Bacterial Lipopolysaccharide Inhibits Influenza Virus Infection of Human Macrophages and the Consequent Induction of CD8+ T Cell Immunity

Kirsty R. Short[a]   Marloes Vissers[b]   Stan de Kleijn[b]   Aldert L. Zomer[b]
Katherine Kedzierska[a]   Emma Grant[a]   Patrick C. Reading[a, c]
Peter W.M. Hermans[b]   Gerben Ferwerda[b]   Dimitri A. Diavatopoulos[b]

[a]Department of Microbiology and Immunology, The University of Melbourne, Melbourne, Vic., Australia;
[b]Laboratory of Pediatric Infectious Diseases, Department of Pediatrics, Radboud University Medical Centre,
Nijmegen, The Netherlands; [c]WHO Collaborating Centre for Reference and Research on Influenza, Victorian
Infectious Diseases Reference Laboratory, Melbourne, Vic., Australia

Abstract

It is well established that infection with influenza A virus (IAV) facilitates secondary bacterial disease. However, there is a growing body of evidence that the microbial context in which IAV infection occurs can affect both innate and adaptive responses to the virus. To date, these studies have been restricted to murine models of disease and the relevance of these findings in primary human cells remains to be elucidated. Here, we show that pre-stimulation of primary human monocyte-derived macrophages (MDMs) with the bacterial ligand lipopolysaccharide (LPS) reduces the ability of IAV to infect these cells. The inhibition of IAV infection was associated with a reduced transcription of viral RNA and the ability of LPS to induce an anti-viral/type I interferon response in human MDMs. We demonstrated that this reduced rate of viral infection is associated with a reduced ability to present a model antigen to autologous CD8+ T cells. Taken together, these data provide the first evidence that exposure to bacte-
rial ligands like LPS can play an important role in modulating the immune response of primary human immune cells towards IAV infection, which may then have important consequences for the development of the host's adaptive immune response.

Copyright © 2013 S. Karger AG, Basel

Introduction

Influenza A virus (IAV) is a negative-sense RNA virus of the Orthomyxoviridae family that causes both seasonal epidemics as well as global pandemics. Every year, on average, influenza virus infects 5–15% of the world's population, resulting in approximately 500,000 deaths [1]. In addition, infection with IAV creates a window of susceptibility to secondary bacterial infections. In recent years, it has become evident that viral-bacterial interactions are not uni-directional, and just as IAV can facilitate bacte-

Dr. Dimitri Diavatopoulos
Laboratory of Pediatric Infectious Diseases
Department of Pediatrics, Radboud University Medical Centre
Kapittelweg 29, NL–6525 EN Nijmegen (The Netherlands)
E-Mail D.Diavatopoulos@cukz.umcn.nl

rial disease, so too may exposure to certain bacterial species affect the pathogenesis of IAV [2].

Historically, bacteria have been thought to aid IAV infection via the production of proteases that cleave the viral haemagglutinin into its active form [3, 4]. Alternatively, bacterial toxins may suppress key components of the anti-viral response such that the pathogenicity of IAV is increased [5]. Recent studies have suggested that the bacterial species that constitute an individual's normal flora represent a key component of the host defence against IAV [6, 7]. Ichinohe et al. [6] demonstrated that commensal flora provide the first signal for inflammasome activation, which is necessary for dendritic cell (DC) migration and the development of an effective adaptive immune response against IAV. Accordingly, antibiotic-treated mice (whose normal flora had been reduced) displayed increased susceptibility to IAV infection. Abt et al. [7] also demonstrated that antibiotic-treated mice displayed increased susceptibility to IAV infection, which was characterised by increased viral replication, decreased CD8+ T cell activation/migration and a suppressed humoral response. This impaired immune response was attributed to the inability of macrophages from antibiotic-treated mice to mediate an appropriate anti-viral response and respond to type I interferons (IFNs). Consistent with these findings, pre-stimulation of certain toll-like receptors (TLRs) with bacterial-pathogen-associated molecular patterns (PAMPs) protected mice from infection with influenza virus [8, 9]. Specifically, treatment of mice with the TLR4 agonist lipopolysaccharide (LPS) protected against a lethal infection with A/Vietnam/1203/04(H5N1), whilst treatment with the TLR2 agonist mycoplasma lipoprotein did not [8], potentially due to the induction of type I IFNs following TLR4 rather than TLR2 stimulation.

Taken together, the above studies suggest that substantive crosstalk exists between bacterial PAMP signalling pathways and the induction of both innate and adaptive anti-viral immunity. However, whilst these studies have highlighted the important role that bacterial species and ligands play in modulating the pathogenesis of IAV, it remains unclear how far these findings can be extrapolated from murine models to human infections. Here, we seek to address the effect of pre-exposure to specific bacterial ligands on IAV infection of primary human macrophages. Macrophages were chosen as the model cell type because of their presence in the nasal cavity [10], which represents the first site of infection for IAV and is an area of high bacterial density and diversity. Macrophages play an integral role in survival of IAV infection [11]. More-over, macrophages are long-lived antigen-presenting cells (APCs) and are crucial for tissue homeostasis [12]. Training of these cell types by bacterial ligands in the nasopharyngeal mucosa could therefore have important consequences on the immune response towards a viral infection.

We show that pre-stimulation of human monocyte-derived macrophages (MDMs) with LPS inhibits IAV replication, which was associated with the induction of a strong anti-viral response and reduced viral transcription. Importantly, we show that LPS-stimulated macrophages are less able to present a model antigen to autologous CD8+ T cells upon IAV infection. Thus, we provide the first evidence using primary human cells that exposure to bacterial ligands may affect the adaptive immune response to IAV.

Materials and Methods

Virus Strains

Influenza virus strains A/PR8/8/34 (PR8/34; H1N1), A/HKx31 (HKx31; H3N2) or A/PR8-GFP/8/34 (GFP-PR8/34; H1N1) [13] were used to model infection with IAV. Virus stocks were prepared in embryonated eggs and titres of infectious virus were determined by three independent plaque assays on Madin-Darby canine kidney (MDCK) cells [14]. Where relevant, PR8/34 was purified by rate zonal sedimentation on 25–80% w/v sucrose gradients as described [15]. Green fluorescent protein (GFP)-labeled RSV A2 (rgRSV30) [16] was cultured on HeLa cells as described previously [17]. Virus concentration was determined by titration on HeLa cells. Briefly, HeLa cells (80–90% confluent) were infected with fivefold viral dilutions for 20–22 h. Virus titre was determined by counting wells with ≥10 and ≤100 infected cells/view (CKX41 microscope; Olympus, Tokyo, Japan) where each data point was the average of an experimental duplicate.

Isolation and Infection of Macrophages

Human peripheral blood mononuclear cells (PBMCs) were isolated from blood obtained from anonymised buffy coats of healthy donors (Sanquin, The Netherlands, or Australian Red Cross, Australia) or from healthy volunteers upon informed written consent. Where relevant, experiments were approved by the Ethics Committee of the University of Melbourne, Australia. PMBCs were isolated from buffy coats by density gradient centrifugation (Lymphoprep; Axis-Shield, Norway), essentially as described previously [17]. PBMCs were then washed, resuspended in 10% DMSO (v/v) with heat-inactivated fetal calf serum (FCS) and frozen in liquid nitrogen until use. Alternatively, washed PBMCs were used fresh and monocytes were isolated by adherence to plastic in the presence of 2% human serum (Sigma, USA) and allowed to differentiate for 6 days in RPMI-1640 medium supplemented with 10% heat-inactivated FCS. MDMs were washed and stimulated with the relevant bacterial ligand (table 1). At the selected time point after stimulation, MDMs were washed and infected with IAV or RSV (multiplicity of infection, MOI, 0.01 and 1, re-

Table 1. Bacterial ligands used

Bacterial ligand	Cognate pattern recognition receptor	Concentration	Company
Lipoteichoic acid	TLR2/6	10 µg/ml	Invivogen, USA
P3C	TRL2/1	10 µg/ml	EMC Microcollections, Germany
High-molecular-weight polyI:C	TLR3	10 µg/ml	Invivogen, USA
LPS (*Escherichia coli* serotype 0111:B4)	TLR4	1 ng/ml	Invivogen, USA
Flagellin (FLA-ST Ultrapure)	TLR5/NLRC4	100 ng/ml	Invivogen, USA
CpG (ODN 2336)	TLR9	1 µg/ml	Invivogen, USA
Muramyl dipeptide	NOD2	5 µg/ml	Sigma-Aldrich, USA

spectively) for 1 h at 37°C. Virus was then removed and cells were incubated for 16 h in RPMI-1640 medium supplemented with 10% heat-inactivated FCS. Cells were then fixed and analysed on a LSRII or FACSCanto flow cytometer (BD Biosciences, USA) with FACSDiva software (BD Biosciences). Collected samples were analysed with FLOWJO, version 8.8.7 (TreeStar, Inc., USA) where IAV infection was determined as the percentage of single cells that were GFP positive.

Virus Adhesion and Internalisation
Purified PR8/34 (1.75 or 0.88 µg) was added to 2×10^5 MDMs 24 h after LPS or mock stimulation. Virus was adhered to cells for a pre-determined period of time at either 4 or 37°C. For uninfected controls, PBS was added in place of purified PR8/34. Cells were then washed and virus particles on the cell surface were stained with monoclonal antibody E2.6 (αPR8/34-haemagglutinin; kindly provided by Prof. Lorena Brown, The University of Melbourne). Following washing, cells were stained with fluorescein isothiocyanate (FITC) goat-anti-mouse IgG (Millipore, USA). Cells were then fixed and analysed on a FACSCantoII with FACSDiva software (BD Biosciences). Collected samples were analysed with FLOWJO, version 8.8.7 (TreeStar, Inc.) where IAV infection was determined as the percentage of single cells that were FITC positive.

Staining of IAV-Infected Cells
Infection of cells with a non-GFP-labelled IAV strain was assessed by staining with an anti-nucleoprotein monoclonal antibody (MP3.10G2.IC7; WHO Collaborating Centre for Reference and Research on Influenza) following cell permeabilisation with 80% (v/v) acetone. Cells were subsequently stained with FITC goat anti-mouse IgG (Millipore), fixed and measured on a FACSCantoII with FACSDiva software (BD Biosciences). Collected samples were analysed with FLOWJO, version 8.8.7 (TreeStar) where IAV infection was determined as the percentage of single cells that were FITC positive.

RNA Extraction, cDNA Synthesis and Quantitative RT-PCR
Total RNA was extracted using the RNeasy kit (Qiagen, USA), genomic DNA was removed using TurboDNase (Ambion, USA) and cDNA was synthesized using SuperScript™ reverse transcriptase (Invitrogen), according to the manufacturer's instructions. cDNA for viral mRNA quantification was synthesised using oligo(dT)$_{20}$ (Roche, USA), cDNA for viral RNA (vRNA) quantifi-

cation was synthesised using the Uni12 primer [18, 19] and cDNA for host gene expression using both oligo(dT)$_{20}$ (Roche) and random hexamers. Quantitative PCR measurements for IFN-β (NM_002176.2), IFN-γ (NM_000619.2), RIG-I (NM_014314.3), IFIT1 (NM_001548.3), TNF-α (NM_000594.2) and GAPDH (NM_002046.4) were performed using commercially available TaqMan gene expression assays (Applied Biosystems, USA). The PCR conditions were as follows: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. Mean relative mRNA expression from two replicate measurements was normalised to GAPDH expression and calculated using the ΔΔCT method. Alternatively, quantitative PCR was performed using SYBR green (Quantance; SensiMix, UK) and primers targeting the matrix gene of PR8/34 (forward: 5′-AAGACCAATCCTGTCACCTCTGA-3′ and reverse: 5′-TCCTCGCTCACTGGGCA-3′) on the Stratagene Mx3005 quantitative PCR thermocycler (Agilent Technologies, USA) as described previously [19]. Cycling conditions were as follows: 95°C for 15 min followed by 38 cycles of 95°C for 15 s, 56°C for 60 s and 72°C for 30 s. In each plate, cDNA levels were calculated using a standard curve created from tenfold dilutions on plasmid DNA (pHW2000 containing the PR8/34 matrix gene).

Microarray Analysis
Four independent samples (each derived from a different donor) were used for each condition and were analysed using Affymetrix human ST 1.0 exon arrays. RNA quality was assessed on the Agilent 2100 bioanalyser with RNA 6000 Nano chips (Agilent). RNA material was amplified, transformed to cDNA and labelled using the Ambion WT expression kit (Ambion) and the Affymetrix terminal labelling kit (Affymetrix, USA). Labelled cDNA was then hybridised for 17 h at 42°C to a human ST 1.0 exon array, washed and stained according to manufacturer's instructions, and scanned on a Genechip® scanner 3000 (Affymetrix). Affymetrix® CEL files from microarray scans were used for quality control and first robust multiarray averaging analysis for normalisation was performed with Affymetrix Expression Console. Data were processed using ArrayStar (DNASTAR, USA). Differential expression tests were performed with a moderated t test implemented in ArrayStar, followed by false discovery rate correction of the p values (q values) according to the method of Storey and Tibshirani [20]. A gene was considered to be differentially expressed when an expression ratio of >4 or <−4 relative to the control was obtained. Differentially expressed genes were mapped to Gene Ontology terms to enrich for gene class using the GeneMANIA online tool

[21]. The proportional Venn diagram was drawn using the eulerAPE application version 2.0.3 [22, 23]. Affymetrix exon array data are available at the Gene Expression Omnibus (GSE41295).

Cytokine Production

Levels of cytokines produced by macrophages were measured using the relevant human ELISA kits (Sanquin, The Netherlands) according to the manufacturer's guidelines.

Viral Production

Virus titres in the culture supernatant at different time points after IAV infection of MDMs was determined by titration of the supernatant on MDCK cells and enumeration of infected MDCK cells. Confluent MDCK cells were infected with twofold viral dilutions in DMEM with trypsin that was treated with 0.2 μg/ml L-1-tosylamido-2-phenylethyl chloromethyl ketone (Worthington, USA) for 1 h at 37°C. After 1 h, fresh DMEM and 10% FCS were added and cells were incubated at 37°C for 16 h. Infected (GFP+) cells were visualised by an Olympus CKX41 microscope and virus titre was determined by counting the wells with ≥10 and ≤100 infected cells/view where each data point was the average of an experimental duplicate.

Antigen Presentation

MDMs were isolated and differentiated from HLA-A2+ human PBMCs obtained from buffy coats of healthy donors (Australian Red Cross). MDMs were stimulated and infected with IAV essentially as described above. CD8+ T cells specific to the Epstein-Barr virus (EBV) peptide EBV-BMLF1$_{280-288}$ (GLCTLVAML; GLC) [24] were in vitro amplified by co-incubation with autologous PBMCs pulsed for 1 h with 10 μM GLC. In order to augment the number of EBV-specific T cells, T cell cultures were further amplified 7 days later by co-incubation for another 7 days with CIR cells that were transfected with HLA-A2, pulsed for 1 h with GLC (10 μM) and then irradiated. The ability of infected/stimulated MDMs to present GLC to autologous, EBV-specific CD8+ T cells was then assessed by flow cytometry. Briefly, MDMs were pulsed with GLC (10 μM) for 90 min. Alternatively, MDMs were pulsed with 10 μM NS3$_{1073-1081}$ CINGVCWTV (CING), a peptide derived from hepatitis C virus [25], as a control to confirm the specificity of the T cells. MDMs were then co-incubated with EBV-specific T cells for 6 h in the presence of FITC-conjugated anti-CD107a (eBioscience, Australia), monensin (5 μM; Sigma, Australia) and BD GolgiPlug (BD Biosciences) [26]. CD8+ T cells were then stained for CD3 (PE-Cy7; eBioscience) and CD8 (PerCP-Cy5.5; BD Pharmingen, USA). Cells were subsequently washed, fixed and permeabilised using the Cytofix/Cytoperm Fixation/Permeabilisation kit (BD Biosciences) and stained with anti-IFN-γ (PE; BD Pharmingen). Cells were then analysed on a FACSCantoII with FACSDiva software (BD Biosciences). Collected samples were analysed with FLOWJO, version 8.8.7 (TreeStar) where the percentage of single cell, CD8+ CD3+ lymphocytes that were either IFN-γ or CD107a positive was determined.

Results

LPS Inhibits IAV Infection of Human MDMs

The nasal cavity contains a variety of different leucocytes situated both between surface epithelial cells and in the sub-epithelial layer [10]. These leucocytes may be exposed to soluble bacterial ligands either directly or following a breach of the epithelial basement membrane. Due to their longevity and crucial role in tissue homeostasis [12], stimulation and infection of macrophages is of particular relevance. In order to assess the role of bacterial ligands in modulating IAV pathogenesis in human macrophages, macrophages were differentiated from the monocytes of healthy donors. MDMs were then stimulated with select bacterial ligands for 24 h and infected with IAV. MDMs were also stimulated with polyinosinic-polycytidylic acid (polyI:C; a TLR3/RIG-I/MDA-5 agonist), a synthetic analogue of dsRNA which triggers type I IFN production in macrophages [27]. As type I IFNs can inhibit IAV infection of leucocytes [28, 29], we reasoned that macrophages pre-stimulated with polyI:C would display reduced IAV infection. As expected, polyI:C treatment resulted in a significant decrease in IAV infection ($p > 0.001$). However, LPS pre-stimulation also significantly reduced IAV infection of MDMs ($p < 0.001$) whilst pre-stimulation with P3C (P3C; TLR2/1 agonist), lipoteichoic acid (TLR2/6 agonist), flagellin (TLR5/NLRC4 agonist), CpG (a TLR9 agonist) or muramyl dipeptide (NOD2 agonist) did not ($p > 0.05$; fig. 1a). This was not due to an increased rate of macrophage apoptosis following LPS stimulation (data not shown) or differential production of IL-6, IL-10 and TNFα (online suppl. fig. S2; for all online suppl. material, see www.karger.com/doi/10.1159/000353905) following stimulation with LPS.

We then assessed whether LPS-induced inhibition of infection was limited to IAV, or if a similar phenotype was observed in response to infection with respiratory syncytial virus (RSV), another enveloped, negative-sense RNA virus. As observed with IAV, pre-stimulation with LPS significantly reduced rgRSV30 infection of human MDMs ($p < 0.06$; fig. 1b). LPS-induced inhibition of infection was not restricted to GFP-labelled virus strains, as LPS pre-stimulation also inhibited infection of MDMs with the non-labelled IAV strain HKx31 (H3N2; $p < 0.01$; fig. 1c). These data suggest that LPS-induced inhibition of infection is not restricted to one IAV strain and may represent a more generalised mechanism of inhibiting infection with enveloped, negative-sense RNA viruses.

To further understand the mechanism by which LPS inhibits IAV infection of MDMs, the kinetics of this interaction was investigated. Co-administration of IAV and LPS (i.e. a pre-stimulation period of 0 h) did not affect the rate of IAV infection (fig. 1d). Instead, a minimum 4-hour pre-stimulation with LPS (prior to infec-
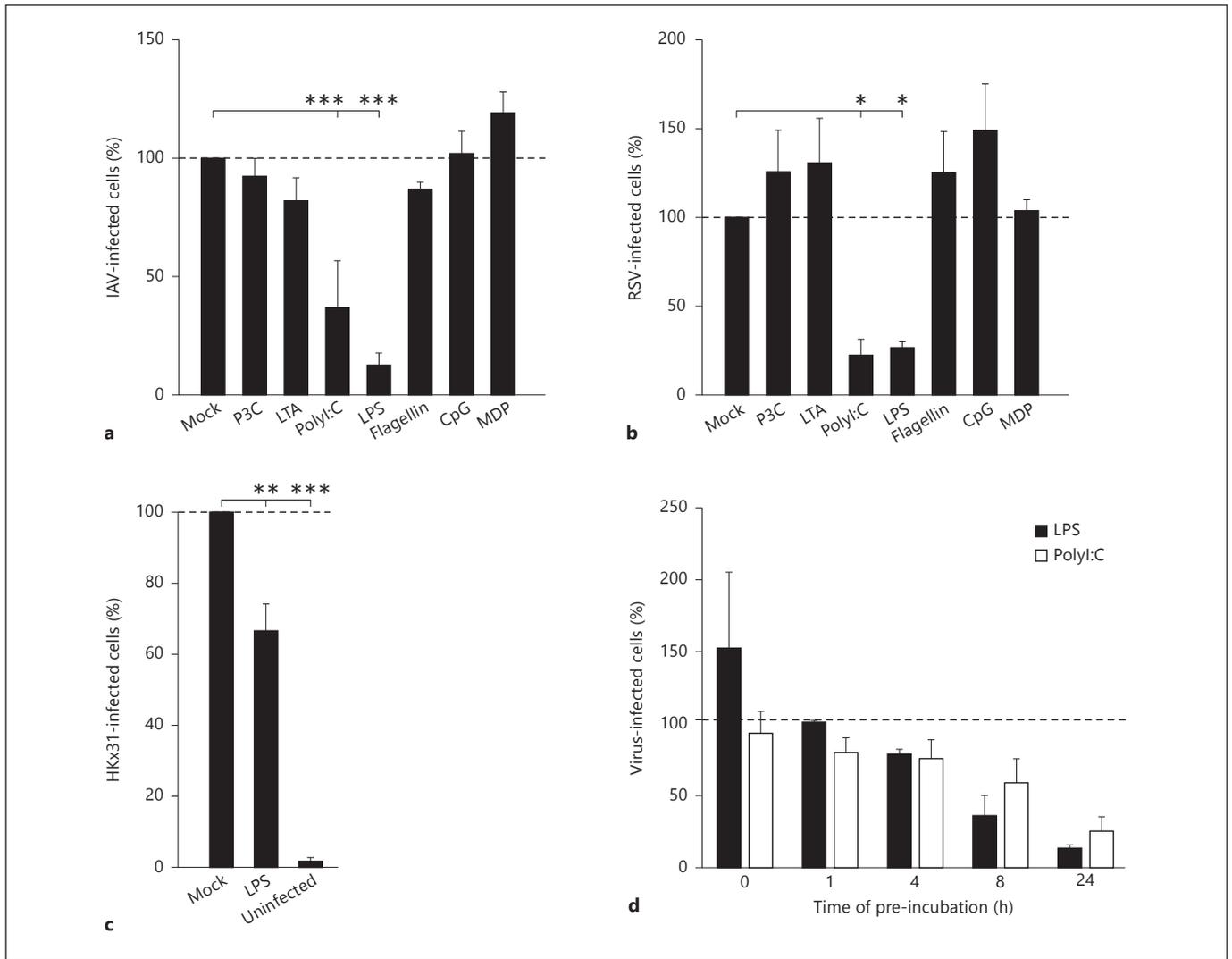
Fig. 1. Pre-stimulation with LPS inhibits IAV (GFP-PR8/34) infection of human MDMs. All data sets were normalised to cells treated with mock infection and IAV, which were defined as 100% infection. Accordingly, an infection rate of >100% indicates enhanced infection rates relative to cells treated with mock infection and IAV. **a** The percentage of MDMs infected with IAV (MOI 0.01) 16 h after infection. Cells were stimulated with the relevant ligands for 24 h prior to IAV infection. **b** The percentage of MDMs infected with RSV (MOI 1) 16 h after infection. Cells were stimulated with the relevant ligands for 24 h prior to RSV infection. **c** The percentage of MDMs infected with IAV (HKx31; MOI 0.1) 16 h after infection. Cells were stimulated with LPS or mock stimulated for 24 h prior to IAV infection. **a–c** Statistical significance was assessed relative to 'mock + virus' cells using one-way ANOVA with Dunnet's post hoc test (* p < 0.05, ** p < 0.01, *** p < 0.001). All data sets were normalised to cells treated with mock + IAV, which were defined as 100% infection. A dashed line indicates an infection rate of 100% (i.e. no change). Data are pooled from a minimum of 3 different donors. **d** The percentage of MDMs infected with IAV (MOI 0.01) 16 h after infection. Cells were stimulated with LPS or polyI:C for the indicated time points prior to IAV infection. A dashed line indicates an infection rate of 100% (i.e. no change). Data are pooled from 3 different donors. The raw (i.e. not normalised) data are shown in online supplementary figure S1. LTA = Lipoteichoic acid; MDP = muramyl dipeptide.

tion with IAV) was necessary to significantly reduce the rate of viral infection (fig. 1d). The rate of IAV infection was inversely proportional to the time of LPS pre-stimulation, such that a 24-hour pre-stimulation period had the greatest effect on IAV infection (fig. 1d). Interestingly, the kinetics of polyI:C-induced viral inhibition were virtually identical to those observed for LPS, and the greatest inhibition of IAV infection occurred when the macrophages were pre-stimulated with polyI:C for 24 h (fig. 1d).
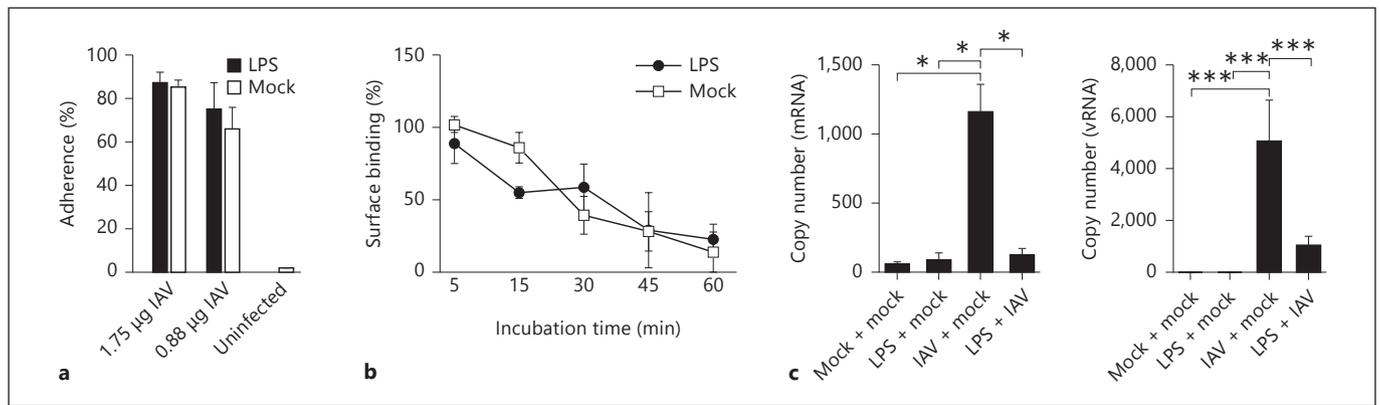
Effect of LPS and Influenza Virus on
Human Macrophages

Fig. 2. Pre-stimulation with LPS reduces the transcription of IAV in human MDMs. **a** The percentage of cell-surface-associated IAV on mock- or LPS-stimulated human MDMs following a 1-hour incubation at 4°C. Data are expressed as the percentage of cells that stained positive for IAV. Statistical significance between LPS- and mock-stimulated cells at each virus concentration was assessed using a Mann-Whitney U test. **b** The percentage of cell-surface-associated IAV on mock- or LPS-stimulated human MDMs following incubation for varying periods at 37°C. Data are normalised to mock- or LPS-stimulated MDMs held at 4°C for 60 min, which was defined as 100%. Statistical significance between LPS- and mock-stimulated cells was assessed using two-way ANOVA. **c** Copy number of vRNA and mRNA in human MDMs. Data show means ± SEM. Data were obtained 16 h after IAV (MOI 0.01) or mock infection. Copy number is expressed per 0.2 µg of RNA. All data were pooled from 3 different donors. Statistical significance was assessed by one-way ANOVA with Bonferroni post hoc correction (* p < 0.05, *** p < 0.001).

LPS Reduces IAV Transcription

There are multiple steps in the IAV replication cycle that LPS may inhibit. As we have measured viral infection by GFP expression (i.e. the production of viral proteins), LPS is likely to inhibit protein production or a process upstream of this in the viral replication cycle rather than a downstream process such as viral assembly or budding. To address how LPS inhibits IAV infection of MDMs, we investigated the effect of LPS pre-stimulation on the first stage of viral replication, i.e. adhesion to the cell surface. No significant difference was observed in virus adherence to MDMs stimulated with LPS or without stimulation for 24 h (p > 0.05; fig. 2a). We then investigated the next stage in the IAV replication cycle, internalisation of virus particles. To measure the rate of virus internalisation, the number of virus particles remaining on the cell surface of LPS- or mock-stimulated MDMs was determined following different incubation periods at 37°C. The rate of virus internalisation was not significantly different between LPS- and mock-stimulated macrophages (p > 0.05; fig. 2b). We then investigated the effect of LPS further downstream in the viral replication cycle by assessing viral transcription. 'Mock- + IAV-'treated MDMs had significantly higher levels of vRNA (p < 0.001) and mRNA (p < 0.05) compared to LPS-stimulated, IAV-infected MDMs (fig. 2c). These data suggest that LPS pre-treatment of human MDMs inhibits viral transcription rather than viral attachment or internalisation.

LPS Induces an Antiviral State in Human Macrophages

PolyI:C pre-stimulation inhibits IAV infection of human leucocytes via triggering the production of type I IFNs and activating an anti-viral state in the cells [28, 29]. Given that the kinetics of LPS-induced inhibition of infection closely mirrored those of polyI:C, we reasoned that pre-stimulation with LPS also triggered an anti-viral state in the MDMs. A component of this anti-viral response may then serve to reduce viral transcription. To further elucidate the response of MDMs to LPS, a whole-genome array was performed on MDMs 4 h after LPS/polyI:C/P3C stimulation. This time point was selected as 4 h represent the minimum pre-stimulation time necessary to inhibit IAV infection (fig. 1d). P3C (which signals via TLR2 and MyD88) was included in this analysis to represent a treatment condition that did not inhibit IAV infection, whilst polyI:C (which signals via TLR3 and TRIF) was included to represent a treatment condition that, like LPS, also inhibited IAV infection. Gene expression was analysed relative to mock-stimulated MDMs and 27 genes were identified as being ≥4-fold up-regulated in LPS and polyI:C pre-stimulated macrophages, but not P3C pre-stimulated macrophages (fig. 3a; online suppl. table S1). These differentially expressed genes were strongly associated with anti-viral/type I IFN responses (fig. 3b) and included a variety of IFN-induced proteins (e.g. IFIT1), 'viral-sensing' molecules (such as RIG-I) and
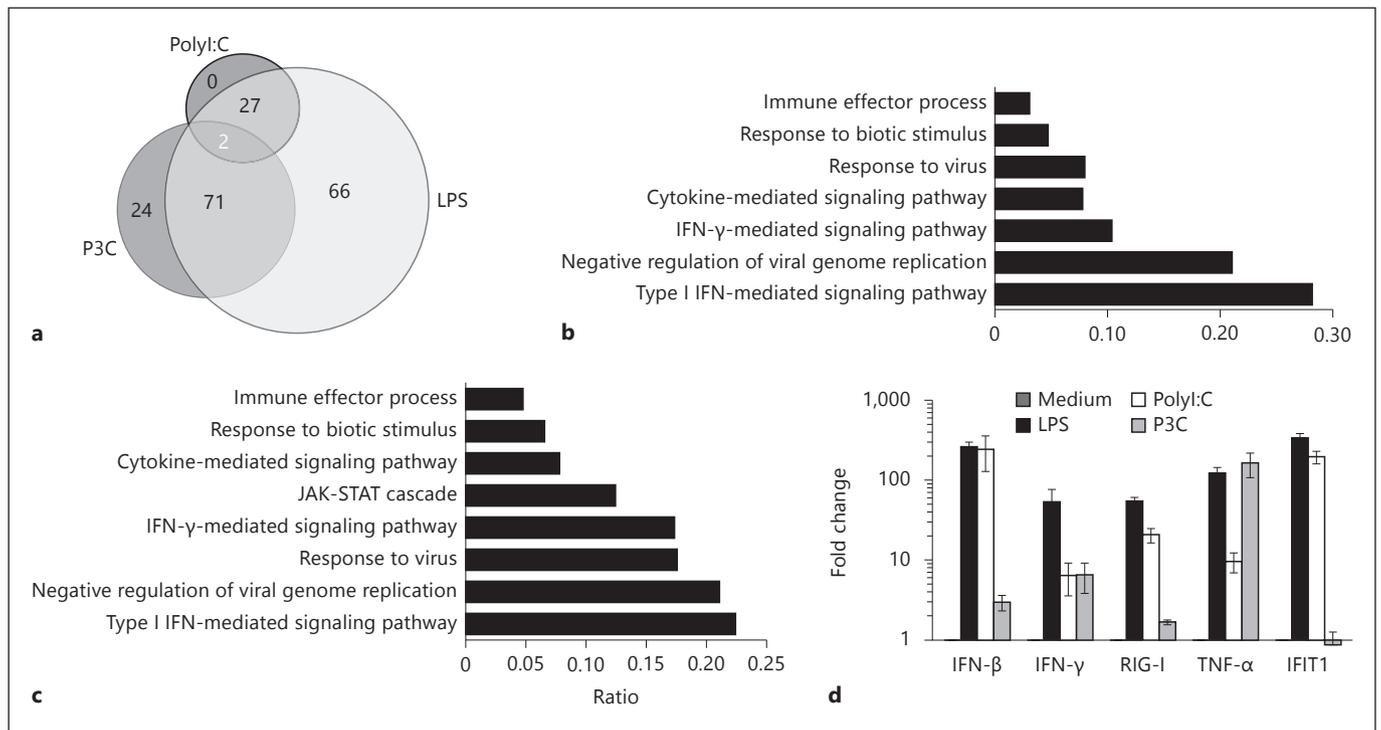
Short et al.

Fig. 3. LPS induces an anti-viral state in human MDMs. **a** Number of genes that are ≥4-fold differentially expressed after stimulation of MDMs with LPS, polyI:C or P3C for 4 h compared to mock-stimulated MDMs. The Euler diagram was made using eulerAPE. Transcription analysis was performed on MDMs from 4 different donors using Affymetrix human ST 1.0 exon arrays. **b** Pathway analysis of the 27 genes up-regulated by LPS and polyI:C but not P3C. Analysis was performed using the online GeneMANIA. Ratios were calculated by dividing the number of up-regulated genes associated with a specific pathway by the number of total genes associated with this pathway. **c** Pathway analysis of the 66 genes up-regulated by LPS but not polyI:C or P3C. Analysis was performed using the online software GeneMANIA. Ratios were calculated by dividing the number of up-regulated genes associated with a specific pathway by the number of total genes associated with this pathway. **d** Fold up-regulation of IFN-β, IFN-γ, RIG-I, TNF-α and IFIT1 after 4 h of stimulation of MDMs with LPS, polyI:C or P3C. Fold up-regulation was compared to medium-stimulated MDMs and calculated using the ΔΔCT method. Data represent the mean ± SEM. Data were pooled from 4 different donors.

anti-viral proteins (e.g. Mx1 and 2′,5′-oligoadenylate synthetase 2; online suppl. table S1). Despite the fact that the kinetics of LPS- and polyI:C-induced inhibition of viral infection were identical, it remains possible that these TLR ligands are acting through different pathways to inhibit IAV infection. We therefore analysed the 66 genes that were specifically up-regulated only by LPS but not P3C or polyI:C (fig. 3c; online suppl. table S2). These genes were also strongly associated with a type I IFN response and the inhibition of viral replication (fig. 3c). The expression of anti-viral/IFN-associated genes (including IFN-β, RIG-I and IFIT1) following LPS pre-stimulation of MDMs was subsequently confirmed by quantitative PCR (fig. 3d). Taken together, these data suggest that LPS may inhibit IAV infection by inducing an IFN/anti-viral state in human MDMs.

Infection of Human MDMs Is Abortive

We then sought to investigate the functional consequences of LPS-suppressed viral infection of MDMs. In this study, IAV infection has been measured by GFP expression, i.e. at the level of viral protein production. There is currently contradicting evidence as to whether IAV infection of MDMs is abortive or productive [30]. We thus investigated whether LPS pre-stimulation resulted in a reduced number of infectious virus particles being produced in the supernatant. However, IAV infection of MDMs was abortive both with and without pre-stimulation with LPS (fig. 4a). Thus, LPS pre-stimulation of MDMs does not affect the rate of infectious virion production.
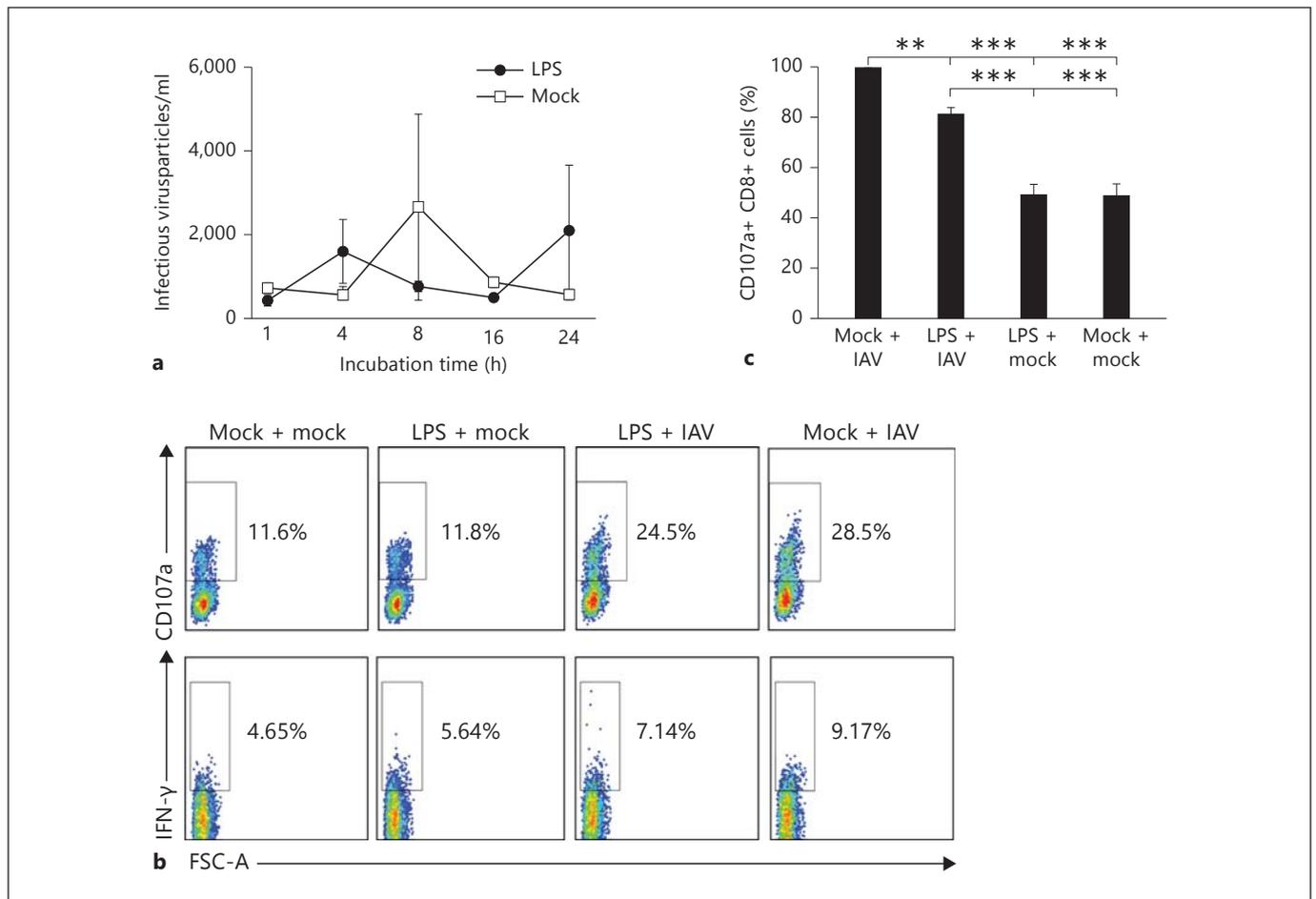
Fig. 4. IAV infection increases antigen presentation by human MDMs. **a** IAV particles in the supernatant of MDMs (±LPS stimulation) at various time points after IAV infection (MOI 0.01). Data represent the mean ± SEM and statistical significance was determined by two-way ANOVA. Data were pooled from 3 different donors. **b** Representative FACS plots showing CD107a and IFN-γ expression by CD8+CD3+ T cells following co-incubation with MDMs. Data are expressed as the percentage of CD107a+ or IFN-γ+ cells among CD8+CD3+ cells. Data are representative of 3 different donors. **c** The percentage of CD8+CD3+ cells which were positive for CD107a. All data sets were normalised to cells treated with mock + IAV, which were defined as 100% CD107a expression. Data are pooled from a minimum of 3 different donors. Statistical significance was assessed by one-way ANOVA with Bonferroni post hoc correction (** p < 0.01, *** p < 0.001).

LPS Stimulation prior to IAV Infection Decreases Antigen Presentation to CD8+ T Cells

Whilst MDMs in this study were unable to produce infectious virus particles, the reduced production of viral proteins in LPS-stimulated MDMs may affect their functionality. Macrophages in IAV-infected lungs can act as APCs and trigger cytolysis and cytokine production by effector CD8+ T cells [31]. Thus, we reasoned that decreased production of viral proteins in mock-stimulated MDMs may alter antigen presentation to CD8+ T cells. To assess this hypothesis, MDMs were differentiated from the monocytes of healthy donors. CD8+ T cells specific to the EBV-derived peptide GLC were also in vitro amplified from autologous PBMCs. In these experiments, the EBV peptide GLC was used as a model antigen to assess antigen presentation. An IAV epitope was not used for these experiments as we wished to assess antigen presentation by IAV-infected MDMs. Hence, if an IAV antigen were used, it would be difficult to differentiate the presentation of the externally added peptide from any peptides derived from the viral infection itself. The ability of LPS- or mock-stimulated MDMs to present antigen following IAV infection was then determined by assessing the percentage of CD8+ T cells expressing the degranulation marker CD107a or producing IFN-γ [26]. MDMs pre-stimulated with LPS and subsequently infected with

IAV induced significantly less T cell degranulation compared to unstimulated MDMs infected with IAV (p < 0.01; fig. 4b, c). A similar trend in IFN-γ production by CD8+ T cells was also observed (fig. 4b). The specificity of this response was confirmed by the negligible CD8+ T cell degranulation and IFN-γ production induced by MDMs pulsed with an irrelevant peptide (online suppl. fig. S3). Interestingly, uninfected MDMs (±LPS pre-stimulated) pulsed with GLC induced less T cell degranulation and IFN-γ production compared to IAV-infected MDMs (±LPS pre-stimulation; p < 0.001; fig. 4b–d). Thus, these data suggest that increased IAV infection of human MDMs results in increased antigen presentation to autologous CD8+ T cells.

Discussion

The nasal cavity is an area of high bacterial density and diversity that contains both Gram-positive (e.g. *Streptococcus* spp.) and Gram-negative (e.g. *Haemophilus influenzae and Moraxella catarrhalis*) bacteria [32]. The nasal cavity is also the first site of infection for IAV. There is now a growing body of evidence to suggest that exposure to bacterial ligands, such as those in the nasal cavity, can affect IAV infection and disease [6–9]. However, the majority of these studies have been performed using murine models of disease, and their applicability to human infections has been unclear [6–9]. Moreover, whilst the effect of TLR pre-stimulation on IAV infection of epithelial cells has been examined [9], to the best of our knowledge this represents the first study to address the interactions between IAV, bacterial PAMPs and primary human MDMs. The ability of IAV to infect human macrophages has important consequences for the pathogenesis of the virus and the subsequent development of an adaptive immune response [30]. How this is altered following priming by soluble bacterial ligands is therefore of considerable interest.

Here, we demonstrated that pre-stimulation of human MDMs with LPS significantly impaired IAV infection of these cells. Transcriptional analysis suggested that this inhibition was associated with the strong anti-viral/IFN response triggered following LPS stimulation. Although the ability of LPS to elicit an IFN response in human MDMs has previously been demonstrated [9], this article represents the first evidence that this has important consequences for IAV infection. This anti-viral response is most likely mediated by an MyD88-independent, TRIF-dependent signalling cascade elicited following engagement of TLR4, which ultimately results in the activation of IFN regulatory factor 3 and the transcription of IFN-β and type I IFN-inducible genes [33, 34]. Unfortunately, attempts to block the IFN response induced by LPS by knocking down IFN regulatory factor 3 were unsuccessful (data not shown). This is most likely due to the redundancies present in the type I IFN pathway (e.g. IFN regulatory factor 7 can also induce an IFN response) and the intrinsic difficulties of using siRNAs to inhibit antiviral responses [35] in primary human cells. The role of a MyD88-independent but TRIF-dependent signalling pathway in inhibiting IAV infection of MDMs is supported by our observation that pre-stimulation with polyI:C (MyD88 independent) inhibited IAV infection, whilst P3C pre-stimulation (MyD88 dependent) did not. It is interesting to speculate which components of the LPS-induced inflammatory response may inhibit IAV infection. One possibility is that the up-regulation of RNase L and 2′,5′-oligoadenylate synthetase 1 following LPS stimulation may serve to inhibit viral transcription by degrading viral mRNA [36]. Interestingly, the inhibitory effects of proteins such as RNAse L on viral replication are not restricted to IAV and also limit the replication of a variety of other viral pathogens [36]. This may explain why the phenotype observed here was not restricted to IAV and that LPS pre-stimulation also inhibited RSV infection of MDMs. The relatively non-specific nature of LPS-induced viral inhibition is further supported by previous studies showing that LPS stimulation of human MDMs inhibits HIV-1 infection [37].

A likely consequence of the reduced viral protein synthesis observed in LPS-stimulated, IAV-infected macrophages is a decreased number of new virions being produced from infected cells. However, consistent with previous reports [38], we found that infection of MDMs with IAV was abortive and that no new virus particles were produced from either mock- or LPS-pre-stimulated MDMs. Nevertheless, we reasoned that the high rate of viral infection in unstimulated MDMs was still relevant to the in vivo situation as it may affect macrophage functionality. Due to the longevity of macrophages, a defect in macrophage function is likely to have long-term consequences for the immune response of the host [39]. Whilst DCs are considered the most 'professional' APCs, one important function of macrophages is to present antigen to specific CD8+ T cells [31]. Here, we have provided, to the best of our knowledge, the first evidence that IAV infection affects antigen presentation by human MDMs. Previous studies investigating the effects of IAV on anti-

gen presentation have largely focused on DCs. Moltedo et al. [40] demonstrated that the high rate of IAV infection of CD103+ DCs (which was due to an impairment in type I IFN signalling) results in increased antigen presentation to CD8+ T cells. Conversely, others have shown that primary human DCs are less able to present antigen to CD8+ T cells following infection with IAV [41]. Similarly, in the absence of bacterial flora mice are more susceptible to IAV infection and display reduced macrophage MHC-I expression and CD8+ T cell activation [7]. In our experiments, the EBV peptide GLC was used as a model antigen to assess antigen presentation by IAV-infected MDMs. This enabled us to directly assess antigen presentation independent of antigen processing. We elected not to use an IAV-derived antigen for these experiments as it would be difficult to differentiate the presentation of the externally added IAV peptide from any peptides derived from the viral infection itself. Thus, if an IAV-derived antigen had been used the results would have been confounded by the amount of available antigen, and the level of antigen processing in IAV-infected macrophages.

We demonstrated that MDMs treated with LPS and IAV induce less degranulation by autologous CD8+ T cells compared to MDMs treated with IAV alone. It is interesting to note that in all experiments there was a higher proportion of CD107a+ CD8+ T cells than IFN-γ+ CD8+ T cells. This is consistent with the two distinct activation thresholds proposed for CD8+ T cells, whereby only in the presence of a mature immunological synapse CD8+ T cells can progress from degranulation (i.e. CD107a positive) to cytokine production [42]. However, more importantly, given the correlation observed between the rate of IAV infection in MDM and CD8+ T cell activation (where increasing the rate of IAV infection increased the amount of CD8+ T cell activation), our data would suggest that infection with IAV increases antigen presentation by human MDMs. At present, it remains unclear how an increased rate of IAV infection facilitates increased antigen presentation. Whilst IAV infection increases MHC-I expression on human DCs [41], we were unable to find any significant differences in MHC-I expression between infected and uninfected MDMs (data not shown). Nevertheless, our data provide the first evidence that priming of primary human macrophages by LPS not only reduces IAV infection, but also affects antigen presentation by macrophages to autologous CD8+ T cells. It remains possible that there are additional functional consequences of increased IAV replication in macrophages aside from those assessed in this study. For ex-

ample, IAV infection is known to affect macrophage phagocytosis [43], thus it is possible that whilst capable of increased antigen presentation, unstimulated macrophages may be less adept at controlling bacterial pathogens following IAV infection. Alternatively, the type I IFNs induced by LPS pre-stimulation may also influence the immune response to other viral infections in vivo. These additional functional consequences remain an area of ongoing research. Nevertheless, this study demonstrates that the microbial context in which viral infections occur can determine the fate of infection through modulation of immune effector functions.

References

1 Fiers W, De Filette M, Birkett A, Neirynck S, Min Jou W: A 'universal' human influenza A vaccine. Virus Res 2004;103:173–176.

2 Short KR, Habets MN, Hermans PWM, Diavatopoulos DA: Interactions between *Streptococcus pneumoniae* and influenza virus: a mutually beneficial relationship? Future Microbiol 2012;7:609–624.

3 Tashiro M, Ciborowski P, Klenk HD, Pulverer G, Rott R: Role of Staphylococcus protease in the development of influenza pneumonia. Nature 1987;325:536–537.

4 Tashiro M, Ciborowski P, Reinacher M, Pulverer G, Klenk HD, Rott R: Synergistic role of staphylococcal proteases in the induction of influenza virus pathogenicity. Virology 1987; 157:421–430.

5 Ayala VI, Teijaro JR, Farber DL, Dorsey SG, Carbonetti NH: *Bordetella pertussis* infection exacerbates influenza virus infection through pertussis toxin-mediated suppression of innate immunity. PLoS One 2011;6:e19016.

6 Ichinohe T, Pang IK, Kumamoto Y, Peaper DR, Ho JH, Murray TS, Iwasaki A: Microbiota regulates immune defense against respiratory tract influenza A virus infection. Proc Natl Acad Sci USA 2011;108:5354–5359.

7 Abt MC, Osborne LC, Monticelli LA, Doering TA, Alenghat T, Sonnenberg GF, Paley MA, Antenus M, Williams KL, Erikson J: Commensal bacteria calibrate the activation threshold of innate antiviral immunity. Immunity 2012;37:158–170.

8 Shinya K, Okamura T, Sueta S, Kasai N, Tanaka M, Ginting TE, Makino A, Eisfeld AJ, Kawaoka Y: Toll-like receptor pre-stimulation protects mice against lethal infection with highly pathogenic influenza viruses. Virol J 2011;8:97–102.

9 Zhao J, Wohlford-Lenane C, Fleming E, Lane TE, McCray PJ Jr, Perlman S: Intranasal treatment with poly(I:C) protects aged mice from lethal respiratory viral infections. J Virol 2012;86:11416–11424.

10 Winther B, Innes DJ Jr, Mills SE, Mygind N, Zito D, Hayden FG: Lymphocyte subsets in normal airway mucosa of the human nose. Arch Otolaryngol Head Neck Surg 1987;113:59–62.

11 Murphy EA, Davis JM, McClellan JL, Carmichael MD, Rooijen NV, Gangemi JD: Susceptibility to infection and inflammatory response following influenza virus (H1N1, A/PR/8/34) challenge: role of macrophages. J Interferon Cytokine Res 2011;31:501–508.

12 Holt P: Down-regulation of immune responses in the lower respiratory tract: the role of alveolar macrophages. Clin Exp Immunol 1986;63:261–270.

13 Manicassamy B, Manicassamy S, Belicha-Villanueva A, Pisanelli G, Pulendran B, García-Sastre A: Analysis of in vivo dynamics of influenza virus infection in mice using a GFP reporter virus. Proc Natl Acad Sci USA 2010;107:11531–11536.

14 Diavatopoulos D, Short K, Price J, Wilksch J, Brown L, Briles D, Strugnell R, Wijburg O: Influenza A virus facilitates *Streptococcus pneumoniae* transmission and disease. FASEB J 2010;24:1789–1798.

15 Anders EM, Hartley CA, Jackson DC: Bovine and mouse serum beta inhibitors of influenza A viruses are mannose-binding lectins. Proc Natl Acad Sci USA 1990;87:4485–4489.

16 Hallak LK, Collins PL, Knudson W, Peeples ME: Iduronic acid-containing glycosaminoglycans on target cells are required for efficient respiratory syncytial virus infection. Virology 2000;271:264–275.

17 Vissers M, Remijn T, Oosting M, de Jong DJ, Diavatopoulos DA, Hermans PWM, Ferwerda G: Respiratory syncytial virus infection augments NOD2 signaling in an IFN-β-dependent manner in human primary cells. Eur J Immunol 2012;42:2727–2735.

18 Hoffmann E, Stech J, Guan Y, Webster R, Perez D: Universal primer set for the full-length amplification of all influenza A viruses. Arch Virol 2001;146:2275–2289.

19 Short KR, Reading PC, Brown LE, Pedersen J, Gilbertson B, Job ER, Edenborough KM, Habets MN, Zomer A, Hermans PW, Diavatopoulos DA, Wijburg OL: Influenza-induced inflammation drives pneumococcal otitis media. Infect Immun 2013;81:645–652.

20 Storey JD, Tibshirani R: Statistical significance for genomewide studies. Proc Natl Acad Sci USA 2003;100:9440.

21 Mostafavi S, Ray D, Warde-Farley D, Grouios C, Morris Q: GeneMANIA: a real-time multiple association network integration algorithm for predicting gene function. Genome Biol 2008;9(suppl 1):S4.

22 Micallef L, Rodgers PR: Application for Drawing 3 Set Area-Proportional Venn Diagrams. Canterbury, University of Kent, 2012.

23 Rodgers P, Flower J, Stapleton G, Howse J: Drawing Area-Proportional Venn-3 Diagrams with Convex Polygons; Diagrammatic Representation and Inference. Berlin, Springer, 2010, pp 54–68.

24 Clute SC, Watkin LB, Cornberg M, Naumov YN, Sullivan JL, Luzuriaga K, Welsh RM, Selin LK: Cross-reactive influenza virus-specific CD8+ T cells contribute to lymphoproliferation in Epstein-Barr virus-associated infectious mononucleosis. J Clin Invest 2005;115:3602–3612.

25 Neveu B, Debeaupuis E, Echasserieau K, le Moullac-Vaidye B, Gassin M, Jegou L, Decalf J, Albert M, Ferry N, Gournay J: Selection of high-avidity CD8 T cells correlates with control of hepatitis C virus infection. Hepatology 2008;48:713–722.

26 Betts MR, Brenchley JM, Price DA, De Rosa SC, Douek DC, Roederer M, Koup RA: Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. J Immunol Methods 2003;281:65–78.

27 Alexopoulou L, Holt AC, Medzhitov R, Flavell RA: Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 2001;413:732–738.

28 Thitithanyanont A, Engering A, Ekchariyawat P, Wiboon-ut S, Limsalakpetch A, Yongvanitchit K, Kum-Arb U, Kanchongkittiphon W, Utaisincharoen P, Sirisinha S: High susceptibility of human dendritic cells to avian influenza H5N1 virus infection and protection by IFN-α and TLR ligands. J Immunol 2007;179:5220–5227.

29 Cella M, Salio M, Sakakibara Y, Langen H, Julkunen I, Lanzavecchia A: Maturation, activation, and protection of dendritic cells induced by double-stranded RNA. J Exp Med 1999;189:821–829.

30 Short KR, Brooks AG, Reading PC, Londrigan SL: The fate of influenza A virus after infection of human macrophages and dendritic cells. J Gen Virol 2012;93(Pt 11):2315–2325.

31 Hufford MM, Kim TS, Sun J, Braciale TJ: Antiviral CD8+ T cell effector activities in situ are regulated by target cell type. J Exp Med 2011;208:167–180.

32 Ingvarsson L, Lundgren K, Ursing J: The bacterial flora in the nasopharynx in healthy children. Acta Otolaryngol 1982;93:94–96.

33 Reimer T, Brcic M, Schweizer M, Jungi TW: Poly(I:C) and LPS induce distinct IRF3 and NF-kappaB signaling during type-I IFN and TNF responses in human macrophages. J Leukoc Biol 2008;83:1249–1257.

34 Honda K, Yanai H, Takaoka A, Taniguchi T: Regulation of the type I IFN induction: a current view. Int Immunol 2005;17:1367–1378.

35 Zamanian-Daryoush M, Marques JT, Gantier MP, Behlke MA, John M, Rayman P, Finke J, Williams BR: Determinants of cytokine induction by small interfering RNA in human peripheral blood mononuclear cells. J Interferon Cytokine Res 2008;28:221–233.

36 Silverman RH: Viral encounters with 2′,5′-oligoadenylate synthetase and RNase L during the interferon antiviral response. J Virol 2007;81:12720–12729.

37 Simard S, Maurais É, Gilbert C, Tremblay MJ: LPS reduces HIV-1 replication in primary human macrophages partly through an endogenous production of type I interferons. Clin Immunol 2008;127:198–205.

38 Friesenhagen J, Boergeling Y, Hrincius E, Ludwig S, Roth J, Viemann D: Highly pathogenic avian influenza viruses inhibit effective immune responses of human blood-derived macrophages. J Leukoc Biol 2012;92:11–20.

39 Didierlaurent A, Goulding J, Patel S, Snelgrove R, Low L, Bebien M, Lawrence T, van Rijt LS, Lambrecht BN, Sirard JC: Sustained desensitization to bacterial Toll-like receptor ligands after resolution of respiratory influenza infection. J Exp Med 2008;205:323–329.

40 Moltedo B, Li W, Yount JS, Moran TM: Unique type I interferon responses determine the functional fate of migratory lung dendritic cells during influenza virus infection. PLoS Pathog 2011;7:e1002345.

41 Smed-Sörensen A, Chalouni C, Chatterjee B, Cohn L, Blattmann P, Nakamura N, Delamarre L, Mellman I: Influenza A virus infection of human primary dendritic cells impairs their ability to cross-present antigen to CD8 T cells. PLoS Pathog 2012;8:e1002572.

42 Faroudi M, Utzny C, Salio M, Cerundolo V, Guiraud M, Müller S, Valitutti S: Lytic versus stimulatory synapse in cytotoxic T lymphocyte/target cell interaction: manifestation of a dual activation threshold. Proc Natl Acad Sci USA 2003;100:14145–14150.

43 Warshauer D, Goldstein E, Akers T, Lippert W, Kim M: Effect of influenza viral infection on the ingestion and killing of bacteria by alveolar macrophages. Am Rev Respir Dis 1977;115:269–277.