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Author(s)	DUTRY, ICA; Li, J; Li, PH; Bruzzone, R; Peiris, JSM; Jaume, MDA
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The Effects of Macrophage Polarity on Influenza Virus Replication and Innate Immune Responses

Isabelle Dutry^{1,2}, Jibin Li¹, Ping Hung Li¹, Roberto Bruzzone^{1,2}, JS Malik Peiris^{1,2} and Martial Jaume^{1,2*}

¹HKU-Pasteur Research Pole, 5 Sassoon Road, Hong Kong SAR, PR China

²School of Public Health, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Pokfulam, Hong Kong SAR, China

*Corresponding author: Martial Jaume, HKU-Pasteur Research Pole, HKJC Building for Interdisciplinary Research, Hong Kong SAR, PR China, Tel: 852-2831 5516; Fax: 852-2872 5782; E-mail: Martial.Jaume@gmail.com

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Abstract

Different pathways of macrophage differentiation and activation lead to diverse macrophage phenotypes including expression of cell surface molecules, cytokine secretion and transcription profiles. Here we investigated *in vitro* the impact of inflammatory or anti-inflammatory polarization of human primary macrophages on their susceptibility to Influenza A virus infection and characterised innate immune responses in infected cells. M2 M-CSF+IL4 macrophages showed greater susceptibility to influenza A infection than M1 GM-CSF+interferon (IFN) γ macrophages.

Keywords: Human macrophages population; *In vitro* polarization; M1; M2; Influenza A virus; Susceptibility; Cytokines

Introduction

Seasonal influenza A viruses are respiratory pathogens causing annual epidemics, typically causing mild illnesses but significant morbidity at the extremes of age. Novel influenza viruses emerge at unpredictable intervals leading to pandemics associated with more widespread and sometimes severe disease. The biological basis for severity of influenza disease remains unclear though it is recognized that the interplay between the influenza viruses and the host immune responses contribute to viral pathogenesis.

Macrophages are key sentinels of the innate immune response and play a crucial role in being the “first responders” as well as contributing to shaping the subsequent (pathogen-specific) adaptive immune response. Upon influenza infection, macrophages are activated and actively phagocytose infected cells thus limiting viral spread. Their activation is also associated with expression of multiple cytokines and chemokines to orchestrate downstream host cellular defences.

Many studies have investigated the expression patterns of antiviral and immunostimulatory cytokines in the macrophages during infection with different strains of influenza A virus [1-5]. Virus infection leads to activation of the NF- κ B pathway, IRF-3 and p38 MAPK pathways, which stimulates the production of type I IFN and enhances the pro-inflammatory response. Induced products include diverse pro-inflammatory cytokines such as TNF α and IL1 β (T-cell stimulatory), IL12 (activates natural killer cells and T lymphocytes), IL8 (recruit neutrophils) and other chemoattractants such as CCL3, CCL2 or CCL7. TNF α is induced in macrophages after infection with some influenza strains [1-5].

It is known that the state of cell activation or differentiation of macrophages *in vitro* generated different subsets of cells that could affect their susceptibility to viral infection [6,7]. Two distinct populations have been reported, one being the pro-inflammatory

classically activated “M1” macrophages described since the nineteen seventies [8], and the other, relatively more recently discovered alternatively activated “M2” macrophages [9]. Other intermediary states of activation have also been documented in response to different growth factors and cytokines [10-12].

M1 macrophages differentiate in culture in response to stimulation by GM-CSF and IFN γ or other pro-inflammatory agents such as tumour necrosis factor- α (TNF α) and bacterial Lipopolysaccharide (LPS). These M1 cells express high levels of pro-inflammatory cytokines such as tumour necrosis factor TNF α , Interleukin (IL) 1 β , IL12, IL18 and the chemokines CCL15, CCL20, CXCL8-11 and CXCL13 in response to challenge. The M1-polarized cells possess enhanced microbicidal capacity and increased secretion of pro-inflammatory cytokines to further strengthen cell-mediated adaptive immunity [6,7,13].

M2 cells are induced by culture with M-CSF and IL4/IL13 or IL-10, the signature cytokines of the CD4+ Th₂ anti-inflammatory response. M2 cells are considered as a continuum of functionally and phenotypically related cells, highly dynamic in type II inflammation and in tissue remodelling, wound healing and allergy. They are subdivided in different groups according to their response to different stimuli [11,12].

In monocytes and macrophages, IL4 or IL13 down-regulates the pro-inflammatory mediators such as CCL2, GM-CSF, IFN γ , IL1 β , IL6, IL8, IL12, TNF α and superoxide anion production [14]. IL4/IL13 also alters the IL1 β activity. In mice, they can produce polyamine and proline for tissue repair, collagen formation and cell growth. Another category includes cells stimulated with IL10, glucocorticoids or TGF β . They are considered as “deactivated macrophages” because of the down regulation of pro-inflammatory cytokines and high expression of scavenger receptors (CD163 for instance). IL10 plays an important role in the homeostatic response to infection and inflammation, inhibiting the pro-inflammatory cytokines. Since those cells are differentiated with M-CSF, which is present at high levels in normal

blood, the drift toward M2 macrophages is believed to be a default pathway.

This study provides a systematic comparison of influenza A virus infection of M1 and M2 subsets of human primary monocyte-derived macrophages and aims to gain insights into the innate immune responses elicited by high and low pathogenic influenza A viruses infections in these different macrophage populations.

Materials and Methods

Isolation and activation of monocyte-derived macrophages: Human peripheral blood cells were extracted from buffy coats provided by the Hong Kong Red Cross Blood Transfusion Service. The protocol of research was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster. Peripheral blood mononuclear cells were separated by Ficoll-Paque™ density gradient centrifugation (GE Healthcare Life Sciences, Piscataway, NJ, USA) and positive CD14 selection was performed by labelling the mononuclear cells with CD14 monoclonal antibodies conjugated to magnetic beads (Miltenyi Biotec, Köln, Germany). Purity and viability of the monocytes were assessed by flow cytometry by staining an aliquot of the purified cells with FITC-conjugated mouse anti-human CD14 mAbs and fixable viability dye eFluor™ 660 (eBiosciences, San Diego, CA, USA). Cells were seeded on coverslips into 24-well tissue culture plates at 0.35×10^6 cells/well for differentiation in RPMI 1640 medium supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and either 5% of autologous plasma or in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 1% non-essential amino-acids, 4 mM L-glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin and 20 µM β-mercaptoethanol and 50 ng/mL of Macrophage-Colony Stimulating Factor (M-CSF) or Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF). All culture media, additives, recombinant growth hormones and cytokines were sourced from Invitrogen, Carlsbad, CA, USA. They were cultured at 37°C in a 5% CO₂ incubator for five days in the respective differentiation media. At day 5, the medium was either refreshed (e.g. cells grown with 5% autologous plasma, GM-CSF or M-CSF only) or different cytokines were added to allow the macrophages to fully differentiate: 20 ng/mL IFNγ + 50 ng/mL GM-CSF, 20 ng/mL IL4 + 50 ng/mL M-CSF or 10 ng/mL IL10 + 50 ng/mL M-CSF. Cells were kept two more days at 37°C in the incubator before being used for infection and characterization. Differentiation of the macrophages was assessed by flow cytometry by staining an aliquot of the polarized cells with PE/Cy7-conjugated mouse anti-human CD86 mAbs, PerCP/Cy5.5-conjugated mouse anti-human CD163 mAbs, APC-conjugated mouse anti-human CD206 (Biolegend, San Diego, CA), FITC-conjugated mouse anti-human CD14 mAbs and fixable viability dye eFluor™ 660 (eBioscience). The donors were included in the study only if all 6 subpopulations of macrophages were correctly polarized and numerous enough to support the whole protocol (i.e., mock infection and infection with three different influenza viruses).

Infection assays with influenza A virus: Pandemic influenza A/California/04/09 (pH1N1), A/Quail/Hong Kong/G1/97 (H9N2/G1) and A/Hong Kong/483/97 (H5N1) were obtained from the virus repository at The University of Hong Kong. Virus stocks were grown in MDCK cells. Three days post-infection, the cell culture supernatants were collected, filtered, centrifuged and then aliquoted for storage at -80°C. Their infectivity was assessed by titration of tissue culture infection dose 50% (TCID₅₀) in MDCK cells. To perform *in*

vitro virus infections, cells were seeded on coverslips, pre-coated if necessary with 50µg/ml poly-L-lysine (Sigma-Aldrich Inc., St. Louis, Missouri, USA) in 24-well culture plates at the usual concentration of 3×10^5 cells/well. The cells were infected with the respective influenza A virus at an Multiplicity of Infection (MOI) of 2. Mock-infected cells were used as controls. After 1h, the cells were washed twice and medium containing 0.5 µg/mL Tosylsulfonyl Phenylalanylchloromethyl Ketone (TPCK)-treated trypsin was added onto the wells together with other macrophage differentiating cytokines as specified. The culture plates were incubated at 37°C in a 5% CO₂ incubator. At specific time points post-infection, cells were either fixed in 4% paraformaldehyde (Sigma-Aldrich Inc., St. Louis, Missouri, USA) for immunofluorescence microscopy, or resuspended in lysis buffer (RLT buffer, RNeasy RNA Mini kit; QIAGEN) for real-time quantitative RT-PCR and stored at -80°C until use.

Virus titration by TCID₅₀ assay: A sub-confluent 96-well tissue culture plate of MDCK cells was prepared 1 day before the virus titration assay. Cells were washed once with PBS and replenished with serum-free Minimal Essential Medium (MEM) supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin and 2 µg/ml of TPCK-treated trypsin. Serial dilutions (from 0.5 log to 7 log) of virus-infected culture supernatants were performed and added onto the cell monolayers in quadruplicate. The plates were observed for a Cytopathic Effect (CPE) daily. The endpoint of viral dilution leading to CPE in 50% of inoculated wells was estimated by using the Karber method.

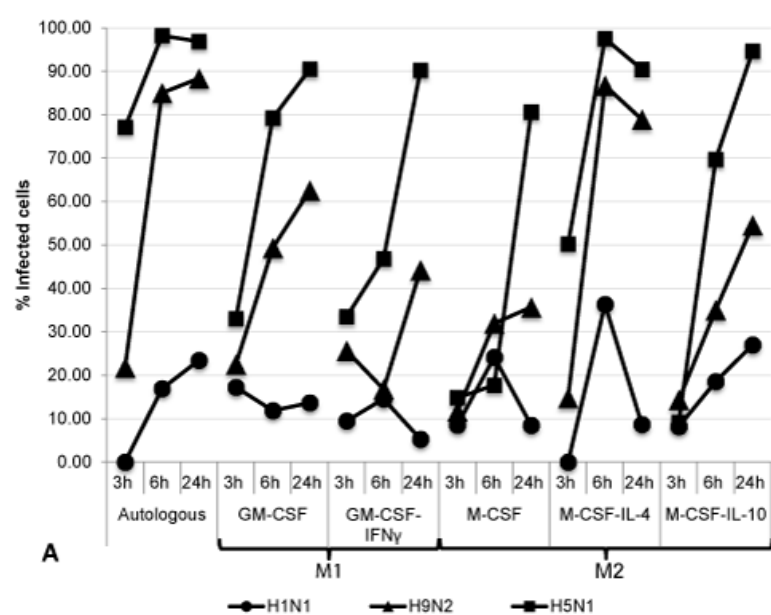
Immunofluorescence microscopy and analysis: The cells were fixed with 4% PFA, free aldehyde groups from PFA treatment were quenched by incubation with a PBS solution containing 50 mM NH₄Cl. The cells were permeabilized with 0.2% Triton X-100 in PBS for 15 minutes. Blocking was performed with 5% goat serum in PBS for 40 minutes. The cells were then incubated for 40 minutes with mouse anti influenza A Nucleoprotein (NP) (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) and rabbit active caspase 3 (BD Biosciences, San Jose, CA, USA). The cells were washed twice with PBS and incubated for 40 minutes with the secondary antibody Alexa Fluor® 488 goat anti mouse and Alexa Fluor® 647 goat anti-rabbit. The cells were washed with PBS and incubated for 5 minutes with 4',6-Diamidino-2-Phenylindole (DAPI) for staining of cell nuclei. After PBS washing, the coverslips were mounted on slides. Image acquisition was performed using fluorescence AxioObserver Z1 microscope (Carl Zeiss, Inc., Thornwood, NY, USA) using 10X objective and 40X oil immersion objective. Images from 5 to 10 random fields were acquired and analysed with Metamorph software (Molecular Devices, Sunnyvale, CA, USA) using either the cell scoring module or the multi-wavelength cell-scoring module.

Real-time quantitative Reverse Transcriptase PCR (RT-PCR): Total RNA was extracted with an RNeasy RNA minikit (Qiagen), with DNase digestion, according to the manufacturer's instructions. Extracted RNA was stored at -80°C until use. Superscript III reverse transcriptase (Invitrogen) and random hexamer primers (Invitrogen) were used to convert RNAs to cDNAs. The quantity of RNA was measured by real-time quantitative PCR using SYBR Green technology on a LightCycler 480-II instrument (Roche). The primers and conditions for detection of the M gene of influenza A virus have been described previously [15]. Positive and negative controls were included in each run and when appropriate, the levels of M gene expression were normalized to those of the 18S rRNA gene, which were determined using 600 nM concentrations of both forward (5'-

CggAggTTCgAAgACgATCA-3') and reverse (5'-ggCgggTCATgggAATAAC-3') primers.

Sialic acid detection and DAS181 treatment: On day 6, macrophages were treated overnight with or without DAS181 (NexBio) at 250 U/mL in RPMI-1640 medium [16]. On day 7, cells were collected for sialic acid detection and Thomsen-Friedenreich (TF) antigen profiling. The sialic acid expression level was examined by staining with lectins. Briefly, cells were distributed in 96 well plates in Tris-Buffered Saline (TBS) at 5×10^5 cells per well. SA α -2,3 and

SA α -2,6 were detected by 2 μ g/mL biotinylated Malckia amurensis lectin II (biotin-MAL, Vector labs) and 0.5 μ g/mL FITC conjugated Sambucus nigra (elderberry) bark lectin (SNA, Vector labs), respectively. PE conjugated streptavidin (STP-PE, Invitrogen) was used to visualize biotin-MAL II staining. After staining the cells with Fixable Viability Dye 660 or 780 (FVD, eBioscience), the data were collected from $\geq 30,000$ singlet living cells on a LSRII flow cytometer (BD Biosciences), and post-acquisition analyses were performed using the FlowJo software (TreeStar).



B

	Autologous 3			Autologous 6			Autologous 24			GM-CSF 3			GM-CSF 6			GM-CSF 24			GM-CSF-IFN γ 3			GM-CSF-IFN γ 6			GM-CSF-IFN γ 24			M-CSF 3			M-CSF 6			M-CSF 24			M-CSF-IL4 3			M-CSF-IL4 6			M-CSF-IL4 24		
	H1N1	H9N2	H5N1	H1N1	H9N2	H5N1	H1N1	H9N2	H5N1	H1N1	H9N2	H5N1	H1N1	H9N2	H5N1	H1N1	H9N2	H5N1	H1N1	H9N2	H5N1	H1N1	H9N2	H5N1	H1N1	H9N2	H5N1	H1N1	H9N2	H5N1	H1N1	H9N2	H5N1	H1N1	H9N2	H5N1	H1N1	H9N2	H5N1						
GM-CSF		**	*																																										
GM-CSF-IFN γ		**	***	***	***	*						*																																	
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M-CSF-IL10		**	*	**	*																																								

Figure 1: Susceptibility of human M1 and M2 macrophages to influenza viruses. (A) CD14⁺-cells were purified from human PBMCs cultured in presence of 5% autologous serum, recombinant GM-CSF, GM-CSF+IFN γ , M-CSF, M-CSF+IL4 or M-CSF+IL10. Cells were incubated with Influenza A/California/04/2009 virus (H1N1), A/Quail/Hong Kong/G1/97 (H9N2) or A/Hong Kong/483/97 (H5N1) at an Multiplicity of Infection (MOI) of 2. At 3, 6 and 24h p.i. cells were fixed with 4% paraformaldehyde, permeabilized and stained for influenza A nucleoprotein and DAPI. The chart shows the median of three different experiments from 3 independent donors. (B) Table shows statistical significance of % of influenza infection of the different macrophages at each time point *p<0.05, **p<0.01, ***p<0.001. Red shows that the reference cell line (listed at the top of column) is significantly higher than the comparator (cells listed on the left) and black shows that it is differently lower, using two-way ANOVA followed by a Bonferroni multiple-comparison test.

Cytokines and Chemokines secretion

The secretion of different cytokines and chemokines from mock and influenza virus-infected macrophages was measured by specific human cytokine beads assay (Flow Cytomix Human Th₁/Th₂ 11-plex kit, eBioscience). The supernatants of mock and influenza virus-infected cells were collected at 6 and 24h p.i. and tested according to

the kit protocol, then fixed with 1% PFA. The data were collected on a LSRII flow cytometer (BD biosciences) and analysis performed using the eBioscience FlowCytomix Software.

Statistical analysis

Viral infection, apoptosis, viral M gene RNA levels and cytokine protein levels were compared in the six differentiated macrophage cell types infected with the three viruses using pairwise two-way Analysis of Variance (ANOVA) followed by a Bonferroni multiple-comparison test. Statistical analysis was carried out using Graph-pad Prism 5. A p value of <0.05 was considered statistically significant.

Results

Subsets of macrophages differ in susceptibility to influenza A infection

Influenza virus protein expression was detected by immunofluorescence staining for viral proteins in six subpopulations of macrophages (Figure 1A and 1B) at 3, 6 and 24 hours after infection at comparable MOIs (MOI=2) with pH1N1, H9N2/G1 or H5N1 virus. The six macrophage subsets were prepared from the buffy coat of three distinct donors. They were all permissive to each of the influenza A

viruses although at different levels. Although there was donor-to-donor variation, the overall trend was consistent across different donors.

M-CSF+IL4 differentiated M2 macrophages were significantly more permissive to H9N2/G1 and/or H5N1 virus infection than M1 macrophages (GM-CSF or GM-CSF+IFN γ) or M-CSF differentiated M2 cells (Figure 1B). Autologous serum differentiated macrophages were more permissive to both viruses compared to M2 (M-CSF or M-CSF+IL10) and M1 (GM-CSF or GM-CSF+IFN γ) cells. In general, H1N1 virus was less efficient at infecting all these types of macrophages and the different types of cell differentiation had no significant impact.

Apoptosis of macrophage subsets

H9N2/G1 and H5N1 virus infected M2 macrophages (M-CSF+IL4) macrophages and H9N2/G1 infected M-CSF+IL10 cells were significantly more susceptible to apoptosis when compared with M1 (GM-CSF or GM-CSF+IFN γ) macrophages (Figure 2A and 2B).

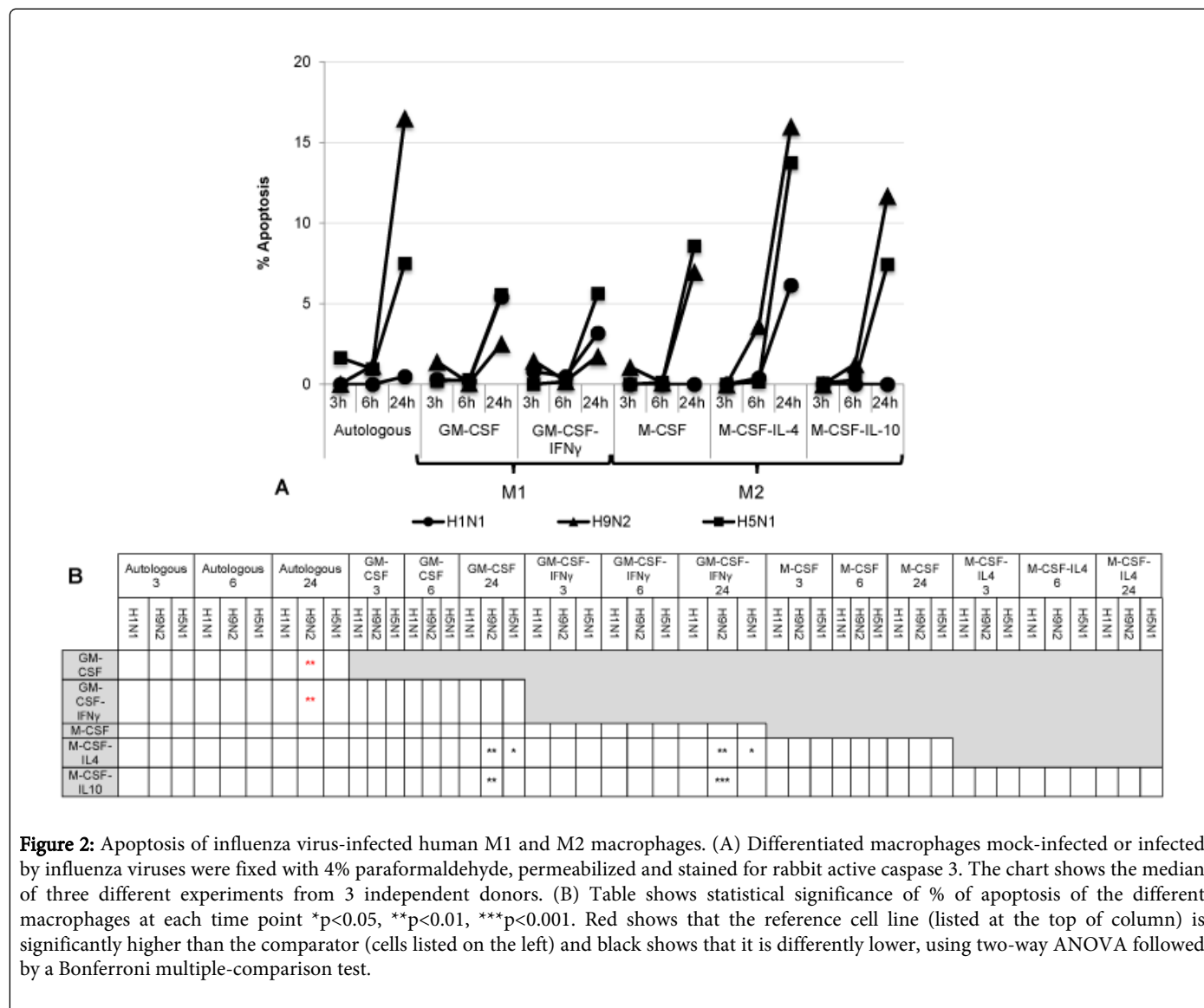


Figure 2: Apoptosis of influenza virus-infected human M1 and M2 macrophages. (A) Differentiated macrophages mock-infected or infected by influenza viruses were fixed with 4% paraformaldehyde, permeabilized and stained for rabbit active caspase 3. The chart shows the median of three different experiments from 3 independent donors. (B) Table shows statistical significance of % of apoptosis of the different macrophages at each time point *p<0.05, **p<0.01, ***p<0.001. Red shows that the reference cell line (listed at the top of column) is significantly higher than the comparator (cells listed on the left) and black shows that it is differently lower, using two-way ANOVA followed by a Bonferroni multiple-comparison test.

Replication of influenza A viruses in the different subsets of macrophages

Viral replication was assessed by quantification of viral RNA (Figure 3A and 3B). RNA samples isolated from either mock- or

influenza virus-infected macrophages were tested by quantitative real-time PCR for expression of the matrix (M) viral gene and viral gene expression was normalized to the housekeeping 18S rRNA gene.

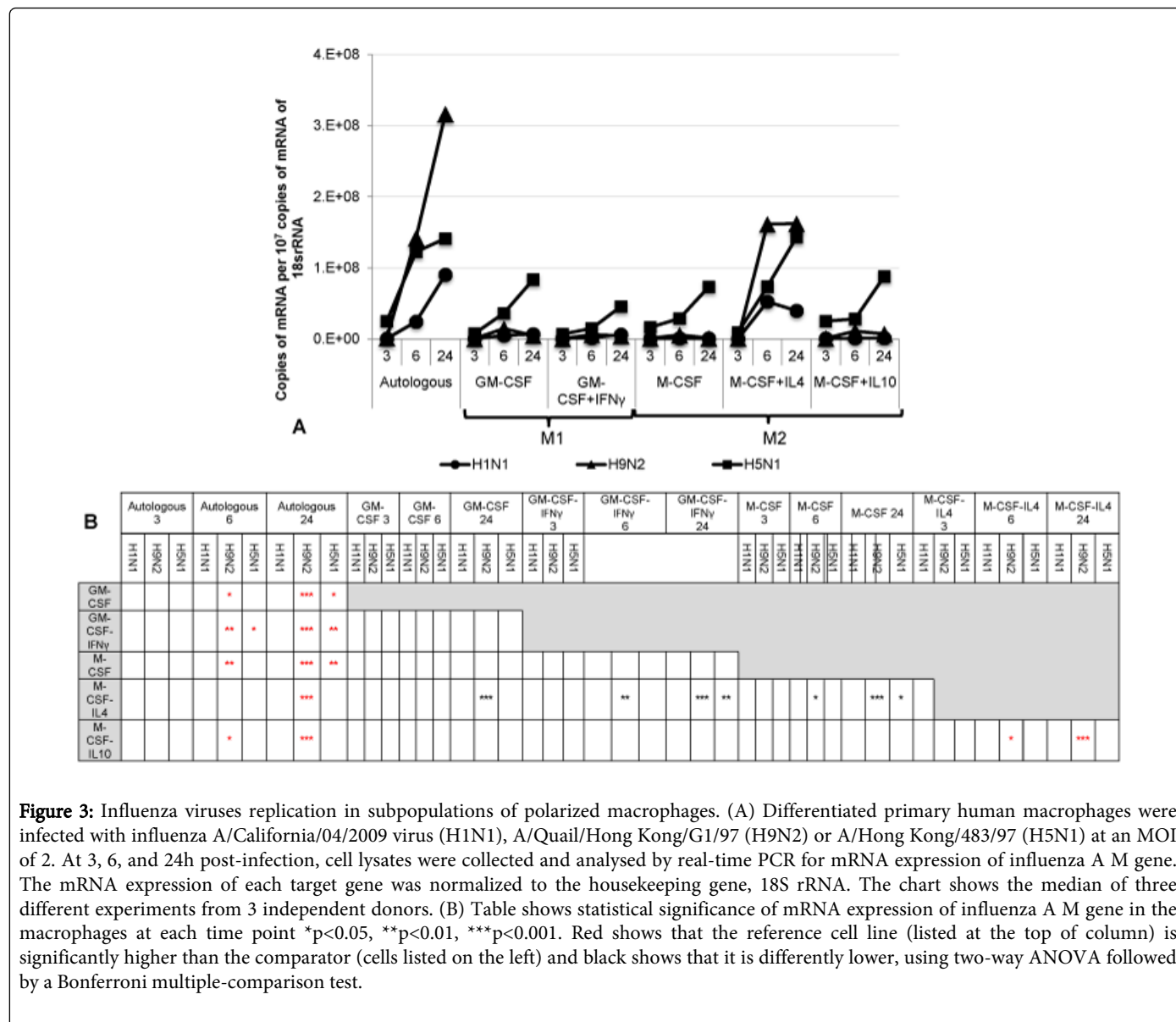


Figure 3: Influenza viruses replication in subpopulations of polarized macrophages. (A) Differentiated primary human macrophages were infected with influenza A/California/04/2009 virus (H1N1), A/Quail/Hong Kong/G1/97 (H9N2) or A/Hong Kong/483/97 (H5N1) at an MOI of 2. At 3, 6, and 24h post-infection, cell lysates were collected and analysed by real-time PCR for mRNA expression of influenza A M gene. The mRNA expression of each target gene was normalized to the housekeeping gene, 18S rRNA. The chart shows the median of three different experiments from 3 independent donors. (B) Table shows statistical significance of mRNA expression of influenza A M gene in the macrophages at each time point *p<0.05, **p<0.01, ***p<0.001. Red shows that the reference cell line (listed at the top of column) is significantly higher than the comparator (cells listed on the left) and black shows that it is differently lower, using two-way ANOVA followed by a Bonferroni multiple-comparison test.

M-CSF+IL4 differentiated M2 cells were significantly more permissive to viral H9N2/G1, and sometimes H5N1, replication compared with M1 (GM-CSF or GM-CSF+IFN γ) differentiated cells. Within the M2 differentiated macrophages, M-CSF+IL4 cells were more permissive than M-CSF or M-CSF+IL10 differentiated cells. Autologous serum differentiated macrophages had higher viral M gene copies than either M1 or M2 differentiated macrophages (Figure 3A and 3B).

The culture supernatants of pH1N1, H9N2/G1 and H5N1-infected activated macrophages were titrated by TCID₅₀ assays to observe virus yield and viral replication kinetics (Figure 4). In the absence of productive viral replication, infectious viral titres decline >2 log₁₀ at 24 hours (unpublished data). Thus, the viral TCID₅₀ titrations confirm

that the M-gene copy numbers shown in Figure 3 is reflected in productive virus replication although differences between macrophages with different differentiation types are less clear (Figure 4).

Sialic acids

It is known that the viral HA mediates primary attachment of influenza virus to host cells via binding to sialic acid residues. Although initial study showed that pH1N1 virus preferentially binds to SA α -2,6, some glycan array studies demonstrated the significant binding of pH1N1 virus to both SA α -2,3 and SA α -2,6 [17]. It could be hypothesized that differential distribution of these sialic acids may

explain the differential susceptibility of macrophages to different avian (H5N1; H9N2/G1) and human (pH1N1) viruses.

The expression profiles of SA at the surface of the different subsets of macrophages were evaluated by using lectins able to preferentially bind to SA with α -2,3 or α -2,6 linkage, MAL II and SNA respectively. Lectin binding was also evaluated on cells either pre-treated or not with DAS181, a sialidase able to remove both α -2,3 and α -2,6 linked glycans [18,19]. As shown in Figure 5, high lectin binding for both MALII (i.e. SA α -2,3) and SNA (i.e. SA α -2,6) was detected for all

subtypes of macrophages despite some slight variation noted in the level of expression between the cell populations. When cells were previously treated with DAS181, all macrophages exhibited a significantly decreased staining for the SNA. However, only a slight decrease in mean of fluorescence intensity of MALII staining was observed following DAS181 treatment of M-CSF, M-CSF+IL4, and M-CSF+IL10 macrophages. A mixed staining was observed for the GM-CSF cells, possibly exemplifying an heterogeneous expression of SA α -2,3 on those cells.

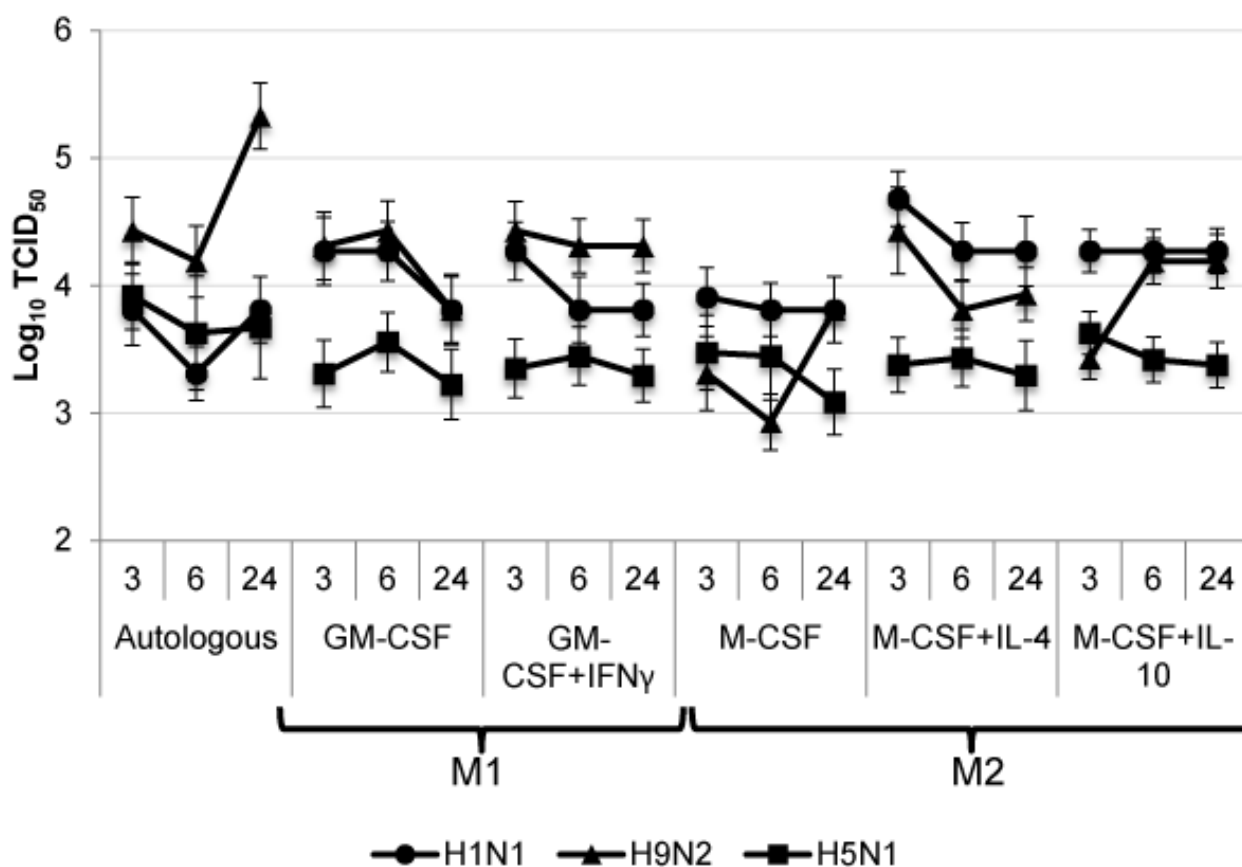


Figure 4: Virus titre detected in the supernatants of influenza virus-infected differentiated macrophages at an MOI of 2. Differentiated primary human macrophages were infected with influenza A/California/04/2009 virus (H1N1), A/Quail/Hong Kong/G1/97 (H9N2) or A/Hong Kong/483/97 (H5N1) at an MOI of 2. At 3, 6, and 24h post-infection, virus-infected culture supernatants were collected. Serial dilutions (from 0.5 log to 7 log) of supernatants were prepared before the addition of the culture supernatant onto a sub-confluent 96-well tissue culture plate of MDCK cells in quadruplicate. The plates were observed for a Cytopathic Effect (CPE) daily. The endpoint of viral dilution leading to CPE in 50% of inoculated wells was estimated by using the Karber method. The chart shows the median and the standard error of the virus titre pooled from three independent experiments.

In order to understand if the detectable binding of MALII on some cells treated with DAS181 was due to non-specific binding of the lectin, thus leading to a high background, or to the inability of the sialidase to cleave SA α -2,3, staining for the residues after cleavage of the branching sialic acid was performed. The Thomsen-Friedenreich (TF) antigen is unmasked only upon cleavage of the SA [20] and TF antigen expression at the surface of the cells was evaluated before and after treatment with DAS181 (data not shown). Compared to native cells, cells treated with DAS181 showed a positive signal for TF

antibody staining, thus demonstrating an efficient cleavage of the SA by DAS181.

Altogether these results illustrate that both SA α -2,3 and SA α -2,6 are expressed at the surface of the differentiated macrophages. Thus, it is unlikely that the variations observed in the sialic acids distribution are sufficient to explain the differential susceptibility of macrophage subtypes to influenza pH1N1.

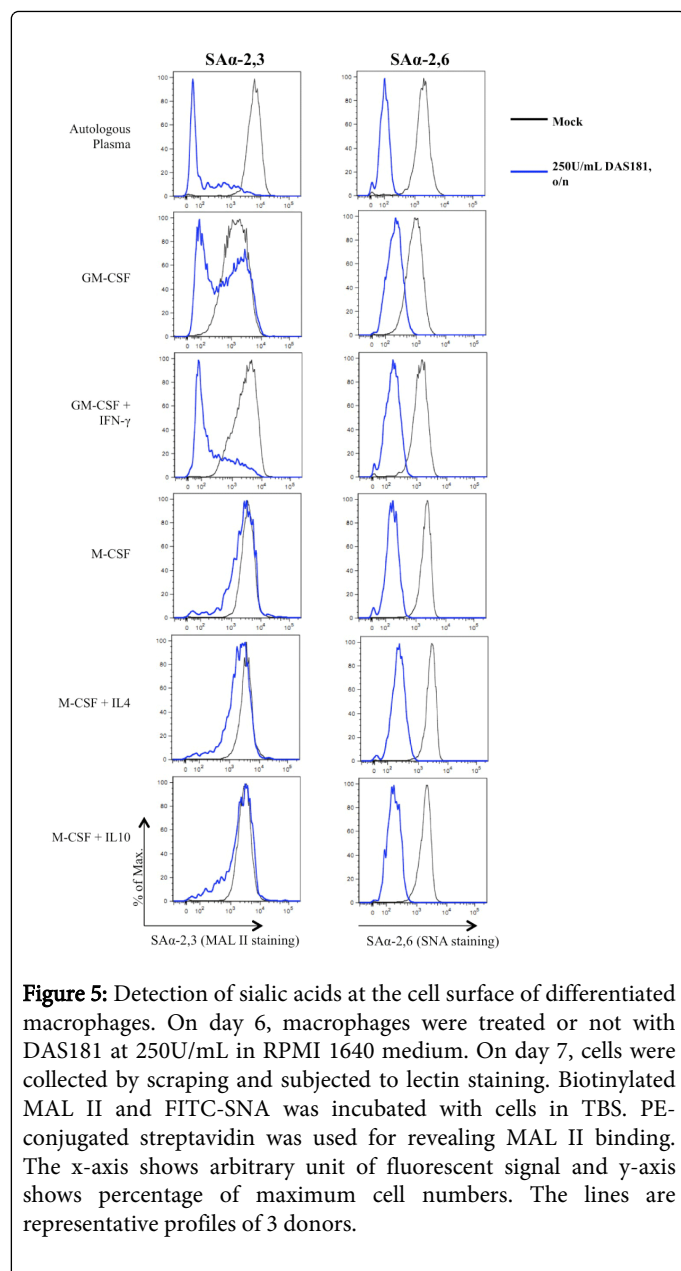


Figure 5: Detection of sialic acids at the cell surface of differentiated macrophages. On day 6, macrophages were treated or not with DAS181 at 250U/mL in RPMI 1640 medium. On day 7, cells were collected by scraping and subjected to lectin staining. Biotinylated MAL II and FITC-SNA was incubated with cells in TBS. PE-conjugated streptavidin was used for revealing MAL II binding. The x-axis shows arbitrary unit of fluorescent signal and y-axis shows percentage of maximum cell numbers. The lines are representative profiles of 3 donors.

Secretion of Cytokines and Chemokines

In order to assess the cytokine and chemokine expression profiles of these different macrophage subsets before and after virus infection, we monitored the cytokines in the cell supernatants using cytokine bead assays. Pro-inflammatory cytokines IL6, CXCL10, CCL3 and TNF α and the anti-inflammatory cytokine IL10 were assayed in mock infected and virus infected cells at 6 and 24h p.i. (Figure 6).

In mock-infected macrophages, baseline expression of CXCL10, CCL3 and TNF α was elevated in GM-CSF+IFN γ differentiated cells. Following virus infection, pH1N1 virus elicited weaker cytokine responses than H9N2/G1 or H5N1 viruses and these results are consistent with other studies [21]. The difference between cell types was also in general more pronounced in H5N1 or H9N2/G1 infected cells. Overall, although GM-CSF+IFN γ differentiated M1

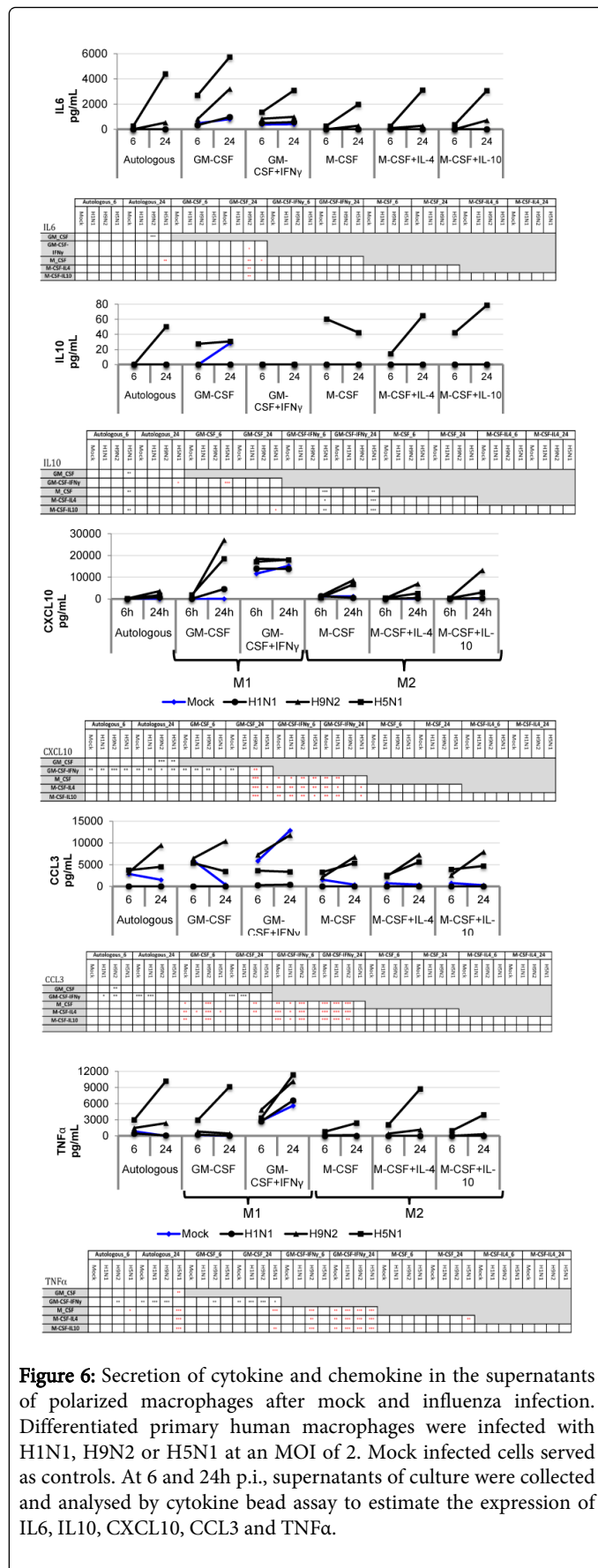
macrophages had higher levels of pro-inflammatory cytokines such as CXCL10, CCL3 and TNF α compared to M2 (M-CSF; M-CSF+IL4; M-CSF+IL10) cells after H5N1 or H9N2/G1 virus infection, this was largely because the baseline (pre-infection) secretion of these cytokines was already high. On the other hand, GM-CSF differentiated macrophages had low baseline levels of IL6 and CXCL10 and H5N1 or H9N2/G1 virus infection led to a significantly more robust response of these cytokines compared with M2 (M-CSF; M-CSF+IL4; M-CSF+IL10) cells. The secretion of anti-inflammatory cytokine IL10 was undetectable in mock infected cells (with the exception of GM-CSF treated cells at 24 hours post mock infection) and secretion was only stimulated by H5N1 virus infection of all cell types other than GM-CSF+IFN γ cells. Interestingly, GM-CSF+IFN γ differentiated cells did not respond to secrete IL10 following infection with any of the viruses studied.

Discussion

The pathogenicity of influenza has been reported to be related to the cell tropism of the virus for respiratory epithelial cells [22]. However, macrophages are also believed to play an important role in the pathogenesis of influenza. It was therefore relevant to investigate the effect of macrophage differentiation on permissiveness to influenza viral infection and on innate immune responses elicited by such infection. The differentiated subpopulations of macrophages we used have been well documented previously [11,13,23]. Thus facilitating comparison and the understanding of the elicited immune responses to influenza infection. The inclusion of monocytes-derived macrophages cultured in medium supplemented with autologous plasma allowed comparison with many previous studies that have used this cell model for studying infection of influenza A viruses [1,24,25].

The results of the infection with different influenza A virus strains showed that all macrophages subsets were permissive to viral infection, more so to avian H5N1 and H9N2/G1 viruses than to pH1N1. The difference between cell types was also more obvious following H5N1 or H9N2/G1 virus infection. M-CSF+IL4 differentiated macrophages and cells differentiated in autologous serum had the greatest susceptibility to both H5N1 and H9N2/G1 viruses while M1 cells were less permissive. This observation correlates well with the results of Hoeve et al. [26] describing a higher level of influenza A/Udorn/72 virus infection in M2 macrophages compared to M1 macrophages. The lower level of infection in GM-CSF+IFN γ subset may be related to the increased baseline levels of antiviral type II interferon [27]. Similarly, it has been demonstrated previously that human DCs and macrophages treated *in vitro* with type I or type III IFN prior to influenza infection elicited potent antiviral activity leading to decreased viral replication [3]. It could be hypothesized that interferon-driven antiviral activities are elicited in these macrophages upon the induction of differentiation/maturation state. Others have reported that GM-CSF activated macrophages showed lower sensitivity to seasonal and influenza pH1N1 infections [4,5,28]. M-CSF+IL4 (M2) differentiated macrophages were also more susceptible to virus induced apoptosis.

The charts in Figure 6 shows the median of three different experiments from 3 independent donors. Tables show statistical significance of cytokine and chemokine secretion * p <0.05, ** p <0.01, *** p <0.001. Red shows that the reference cell line (listed at the top of column) is significantly higher than the comparator (cells listed on the left) and black shows that it is differently lower, using two-way ANOVA followed by a Bonferroni multiple-comparison test.



Sialylated residues, and in particular the α 2,3 and α 2,6-linked sialic acids, are considered the natural receptors for influenza A viruses. Changes in their expression may explain differences in permissiveness for influenza. In our study we have found that all 6 subsets of macrophages efficiently bound MAL II and SNA, the plant lectins used to characterize expression of the α 2-3 and α 2-6 SA respectively. SA expression was previously reported only for GM-CSF differentiated macrophages [4] but our study now provides a systematic comparison of SA expression between the different types of M1 and M2 macrophages. The results indicate that both SA α -2,3 and SA α -2,6 are distributed on all the macrophage subsets we studied. Desialylation of the macrophages with DAS181 pre-treatment removed all SA α -2,6. The MAL II positive staining after DAS181 treatment is probably due to non-specific binding as MALII has been described as being able to highly bind sulfonated moieties after desialylation (JM Nicholls's – personal communication). We observed differential susceptibility of the macrophages to influenza A virus infection by immunofluorescence despite minimal variation in cell surface expression of SA among the different cell subsets. Thus it is unlikely that differences in infection pattern rely on the profile of sialic acid receptors. However, other receptors such as mannose receptor or DC-sign may be relevant in this regard and need to be investigated. Mannose receptor and DC-sign have been shown to contribute to virus attachment and entry in mouse macrophages [29].

Susceptibility of a cell to a pathogen is not only defined by cell surface receptors, but also by the cell being permissive to virus uncoating and replication once inside the cell. In our study, infections by different influenza A viruses led to significant increase in detection of the viral M-gene and also to productive viral replication. This is similar to other studies where monocytes-derived macrophages were differentiated on plastic [4,30] but differs from studies where the cells were differentiated on teflon [24]. Thus the manner of macrophage differentiation can have a significant effect of the permissiveness of macrophages to viruses [5].

A number of studies have reported that infection of monocyte-derived macrophages with low (seasonal H1N1, H3N2, pH1N1) or high (H5N1, H9N2/G1) pathogenic strains leads to the expression of type I and type III IFNs, IL1 α , IL1 β , IL6, TNF α , CXCL8, CCL2, CCL3, CCL4, CCL5 and CXCL10 with evidence for involvement of ERK1/2, p38 and JNK signal transduction pathways in their production [1,30-32]. The level of cytokine and chemokine expression depended on the influenza strain, with H5N1 or H9N2/G1 (which shares 6 internal gene segments with H5N1 viruses in Hong Kong in 1997) inducing more potent responses than pH1N1. The results from our study are thus concordant with data from these previous studies [33,34].

However, there were important differences in cytokine expression when comparing the results from M1 and M2 macrophages, following virus infection. As expected, we found that pro-inflammatory macrophages (M1) express higher levels of pro-inflammatory cytokines and chemokines within the first hours of infection, compared with M2 macrophages. However, although GM-CSF+IFN γ differentiated macrophages had high levels of pro-inflammatory cytokines such as CXCL10, CCL3 and TNF α , this was due to high constitutive expression rather than being elicited by viral infection. In contrast, GM-CSF differentiated macrophages had low baseline levels of pro-inflammatory cytokine expression. But following viral infection, these cells responded with more potent pro-inflammatory cytokine responses compared with M2 macrophages.

It is striking that the anti-inflammatory cytokine IL10 appeared to be elicited only by H5N1 virus. Whether this contributes to its pathogenesis by blunting protective immune responses deserves to be further investigated. It has been shown that IL10 plays a role in CD8 cytotoxic vs T.reg cell functional balance and may indeed be relevant in pathogenesis [35]. Interestingly GM-CSF+IFN γ differentiated cells did not respond with an IL10 response in response to any virus. These findings contrast sharply with the distinct cytokine profiles of M1 and M2 macrophages exposed to bacterial products, infection with live bacteria [36] or HIV [7]. The pattern recognition receptors sensing influenza A viruses might be differentially expressed by M1 and M2 macrophages.

M-CSF+IL4 differentiated M2 subset macrophages are believed to be anti-inflammatory cells inducing anti-inflammatory cytokines and to promote tissue repair and limit injury than to promote pro-inflammatory responses. However, these cells did respond to H5N1 virus infection with detectable pro-inflammatory responses (IL6, CCL3, TNF α), albeit less in magnitude than M1 GM-CSF+IFN γ differentiated cells. Thus H5N1 virus infection appears to be able to override the anti-inflammatory phenotype by re-polarizing these cells from Th₂ to Th₁ cytokines as it has been shown in other studies [37,38]. Monitoring the expression of receptors and some other characteristic signalling molecules such as SOCS1, SOCS3 may give us insights into such a de-differentiation of M2 cells following H5N1 virus infection. Recently, simultaneous expression of both M1- and M2-like macrophage markers has been identified in inflammatory lung monocytes *ex vivo* [39], confirming the concept that macrophages are in a dynamic continuum of functional states [40,41]. For instance, macrophages with mixed profiles have been observed in obese mice [42].

Viral adhesion to different sialic acid residues could affect viral distribution within the lung and in the environment of the host cell responses. Avian influenza strains capable of binding avian-like sialic acid residues, such as H5N1 strains, seem to induce higher levels of pro-inflammatory cytokines and IFN-inducible genes compared with strains binding human-like sialic acid residues [40].

GM-CSF differentiated macrophages have been thought to be similar to alveolar macrophages since the alveoli have an abundance of GM-CSF. However, Van Riel et al observed that alveolar macrophages were less permissive to both H5N1 and pH1N1 viruses than macrophages differentiated in autologous serum or GM-CSF [32,33]. Yu et al have also shown that alveolar macrophages harvested from human lung were less efficiently infected and less potent at releasing CXCL10, CCL5 and TNF α compared to peripheral blood monocyte derived macrophages [31]. Thus *in vitro* differentiated M1 or M2 macrophages do not precisely reflect resting alveolar macrophages and this is a limitation of our study. However, it is relevant to note that during infection of the lung, there will be an influx of cells from the peripheral blood including monocytes which will differentiate into macrophages, explaining the increased numbers of macrophages in lungs of patients with severe viral pneumonia including H5N1. It is recognized that the M1 and M2 macrophages exhibit a functional plasticity [43] and this suggests that the differentiation state of the cells may lead to different disease outcome following infection. It will be useful to correlate these *in vitro* results with *in vivo* observations.

In conclusion, these data suggest that different subsets of human blood-derived macrophages differ in their permissiveness to influenza A viruses and elicit different innate immune responses. The host responses of macrophages to virus infection depend on several factors

including the virus subtype, the differentiated macrophage phenotype and the microenvironment within which the infection is occurring. Thus, it could be hypothesized that the cellular differentiation state might trigger very different outcomes with important downstream consequences of the infection. Highly pathogenic viruses such as H5N1 appear to have the capacity to elicit pro-inflammatory responses from even M2 macrophages. On the other hand, these viruses have the capacity to elicit IL10 responses from M2 as well as GM-CSF differentiated macrophages and this may be relevant in the pathogenesis of H5N1 disease. A mechanistic understanding of the macrophage differentiation states *in vitro* and *in vivo* may allow interventions that switch the phenotype of the monocytes-derived macrophage during infection thus modulating the outcome of the disease.

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