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Toll-like receptor 5 agonist flagellin reduces influenza A virus replication independently of type I interferon and interleukin 22 and improves antiviral efficacy of oseltamivir

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ABSTRACT

Influenza infections remain a burden on health care systems despite vaccination programs and marketed antiviral drugs. Immunomodulation through activation of innate sensors could represent innovative approaches to fight the flu. This study evaluated the ability of flagellin, agonist of Toll-like receptor 5 (TLR5), to control the replication of influenza A virus (IAV) in mice. First, we showed that systemic or intranasal administration of flagellin activated transcription of anti-viral genes in lung tissue. Prophylactic and therapeutic flagellin administration resulted in decreased levels of viral RNA and infectious virus in the lungs of H3N2 IAV-infected mice. The effect of the flagellin on viral replication was also observed in *Ifnar*$^{-/-}$ and *Il22*$^{-/-}$ IAV-infected mice, suggesting a mechanism independent of type I interferon and interleukin 22 signaling. In addition, a combination therapy associating the neuraminidase inhibitor oseltamivir and flagellin was more effective than standalone treatments in reducing pulmonary viral replication. Thus, this study highlights the therapeutic potential of the flagellin to control the replication of the influenza virus.

1. Introduction

Influenza viruses are responsible for human seasonal epidemics and pandemic outbreaks and cause respiratory illness in humans with the potential for severe complications in young children, the elderly, immuno-compromised individuals, and patients with chronic cardiovascular or respiratory diseases. Annually, 3–5 million cases of serious illness are caused by influenza virus infections resulting in 250,000 to 500,000 deaths worldwide (Horimoto and Kawaoka, 2005). Therapeutic interventions are limited to sub-optimal vaccination and few drugs. Among them, neuraminidase inhibitors such as oseltamivir are widely used for the oral treatment of uncomplicated acute illness due to influenza but are also recommended for pre-exposure prophylaxis among at-risk individuals in contact with flu patients (Fiore et al., 2011).

Because of the narrow therapeutic window of the neuraminidase inhibitors and the recurrent emergence of drug-resistant strains, new antiviral strategies are needed (Govorkova et al., 2013). The stimulation of innate immunity could be an alternative therapeutic approach to current treatments as it induces local production of antimicrobial molecules and the recruitment of effector cells involved in controlling infection (Hancock et al., 2012; Iwasaki and Pillai, 2014). The Toll-like receptors (TLRs) are sensors of innate immunity involved in the recognition of conserved microbe-associated molecular patterns (Kawai and Akira, 2010). The activation of TLRs by microbial components triggers signaling cascades and promotes the archetypal pro-inflammatory responses involved in antimicrobial defense (Kawai and Akira, 2010). In view of their broad cellular distribution and important role in immunity, TLRs have emerged as therapeutic targets against bacterial and viral infections (Hancock et al., 2012; Savva and Roger, 2013; Hedayat et al., 2011; Mifsud et al., 2014).

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In this context, experimental models showed that various agonists of TLRs could protect against influenza virus infections: agonists for TLR2, TLR3, TLR7, TLR8 and TLR9, as standalone treatments or in combination, stimulate innate immune responses able to protect against influenza A virus (IAV) infection in mammals as well as in poultry (Tan et al., 2012; Mifsud et al., 2016; Tuvim et al., 2012; Wong et al., 2009; Lau et al., 2010; Zhao et al., 2012; Jiang et al., 2011; Norton et al., 2010; Wu et al., 2007; Hammerbeck et al., 2007; Barjesteh et al., 2015). Interestingly, most of the agonists were administrated through the respiratory route as prophylaxis, indicating that the pre-stimulation of the local innate immune response can control the viral replication.

TLR5 recognizes flagellin, the structural protein of bacterial flagella and is expressed at the surface of macrophages, dendritic cells and epithelial cells. Like other TLR agonists, flagellin has immunomodulatory properties that can control numerous bacterial infections (Munoz et al., 2010; Yu et al., 2010; Jarchum et al., 2011; Kinnebrew et al., 2010; Vijayan et al., 2018). Recently, it has also been shown that repeated systemic administration of flagellin prevents rotavirus infection in mice through a mechanism dependent on interleukin 22 (IL-22) and IL-18 (Zhang et al., 2014). Prophylactic systemic administration of flagellin also protects mice against lethal infection with cytomegalovirus (Hossain et al., 2014). We recently demonstrated in mice that intranasal administration of flagellin improves the therapeutic index of antibiotics in the treatment of post-influenza virus bacterial pneumonia (Porte et al., 2015), but the role of flagellin in the control of influenza virus replication has never been studied. However, the capacity of flagellin to trigger the production of host antimicrobial molecules and the recruitment of innate cells after intranasal administration presages a role for flagellin against influenza viruses (Porte et al., 2015; Vijayan et al., 2018; Van Maele et al., 2014a).

In the present study, we analyzed the pulmonary antiviral response in mice upon respiratory or systemic flagellin administration, and tested the ability of flagellin to interfere with IAV replication. We also evaluated the role of type I interferons and IL-22 in the flagellin-mediated antiviral mechanism, as well as the potential impact of a combination of flagellin with oseltamivir on IAV production.

2. Materials & methods

2.1. Mouse model of infection

Female or male C57BL/6J (6- to 12-week-old) mice (Janvier Laboratories, Saint-Berthevin, France) and female or male *Il22*$^{-/-}$ and *Ifnar*$^{-/-}$ mice (backcrossed on C57BL/6J) were maintained in individually ventilated cages and handled in a vertical laminar flow cabinet (class II A2; Esco, Hatboro, PA). All experiments complied with current national and institutional regulations and ethical guidelines (B59-350,009, Institut Pasteur de Lille protocol number: 2015121722376405). For the viral infection, mice were anesthetized by intraperitoneal injection of 1.25 mg of ketamine plus 0.25 mg of xylazine in 250 µl of phosphate buffered saline (PBS), and then intranasally infected with 50 µl of PBS containing 30 plaque forming units (PFU) of the highly pathogenic murine-adapted H3N2 influenza A virus strain Scotland/20/74 (Ivanov et al., 2013; Paget et al., 2012). The lethal dose 50 (LD50) of the H3N2 strain is 68 PFU in C57BL/6 mice. At selected time-points, mice were sacrificed through intraperitoneal injection of 5.47 mg of sodium pentobarbital in 100 µl of PBS, and their lungs were aseptically recovered.

2.2. Flagellin administration

The native flagellin (FliC, GenBank accession no. **AAL20871**) was isolated from the *Salmonella enterica* serovar Typhimurium strain SIN22, as described previously (Nempont et al., 2008; Didierlaurent et al., 2008). Using the Limulus assay (Associates of Cape Cod, Inc., East Falmouth, MA), the residual LPS concentration was determined to be < 20 pg per µg of flagellin. To ensure that flagellin was mostly monomeric, it was heated for 10 min at 65 °C before use. Flagellin was administered either intranasally (5 µg of FliC in 30 µl of PBS), under light anesthesia by inhalation of isoflurane (Axience, Pantin, France) either intraperitoneally (5 µg of FliC in 200 µl of PBS).

2.3. Oseltamivir administration

Tablets of oseltamivir phosphate (Tamiflu® 30 mg, Roche Pharma AG, Grenzach-Wyhlen, Germany) were solubilized in 1 ml of sterile water to a 30 mg/ml stock concentration, vortexed, and sonicated in a water bath at room temperature for 5 min. Mice were given oseltamivir by oral gavage once a day at 4 mg/kg/day diluted in sterile water, in a total volume of 200 µl.

2.4. Viral and murine RNA analysis

Total RNA was extracted with the NucleoSpin RNA II kit (Macherey-Nagel, Duren, Germany). For H3N2 RNA detection, 500 ng of total lung RNA were reverse transcribed with Superscript II Reverse Transcriptase (Invitrogen) in the presence of the IAV specific primer targeting the segment 7 which encodes the matrix protein 1 (M1) (5′ TCTAACCGA GGTCGAAACGTA 3'). The cDNA was amplified by Taqman real-time PCR using Taqman probe FAM-TTTGTGTTCACGCTCACCGTGCC-TAMRA with forward primer: AAGACCAATCCTGTCACCTCTGA and reverse primer: CAAAGCGTCTACGCTGCAGTCC. A plasmid coding the M1 gene was serially diluted to establish a standard curve (Ct values/plasmid copies) (Meyer et al., 2017). Equivalent of 12.5 ng of total lung RNA was thoroughly used to determine the level of M1 RNA in the lung of infected animals by absolute quantification (Meyer et al., 2017).

For mouse gene expression quantification, total lung RNAs were reverse transcribed with the high-capacity cDNA archive kit and random primers (Applied Biosystems, Foster City, CA). The cDNA was amplified using SYBR green-based real-time PCR using primers listed in Supplementary Table 1. Relative mRNA levels ($2^{-\Delta\Delta Ct}$) were determined by comparing first the PCR cycle thresholds (Ct) for the gene of interest and β-actin, the housekeeping gene (ΔCt) and second, the ΔCt values for the treated and untreated (mock) groups (ΔΔCt). The Ct threshold was set to 35 cycles.

PCR experiments were all performed and analyzed using a QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems).

2.5. Determination of infectious lung viral titers

For determination of the viral load in the lungs, mice were sacrificed at the indicated time point and lungs were collected, weighted and immediately frozen in liquid nitrogen. Lungs were then homogenized with an Ultra-Turrax homogenizer (IKA-Werke, Staufen, Germany), centrifuged (2500 × *g*, 5 min, 4 °C), and the supernatants were kept at 80 °C until viral titration by standard plaque assay (Hatakeyama et al., 2005). Briefly, 6 serial 10-fold dilutions of samples were prepared in Eagle's Minimum Essential Medium (EMEM, Lonza, Verviers, Belgium) complemented with trypsin 1 µg/ml, and subsequently inoculated onto confluent Madin-Darby Canine Kidney (MDCK) cell monolayers prepared in 6-well plates. After 1 h incubation for viral adsorption, the inoculum was removed and wells were covered with 2 ml of overlay medium (1% Noble agar in EMEM) with trypsin 1 µg/ml. The plates were incubated at 37 °C under 5% CO2 and viral titers in plaque forming units (PFU) were determined 3 days after inoculation.

2.6. Cytokine assays

Serum IL-22 levels were measured by enzyme-linked immunosorbent assay (ELISA) following manufacturer's instructions (eBioscience, Thermo Fisher Scientific, Waltham, MA).

2.7. Statistical analysis

The results are expressed as means or medians ± standard deviations (SD). Statistical differences were analyzed using the Mann-Whitney test or one-way ANOVA assay with Dunnett's multiple comparison test (GraphPad Prism5.0a) and were considered to be statistically significant for P values < 0.05.

3. Results

3.1. Flagellin induces expression of anti-viral genes in the lung

Previously, microarrays were performed on lungs of C57BL/6 mice at 2, 4 and 18 h after a single intranasal administration of flagellin. Analysis of the transcriptional changes upon flagellin treatment were compared to PBS (mock)-treated animals and highlighted gene pathways related to granulocyte adhesion and diapedesis, acute phase response, or recognition of microbes through pattern recognition receptors, among others (http://mace.ihes.fr, accession no. 2176499768) (Fougeron et al., 2015). The transcriptomic analysis also revealed that flagellin activates the pathways involved in antiviral responses, the impact of flagellin on these pathways appearing to be greater at 4 h than at 2 or 18 h (Table 1). We then focused on the genes involved in the different pathways and identified a set of genes which the expression significantly increased upon flagellin stimulation (fold increase > 2) (Table 2). Different profiles of gene expression were observed. The gene encoding for the cholesterol 25-hydroxylase (*Ch25h*), which converts cholesterol to a soluble 25-hydroxycholesterol and blocks viral fusion (Blanc et al., 2013; Liu et al., 2013) was found to be upregulated at 2 and 4 h compared to the mock group. Expression of some genes was gradually upregulated from 2 to 4 h post-treatment. This includes the interferon gamma inducible 47 and 204 genes (*Ifi47* and *Ifi204*) encoding antiviral GTPase and an antiviral innate immune sensor (Collazo et al., 2001; Conrady et al., 2012), and the interferon-induced transmembrane protein 5 and 6 *(Ifitm5* and *Ifitm6*) that belong to a family of inhibitors of viral entry (Huang et al., 2011; Brass et al., 2009). Finally, other genes were exclusively upregulated at 4 h post-treatment. This is the case of the genes encoding the radical S-adenosyl methionine domain containing 2 (*Rsad2)* and the interferon-stimulated gene 15 ubiquitin-like modifier *(Isg15)* which products interferes with the formation of the lipid raft during the virus budding (Wang et al., 2007) and targets newly translated viral proteins for modification, respectively (Lenschow et al., 2007; Morales and Lenschow, 2013). For all the genes, the baseline expression was recovered after 18 h indicating a transient effect of the flagellin.

The transcriptomic study was confirmed by testing the expression of the identified genes by real time qPCR, in the lung at 2 and 4 h after intranasal instillation of flagellin (Fig. 1A). We also analyzed the transcriptional response of the selected genes after systemic administration of flagellin (Fig. 1B). Interestingly, the transcriptomic response for the selected genes was globally similar between the two routes of administration, demonstrating that a systemic administration of flagellin can activate antiviral genes in the lung tissue. We also observed (i) a reduced variation in the gene expression after the systemic route compared to the intranasal route and (ii) a peak expression at 4 h post-flagellin administration thus confirming the transcriptomic analysis. As expected, flagellin was able to stimulate > 50-fold expression of *Ccl20* and *Saa3*, surrogate markers of effective stimulation of lung innate immunity by flagellin (Van Maele et al., 2010).

3.2. Systemic and mucosal flagellin treatment induces a decrease of influenza genomic RNA levels

We then tested in an experimental model of infection whether the flagellin-mediated antiviral transcriptomic response was associated with a decrease in IAV replication in the lung. The model consists of the intranasal inoculation of mice with a sublethal dose of H3N2 influenza A virus (IAV, 30 PFU), combined with intranasal treatments with 5 µg of flagellin (or PBS as control intervention) every 24 h, starting 12 h prior infection (Fig. 2A). Viral replication was followed by measuring the RNA levels of M1 genomic RNA in lung homogenates at 24, 48 and 96 h post-infection, through absolute quantification using Taqman-based real time PCR with a calibrating curve. We found an increase in H3N2 M1 RNA levels from 24 to 96 h post-infection, hence indicating efficient viral replication (Fig. 2B). At 48 h post-infection, the level of M1 RNA copies in lung of flagellin-treated animals was 7.3 fold lower than in mice receiving PBS, indicating an effect of the flagellin on the viral RNA replication. A similar yet milder trend (2.3 fold decrease compared to PBS-treated mice) was also observed at 96 h post-infection (Fig. 2B).

As flagellin showed antiviral properties through the systemic route (Zhang et al., 2014; Hossain et al., 2014), we also treated the H3N2-infected animals with 5 µg of flagellin through the intraperitoneal route (Fig. 2C). Interestingly, when IAV-infected animals were treated by flagellin, we observed an 18.3 fold decrease on viral RNA levels at 48 h post-infection compared to the PBS-treated group. A significant decrease (1.9 fold) of the M1 RNA levels was also observed at 96 h post-infection in the flagellin-treated group. These results indicate that systemic administration of flagellin has a stronger effect on viral replication than intranasal administration.

3.3. The decrease of viral RNA levels upon flagellin administration is independent of type I interferon signaling

Most of the genes identified in the transcriptomic study (Table 2) are under the control of type I interferon (IFNα and IFNβ), therefore suggesting a role of these interferons in the flagellin-mediated effect. To assess the putative role of type I IFN signaling, the levels of viral RNA were quantified after flagellin treatment in mice deficient in the type I interferon receptor *($Ifnar^{-/-}$)*. These mice were infected by IAV H3N2 and treated with flagellin by systemic administration before monitoring viral RNA in lungs as mentioned before (Fig. 2A). Flagellin induced significant 3.6 and 9 fold decrease of IAV M1 RNA at 48 and 96 h, respectively (Fig. 3). These results show that type I IFN signaling is not

Table 1
Antiviral canonical pathways up-regulated by flagellin.

Canonical pathways	p-values[a]		
	2 h	4 h	18 h
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	3.55×10^{-9}	7.08×10^{-10}	7.24×10^{-4}
Activation of IRF by Cytosolic Pattern Recognition Receptors	4.68×10^{-7}	3.31×10^{-9}	4.92×10^{-1}
Role of PKR in Interferon Induction and Antiviral Response	1.02×10^{-6}	7.94×10^{-12}	2.30×10^{-2}
Role of RIG1-like Receptors in Antiviral Innate Immunity	3.63×10^{-6}	9.33×10^{-6}	1.12×10^{-1}
NF-κB Activation by Viruses	6.76×10^{-3}	1.29×10^{-6}	1.82×10^{-4}

[a] Whole-genome expression microarrays were performed using lungs from C57BL/6 mice (n = 3–5 per group) that were treated intranasally with flagellin for 2 h, 4 h and 18 h and compared to mock-treated mice (http://mace.ihes.fr; accession no. **2176499768**) (Fougeron et al., 2015). After NeoNORM analysis, the gene lists containing group means of expression, p-values and standard fold changes were utilized as input for analysis of canonical pathways with Ingenuity Pathway Analysis 1.0 software (IPA, Ingenuity Systems).

Table 2
Intranasal administration of flagellin induces the transcription of anti-viral genes in the lung.

Gene ID	Gene symbol	Gene name	Fold increase[a]		
			2 h	4 h	18 h
12642	*Ch25h*	cholesterol 25-hydroxylase	13.44	12.42	2.88
875318	*Ifi204*	interferon gamma-inducible protein 16	4.64	7.07	< 2
213002	*Ifitm6*	interferon induced transmembrane protein 6	4.23	7.82	< 2
73835	*Ifitm5*	interferon induced transmembrane protein 5	3.24	3.45	< 2
15953	*Ifi47*	interferon gamma inducible protein 47	3.08	4.42	< 2
58185	*Rsad2*	radical S-adenosyl methionine domain containing 2	< 2	10.94	< 2
100038882	*Isg15*	ISG15 ubiquitin-like modifier	< 2	6.93	< 2

Whole-genome expression microarrays were performed using lungs from C57BL/6 mice (n = 3–5 per group) that were treated intranasally with flagellin for 2 h, 4 h and 18 h and compared to mock-treated mice (http://mace.ihes.fr; accession no. 2176499768) (Fougeron et al., 2015).

[a] The fold increase values correspond to the differential expression of genes at the indicated time after flagellin stimulation compared to mock-treated condition.

required for the flagellin-mediated reduction of IAV replication. This is also consistent with microarray data that did not indicate any upregulation of IFNα − or IFNβ-specific transcripts in the lungs of flagellin-treated animals (http://mace.ihes.fr, accession no. 2176499768). Furthermore, systemic or respiratory administration of flagellin did not increase the pulmonary expression of *Ifnl2* or *Ifnl3* coding for type III IFN that were previously associated with influenza virus clearance in mice (data not shown) (Mordstein et al., 2008). Altogether, these results strongly suggest that interferons are not associated with the flagellin-mediated antiviral effect.

3.4. The effect of flagellin on viral RNA replication is independent of interleukin 22 (IL-22)

Flagellin has previously been shown to protect against rotavirus intestinal infection through production of IL-22 together with IL-18 (Zhang et al., 2014). First, we found that flagellin did not stimulate *Il18* transcription in the lungs after intraperitoneal administration of flagellin both in

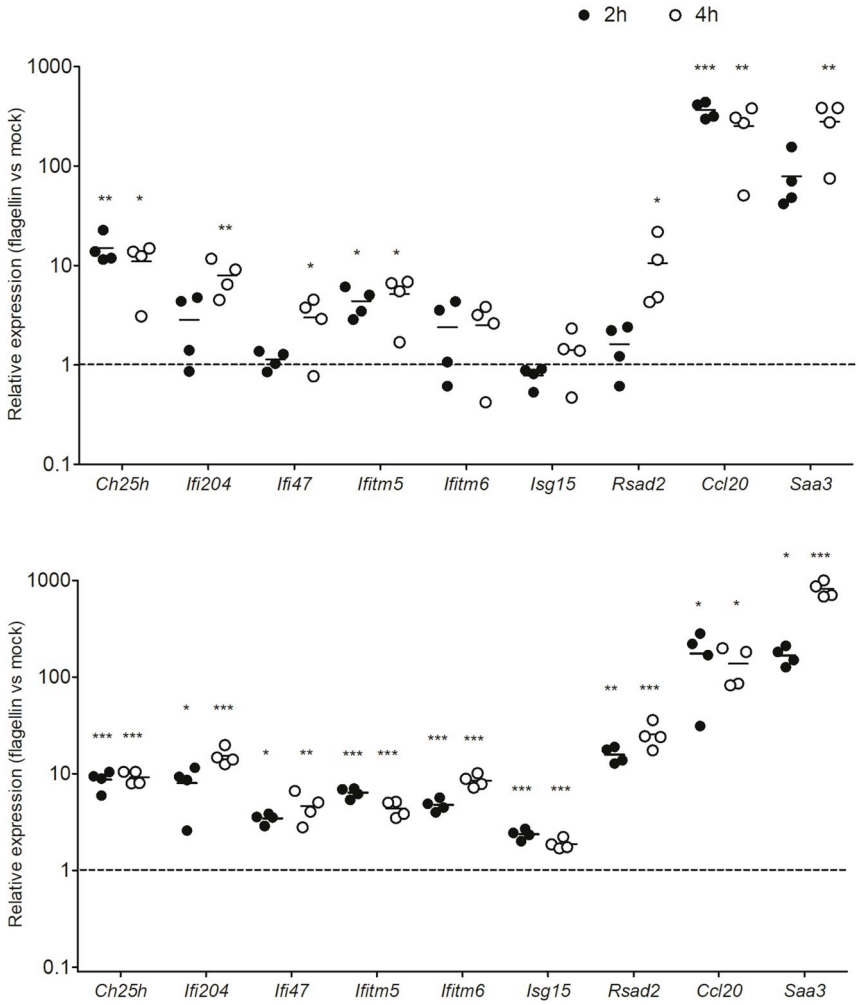

Fig. 1. Intranasal or systemic administration of flagellin activates antiviral genes in the lung. C57BL/6 male mice (n = 4) were treated intranasally (A) or intraperitoneally (B) with 5 μg of flagellin in phosphate-buffered saline. Lungs were sampled at 2 h and 4 h and mRNA was quantified by qRT-PCR. Relative expression was normalized to the expression of mock animals (arbitrarily set to a value of 1). Results are represented as the mean. Statistical significance relative to mock group was assessed by one-way ANOVA with Dunnett's multiple comparison test (*: P < 0,05; **: P < 0,01, ***: P < 0.001).

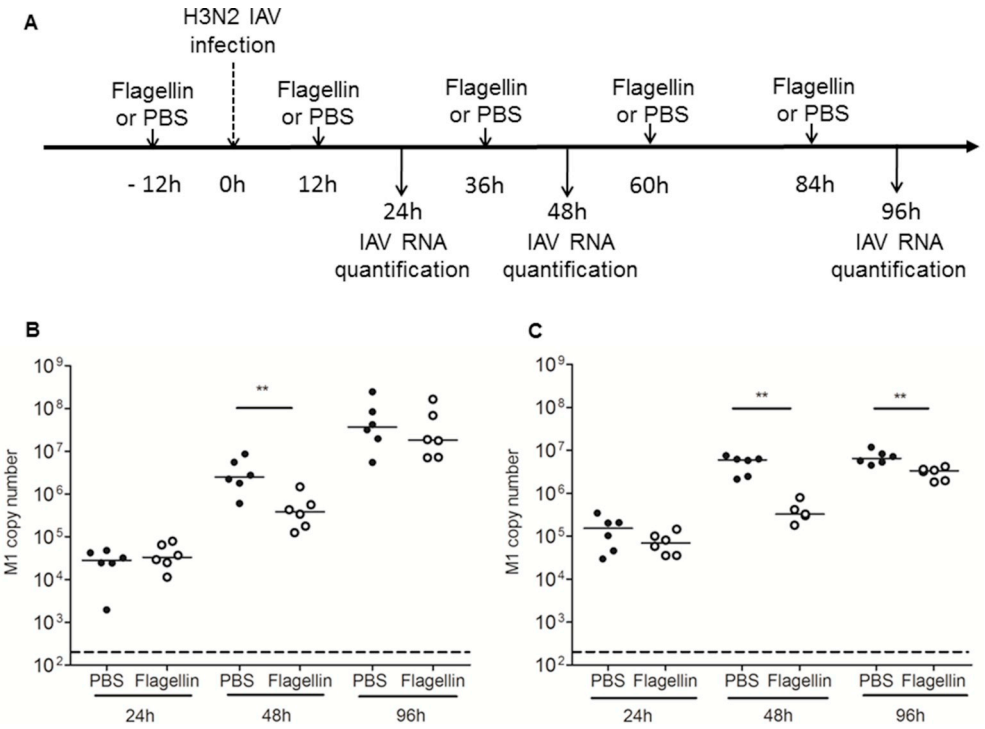

Fig. 2. Systemic and mucosal administration of flagellin altered viral RNA replication in the lung. (A) Male C57BL/6 mice (n = 5 to 6) were infected intranasally with H3N2 (30 PFU). Twelve hours before infection and 12, 36, 60 and 84 h post-infection, mice received 5 µg of flagellin intranasally (B) or intraperitoneally (C). Lungs were sampled 24, 48 or 96 h post infection for viral RNA quantification using quantitative RT-PCR. Values correspond to the absolute copy number of the M1 viral RNA/12.5 ng of total lung RNA. The solid line corresponds to the median value and the dashed line represents the detection threshold. A representative experiment out of two is shown. Statistical significance was assessed by Mann-Whitney test (**: P < 0,01).

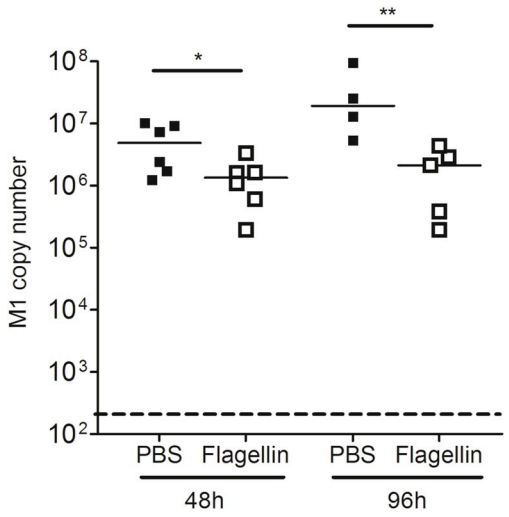

Fig. 3. The type I interferon pathway is not required for the flagellin-mediated effect on viral RNA production. *Ifnar*$^{-/-}$ mice (n = 4 to 6) were infected intranasally with H3N2 (30 PFU) and treated intraperitoneally with 5 µg of flagellin 12 h before infection and 12, 36, 60 and 84 h post-infection. Lungs were sampled 48 and 96 h post infection for viral RNA quantification using quantitative RT-PCR. Values correspond to the absolute copy number of the M1 viral RNA/12.5 ng of total lung RNA. The solid line corresponds to the median value and the dashed line represents the detection threshold. Statistical significance was assessed by Mann-Whitney test (*: P < 0.05; **: P < 0.01).

naive and IAV-infected animals, thus ruling out the possible role of IL-18 in flagellin-mediated anti-IAV effect (Supplementary Fig.). We next evaluated the role of IL-22 upon flagellin-administration in the IAV respiratory infection context. We first showed that systemic administration of flagellin induced a strong production of IL-22 in the blood 2 h post administration both in non-infected and H3N2-infected animals (Fig. 4A and B). Strong expression of *Il22* was also observed in the lungs of naïve mice treated with flagellin (Supplementary Fig.). The contribution of IL-22 in the flagellin-mediated anti-viral effect was then analyzed in *Il22*$^{-/-}$ mice by measuring the levels of viral RNA at 48 h post infection after flagellin treatments (Fig. 4A). As in IL-22 proficient mice, IAV RNA levels were significantly lower (7.5 fold decrease) in flagellin-treated *Il22*$^{-/-}$ mice than in PBS-receiving *Il22*$^{-/-}$ mice (Fig. 4C). These results indicate that IL-22, which is produced upon flagellin stimulation, is not required for the effect of flagellin on viral RNA production.

3.5. Systemic administration of flagellin boosts the anti-viral effect of oseltamivir

We then investigated whether the reduction of viral RNA induced by flagellin could be improved by combination with a specific virus-targeted antiviral drug such as the neuraminidase inhibitor oseltamivir. This first line antiviral agent is recommended as *per os* administration to populations exposed to IAV-infected individuals for either prophylaxis and/or therapy. Mice were treated 12 h prior H3N2 infection and then 12 and 36 h post infection with either flagellin, oseltamivir or a combination of both compounds. A group of mice received PBS as untreated control. Viral RNA replication and virus production were analyzed 48 h post viral challenge (Fig. 5A). First, we found that flagellin treatment statistically decreased the infectious virus titer by 4.5 fold thus confirming the impact of flagellin on the viral RNA levels (Fig. 5B and C). Interestingly, 5 µg of intranasal flagellin was as efficient as 4 mg/ml of oral oseltamivir to control the RNA replication and IAV production. The combination of flagellin and oseltamivir significantly reduced the levels of viral RNA and viral infectious particles by 38 and 163 fold, respectively, compared to untreated animals (Fig. 5B and C). The combination therapy is also more effective at reducing RNA replication than flagellin or oseltamivir standalone treatments. We did not observe any significant reduction of the infectious particles with the combination therapy compared to the standalone treatments. However, in 50% of flagellin + oseltamivir-treated animals, PFU numbers were below the limit of detection compared to 28% for the oseltamivir group and 0% for the flagellin group. These results suggest that a combination of flagellin and oseltamivir may enhance the antiviral effect of each compound taken separately.

4. Discussion

Immunomodulation targeting TLR signaling represents a promising

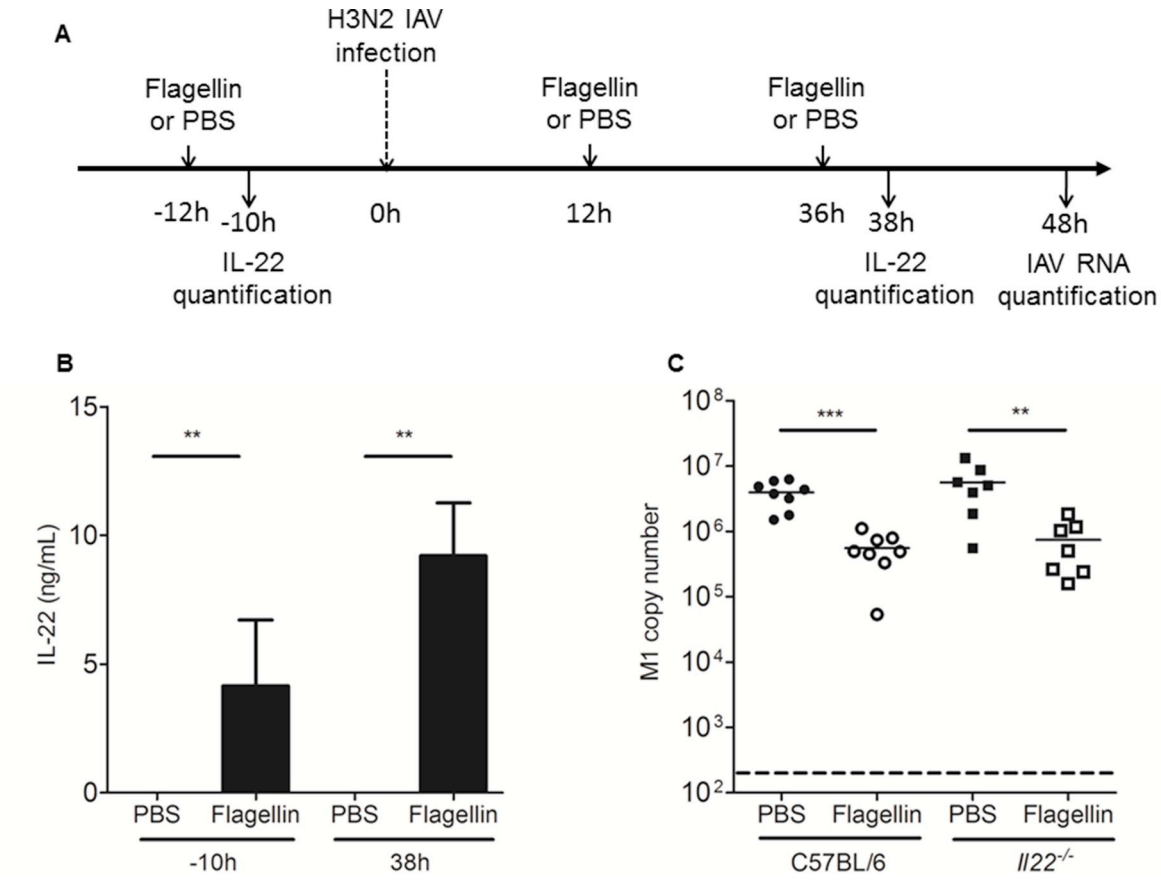

Fig. 4. IL-22 is not required for flagellin-mediated decrease of viral H3N2 M1 RNA levels. (A) Male C57BL/6 or *Il22*$^{-/-}$ mice (n = 7) were infected intranasally with H3N2 (30 PFU). Twelve hours before infection and 12 and 36 h post-infection, mice received intraperitoneally 5 µg of flagellin or PBS as control. (B) Blood was sampled in C57BL/6 mice 10 h before infection and 38 h post-viral challenge for serum cytokine analysis. IL-22 levels in serum were measured by ELISA. Results are given as mean with standard deviation. As expected IL-22 was not detected in *Il22*$^{-/-}$ mice (data not shown). Statistical significance (**: $P < 0.01$) compared to PBS-treated group was assessed by Mann-Whitney test. (C) Lungs were sampled at 48 h post-infection for viral RNA quantification using quantitative RT-PCR. Values correspond to the absolute copy number of M1 viral RNA/12.5 ng of total lung RNA. The solid line corresponds to the median value and the dashed line represents the detection threshold. One representative experiment out of two is shown. Statistical significance was assessed by Mann-Whitney test (**: $P < 0.01$, ***: $P < 0.001$).

approach to control infectious diseases (Savva and Roger, 2013; Hedayat et al., 2011; Mifsud et al., 2014). In the present study, we showed for the first time that treatment of mice with the TLR5 agonist flagellin alters influenza virus replication in the lung, independently of the route of administration (systemic or respiratory). We know from several infectious models that the biological effect of flagellin is at least in part dependent on the administration route (Vijayan et al., 2018): mucosal administration rather stimulates the epithelium, resulting in the recruitment of neutrophils and the production of antimicrobial molecules, while a systemic treatment activates dendritic cells that lead to the activation of T helper and type 3 innate lymphoid cells.

Nevertheless, the mechanism by which flagellin reduces influenza replication is yet unknown. As flagellin mainly affects viral RNA production, one can speculate that the TLR5 agonist affects the genomic transcription and replication of the influenza virus. Replication of IAV is a complex mechanism involving many host factors (Villalon-Letelier et al., 2017). It would be relevant to know whether these molecules are modulated upon flagellin stimulation and what stage(s) of the viral RNA production is impacted by flagellin (negative-sense viral RNA replication, complementary positive sense RNA production or viral RNA transcription).

Our results also reinforced the proof-of-concept that flagellin is able to trigger antiviral defenses (Zhang et al., 2014; Hossain et al., 2014). Hossain et al. showed in a model of liver colonization by the murine CMV that the decrease of viral load by flagellin correlated with the presence of NK cells suggesting a major role of these cells (Hossain et al., 2014). A detailed analysis of pulmonary cellular populations is required to evaluate the participation of NK cells but also other immune cells in flagellin-mediated control of IAV replication. In the case of rotavirus intestinal infection, Zhang et al. demonstrated that IL-22 was required for flagellin-mediated protection (Zhang et al., 2014). IL-22 is involved in host defense mechanisms at the respiratory and intestinal mucosal surfaces and is produced after systemic administration of flagellin in mice. (Van Maele et al., 2010, 2014b; Kinnebrew et al., 2012). In our study, although we confirmed the IL-22 production after flagellin administration, IL-22-deficient mice still managed to reduce M1 RNA upon flagellin treatment, indicating that this cytokine was dispensable for the antiviral effect of flagellin. These results are in accordance with prior studies showing that IL-22, produced in the blood and lungs during IAV infection, is not involved in the control of the IAV replication (Guo and Topham, 2010; Ivanov et al., 2013).

Surprisingly, we also demonstrated that the effect of flagellin on viral replication was independent of type I IFN, yet recognized as the main first line of defense against viruses. This type I IFN independent anti-influenza response was also demonstrated with TLR2 agonists as well as with the association of TLR2/6 and TLR9 agonists, hence suggesting that antiviral pathways induced through stimulation of these TLRs and TLR5 are independent of the canonical IFN response (Tan et al., 2012; Tuvim et al., 2012). Nevertheless, we found that flagellin activates several interferon-stimulated genes (ISG), which may indicate the possibility of a TLR5-dependent, interferon-independent activation of these genes.

We previously showed that administration of flagellin through the respiratory tract could improve the therapeutic index of antibiotics in the

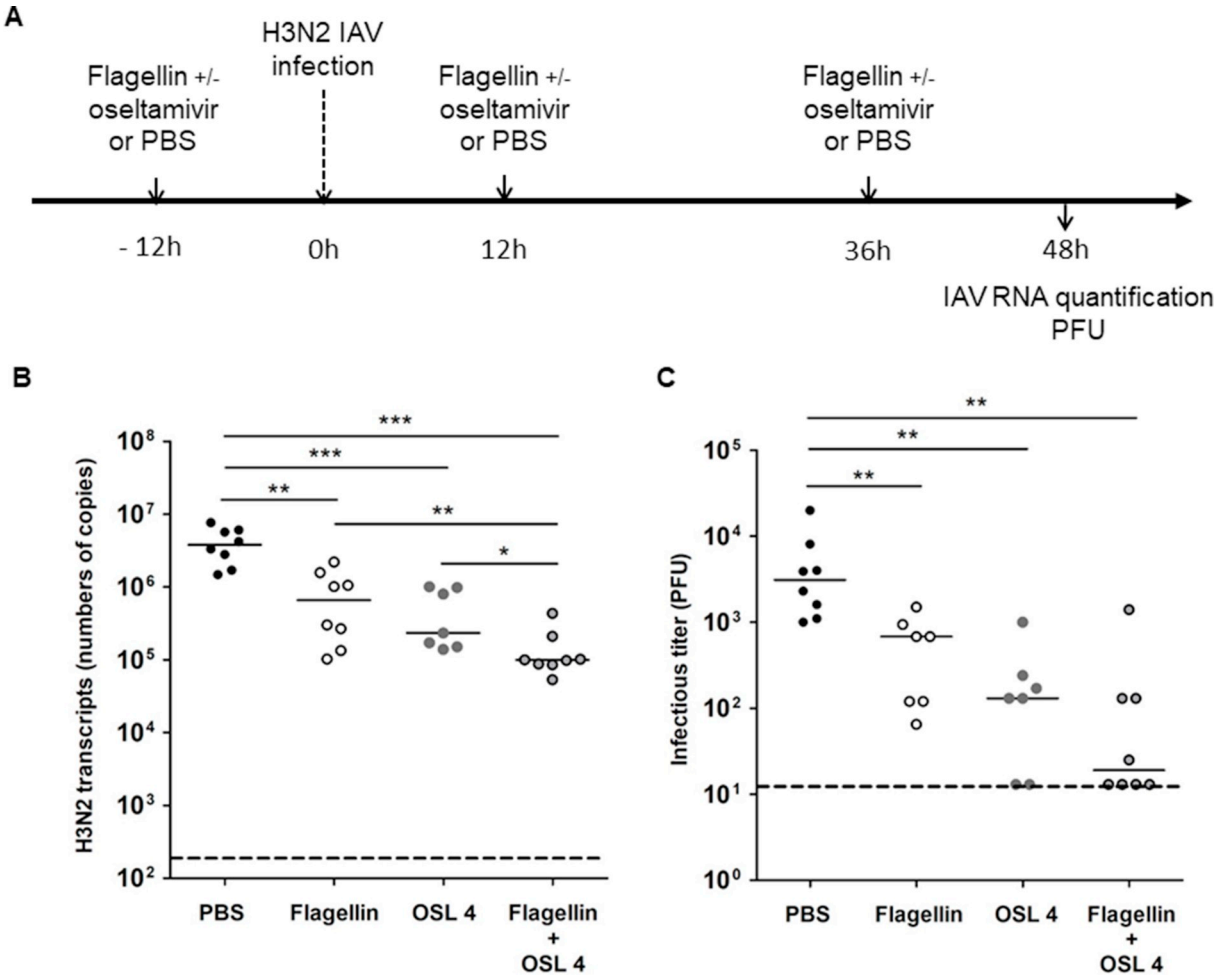

Fig. 5. Systemic administration of flagellin boosts the effect of oseltamivir. (A) Male C57BL/6 mice (n = 11) were infected intranasally with H3N2 (30 PFU). Twelve hours before infection and 12 and 36 h post-infection, mice received either 5 μg of flagellin intraperitoneally, 4 mg/kg of oseltamivir (OSL 4) intragastrically, or a combination of flagellin with oseltamivir. A group of mice was left untreated (PBS). Lungs were sampled 48 h post infection. (B) Viral RNA quantification using quantitative RT-PCR. Values correspond to the absolute copy number of M1 viral RNA/12.5 ng of total lung RNA. (C) Infectious viral titers defined as plaque forming units (PFU)/g of lung tissue. The solid line corresponds to the median value. The dashed line represents the detection threshold. Statistical significance was assessed by Mann-Whitney test (*: P < 0.05; **: P < 0.01; ***: P < 0.001).

context of pneumococcal infection and post-IAV bacterial pneumonia (Porte et al., 2015). Here, we demonstrated that the combination of flagellin and oseltamivir strongly decreases the level of viral M1 RNA, significantly enhancing the antiviral effect of each standalone treatment. In that regard, the effect of a therapy combining a TLR agonist and a specific influenza-targeted antiviral molecule like oseltamivir has been described elsewhere. Indeed, Lau et al. showed that co-administration of TLR3 agonist with oseltamivir reduced pulmonary viral titers of mice infected with H5N1 virus, compared to oseltamivir alone (Lau et al., 2010). More recently, Leiva-Suarez et al. reported that aerosol administration of TLR2/6 and TLR9 agonists associated with oseltamivir improved mouse survival after lethal influenza A pneumonia (Leiva-Juarez et al., 2018). Altogether, our data contribute to highlight the therapeutic potential of the association of molecules with distinct modes of action: a neuraminidase inhibitor with a direct effect on viral infectivity but with a narrow therapeutic window, and stimulators of innate immunity acting on host cells by inducing antiviral pathways with longer lasting effect.

Declaration of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.05.002.

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