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COVID-19 adenovirus vector vaccine induces higher interferon and pro-inflammatory responses than mRNA vaccines in human PBMCs, macrophages and moDCs

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ABSTRACT

Background: During the COVID-19 pandemic multiple vaccines were rapidly developed and widely used throughout the world. At present there is very little information on COVID-19 vaccine interactions with primary human immune cells such as peripheral blood mononuclear cells (PBMCs), monocyte-derived macrophages and dendritic cells (moDCs).
Methods: Human PBMCs, macrophages and moDCs were stimulated with different COVID-19 vaccines, and the expression of interferon (IFN-λ1, IFN-α1), pro-inflammatory (IL-1β, IL-6, IL-8, IL-18, CXCL-4, CXCL-10, TNF-α) and Th1-type cytokine mRNAs (IL-2, IFN-γ) were analyzed by qPCR. In addition, the expression of vaccine induced spike (S) protein and antiviral molecules were studied in primary immune cells and in A549 lung epithelial cells.
Results: Adenovirus vector (Ad-vector) vaccine AZD1222 induced high levels of IFN-λ1, IFN-α1, CXCL-10, IL-6, and TNF-α mRNAs in PBMCs at early time points of stimulation while the expression of IFN-γ and IL-2 mRNA took place at later times. AZD1222 also induced IFN-λ1, CXCL-10 and IL-6 mRNA expression in monocyte-derived macrophages and DCs in a dose-dependent fashion. AZD1222 also activated the phosphorylation of IRF3 and induced MxA expression. BNT162b2 and mRNA-1273 mRNA vaccines failed to induce or induced very weak cytokine gene expression in all cell models. None of the vaccines enhanced the expression of CXCL-4. AZD1222 and mRNA-1273 vaccines induced high expression of S protein in all studied cells.
Conclusions: Ad-vector vaccine induces higher IFN and pro-inflammatory responses than the mRNA vaccines in human immune cells. This data shows that AZD1222 readily activates IFN and pro-inflammatory cytokine gene expression in PBMCs, macrophages and DCs, but fails to further enhance CXCL-4 mRNA expression.

1. Introduction

Although we have entered the fourth year of the COVID-19 pandemic the virus - severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is still actively spreading throughout the world. As of April 2023 there has been over 684 million cases and over 6.8 million deaths caused by the pandemic (https://www.worldometers.info/coronavirus/) which has brought massive medical, social and economic burden to our life.

Fortunately, the development of COVID-19 vaccines was very active and led to a rapid and successful development of a wide

Abbreviations: **Ad CV**, Adenovirus 5 control vector; **Ad-vector**, Adenovirus vector; **COVID-19**, Coronavirus disease 2019; **CXCL**, Chemokine (C-X-C motif) ligand; **HIT**, Heparin-induced thrombocytopenia; **IFN**, Interferon; **IL**, Interleukin; **moDC**, Monocyte-derived dendritic cell; **MΦ**, Macrophage; **PBMC**, Peripheral blood mononuclear cell; **SARS-CoV-2**, Severe acute respiratory syndrome coronavirus 2; **TTS**, Thrombosis with thrombocytopenia syndrome; **VITT**, Vaccine-induced thrombotic thrombocytopenia.

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range of vaccine platforms. Currently, there are several types of COVID-19 vaccines authorized and widely used, including genetic vaccines, protein-based vaccines and live-attenuated vaccines [1]. The protein-based COVID-19 vaccines include inactivated virus vaccines and protein subunit vaccines [2,3]. The global demand for huge amounts of pandemic vaccines resulted in a successful development of genetic vaccines with advanced technologies. Although the principle of an mRNA vaccine as one of the genetic vaccines was introduced over 30 years ago [4] and has been studied for decades afterwards [5,6], COVID-19 mRNA vaccines were the first mRNA vaccines that were approved for human use [7]. COVID-19 mRNA vaccines are relatively easy and fast to produce and the S protein encoding mRNA induces the expression S protein followed by the degradation of injected mRNA and induction of strong anti-S specific immune responses [7,8]. As another genetic vaccine, adenovirus (Ad)-vector vaccine has already been developed against several different pathogens, including influenza virus [9], Ebola virus [10], human immunodeficiency virus (HIV) [11], tuberculosis [12] and malaria parasite *Plasmodium falciparum* [13]. Not all of these vaccines, however, have been adapted to general use. The deletion of adenoviral E3 gene and the replacement of the E1 gene with SARS-CoV-2 S gene prevents the replication of the virus but allows the vector to readily express SARS-CoV-2 S as an immunogen, respectively [1,14]. Ad-vector vaccine has several advantages over mRNA vaccines, such as lower cost, thermostability and scalable production [15]. It is safer and provides stronger expression of the target antigens compared to some other viral vector vaccines [14]. Moreover, COVID-19 Ad-vector vaccine was reported to express S protein for a long time, which may induce more durable antigen-specific cellular and/or humoral immune responses against SARS-CoV-2 [16].

Human peripheral blood mononuclear cells (PBMCs) consist of several types of immune cells, including T and B cells, natural killer cells, monocytes and dendritic cells [17]. T cells, including CD4 + helper cells and CD8 + cytotoxic cells, are involved in cell-mediated immune responses, B cells are responsible for humoral immunity and natural killer cells are able to directly destroy virus-infected cells. Monocytes can sense the environmental changes, regulate inflammatory responses and differentiate into macrophages (MΦs) or antigen presenting dendritic cells (DCs) [18]. MΦs and DCs play central roles in regulating innate immunity and also bridge innate and adaptive immune responses during microbial infections [19]. These cells capture and process antigens and present them to T cells and initiate immune responses. The purpose of vaccination is to induce protective immunity against microbial pathogens and at the same time avoid potential side effects generated by the delivery of the vaccine. PBMCs, MΦs, and DCs are the key types of cells that are involved in vaccine induced immunity.

Due to the complexity of the vaccine development and rigorous safety and efficacy assessment processes vaccine development may take several years or even up to decades [20,21]. However, several COVID-19 vaccines obtained their market authorization within the first year of the pandemic. Even though COVID-19 vaccines are generally considered safe and effective, the long-term safety and the persistence of protective immunity induced by COVID-19 vaccines need to be carefully monitored. Within European Union, presently, two mRNA vaccines, two Ad-vector vaccines and four protein-based vaccines have been approved. Among these vaccines, mRNA vaccines BNT162b2 and mRNA-1273 and Ad-vector vaccine AZD1222 have been the most widely used COVID-19 vaccines. The protective efficacy of them has been reported to be high, with more than 95% efficacy against a severe disease after two doses of the vaccine [22–25]. COVID-19 vaccines have been reported to cause local side effects [22,23,26–28], but occasionally more severe adverse effects like myocarditis and vaccine-induced thrombotic thrombocytopenia (VITT) have been reported [29–32]. In order to provide more information on possible vaccine-induced inflammatory responses we have analyzed Ad-vector vaccine AZD1222 and mRNA vaccines BNT162b2 and mRNA-1273-induced pro-inflammatory cytokine responses in *in vitro*-stimulated human immune cells and A549 lung epithelial cell line.

2. Materials and methods

2.1. Cell cultures

Human primary monocytes were purified from freshly collected, leukocyte-rich buffy coats obtained from healthy blood donors as described previously [33]. The serum of the blood donors was analyzed for the presence of SARS-CoV-2 antibodies, and all tested samples were negative. PBMCs were obtained after Ficoll gradient centrifugation and were maintained in RPMI 1640 medium (Sigma-Aldrich) supplemented with 60 μg/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 20 mM HEPES, and 10% (vol/vol) fetal bovine serum (Sigma Aldrich). Monocytes were differentiated into MΦs or immature DCs as described previously [34]. For MΦ differentiation monocytes were allowed to adhere to plates (Sarstedt) for 1 h at 37 °C in RPMI 1640 medium, cells were washed with cold phosphate-buffered saline (PBS; pH 7.35), and the remaining monocytes were cultured in MΦ/serum-free medium (Life Technologies) supplemented with recombinant human granulocyte-MΦ colony-stimulating factor (GM-CSF) (10 ng/ml; Gibco Invitrogen). Cells were differentiated into MΦs for 6 days, with a change to fresh culture medium every 2 days.

Monocyte-derived DCs (moDCs) were obtained by cultivating adhered monocytes in the presence of 10 ng/ml of recombinant human GM-CSF (Gibco Invitrogen) and 20 ng/ml of recombinant human interleukin-4 (IL-4) (GenScript) in RPMI 1640 medium supplemented as above. The cells were cultivated for 6 days, and fresh medium was added every 2 days.

Cultured human airway epithelial A549 (ATCC CCL185) cells were grown in Eagle minimal essential medium (Eagle-MEM) (Sigma-Aldrich). Cell culture media was supplemented as described above for RPMI. Cells were maintained at 37 °C in a humidified atmosphere in the presence of 5% CO_2.

2.2. Vaccines

All vaccines were purchased in association with the national COVID-19 vaccination programme. The vaccines for this study were kindly provided by the Finnish Institute for Health and Welfare to be used solely for research purposes. Ad-vector vaccine AZD1222 was from AstraZeneca (UK, LOT ABV7764), mRNA vaccine BNT162b2 was from BioNTech-Pfizer (Germany, USA, LOT EY3014) and mRNA-1273 was from Moderna-NIAID (USA, LOT 3001940). Human adenovirus type 5 vector expressing beta-galactosidase gene (Ad5-CMV-lacZ) [35] was used as an Ad-vector control in stimulation experiments. According to the instruction of the vaccine manufacturers, AZD1222 contains not less than 2.5×10^8 infectious units per dose (500 μl), BNT162b2 contains 30 μg of COVID-19 mRNA per dose (300 μl) and mRNA-1273 contains 100 μg of COVID-19 mRNA per dose (500 μl). The amount of Ad5-CMV-LacZ control used in this study was $1.8x10^8$ pfu/ml, and by adenovirus hexon-specific qPCR, the virus copy numbers of the Ad-CMV-LacZ control vector and the AZD1222 vaccine were similar.

2.3. The preparation of vaccines and stimulation of human primary immune cells and A549 cells.

Vaccines were prepared and diluted according to the instruction of the manufacturers. The primary human immune cells and A549 cell line were stimulated with indicated vaccines in different dilutions starting from 100 µl of the original vaccine (as shown in the figures) in the cell growth media. For the samples stimulated with undiluted mRNA vaccines in A549 cells, fresh media was added 90 minutes after the stimulation.

2.4. Ultracentrifugation of Ad-vector vaccine AZD1222

To separate Ad-vector virus from solvent and other vaccine components in AZD1222, the vaccine was ultracentrifuged through a 20% sucrose cushion at 31000 rpm for 90 minutes in a SW50.1 rotor (Beckman Coulter) to spin down the virus. Supernatant samples with vaccine components were collected as 700 µl fractions above the sucrose cushion and the pelleted virus was resuspended into PBS to obtain 10 × concentrated virus compared to the original vaccine. The concentrated Ad-vector virus fraction was used in the experiments with the same concentration as the original vaccine. Dilutions are indicated in the figures.

2.5. Immunofluorescence assay

For immunofluorescence microscopy A549 cells were grown on black 96-well plates (Thermo Fisher Scientific) for 24 h and stimulated with different SARS-CoV-2 vaccines with different time periods. After stimulation, the cells were fixed with 4% formaldehyde at RT for 30 minutes and permeabilized with 0.1% Triton X-100 for 5 minutes before staining with guinea pig anti-SARS-CoV-2 S1 protein-specific antisera. Antibodies were produced by immunizing one guinea pig with 50 mg of purified S1 protein [36] per dose in AS03 adjuvant (GSK) for 4 times at 2-week intervals. The animal was bled 10 days after the last immunization. The guinea pig immune serum was used at a dilution of 1:5000 for staining the cells at RT for 1 h. Alexa-488-labeled goat anti-guinea pig antibody (Invitrogen) and DAPI (Thermo Fisher Scientific) were used in secondary staining. The cells were imaged using EVOS FL Auto Fluorescence Inverted Microscope (Life Technologies) with a 20× objective.

2.6. RNA isolation and qRT-PCR

Cells were harvested and total cellular RNAs were isolated using the RNeasy Mini kit (Qiagen) including DNase digestion (RNase-free DNase kit, Qiagen). 500 ng of total cellular RNAs were transcribed to cDNAs using TaqMan Reverse Transcriptase kit (Applied Biosystems) with random hexamers as primers. cDNAs were amplified by PCR using TaqMan Universal PCR Master mix and Gene Expression Assays (Applied Biosystems).

The commercially available primers (Applied Biosystems) were used for analyzing the expression levels of IL-1β, IL-6, IL-8, IL-18, CXCL-4, CXCL-10, TNF-α, IFN-λ1, IFN-α1 and IFN-γ mRNAs. Cytokine mRNA levels were normalized against human β-ACTIN mRNA. Gene expression data is presented as a relative gene expression in relation to unstimulated cell samples in order to calculate the fold changes seen in vaccine stimulation experiments.

2.7. Immunoblotting

For protein expression analyses the cells from different blood donors were pooled to obtain sufficient amounts of protein. The whole cell lysates from pooled primary immune cells or A549 cells were prepared in the passive lysis buffer of Dual Luciferase Assay Kit (Promega) containing 10 mM Na_3VO_4. Equal amounts of proteins (10–30 µg/lane) were separated on SDS-PAGE and transferred to Hybond-P polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences). The membranes were blocked with 5% milk protein in PBS. Rabbit antibodies against IRF3 and MxA [37,38] and SARS-CoV-2 S1 protein [19] have been previously described. To obtain adenovirus hexon-specific antibodies one rabbit was immunized with a human dose of AZD1222 for three times at 3 week intervals followed by the collection of immune serum 10 days after the last dose. All antibody stainings were done in blocking buffer at RT for 1 h. Antibodies against phosphorylated IRF3 (P-IRF3, #4947), and GAPDH (#2118) were from Cell Signaling Technology and staining was done in Tris-buffered saline, pH 7.4 containing 5% BSA at + 4 °C overnight. HRP-conjugated antibodies (Dako) were used in the secondary staining at RT for 1 h. Protein bands were visualized on HyperMax films using an ECL plus system (GE Healthcare).

2.8. Elisa

Cytokine levels of IFN-λ1, IL-6 and TNF-α from PBMC, MΦ and moDC culture supernatants were analyzed using Ab pairs and standards from R&D systems (Bio-Techne Corporation, US).

3. Results

3.1. IFN and pro-inflammatory responses induced by COVID-19 Ad-vector vaccine AZD1222 in human PBMCs, MΦs and moDCs

To analyze whether COVID-19 Ad-vector vaccine can induce notable IFN and pro-inflammatory responses in human PBMCs we added AZD1222 vaccine in a final dilution from 1:10 to 1:1000 directly into the cell growth media. The expression of IFN-λ1, pro-inflammatory (IL-6, TNF-α, IL-1β) and Th1-type (CXCL-10, IL-2, IFN-γ) cytokine mRNAs were analyzed at three time points (8 h, 24 h and 48 h) in response to AZD1222 stimulation (Fig. 1A). AZD1222 strongly induced the expression of IFN-λ1, CXCL-10 and IL-6 mRNAs and this phenomenon was dose-dependent and high vaccine doses induced faster mRNA expression. In addition, AZD1222 induced moderate TNF-α, IFN-γ and IL-2 mRNA expression and the expression of IFN-γ and IL-2 genes became stronger at late time points after the vaccine stimulation. All doses of AZD1222 failed to induce IL-1β gene expression in PBMCs (Fig. 1A).

We also investigated the ability of AZD1222 to induce IFN and pro-inflammatory cytokine gene expression in MΦs and moDCs. IFN-λ1 and CXCL-10 mRNA expression was strongly induced with a high dose of the vaccine in both cell types, while the expression of IL-6 gene was only induced with a high dose of AZD1222 in MΦs (Fig. 1B). All inductions were vaccine dose dependent. AZD1222 failed to induce or induced very weak expression of TNF-α and IL-1β mRNAs in both cell types (data not shown).

3.2. Immune responses induced by COVID-19 Ad-vector vaccine in PBMCs were associated with virus particles in the vaccine

In order to further study whether the virus particles are responsible for Ad-vector vaccine-induced cytokine responses in human immune cells, AZD1222 vaccine was ultra-centrifuged to pellet adenovirus particles in the vaccine. The vaccine supernatant above the sucrose cushion and virus fractions (resuspended and diluted to the original vaccine concentration) were then used to stimulate PBMCs with different amounts. IFN-λ1, CXCL-10 and IL-6 mRNA expression was analyzed at three time points (8 h, 24 h and 48 h) after the stimulation (Fig. 2). Human adenovirus type 5 vec-

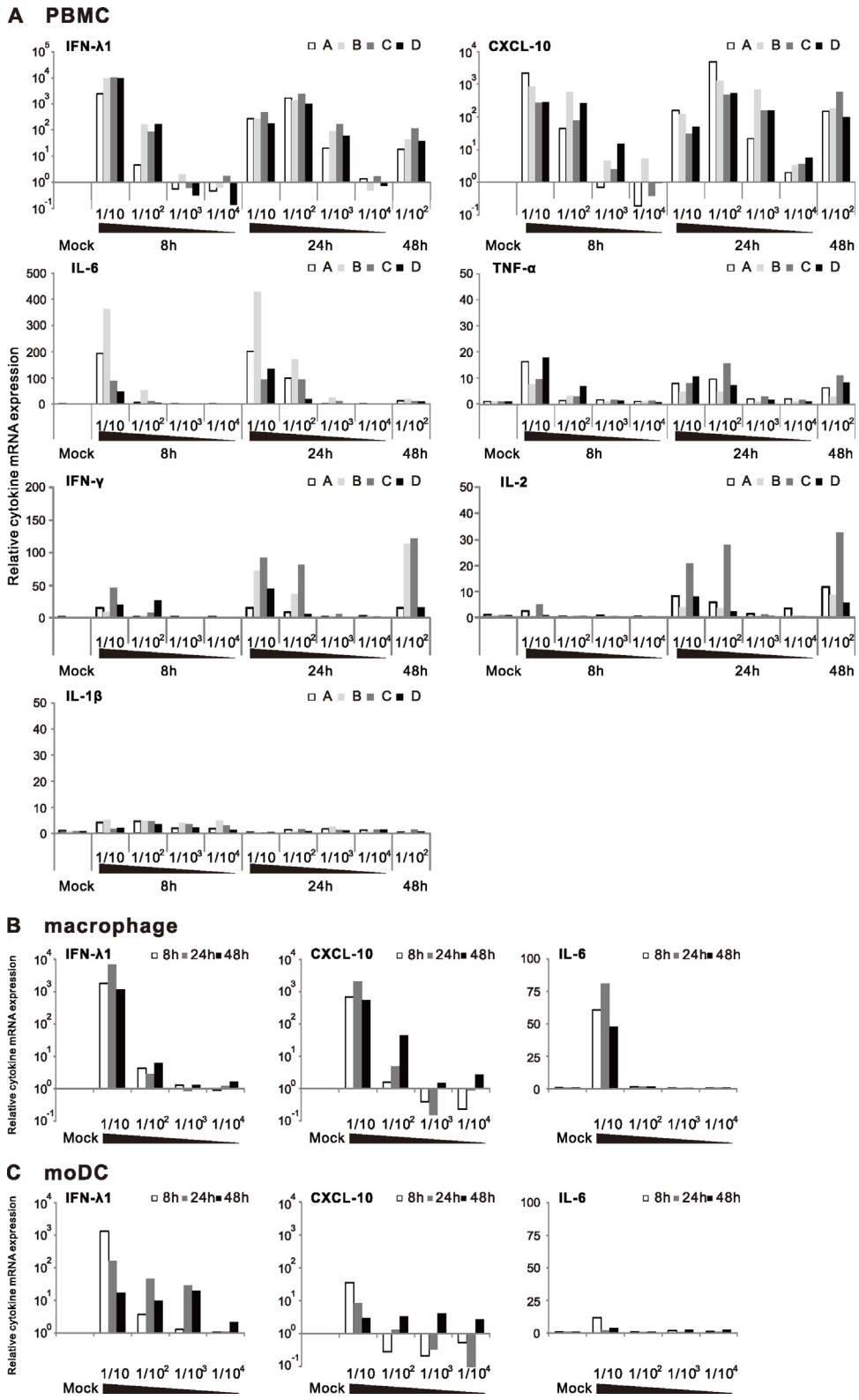

Fig. 1. Dose-dependent expression of IFN and pro-inflammatory mRNAs in Ad-vector vaccine-stimulated human PBMCs, MΦs and moDCs. (A) Human PBMCs from four different blood donors were separately stimulated with AZD1222 in different dilutions and cellular RNA samples were collected at different times after stimulation as indicated in the figure. Relative mRNA expression of IFN and pro-inflammatory cytokines IFN-λ1, CXCL-10, IL-6, TNF-α, IFN-γ, IL-2 and IL-1β were analyzed by quantitative RT-PCR. Human MΦs (B) and moDCs (C) from four donors were separately stimulated with AZD1222 in different dilutions for 8, 24 or 48 h. Cellular RNA samples from different donors were pooled for quantitative RT-PCR to analyze the relative mRNA expression of IFN-λ1, CXCL-10 and IL-6. The values were normalized against β-ACTIN mRNA, and relative expression levels were calculated with the ΔΔCt method using unstimulated cells as a calibrator. The data is representative of two individual experiments. The results are shown as a relative fold induction of each cytokine mRNA compared to the mock cells.

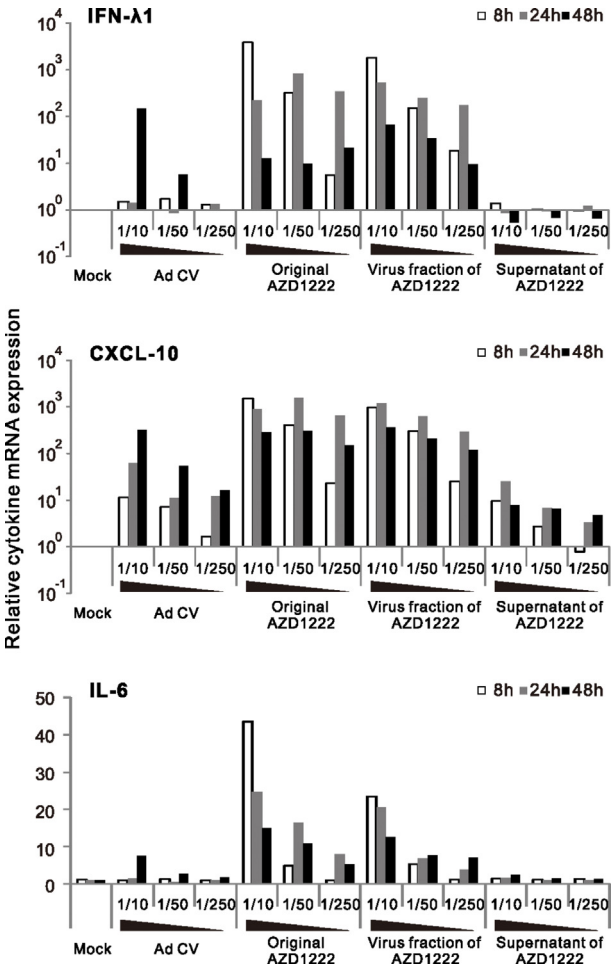

Fig. 2. Induction of IFN and pro-inflammatory cytokine gene expression by virus fraction or supernatant fraction of Ad-vector vaccine in human PBMCs. Human PBMCs from four different blood donors were separately stimulated with Ad CV, original AZD1222 vaccine, the virus fraction or the supernatant of AZD1222 vaccine with different dilutions. Cells were collected at different time points after stimulation and total cellular RNAs were isolated. RNA samples from different donors were pooled. Relative mRNA expression of IFN-λ1, CXCL-10 and IL-6 were analyzed by quantitative RT-PCR. The values were normalized against β-ACTIN mRNA, and relative expression levels were calculated with the ΔΔCt method using untreated cells as a calibrator. The data is representative of two individual experiments. The results are shown as a relative fold induction of each cytokine mRNA compared to the mock cells.

tor with similar infectious unit levels as Ad-vector vaccine was used as a control in the experiment. AZD1222 virus fraction in different dilutions (equivalent to the original vaccine doses) induced cytokine mRNA expression at similar levels as the original vaccine, while the supernatant of Ad-vector vaccine failed to induce any notable expression of cytokine mRNAs. However, the adenovirus 5 control vector (Ad CV) could also induce some expression of IFN-λ1 and CXCL-10 mRNAs but failed to induce IL-6 gene expression. All inductions were dose dependent (Fig. 2).

3.3. COVID-19 Ad-vector vaccine induced stronger IFN and pro-inflammatory responses than mRNA vaccines in human PBMCs, MΦs and moDCs

Currently, Ad-vector vaccine AZD1222 and mRNA vaccines BNT162b2 and mRNA-1273 are widely used COVID-19 vaccines throughout the world. Therefore, we compared the ability of these vaccines to induce IFN and pro-inflammatory cytokine gene expression in human primary immune cells. In PBMCs, stimulation of cells with AZD1222 or Ad CV induced the expression of IFN-λ1, IFN-α1 (only by AZD1222), IL-6, TNF-α, IFN-γ and IL-2 mRNAs, especially at an early time point (8 h after stimulation), while both mRNA vaccines BNT162b2 and mRNA-1273 failed to induce the expression of these genes (Fig. 3A). All vaccines induced the expression of CXCL-10 mRNA, but AZD1222 and Ad CV induced higher level of the expression than the ones induced by both mRNA vaccines (Fig. 3A). Similar results were also seen in MΦs and moDCs, where AZD1222 and Ad CV readily induced IFN-λ1 and CXCL-10 mRNA expression. The mRNA vaccines, instead, induced clearly detectable CXCL-10 mRNA expression, but very low or undetectable expression of other analyzed cytokine genes (Fig. 3B, C). Moreover, all three vaccines failed to induce pronounced IFN-α1 mRNA expression in MΦs and moDCs (Fig. 3B, C), and also failed to induce any IL-18 mRNA expression in PBMCs, MΦs and moDCs (data not shown). ELISA result also showed that only AZD1222 stimulation induced IFN-λ1, IL-6 and TNF-α cytokine production in the supernatants of PBMCs, while BNT162b2 and mRNA-1273 failed to induce or induce very moderate cytokine production of IFN-λ1, IL-6 and TNF-α (Fig. 4B) in PBMCs, MΦs and moDCs (Fig. 4B).

Next we analyzed whether the vaccines were able to express SARS-CoV-2 S protein and adenovirus hexon or stimulate IRF3 phosphorylation and express type I and III IFN-induced antiviral MxA protein. PBMCs, MΦs or DCs were stimulated with different vaccines and whole cell protein lysates from the vaccine-stimulated cells were analyzed by Western blotting. The results showed that the AZD1222 and mRNA-1273 induced much higher levels of SARS-CoV-2 S protein expression than BNT162b2 in all studied cell types, while only the AZD1222 vaccine induced notable phosphorylated of IRF3 and the expression of MxA protein (Fig. 4A).

3.4. All studied COVID-19 vaccines failed to enhance the expression of CXCL-4 gene in human PBMCs, MΦs and moDCs

Recently, Ad-vector vaccines were linked with a rare adverse effect called vaccine-induced thrombotic thrombocytopenia (VITT) [31,39,40]. This syndrome is similar to heparin-induced thrombocytopenia (HIT), where the chemokine (C-X-C motif) ligand 4 (CXCL-4), also called platelet factor 4 (PF4), is released by activated platelets followed by binding to HIT antibodies developed after the administration of heparin [41]. Moreover, the change of expression of CXCL-4 has been associated with COVID-19 [42]. Increased level of CXCL-4 protein was found in the plasma of severe COVID-19 patients, indicating the potential role of CXCL-4 triggering thrombosis in VITT [43]. We studied the expression of CXCL-4 mRNA after stimulation with COVID-19 vaccines in human PBMCs, MΦs and moDCs. None of the studied vaccines enhanced pronounced CXCL-4 mRNA expression in any of the cell types, indicating that the stimulation with vaccines does not have a direct link with the regulation of CXCL-4 gene expression in these cells (Fig. 5). All analyzed cell types showed high constitutive CXCL-4 mRNA expression and spontaneous secretion of CXCL-4 protein (data not shown).

3.5. AZD1222 induced COVID-19 S protein expression and pro-inflammatory responses in A549 lung epithelial cells

Next we studied COVID-19 S protein expression and IFN and pro-inflammatory responses induced by different COVID-19 vaccines in A549 cells. The immunofluorescence result showed that the COVID-19 S protein started to be expressed at 8 h after the stimulation with AZD1222 or mRNA-1273 in A549 cells and the S protein expression continued to reach the peak at 48 h (Fig. 6A). However, very faint S protein expression was seen in BNT162b2 stimulated

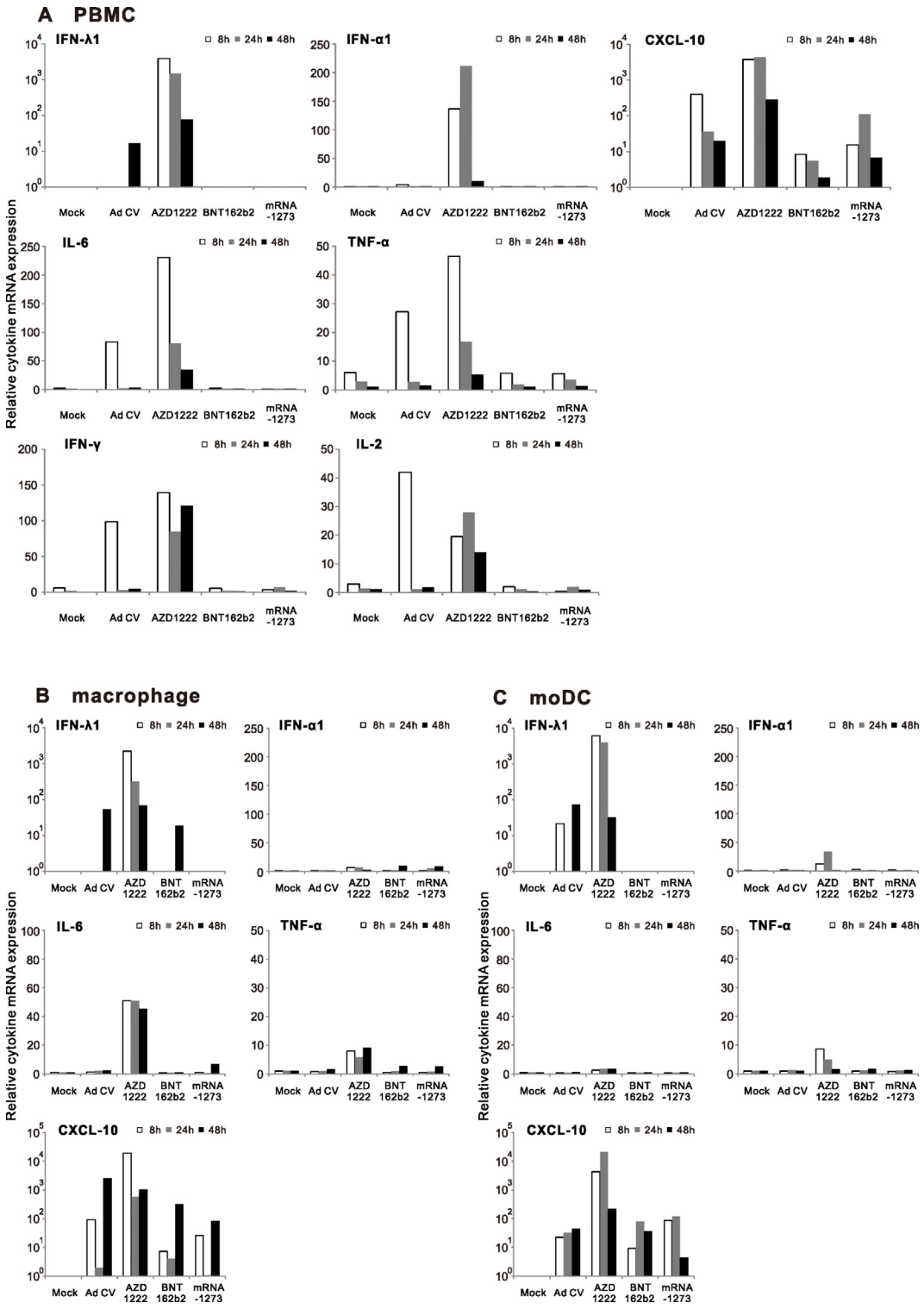

Fig. 3. Induction of IFN and pro-inflammatory responses by different COVID-19 vaccines in human PBMCs, MΦs and moDCs. Ad CV, AZD1222, BNT162b2 or mRNA-1273 (1:10 dilution in the media) were used to stimulate human immune cells from four different blood donors. Cellular RNA samples were collected at three time points after stimulation as indicated in the figure, total cellular RNAs were isolated and the samples from different donors were pooled. Relative mRNA expression of IFN and pro-inflammatory cytokine mRNAs for IFN-λ1, IFN-α1, CXCL-10, IL-6, TNF-α, IFN-γ and IL-2 in human PBMCs (A) or IFN-λ1, IFN-α1, CXCL-10, IL-6, and TNF-α in MΦs (B) and moDCs (C) were analyzed by quantitative RT-PCR. The values were normalized against β-ACTIN mRNA, and relative expression levels were calculated with the ΔΔCt method using untreated cells as a calibrator. The data is representative of two individual experiments. Cytokines and time points are indicated in the figure. The results are shown as a relative fold induction of each cytokine mRNA compared to the mock cells.

A549 cells only at 48 h after the stimulation (Fig. 6A). Western blotting results showed that the AZD1222 and mRNA-1273 induced much higher levels of SARS-CoV-2 S protein expression than BNT162b2 starting from 8 h after the vaccine stimulation (Fig. 6B). qPCR results showed that AZD1222 induced much higher expression of IL-6 and IL-8 mRNAs than the other two mRNA vac-

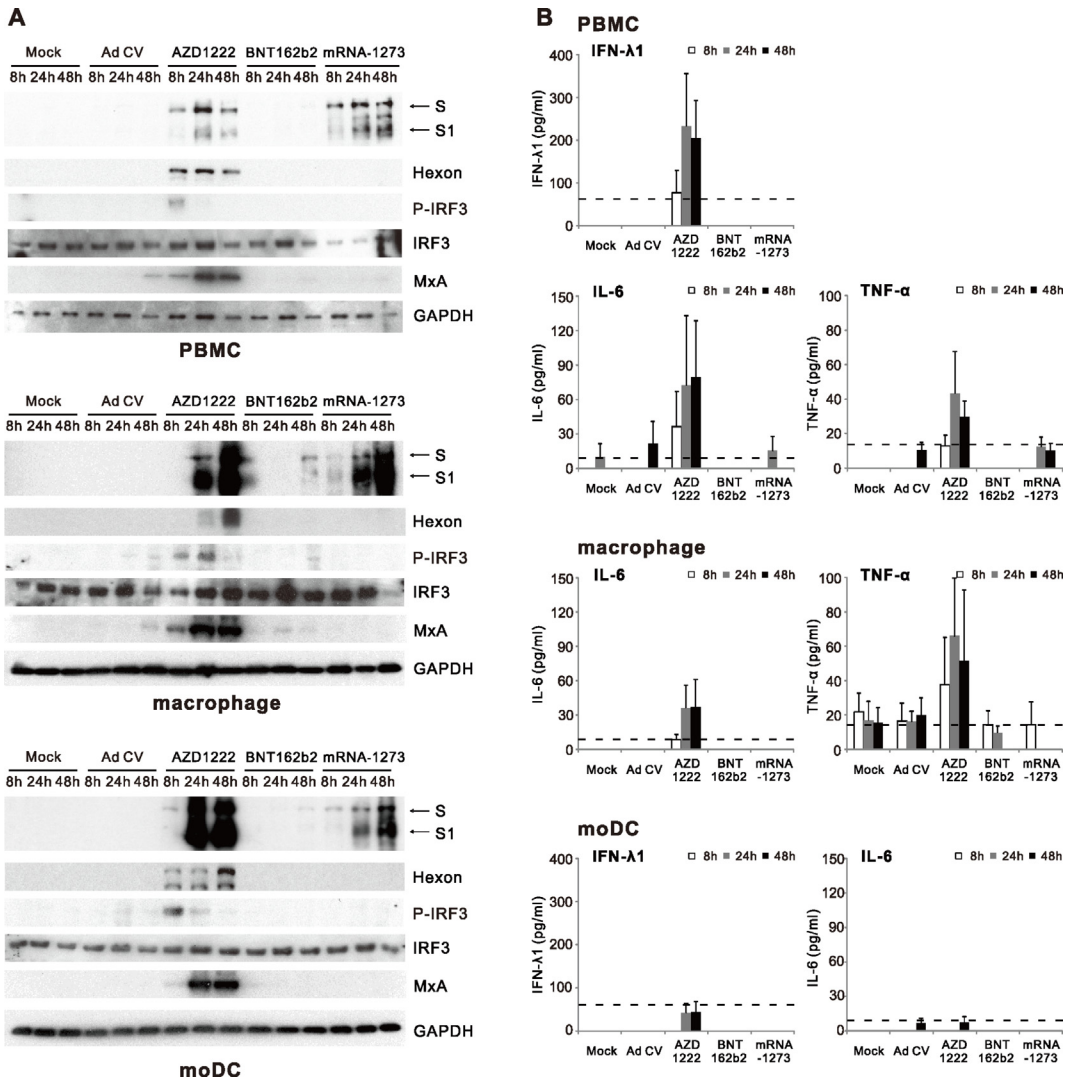

Fig. 4. Comparison of three COVID-19 vaccine-induced expression of SARS-CoV-2 S protein and IFN signaling molecules and cytokine production in human immune cells. Human PBMCs, MΦs and moDCs from four different blood donors were separately stimulated with Ad CV, AZD1222, BNT162b2 or mRNA-1273 (1:10 dilution in the media). (A) Cells from different donors were pooled when collected at 8 h, 24 h or 48 h after vaccine stimulation, and whole-cell lysates were prepared for immunoblotting against SARS-CoV-2 S protein, adenoviral hexon, phospho-IRF3 (P-IRF3), IRF3, and antiviral MxA. GAPDH was stained as a loading control. One representative experiment out of two is shown. (B) Cell culture supernatants were collected at 8 h, 24 h and 48 h after vaccine stimulation and protein levels of IFN-λ1, IL-6 and TNF-α were determined by ELISA. The minimum detected amount of the indicated cytokine is shown as discontinuous line. The mean (±SD) cytokine levels produced by the cells of four blood donors are shown.

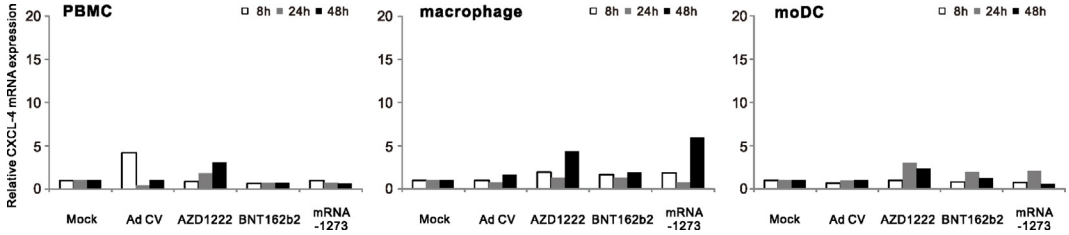

Fig. 5. Relative expression of CXCL-4 gene in COVID-19 vaccine-stimulated human PBMCs, MΦs and moDCs. Human immune cells from four different blood donors were separately stimulated with Ad CV, AZD1222, BNT162b2 or mRNA-1273 (1:10 dilution in the media), total cellular RNAs were isolated at 8 h, 24 h and 48 h time points and cellular RNA samples from different donors were pooled for quantitative RT-PCR. The mRNA expression of CXCL-4 was analyzed in PBMCs, MΦs and moDCs. The values were normalized against β-ACTIN mRNA, and relative expression level was calculated with the ΔΔCt method using untreated cells as a calibrator. The data is representative of two individual experiments.

cines BNT162b2 and mRNA-1273 especially at late time points after the stimulation (Fig. 6C), while all vaccines failed to induce the expression of IFN-λ1 and CXCL-10 mRNAs in A549 cells (data not shown).

4. Discussion

Currently, the information on head-to-head comparisons of COVID-19 vaccines regarding to the expression of S protein and

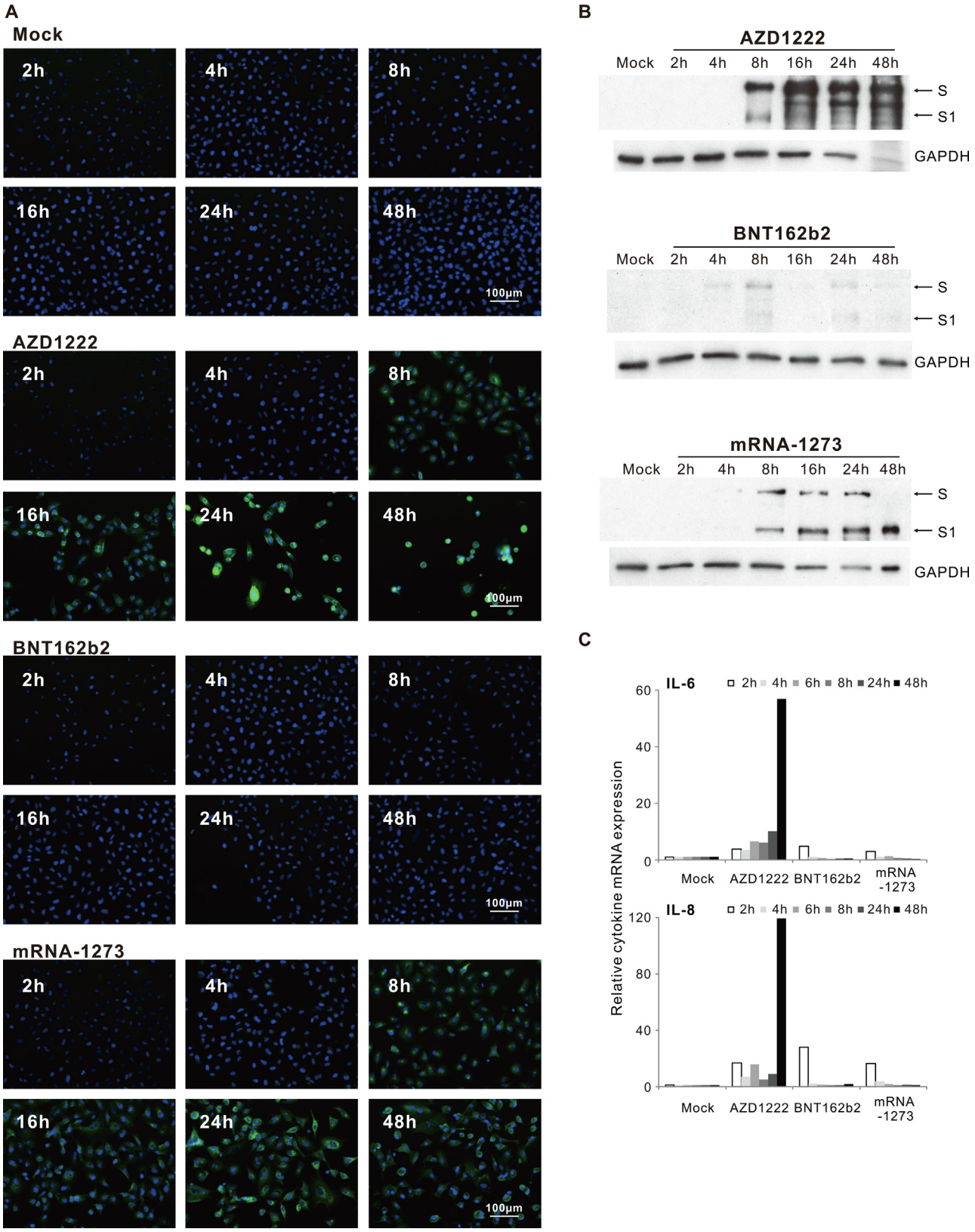

Fig. 6. The expression of SARS-CoV-2 S protein and expression of pro-inflammatory cytokine mRNAs in COVID-19 vaccine stimulated A549 cells. (A) A549 cells ($1x10^4$ cells/well) were grown on 96-well plate one day before stimulation with different COVID-19 vaccines (AZD1222, 1:10 dilution in the media, BNT162b2 and mRNA-1273, undiluted). After stimulation A549 cells were fixed at different time points as shown in the figure and labeled with guinea pig antibody against SARS-CoV-2 S1 protein for immunofluorescence microscopy. (B, C) A549 cells ($3x10^5$ cells/well) were seeded in 6-well plates one day before stimulation with different COVID-19 vaccines (AZD1222, 1:10 dilution in the media, BNT162b2 and mRNA-1273, undiluted). Cells were collected at 8 h, 24 h or 48 h after vaccine stimulation, and whole-cell lysates were prepared for immunoblotting against SARS-CoV-2 S protein, GAPDH was stained as a loading control (B). Cellular RNA samples were collected at different time points after vaccine stimulation as indicated in the figure. Relative expression of IL-6 and IL-8 mRNAs was analyzed by quantitative RT-PCR. The values were normalized against β-ACTIN mRNA and relative expression levels were calculated with the ΔΔCt method using untreated cells as a calibrator. Cytokines and time points are indicated in the figure (C). The results are shown as a relative fold induction of each cytokine mRNA compared to the mock cells. The data is representative of two individual experiments.

the stimulation of innate immune responses are still very limited, especially in human immune cells. In our study, IFN and pro-inflammatory responses induced by three widely used COVID-19 vaccines were compared in human PBMCs, MΦs, moDCs as well as in A549 cells. Among the three vaccines analyzed, AZD1222 induced much higher IFN and pro-inflammatory responses than the other two mRNA vaccines, while both AZD1222 and mRNA-1273 induced stronger S protein expression than BNT162b2 in human immune cells. All vaccines failed to directly enhance the expression of CXCL-4 gene and the secretion of CXCL-4 protein, the potential inducer for thrombosis in VITT, in studied human primary cells. Moreover, AZD1222 and mRNA-1273 induced strong expression of SARS-CoV-2 S antigen while only AZD1222 induced stronger pro-inflammatory responses than mRNA vaccines in A549 cells.

An optimal (viral) vaccine is able to deliver or translate pathogen-specific antigen(s) to stimulate adaptive immunity, including the production of neutralizing antibodies and activation of cell-mediated immunity to resist an infection caused by the respective pathogen. Some vaccines contain adjuvants which are inducing controllable innate immune responses to provide necessary secondary signals for activating T cell-mediated responses [25]. However, the activation of innate immunity by the vaccine needs to be weak or moderate in order to circumvent systemic inflammation which may lead to severe side effects. Therefore, different types of COVID-19 vaccines have been designed and modified in order to follow this principle. For example, since the mRNA serves as an immunogen and adjuvant in mRNA vaccines, N1-methylpseudouridines (m1Ψ) substitute all uridine residues of mRNA in both BNT162b2 and mRNA-1273 vaccines in order to minimize innate immune responses and at the same time maintaining prolonged mRNA stability [44]. Indeed, both BNT162b2 and mRNA-1273 mRNA vaccines induced weak or moderate IFN and pro-inflammatory responses compared to the higher immune responses induced by AZD1222 Ad-vector vaccine in all types of cells we analyzed. However, the expression of SARS-CoV-2 S antigen was much higher in mRNA-1273 vaccine-stimulated cells compared to the cells stimulated by BNT1162b2. This may be due to the overall composition of the vaccines including the lower amount of mRNA per dose in BNT1162b2 (30 μg) [22] than in mRNA-1273 vaccine (100 μg) [23].

Ad-vector vaccine AZD1222 dose-dependently induced innate cytokine responses in human immune cells including the expression of IFN-λ1 and pro-inflammatory cytokine genes, phosphorylation of IRF3 and expression of MxA antiviral protein, although the level of S protein expression in AZD1222 stimulated cells was similar to the one seen in mRNA-1273 stimulated cells. The manufacturing process of Ad-vector vaccine is more complicated than that of mRNA vaccines since the Ad-vector vaccine is grown in cell culture. Even though Ad-vector vaccine virus does not replicate in target cells, it is transported into the nucleus and it expresses other genes apart from the vaccine antigen (spike protein) which may increase the possibility of activating innate immune responses [1]. Indeed, the Ad CV (human adenovirus type 5 vector) also induced IFN and pro-inflammatory mRNA expression in our cell models, albeit at low levels, indicating that the Ad-vector alone is able to induce immune responses. As an adenovirus vector vaccine, AZD1222 is designed based on the chimpanzee adenovirus vector ChAdOx1, which may be the reason why AZD1222 could induce such strong IFN and pro-inflammatory responses in human immune cells.

Both mRNA and Ad-vector vaccines target innate immune cells such as MΦs and DCs, and this may trigger innate immune responses through different signaling pathways. However, the current mRNA vaccines contain purified and modified mRNAs which can at least partially avoid the recognition by RNA sensors in endosomes (mainly TLR-3 and TLR-7) or in the cell cytoplasm (mainly RIG-I and MDA5). DNA virus such as the Ad-vector vaccine is recognized by TLR-9 and/or other DNA sensors, which trigger downstream IFN signaling pathway and elicit IFN and inflammatory responses [25]. AZD1222 does not contain S protein stabilizing mutations which may avoid the formation of post-fusion structure of S2 [45], thus allowing the shedding of the cleaved S1 protein in AZD1222 stimulated cells [46]. The post-fusion structure of S2, after the release of S1 domain, may mediate cell–cell fusions and increase the risk of syncytia formation leading to IFN and inflammatory responses [47]. This may be one of the reasons why Ad-vector vaccine induces higher IFN and inflammatory responses in human immune cells.

We also observed that AZD1222 induced stronger expression of IL-2 and IFN-γ genes in PBMCs, especially at late time points after stimulation. This suggests that Ad-vector vaccine may induce better T cell activation and cell-mediated responses than mRNA vaccines. This may be due to the persistence of antigen expression by Ad-vector vaccine which may then lead to more long-lasting adaptive immune responses as compared to that induced by mRNA vaccines [16].

As mentioned above, several cases of VITT or thrombosis with thrombocytopenia syndrome (TTS) have been reported after vaccination with Ad-vector vaccine AZD1222 [31,39,40,48]. Although the exact mechanisms behind these events are still elusive, it has been suggested that the components of Ad-vector vaccine may trigger autoantibody formation to CXCL-4 in immune complexes leading to platelet activation, thrombosis and complement activation [48–50]. In addition, CXCL-4 is capable of binding to the surface of Ad-vector AZD1222 at the interfaces of three hexon molecules [51], which may together with other proteins or free DNA generated during the culture in HEK293 cells play a role in triggering the platelet activation [49]. In our study, we failed to find a direct link between the COVID-19 vaccines and the expression of CXCL-4 gene. However, AZD1222 induced expression of pro-inflammatory cytokines is consistent with the hypothesis that increased cytokine production may contribute to the formation of anti-CXCL-4 antibodies and platelet activation [49]. In conclusion, our analyses demonstrate that AZD1222 COVID-19 vaccine can induce relatively strong pro-inflammatory cytokine responses in human PBMC, MΦs and DCs, but further studies are needed to reveal the precise mechanism of AZD1222 associated thrombotic syndrome.

5. Conclusions

In summary, we compared the ability of three commonly used COVID-19 vaccines to induce pro-inflammatory cytokine responses in human immune cells. Ad-vector vaccine, AZD1222 induced much stronger interferon and pro-inflammatory cytokine gene expression than BNT162b2 and mRNA-1273 mRNA vaccines in *in vitro*-stimulated primary human PBMCs, MΦs or moDCs. Even though we did not observe a direct link between AZD1222 vaccine and CXCL-4 gene expression that could explain the mechanisms of VITT, we have provided detailed information of Ad-vector and mRNA vaccine interactions with primary human immune cells.

CRediT authorship contribution statement

Miao Jiang: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Elina Väisänen:** Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Writing – review & editing. **Pekka Kolehmai-**

nen: Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Writing – review & editing. **Moona Huttunen:** Data curation, Formal analysis, Investigation, Methodology, Resources, Software. **Seppo Ylä-Herttuala:** Conceptualization, Investigation, Resources, Writing – review & editing. **Seppo Meri:** Conceptualization, Investigation, Writing – review & editing. **Pamela Österlund:** Conceptualization, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing. **Ilkka Julkunen:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing.

Data availability

Data will be made available on request.

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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Ethics statement

Adult human PBMCs and blood-derived MΦs and DCs used in the experiments were obtained from anonymous healthy blood donors through the Finnish Red Cross Blood Service. The use of buffy coats for research purposes was approved by the Finnish Red Cross Blood Service Institutional review board (license number 32/2022, renewed yearly) by which the need for informed consent was waived. All experimental protocols were approved and performed in accordance with the guidelines of the Finnish Institute for Health and Welfare (THL), Helsinki, Finland.

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