESION

It has been proposed that influenza infection may predispose to secondary Pneumococcal infection due to epithelial damage. The explanation for this was that the injured epithelium would increase the susceptibility of bacterial adhesion. However, the exact mechanism by which the influenza virus may cause this is still unclear. Many studies have shown that different viral, bacterial and epithelial factors are involved in the process leading to Pneumococcal pneumonia. Two of these factors are the platelet-activating factor receptor (PAFr) and neuraminidase.

2.1 Platelet-activating factor receptor

As explained before, binding to PAFr by S. *pneumoniae* stimulates epithelial adherence, thereby causing infection. Besides, through this binding, the pneumococcus can enter cells and get through the epithelial barrier. By this way the bacterium can invade into other body compartments which might lead to complications like sepsis or meningitis.

Studies have suggested that during and after influenza infection the expression of PAFr on epithelial cells is upregulated, thereby causing a higher susceptibility to the development of pneumococcal pneumonia [22]. Indeed it was shown that PAFr-gene deficient mice had a longer survival rate after pneumococcal coinfection. This implies that, as hypothesized, adhesion and invasion of the bacterium is impaired due to a reduced availability of PAFr. Van der Sluijs et al. elucidated the mechanism by which PAFr affects pneumococcal infection [29]. They showed a lower production of the chemokines and cytokines KC (belonging to the CXC chemokine family), IL6, TNFα and IL10 in PAFr-gene deficient mice. This observation can be explained by the fact that when S. *pneumoniae* is less able to enter epithelial cells, the binding of bacterial antigens to intracellular Toll-like receptors is declined, causing a reduced production of chemokines and cytokines. However, it is not clear how this disturbed reaction affects pneumococcal infection due to influenza. In the next chapter this subject will be discussed more detailed.

Nevertheless, a recent study by McCullers et al. was contrary to these findings [30]. No reduction of bacterial load in the lungs was observed in PAFr-gene deficient mice. However, they did find that the absence of PAFr prevented invasion of pneumococcus and thereby death due to meningitis. The result of McCullers et al. may reduce the importance of PAFr, but it is still an interesting target for research.

2.2 Neuraminidase

Neuraminidase is one of the two glycoproteins present on the surface of influenza. It functions as a mediator to release newly synthesized virus. NA cleaves sialic acid from host cell glycoproteins thereby releasing the virus. Many cellular structures that can act as Pneumococcal adhesion molecules are covered by sialic acid. Cleavage of these molecules by NA can help bacteria in binding and invading epithelial cells. Therefore it was proposed that NA from influenza primes the lung epithelium for Pneumococcal infection. Studies have shown that administration of viral NA stimulates adhesion of pneumococcus [14]. Also, when NA activity of influenza was inhibited a reduction of bacteria number was observed.

These results agree with studies which investigated the differences between several (pandemic) influenza stains [21]. The annual epidemic-causing H3N2 virus is associated with a higher mortality rate compared with H1N1, which also presently circulates in the human population. When investigating the NA activity, it was shown that this is clearly higher for the

H3N2 strain. The same applies for the activity of N2 NA in different H2N2 (1957-1968) and H3N2 (1968-now) strains, which was measured between 1957 until 2004. In the period 1957-1968 the mortality rate of the H2N2 strain decreased, combined with the observation of a reduced NA activity. During 1968 until 2004 both the mortality and the NA activity of H3N2 were increased until 1997. More importantly, in all the strains where this effect was observed, the higher mortality rate was explained by a higher incidence of Pneumococcal pneumonia.
If the mechanism of viral NA indeed leads to increased susceptibility to a secondary bacterial infection, the importance of the use of NA inhibitors as an anti-influenza drug may become more evident. Bhatia et al. [17] showed that treatment with NA inhibitors like oseltamivir has a double effect because at first, it decreases influenza replication due to the blocking of release of the virus from the host cell. Secondly it prevents the virus from cleaving sialic acid on epithelial cells, thereby decreasing the bacterial ability to adhere to these cells.

Nevertheless, in the last influenza epidemics virus strains have been found which appeared to be resistant against oseltamivir. Up to 16 percent of children infected with H1N1 who received this drug did not heal due to the treatment [1]. This H1N1 strain appeared to have a single amino acid substitution (His274Tyr) in its N1 NA. Later this mutant was also found in several patients infected with H5N1. It may be necessary to examine the differences between NA molecules of multiple influenza types.

3 DISTURBANCE OF THE IMMUNE RESPONSE

It is known that a clinical secondary bacterial infection occurs at a time point when virus titers are decreasing due to recovery of the patient to influenza infection [19]. This implicates that the immunological reaction against the virus has an adverse effect on the anti-bacterial challenge [20]. As the innate immune system is the first line of defense against pathogens, the role of alveolar macrophages may be interesting.

After an influenza infection, T-lymphocytes react by producing Interferon-γ (IFNγ). One of the functions of this cytokine is the triggering of the adaptive immune system in order to eradicate viruses. Sun et al. [20] tested the effect of IFNγ on alveolar macrophage-mediated pneumococcal clearance. They showed that IFNγ–deficient mice infected with influenza had a higher bacterial clearance activity. Further analysis proved that IFNγ causes a downregulation of the expression of the macrophage receptor with collagenous structure (MARCO). The MARCO-receptor is a class A scavenger membrane protein which is critical for the binding and uptake of microbes by macrophages. Previous studies demonstrated that this receptor is also of importance for the uptake of S. *pneumoniae* [28]. These results indicate that the IFNγ-mediated reaction against influenza indeed has a beneficial effect on the survival of S. *pneumoniae*, thereby stimulating the development of a secondary bacterial infection.

Another pathway by which IFN disturbs the anti-bacterial innate immune response, is the induction of apoptosis of bone-marrow neutrophils by type 1 IFN. Eventually this will lead to granulocytopenia; an abnormally low concentration of granulocytes in the blood [6]. Neutrophils are, like macrophages, very important in the initial defense against a bacterial infection. So besides the function of IFN as an immune system activating protein, it appears that it has an inhibitory effect on the scavenger role of neutrophils and macrophages.

The reason why an immunological mechanism would cause increased susceptibility to bacterial infections is unclear. A possible explanation could be the fact that an inflammatory reaction in the normally sterile lungs causes tissue damage and an impaired lung function. Therefore, during an influenza infection, part of the innate immune system is suppressed and viral clearance is performed by the adaptive system, causing less inflammation and less

recruitment of neutrophils. The disadvantage of this mechanism is an increased susceptibility to a secondary bacterial infection.

A study showing that this theory is plausible was performed by Didierlaurent et al. [8]. They tested the effect of an influenza infection on the activity of Toll-like receptors (TLRs). TLRs form a family of proteins located on the membranes of different immune cell types. Their function is the recognition of Pathogen Associated Molecular Patterns (PAMPs). PAMPs are molecules which especially originate from bacteria but also from viruses. Examples of PAMPs are flagellin, LPS, and lipoteichoic acid. Ligand binding to the TLR is associated with intracellular signaling leading to activation of the transcription factor NF-κB, which triggers cytokine and chemokine production. This should eventually lead to elimination of the pathogen. When investigating the effect of different PAMP's on alveolar macrophages isolated from post-influenza mice, a reduced production of KC, MIP-2α, and TNF-α was observed [8]. Measurement of a decline in NF-κB-activation proved the role of TLRs in this process. These results indicate that the sensitivity of TLRs on macrophages is reduced, thereby downregulating the inflammatory reaction.

Different studies confirm that influenza infection has an inhibitory effect on specific functions of the immune system. Indeed, reduction of neutrophil and macrophage numbers might have a physiological advantage by preventing inflammatory damage. Nevertheless, it is also well known that invasion of pneumococcus is stimulated by epithelial injury caused by inflammation. These two observations seem to contradict each other, however it may be possible that they both play a role in development of a secondary bacterial infection.

4 INFLAMMATORY DAMAGE

After a viral or bacterial infection, an inflammatory reaction causes release of cytokines and chemokines leading to migration of lymphocytes and macrophages to the site of infection. It has been proposed that a positive cytokine feedback loop mediated by influenza causes the accumulation of chemokines, cytokines. Due to these signals increased activated lymphocyte and macrophage numbers may cause an adverse outcome of the goal of eliminating pathogens.

Smith et al. [27] measured multiple cytokines and chemokines after influenza infection and pneumococcal challenge in mice. Although it is not unexpected that levels of immunomodulating agents are raised after infection, the result was remarkable. IL10, IL6, KC, and MIP1α concentrations were significantly enhanced. IL10 is known to have pleiotropic effects on the inflammatory response, but is generally anti-inflammatory. The role of IL10 was also investigated by van der Sluijs et al. [4], who observed a positive correlation between IL10 and bacterial outgrowth. The increase of IL10 was explained by expression of Indoleamine 2,3-Dioxygenase (IDO) by macrophages after influenza infection. IDO is a tryptophan-catabolizing enzyme which enhances inflammatory mediator responses. It is likely that the effect of IL10 on bacterial survival is mainly caused by inhibition of TNFα-production which is known to be an inflammation enhancing cytokine.

The high levels of KC and MIP1α were also interesting. These proteins are chemokines which stimulate the migration of macrophages and neutrophils to the site of inflammation. According to Smith et al. the release of KC and MIP1α will cause accumulation of neutrophils in the lungs thereby inducing acute inflammatory tissue damage.

This is contradictory to the previously mentioned observation of lower chemokine levels leading to a decrease of neutrophils and macrophages. However, the studies that showed an increased susceptibility to S. *pneumoniae* after influenza infection measured the innate immune system suppressing factors before Pneumococcal challenge. In this study inflammatory agents were shown afterwards, when epithelial damage and pneumococcal

adhesion were already initiated. This difference could mean that both mechanisms are responsible for the development of a severe Pneumococcal pneumonia.

A pathological model in which stimulation of susceptibility to bacterial infection is followed by an influenza induced mechanism which increases the severity of the infection can explain these findings. In this model influenza causes adhesion of S. *pneumoniae* both due to activation of specific receptors and by suppression of the innate antibacterial immune system. When a bacterial infection is initiated, a positive cytokine feedback loop will lead to a disturbed immune system and severe inflammatory damage.

5 PB1-F2

The PB1-F2 protein was discovered in 2001 by Chen et al. [25]. In their search for unknown influenza virus proteins encoded by alternate reading frames, they found this protein (Fig. 1). PB1-F2 originates from an open reading frame of the PB1 gene, one of the influenza polymerase proteins essential for virus replication [15]. After more investigation on the protein, several unusual features were observed [25]. An interesting result was the fact that different subtypes of PB1-F2 were found when host cells were infected with different influenza-types. This was recently confirmed by Conenello et al, who also showed that a single amino acid change (N66S) in the PB1-F2 sequence increases virulence in mice [24]. The N66S mutation was found in both the pandemic H1N1 'Spanish Flu' virus and in the H5N1 'Bird Flu' virus. This observation implies that the virulence of H5N1 is based on the same mechanism as the pandemic 1918 H1N1 variant.

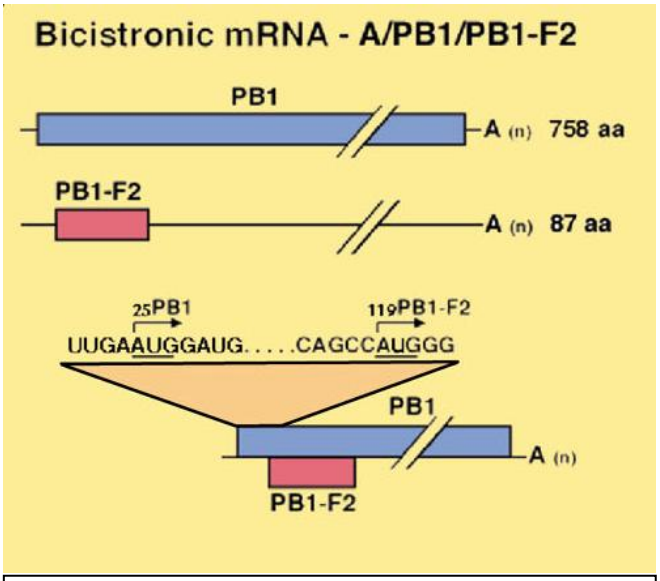

Figure 1. PB1-F2, 87-residue protein. Encoded by the +1 reading frame of PB1 [15].

After the discovery of the PB1-F2 protein, it was shown that PB1-F2 localizes to the inner and outer membrane of the mitochondria. Here, PB1-F2 causes permeabilization of the mitochondrial membrane, thereby reducing the membrane potential. This will eventually lead to apoptosis of the infected cell [23]. When a cell receives apoptosis stimulating signals, intracellular pro-apoptotic proteins are activated. The mitochondria thereby releases apoptotic mediators, such as cytochrome C. Eventually, this pathway is followed by the constitution of a permeability transition pore complex (PTPC). It is known that the proteins adenine nucleotide translocator 3 (ANT3) in the inner mitochondrial membrane and the voltage-dependent anion channel 1 (VDAC1) in the outer mitochondrial membrane are of major importance in the formation of the PTPC. In the early stage of an influenza infection, a higher sensitivity to stimulation by mitochondrial apoptotic mediators was observed [26]. However, no direct apoptosis by PB1-F2 occurred. This implicates that in early stages of infection no pore complexes are formed, possibly due to low PB1-F2 levels. In the later stage of infection apoptosis is directly caused by PB1-F2. The induction of apoptosis could proceed through three mechanisms (Fig. 2). PB1-F2 can act as a bridge between ANT3 and VDAC1, causing the formation of the pore complex. Secondly, PB1-F2 can interact with the two proteins

separately in the inner and outer mitochondrial membrane. Another possible mechanism is the formation of a multimeric complex by several PB1-F2 proteins.

Besides being a cause of increased virulence of some influenza variants, PB1-F2 might also facilitate secondary bacterial infection. It is proposed that the apoptosis-inducing mechanism explained before especially occurs in alveolar macrophages [23]. Alveolar macrophages are important cells of the respiratory innate immune system, so induction of apoptosis of these cells could cause increased bacterial survival. Apoptosis might more frequently occur in alveolar macrophages due to higher levels of PB1-F2 because it is also taken up by phagocytosis of other infected cells. However, there possibly is a more precise mechanism. Chen et al. showed that PB1-F2 induces apoptosis in a cell specific manner [25]. When comparing the effect of influenza infection on lung epithelial cells to monocytes, they found that epithelial cells survived while monocytes were highly affected and died through apoptosis. Although the exact mechanism behind this observation is yet to be discovered, it might be an important link to the cause of secondary bacterial infection.

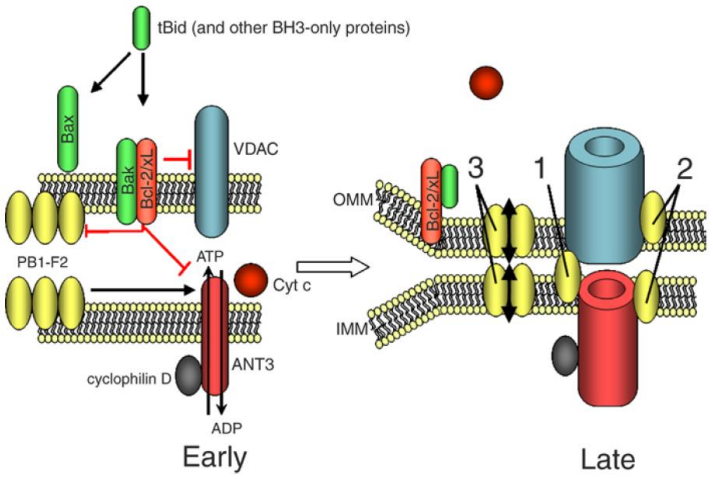

Figure 2. PB1-F2-induced mitochondrial permeabilization can proceed through three possible mechanisms, as indicated on the right graphic: (1) enhancement of the pore complex formation through direct interaction with ANT3 and VDAC1; (2) independent permeabilization of the inner and outer mitochondrial membranes with the help of ANT3 and VDAC1, respectively; and (3) direct permeabilization of the mitochondrial membranes [26].

McAuley et al [7] proposed that PB1-F2 may also contribute to the earlier mentioned positive feedback cytokine loop. Bacterial cell wall components such as lipoteichoic acids and peptidoglycan are known to activate the innate immune system through TLRs, which causes production of pro-inflammatory cytokines. This leads to apoptosis of cells sensitized by PB1-F2, thereby causing extra damage due to pneumococcal infection. Another interesting theory is the induction of production of cytokines due to direct recognition of PB1-F2 by TLRs [7].

The findings on the effects of this relatively new protein may be very relevant in the explanation of the mechanism by which influenza induces pneumococcal pneumonia. Both inflammatory damage and decreased macrophage and neutrophil numbers may not only be caused by the immune response after influenza infection. PB1-F2 is shown to affect these aspects in an independent way. The delayed effect of the influenza virus on bacterial infection can also be clarified by apoptosis-induction by PB1-F2. As explained, the formation of pore complexes in the mitochondrion was observed in the later stage of the infection. In this stage the bacterial infection is also initiated. By this mechanism the already increased susceptibility to pneumococcal invasion is worsened due to epithelial cell death, directly induced by influenza.

6 CONCLUSION

With the results of many different studies one can conclude that the synergistic interaction between the influenza virus and S. *pneumonia* is a complex, multifactorial process. The explanation of epithelial damage aiding the streptococcus in the adherence to lung epithelium is clearly not sufficient. Several specific factors induced by infection with influenza enhance the risk of development of a bacterial pneumonia.

PAFr is known to be an important receptor for pneumococcus to adhere and invade the lung epithelium. The finding that PAFr is upregulated by epithelial cells after influenza infection implicates that this protein plays a major role in the interaction between the virus and bacterium. Another factor which enhances the potential of pneumococcus to adhere to the lung tissue is NA. This sialic acid-cleaving molecule of influenza virus primes the epithelium for pneumococcal infection. The observed relation between mortality and viral NA activity indicates that this molecule indeed stimulates bacterial adhesion.

However, bacterial adherence is not the only important way by which pneumococcal pneumonia is induced. The fact that a clear increase in bacterial number is frequently observed at the time point that virus titers start to decrease, suggests an immunological reaction of the host which impairs bacterial elimination. Both type 1 and type 2 interferon are shown to be released after influenza infection, followed by reduced neutrophil and macrophage numbers in the lungs. These immune cells are important for phagocytosis of bacteria. Also, the immunological response of the host may be a cause of increased pneumococcal damage. Inflammatory damage after a bacterial infection is induced by a positive cytokine feedback loop. Studies have shown very high levels of chemokines and cytokines after influenza infection followed by pneumococcal challenge. Both these host reactions show to have an adverse effect against pneumococcal infection. However, many different factors are involved in this process.

The PB1-F2 influenza protein is also a significant factor in the relation between influenza and pneumococcus. Its pro-apoptotic effect on macrophages shows a direct way by which influenza causes pneumococcal pneumonia. Besides, cell-death after pneumococcal infection is increased due to the apoptosis sensitizing effect of the protein. This may also lead to a feedback loop where cell-death and inflammation both trigger each other.

With the threat of the development of the avian H5N1 strain into a new pandemic influenza virus, possibly as dangerous as the 1918 H1N1, we cannot underestimate the importance of studies investigating the interaction between influenza and pneumococcus. Different influenza strains with a high mortality rate showed similar factors that may influence susceptibility to pneumococcal infection. This threat raises concerns about the limited knowledge of the exact mechanisms involved in the pathway leading to pneumococcal pneumonia. As a secondary bacterial infection is a common cause of death after influenza infection, this knowledge is of major importance for the development of therapies and methods to prevent death by influenza. This paper reviewed several influenza-induced factors that are shown to stimulate infection of S. *pneumoniae.* It is very well possible that these factors influence each other in the pathway leading to damage caused by pneumococcal pneumonia. The increased bacterial adhesion may have a combined action with the reduction of neutrophil and macrophage numbers due to PB1-F2 and IFN, thereby worsening the infection. These different factors may even act synergistically. The relation between these mechanisms and their exact effect should be investigated.

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