

The effect of obesity on the host innate immune response to influenza infection

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For James and Becca

Statement of Originality

I declare that the work presented in this thesis was undertaken by myself, under the supervision of Professor Sebastian Johnston, Professor Wendy Barclay and Doctor Michael Edwards. This thesis, and the work described herein, is my own except where explicitly referenced, and has not been previously submitted for a higher degree.

Dr Mark H Almond

October 1st 2015

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Abstract

Background: We live in an ‘obesogenic environment’ in which overconsumption of affordable, energy-dense, processed foods has resulted in the prevalence of obesity more than doubling since 1980, such that it is now the commonest nutritional disorder worldwide. In March 2009, a novel strain of influenza A virus (pH1N1/09) emerged in Mexico, rapidly spreading around the world to cause the first influenza pandemic of the 21st century. For the first time during an influenza pandemic, obesity was convincingly identified as a novel, independent risk factor for multiple markers of disease severity, including hospitalisation, intensive care unit admission and death following infection.

The aim of this project was to investigate the mechanisms underlying this increased susceptibility to severe outcomes following pH1N1/09 infection in morbidly obese individuals using primary human tissues.

We hypothesised that obesity-associated hyper-leptinaemia would increase constitutive expression of the negative regulator, suppressor of cytokine signalling 3 (SOCS3), in key pulmonary structural and immune cells and that this, in turn, would attenuate interferon (IFN) expression and signal transduction, thus reducing induction of IFN stimulated genes (ISGs), the main antiviral effectors.

Methodology: A case-control study design was adopted in which bronchial epithelial cells (hBECs), bronchoalveolar lavage (BAL) cells, peripheral blood mononuclear cells (PBMCs) and peripheral blood dendritic cells (DCs) were isolated from 15 morbidly obese individuals (body mass index (BMI) ≥ 35 kg/m²) and 15 age, gender and ethnicity-matched healthy weight (‘lean’) controls (BMI 20-25 kg/m²) by bronchoscopy and venesection to allow comparison of the type I and III IFN and pro-inflammatory cytokine responses following pandemic and seasonal influenza infection. Viral replication, ISG and SOCS expression profiles were also quantified in the primary cells and the constitution of the inflammatory environment in which the cells reside was evaluated through measurement of adipokines, cytokines, acute phase proteins and chemokines.

Results: BAL cells from morbidly obese individuals exhibited deficient type I and III IFN responses to both pandemic and seasonal influenza virus infection. This IFN-deficiency was BAL cell specific and therefore not observed following infection of primary hBECs, PBMCs or DCs. Although unlikely, it is possible that the observed IFN deficiency may have been due to the confounding effects of the general anaesthesia that the obese subjects underwent during their bronchoscopy. Elevated leptin levels were observed in the lung and serum of the obese subjects; however, baseline constitutive SOCS3 mRNA expression was not increased in the obese subjects and we were unable to demonstrate leptin-induced upregulation of SOCS3. For the first time, we report relatively high expression of the lesser-studied adipokines resistin, visfatin and adiponin in the lung and nose. Additionally, we report that the acute phase protein, C-reactive protein (CRP), a biomarker of inflammation, was significantly elevated in the BAL fluid of the obese subjects.

Conclusion: Obesity-associated BAL cell IFN deficiency may contribute to the increased susceptibility to severe outcomes seen in the obese population following influenza infection.

Declaration of work

The author:

- Devised the overall research question
- Obtained funding and ethics approval for the project
- Screened and recruited all the study subjects and procured all the clinical samples
- Grew the three influenza viral stocks
- Isolated, cultured and stimulated/infected the four primary cell types
- Performed the flow cytometry and mRNA & protein quantification for all the study subjects
- Carried out all data and statistical analysis

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Abbreviations

α2M	Alpha-2 macroglobulin
A549	Human alveolar adenocarcinoma cell line
AM	Alveolar macrophage
ARDS	Acute respiratory distress syndrome
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
BDCA	Blood dendritic cell antigen
BEAS-2B	Human bronchial epithelial cell line
BEBM	Bronchial epithelial basal medium
BEGM	Bronchial epithelial growth medium
BLF	Bronchial lining fluid
BMI	Body Mass Index
BSA	Bovine serum albumin
BTS	British Thoracic Society
CARD	Caspase activation and recruitment domain
cDNA	Complementary deoxyribonucleic acid
CF	Cystic Fibrosis
CI	Confidence Interval
CID	Central interacting domain
CINC	Cytokine-induced neutrophil chemoattractant
CKD	Chronic kidney disease
COPD	Chronic obstructive pulmonary disease
CPE	Cytopathic effect
CPG-A	CpG oligodeoxynucleotide class A
CPSF	Cleavage and polyadenylation specificity factor
CRP	C-reactive protein
CST	Cytometer setup and tracking
CXCL	Chemokine (C-X-C motif) ligand
DC	Dendritic cell
DIO	Diet-induced obesity
DM	Diabetes Mellitus
dNTP	Deoxynucleotide
dsRNA	Double stranded ribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
eIF2α	Eukaryotic initiation factor 2 alpha
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FEV1	Forced expiratory volume in one second
FGF	Fibroblast growth factor
FIZZ	Found in inflammatory zone
FPFS	Farnesyl diphosphate synthase

FVC	Forced vital capacity
HA	Haemagglutinin
hBEC	Human bronchial epithelial cell
HEK	Human embryonic kidney
HeLa	Human cervical adenocarcinoma cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
hpi	Hours post infection
HRV	Human rhinovirus
HUVEC	Human umbilical vein endothelial cells
GA	General anaesthetic
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GRADE	Grading of recommendations, assessment, development & evaluations
GTP	Guanosine triphosphate
IAV	Influenza A virus
IBV	Influenza B virus
ICAM	Intercellular adhesion molecule
ICRRU	Imperial College Respiratory Research Unit
ICU	Intensive care unit
IFITM	Interferon-induced transmembrane protein
IFN	Interferon
IFNAR	Interferon- α/β receptor
IgE	Immunoglobulin E
IKK-β	Inhibitor of nuclear factor kappa-B kinase subunit beta
IL-1RA	Interleukin 1 receptor antagonist
IL-8	Interleukin 8
IL-6	Interleukin 6
IR	Infrared
IRF	Interferon regulatory factor
IRP	Influenza related pneumonia
ISG	Interferon stimulated gene
ISGF	Interferon stimulated gene factor
ISRE	Interferon stimulated response element
iU	International unit
JAK	Janus Kinase
KIR	Kinase inhibitory region
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
LZ	Leucine zipper
MACS	Magnetic activated cell sorting
MCP	Monocyte chemoattractant protein
MDCK	Madin-Darby canine kidney

MDM	Monocyte-derived macrophage
MDT	Multi-disciplinary team
MFI	Median Fluorescence Intensity
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MOI	Multiplicity of Infection
mRNA	Messenger ribonucleic acid
MUAC	Mid-upper arm circumference
MyD88	Myeloid differentiation primary response 88
MxA	Myxovirus resistance gene A
NA	Neuraminidase
NAMPT	Nicotinamide phosphoribosyltransferase
NEP	Nuclear export protein
NF-κB	Nuclear factor kappa B
NHS	National Health Service
NLF	Nasal lining fluid
NLR	Nod-like receptor
NK	Natural killer
NMR	Nuclear magnetic resonance
NP	Nucleoprotein
NS	Non-structural
OA	Osteoarthritis
OAS	Oligoadenylate synthase
ObFlu	The shortened name given to the clinical study
OR	Odds ratio
OSA	Obstructive sleep apnoea
PAMP	Pathogen-associated molecular pattern
PB	Polymerase basic
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
PFA	Paraformaldehyde
PMT	Photomultiplier tube
PKR	Protein kinase R
Poly(I:C)	Polyinosinic:polycytidylic acid
POMC	Pro-opiomelanocortin
PPAR	Peroxisome proliferator-activated receptors
PRR	Pathogen recognition receptor
RANTES	Regulated on activation, normal T cell expressed and secreted
R848	Resiquimod
RBX2	RING-box protein 2
RD	Repressor domain
RIG-I	Retinoic acid inducible gene-1
RLR	RIG-I like receptors

RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RPMI	Roswell Park Memorial Institute medium
RR	Relative risk
RT-qPCR	Real time quantitative polymerase chain reaction
RV	Rhinovirus
SA	Sialic acid
SAM	Synthetic absorptive matrix
SAP	Serum amyloid P
SA-PE	Streptavidin-Phycoerythrin
SEM	Standard error of the mean
SH2	Src Homology 2
SHP	SH2 domain-containing protein tyrosine phosphatase
SOCS	Suppressor of cytokine signalling
ssRNA	Single stranded ribonucleic acid
STAT	Signal transducer and activator of transcription
TIA	Transient ischaemic attack
TIR	Toll/interleukin-1 receptor (TIR) homology domain
TLR	Toll like receptor
TNF α	Tumour necrosis factor alpha
TRIF	TIR-domain-containing adapter-inducing interferon- β
TRIM	Tripartite motif
UK	United Kingdom
USA	United States of America
VEGF	Vascular endothelial growth factor
Viperin	Virus inhibitory protein, ER-associated, IFN-inducible
vRNA	Viral ribonucleic acid
WHO	World Health Organisation

Chapter 1 Background

In this chapter the rationale for the study, along with the study hypotheses, is explained. After a brief review of influenza virology the origins, epidemiology and burden of the 2009 H1N1 pandemic are described, followed by a summary of the epidemiological evidence identifying obesity as a novel, independent risk factor for adverse outcomes following influenza infection. The evidence for obesity as an inflammatory state is then presented followed by a brief outline of non-immune factors that may contribute to obesity-associated adverse outcomes. The hypothesised mechanism by which obesity-associated innate immune deficiency may predispose to these outcomes is subsequently explained, including brief reviews of the innate immune response to influenza and the roles of suppressor of cytokine signalling (SOCS) proteins in both obesity and influenza. Finally, a comprehensive outline of the study hypotheses, sub-hypotheses and aims is presented.

1.1 Influenza A viruses

1.1.1 Structure

Influenza A virions are predominantly spherical particles, 80-120nm in diameter, comprising a host cell-derived lipid bilayer enveloping a central core¹ (Figure 1-1). The external envelope is characteristically studded with spike-like projections comprising haemagglutinin (HA) and neuraminidase (NA) membrane glycoproteins. The genome contained within the core comprises eight segments of negative-sense, single-stranded ribonucleic acid (RNA) encoding eleven proteins¹.

The HA is the pre-dominant membrane glycoprotein, outnumbering the NA approximately four to one². Individual HA projections are homotrimeric, composed of individual HA monomers, each of which contains two subunits, HA1 and HA2. The globular HA1 domain resides at the distal end of the spike and is responsible for viral attachment to host cells via surface sialic acid (SA) receptors. The HA2 domain forms the stem of the spike and contains a fusion peptide that undergoes a conformational change upon acidification within the endosome, enabling the viral envelope to merge with the endosomal membrane, allowing release of viral ribonucleoproteins (RNPs) into the cytosol³. The tetrameric matrix protein 2 (M2), also located within the viral envelope, assists this process by forming a proton channel that mediates virion

acidification whilst in the endosome, resulting in viral uncoating. Unlike the HA, the NA is tetrameric and responsible for release of newly formed virions from the cell surface, via enzymatic cleavage of sialic acid-HA anchors. The matrix 1 (M1) protein is the most abundant protein within the virion, lining the internal aspect of the viral envelope to provide structural support¹.

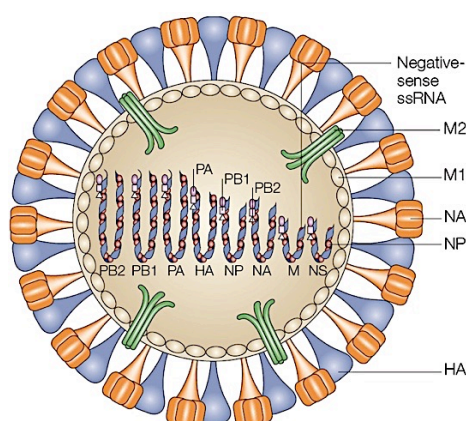


Figure 1-1 The structure of influenza A viruses

A central core containing eight, negative sense single-stranded RNA segments is surrounded by a lipid bilayer characteristically studded with haemagglutinin (HA) and neuraminidase (NA) glycoproteins. The viral matrix protein (M1) provides structural support to the virion whilst the M2 protein forms a proton channel to facilitate viral uncoating (adapted from Horimoto 2005⁴).

Table 1-1 Influenza A gene segments, encoded proteins and functions

The influenza A viral core contains eight single stranded, negative sense RNA segments encoding eleven proteins. Segment 7 encodes two proteins, M1 and M2, with overlapping reading frames. Similarly, segment 8 encodes the non-structural proteins NS1 and NS2, again with superimposed reading frames (adapted from Hale *et al*⁵).

Gene Segment	Encoded protein (s)	Protein function
1	PB2	Polymerase subunit basic 2 - part of the polymerase complex
2	PB1/PB1-F2	PB1 – Part of the polymerase complex PB1-F2 – Binds mitochondria and induces apoptosis
3	PA	Polymerase subunit acidic – Part of the polymerase complex
4	HA	Haemagglutinin – Facilitates viral entry and endosomal fusion
5	NP	Nucleocapsid protein – RNA encapsidation
6	NA	Neuraminidase – Facilitates cellular release of virion by cleaving sialic acid-HA anchors
7	M1/M2	M1 – Viral matrix protein – provides structural support to the virion M2 – Proton channel – facilitates viral uncoating
8	NS1/NS2	NS1 – Interferon antagonist NS2 – Nuclear export protein (NEP) – facilitates nuclear export of RNPs

The eight segments of viral RNA (vRNA) are housed within the viral core (Table 1-1). Each vRNA segment forms an RNP complex that creates a twisted, rod-like structure that is folded back and coiled on itself⁶ (Figure 1-2). Each RNP comprises vRNA in association with nucleoprotein (NP) and a heterotrimeric RNA-dependent RNA polymerase complex (PB1, PB2 and PA), essential for the translation of negative sense vRNA into viral proteins.

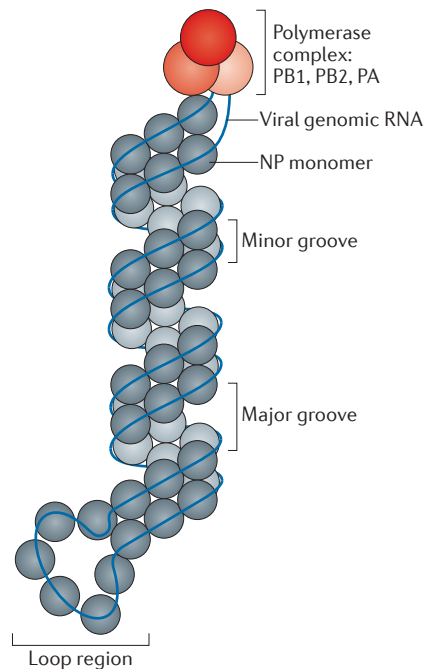


Figure 1-2 The influenza viral ribonucleoprotein

Each viral RNA (vRNA) segment forms a ribonucleoprotein (RNP) complex that creates a twisted, rod-like structure that is folded back and coiled on itself. Each RNP comprises vRNA in association with nucleoprotein (NP) and a heterotrimeric RNA-dependent RNA polymerase complex (PB1, PB2 and PA), essential for the replication of viral RNA and generation of positive sense RNA that is translated into protein (adapted from Einfeld *et al*⁷).

Although the influenza genome contains eight segments, alternative splice reading of three gene segments results in the production of a total of 11 proteins. Segment 7 encodes two proteins, M1 and M2, with overlapping reading frames. Similarly, segment 8 encodes the non-structural proteins NS1 and NS2, again with superimposed reading frames. Whilst the M2 protein assists in endosomal viral uncoating, NS2, also known as nuclear export protein (NEP), mediates the nuclear export of virion RNAs by acting as an adaptor between vRNP complexes and the nuclear export machinery of the cell⁸. The eleventh influenza A virus gene product, PB1-F2, encoded by gene segment two, is only produced by certain strains of influenza and is thought to be an

important determinant of influenza virus virulence⁹. PB1-F2 binds to mitochondria, leading to a release of cytochrome c and induction of apoptosis in CD8 T-cells and alveolar macrophages. The absence of a full-length PB1-F2 has been suggested as one possible determinant for the relatively low pathogenicity of the 2009 influenza H1N1 pandemic strain¹⁰.

1.1.2 *Classification*

Influenza viruses are differentiated according to the antigenicity of their NP and M proteins into three genera: influenza A, B and C. Influenza A viruses (IAVs) are further sub-classified according to the antigenicity of their HA and NA proteins. To date, 18 HA and 11 NA subtypes have been identified, all of which circulate in wild, aquatic bird populations, except subtypes H17N10 and H18N11 which have only been described in bats¹¹. Only two IAV subtypes (H1N1 and H3N2) are currently in general circulation among the human population.

1.1.3 *Life Cycle*

To initiate viral replication, the influenza HA binds to glycoproteins containing sialic acid (SA) residues located on the host cell surface (Figure 1-3). IAVs that circulate in humans preferentially bind to SAs that are attached to galactose in an α -2,6 configuration (found predominantly in the human upper respiratory tract) whilst avian influenza viruses prefer SAs attached to galactose in an α -2,3 configuration (located in the avian gut)¹². Upon adsorption, mediated by the HA1 receptor binding domain, the virion is internalized by both clathrin and non-clathrin pit dependent endocytosis. The low pH (pH 5-6) of the endocytic vesicle subsequently induces a major conformational change in the HA such that the fusion peptide of the HA2 domain moves from the stem of the HA spike to the distal tip, facilitating insertion into the endosomal membrane and virus-host membrane fusion. Concurrently, the M2 ion channel allows hydrogen ions to pass into the virion, disrupting protein-protein interactions, resulting in viral uncoating and release of the viral RNPs into the cytosol¹.

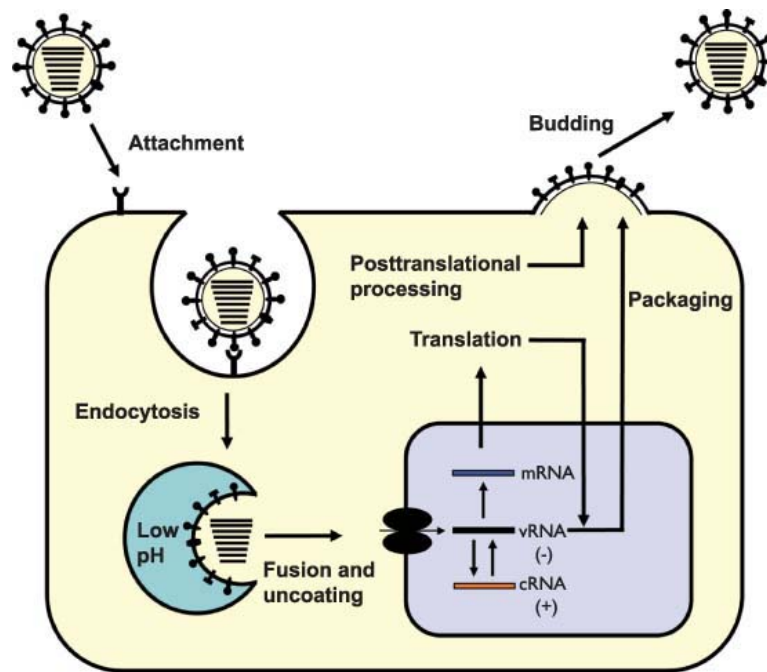


Figure 1-3 Influenza life cycle

After binding the viral HA protein the influenza A virus enters the cell by endocytosis. Following internalization, the endosome is acidified, which in turn results in conformational changes in the HA leading to fusion between the virion and the endosomal membrane. This releases the viral genome into the cytoplasm. vRNPs are then transported into the nucleus and primary transcription results in the production of viral mRNAs, which are exported to the cytoplasm and translated into proteins by ribosomes. Newly translated viral proteins are then transported to the nucleus. After translation and nuclear entry of PB1, PB2, PA and NP, new copies of the viral genome are made and incorporated into vRNPs. These vRNPs are then exported into the cytoplasm and incorporated into progeny virions that are released from the cell surface by budding (adapted from Palese *et al*¹).

In contrast to the replication of other RNA viruses, which occurs exclusively in the cytoplasm, influenza viruses have evolved to replicate in the nucleus, thus minimizing exposure of viral replication products to cellular immune sensors. Following nuclear translocation, vRNPs serve as templates for the production of two types of positive-sense RNA: viral messenger RNA (mRNA) and complementary RNA (cRNA). The virus-derived RNA-dependent RNA polymerase complex (comprising PA, PB1 and PB2) catalyzes viral mRNA synthesis (primary transcription). These viral mRNAs are subsequently processed in a similar fashion to other eukaryotic mRNAs namely, they are capped, polyadenylated and exported from the nucleus for translation by cytoplasmic ribosomes⁷.

Following translation in the cytosol, newly formed viral proteins are imported back into the nucleus for the final stages for vRNP assembly. Unlike viral mRNA, viral cRNA is neither capped nor polyadenylated, instead forming a perfect copy of the

initial vRNA template. These cRNAs serve as templates for the synthesis of further negative-sense genomic vRNA segments that are packaged into progeny virions. Notably, both cRNA and vRNA molecules contain a 5' triphosphate group which, as discussed later, form a major stimulus for the host innate immune response¹³. Newly synthesized RNPs are exported from the nucleus and directed to the inner surface of the plasma membrane where they attach to M1 proteins. Newly formed virions are creating by budding from the plasma membrane and are subsequently released from the host cell following cleavage of the SA-HA anchor by NA¹.

1.2 Influenza Pandemics

1.2.1 *Influenza pandemics of the 20th century*

Influenza pandemics occur when a virus expressing a novel HA or NA surface glycoprotein is introduced into a population with no prior immunity (Table 1-2).

Table 1-2 Origins of the influenza pandemics of the 20th and 21st centuries

Year	Name	Origin	Genetic composition	Estimated mortality
1918	Spanish influenza	Avian to human transmission of H1N1	All 8 gene segments from avian influenza virus	20-50 million ¹⁴
1957	Asian influenza	Reassortment between avian H2N2 and human H1N1 virus	5 gene segments from 1918 H1N1 virus and 3 segments from avian virus (HA, NA, PB1)	>1 million ¹⁵
1968	Hong Kong influenza	Reassortment between avian H3 virus and human H2N2 virus	5 segments from 1918 H1N1 virus. 1 segment from H2N2 (N2), 2 segment from avian virus (HA, PB1)	1-2 million ¹⁵
2009	Mexican influenza	Reassortment between Eurasian, avian-like H1N1 virus and 'triple reassortment' virus (avian PB2 and PA, PB1 and NA from human H3N2 and 4 genes from classical swine virus)	2 gene segments (NA, M) from the Eurasian virus and 6 segments from the 'triple reassortant' virus	123,000– 395,600 ^{16,17}

The catastrophic effects of this were demonstrated by the 1918 H1N1 pandemic during which approximately 20–50 million people died¹⁴. Reassortment of the segmented genome of the influenza virus and interspecies transmission (antigenic shift) results in the introduction of pandemic influenza viruses whereas seasonal, epidemic influenza viruses are derived from pandemic viruses by antigenic drift (gradual minor antigenic changes caused by point mutations in the HA and NA). Influenza A and B viruses cause epidemics, whereas only influenza A has pandemic potential¹⁸.

The 1918 ‘Spanish’ influenza pandemic, in which approximately 20 - 40% of the global population became infected, was most likely caused by interspecies transmission of an avian H1N1 virus¹⁴. Following this pandemic, H1N1 continued to circulate in humans resulting in annual epidemics. Influenza viruses are especially prone to reassortment events if one host is co-infected by two different viruses, due to the segmented nature of the genome¹⁸. This allows the genetic material from avian influenza viruses to be introduced to human adapted viruses and the species barrier to be overcome. Indeed, the 1957 ‘Asian’ pandemic was initiated by a novel H2N2 virus that retained five genome segments from the 1918 H1N1 virus, but gained three new segments from an avian H2N2 virus. Similarly, the 1968 H3N2 ‘Hong Kong’ pandemic occurred when a circulating human H2N2 reassorted with the HA from an avian H3 virus. The pandemic H3N2 and H1N1 subtypes have continued to co-circulate and to cause human seasonal influenza outbreaks into the 21st century¹⁹.

1.2.2 *Origins of the 2009 H1N1 pandemic*

The 2009 H1N1 pandemic was unexpected (an H5N1 pandemic was predicted) and challenged the pre-existing paradigm that the introduction of a new HA subtype into the population is required to initiate a pandemic²⁰. The eight gene segments present in the 2009 H1N1 pandemic virus (pH1N1/09) originated from at least four different sources and three different hosts (pigs, birds & humans). In the late 1990s a ‘triple reassortant’ virus emerged in pigs, comprising the PA & PB2 genes from an avian reservoir, the PB1 and NA from a human H3N2 virus and four other genes, including the HA, from the ‘classical’ swine virus that had been circulating since the 1918 pandemic (Figure 1-4). This ‘triple reassortant’ virus subsequently recombined with a Eurasian, avian-like, H1N1 virus that had been circulating in pigs since the 1970s (receiving the NA and M genes), resulting in the 2009 pandemic strain¹⁸. The pH1N1/09 virus differed from the seasonal H1N1 influenza A virus that had been circulating in humans since the 1918 pandemic; although both viruses had HA proteins derived from the 1918 pandemic, the pH1N1/09 virus contained the HA from the ‘classical’ swine influenza virus, which, unlike the seasonal human H1, had been under relatively little antigenic pressure, hence the introduction of a novel antigen into the human population¹⁸.

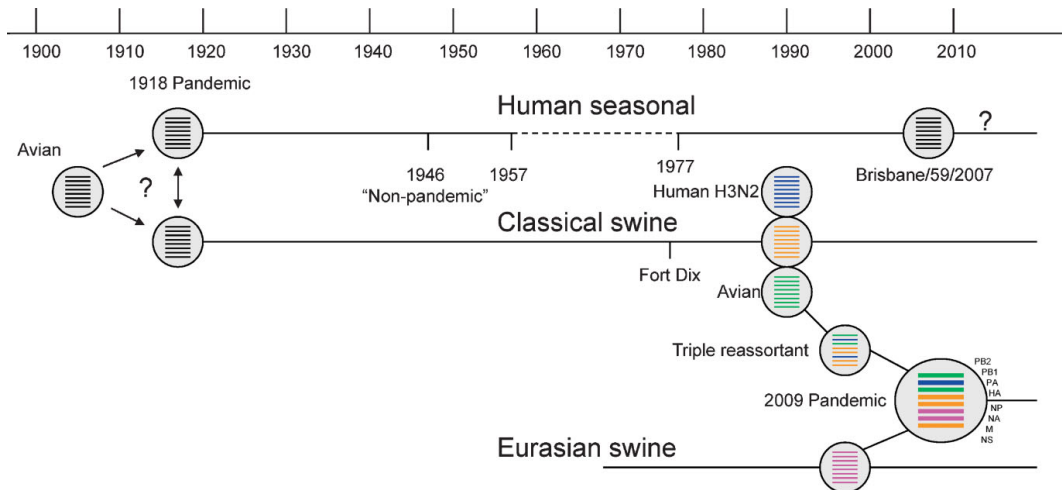


Figure 1-4 Origins of the 2009 pandemic influenza A virus

Introduction of an avian virus into the human population is thought to have caused the 1918 pandemic, from which emerged H1N1 viruses causing seasonal epidemics that disappeared in 1957 and re-emerged in 1977. An H1N1 virus, related to the 1918 pandemic virus, co-emerged in pigs and continued to circulate as “classical swine virus”. In the late 1990s a “triple reassortant” emerged in pigs comprising an avian virus-derived PB2 and PA, PB1 and NA of a human H3N2 virus, and 4 genes of the classical swine virus. The 2009 pandemic virus was a derivative of this triple reassortant with the NA and M genes from an Eurasian avian-like H1N1 lineage that emerged around 1970 in pigs (adapted from Klenk *et al*¹⁸).

1.2.3 *Epidemiology of the 2009 H1N1 pandemic*

The first laboratory-confirmed case of pH1N1/09 arose in a six-month old girl in San Luis Potosi State, Mexico during late-February 2009²¹; two months later, Mexico declared a national alert with 1918 suspected cases and 84 deaths occurring between March 1 and April 30. Observational data from early on in the Mexican outbreak was concerning as 6.5% of hospitalised patients became critically ill and, of those, 41% died²².

The pH1N1/09 virus spread rapidly such that, by the end of April 2009, seven countries on different continents (Canada, Israel, Mexico, New Zealand, Spain, United Kingdom and the United States of America (USA)) had reported laboratory-confirmed cases. On April 25, the World Health Organisation (WHO) declared a public health emergency of international concern and on June 11 raised the pandemic alert to phase six, indicating the start of a fully-fledged pandemic²². By this point, 73 countries had reported more than 26,000 laboratory-confirmed cases in total. Although by October 2009 the pandemic had passed its peak, transmission was sustained in many regions of the world. Over the course of 2010, incidence continued to decline and the pandemic was declared over on 10 August 2010²².

England experienced the pandemic in three separate waves (Figure 1-5); the first wave occurred in July-August 2009, peaking twelve weeks after the first English case of pH1N1/09 was reported²³. The second wave occurred in autumn 2009, manifesting as a gradual and prolonged rise in influenza activity that lasted for several weeks. Based upon general practice consultations, the incidence of influenza in the community was lower than in the first wave; however, rates of influenza activity in hospital were much greater than those in the first wave. The third wave presented in December 2010 to January 2011 (during the post-pandemic period) and was characterised by a short sharp rise and fall in influenza activity associated with greater peaks in hospitalisation and critical care admission than in either of the two preceding waves, placing a considerable burden on National Health Service (NHS) resources²³.

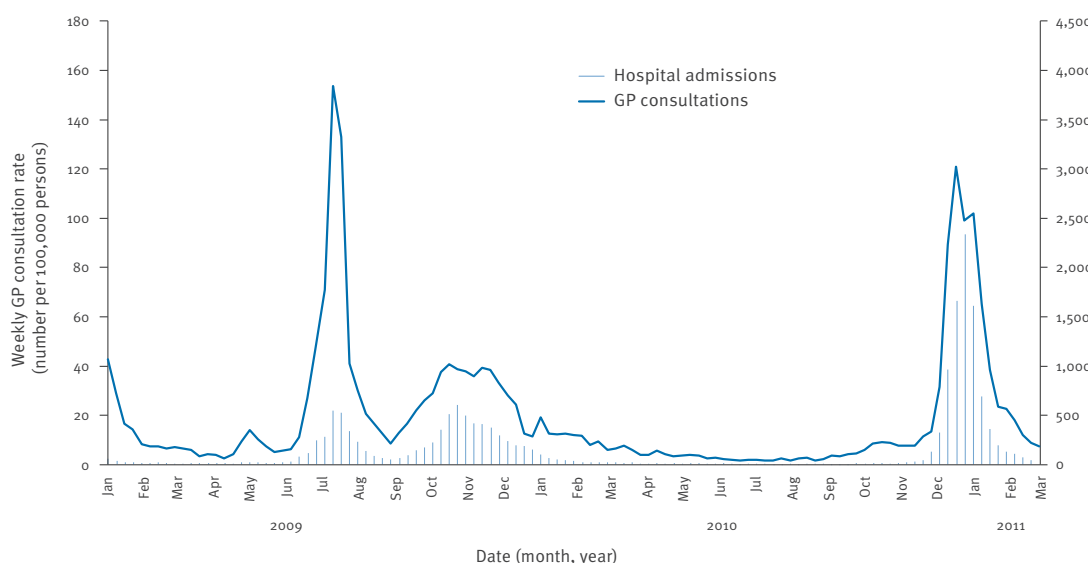


Figure 1-5 The three waves of the pH1N1/09 pandemic in England

Influenza-related general practice consultations and hospital admissions in England between 5th January 2009 and 13th March 2011. Three distinct waves of influenza activity occurred over two years: two during 2009/10 and a single wave in 2010/11. The first wave peaked 12 weeks after the first case of pH1N1/09 was reported in England and was characterised by a short sharp rise and fall in influenza activity. The second wave, a gradual and prolonged rise in influenza activity that lasted for several weeks, occurred in autumn 2009. The third wave, associated with the greatest peak in hospitalisation and ICU admission, occurred in late 2010 and was characterised by a short sharp rise and fall in influenza activity (adapted from Mytton *et al*²³).

1.2.4 Mortality burden of the 2009 H1N1 pandemic

The WHO reported 18,631 laboratory-confirmed deaths secondary to pH1N1/09¹⁶; however, the actual 2009 global pandemic mortality has been variously reported as between 123,000 to 203,000 deaths¹⁶ and 105,700 to 395,600 deaths¹⁷, approximately ten-fold higher than the WHO estimate¹⁶. This discordance may have been due to the fact that only a small proportion of influenza-related deaths are ever definitively diagnosed as such and fatalities following influenza-induced secondary bacterial infections or exacerbations of pre-existing conditions are commonly not recorded as being in any way related to influenza.

The influenza-related mortality rate observed in 2009 was well below that of previous influenza pandemics, which has varied from one million deaths in 1968 to approximately 50 million deaths in 1918¹⁴. Indeed, the total number of influenza-related deaths worldwide proved similar to the number in a relatively mild year of seasonal influenza. However, due to the proportionately higher mortality among young children and adults, the severity in terms of life-years lost was greater than in a

typical seasonal influenza year²²; The majority (62% – 85%) of the pH1N1/09 deaths occurred among persons under 65 years of age, compared to only 19% of the 148,000 to 249,000 seasonal influenza respiratory deaths per season¹⁶.

The first UK death attributed to pH1N1/09 occurred on 15th June 2009 with 360 subsequent deaths occurring during the first two waves and 474 during the third wave in 2011²⁴.

1.2.5 Risk factors for severe outcomes following pH1N1/09 infection

Although it is now generally considered that the 2009 pandemic resulted in mild, self-limiting disease in the majority of individuals²⁴, serious complications, both pulmonary and extra-pulmonary, still occurred. The risk factors for adverse outcomes following influenza infection, as reflected by current UK vaccination policy²⁵ are detailed in Table 1-3. However, despite the widely accepted public health policy of recommending vaccination to groups believed to be at high risk for complications of influenza, the strength of evidence upon which these recommendations are based is relatively weak²⁶.

Table 1-3 Clinical risk groups for adverse outcomes following influenza infection

At the time of study conception obesity was not listed as a clinical risk category by the UK government. COPD=Chronic obstructive pulmonary disease, CKD=Chronic kidney disease, TIA=Transient ischaemic attack (adapted from the Green Book, chapter 19²⁵).

Clinical risk category	Examples (not exhaustive)
Age	≥65 years, >6 months if in clinical risk group, children aged 2,3 or 4 years
Chronic respiratory disease	Asthma, COPD, Cystic Fibrosis
Chronic heart disease	Congenital heart disease, chronic heart failure
Chronic kidney disease	CKD stage 3,4,5, kidney transplantation
Chronic liver disease	Cirrhosis, biliary atresia, chronic hepatitis
Chronic neurological disease	Stroke, TIA
Diabetes Mellitus	Type I & II
Pregnant women	At any stage of pregnancy
Asplenia or splenic dysfunction	Sickle cell disease
Morbid obesity (class III)	BMI ≥40kg/m ²
Immunosuppression	HIV, chemotherapy, systemic steroids

Mertz *et al* recently published a WHO-funded systematic review and meta-analysis of observational studies evaluating the risk factors for severe outcomes (including pneumonia, hospitalisation, ICU admission, need for ventilator support and death) in patients with seasonal or pandemic influenza²⁶. 234 articles, with a total of 610,782

participants were included in the analysis. Three key messages emerged: firstly, obesity was identified as an important risk factor for mortality following influenza infection (OR 2.94, (95% CI 1.71-4.74), $I^2 = 93\%$, seasonal and pandemic influenza combined); notably, at the time of study conception obesity was not listed as a clinical risk category by the UK government. Secondly, no association between ethnic origin and severe outcomes, aside from hospitalisation, following pandemic influenza was identified. Finally, and interestingly given worldwide vaccination policy, no association between pregnancy and influenza-associated mortality was identified (although the first month post-partum was associated with increased risk of death). The authors found that the quality of evidence reviewed was generally very low, with limitations in the published literature including lack of power and lack of adjustment for confounders. Additionally, there was a scarcity of data for non-2009 H1N1 pandemics and seasonal influenza epidemics²⁶.

1.2.6 *Obesity as a novel risk factor for severe outcomes following pH1N1/09 infection*

Obesity is defined by the WHO as ‘abnormal or excessive fat accumulation that may impair health’²⁷ and is classified according to body mass index (BMI); determined by dividing an individual’s weight (in kilograms) by the square root of their height (in metres). A BMI ≥ 25 kg/m² is deemed ‘overweight’, whereas a BMI ≥ 30 kg/m² defines ‘obese’ (Table 1-4).

Table 1-4 The WHO international classification of adult obesity according to BMI
These BMI values are age-independent and the same for both sexes (adapted from Schmatz *et al*²⁸).

Classification	Body Mass Index (BMI) (kg/m ²)
Underweight	< 18.50
Normal range	18.50 - 24.99
Overweight	≥ 25.00
Pre-obese	25.00 - 29.99
Obese	≥ 30.00
Obese class I	30.00 – 34.99
Obese class II	35.00 – 39.99
Obese class III	≥ 40.00

The prevalence of obesity has more than doubled since 1980, such that it is now the commonest nutritional disorder worldwide²⁷. 2.8 million deaths annually, and a significant proportion of diabetes mellitus, ischaemic heart disease and malignancy have been attributed to being overweight; however, obesity is also associated with significant psychological, social and financial burdens.

As of 2008, 502 million adults were classified obese and it has been estimated that by 2030 this number will increase by 65 million adults in the USA and 11 million in the United Kingdom (UK), adding £5.5 billion to NHS medical costs²⁹. The rising incidence over the past several decades is, in part, due to increased consumption of cheap, energy-dense foods in combination with a general reduction in daily physical activity. Societal changes and increased availability of affordable, processed foods has created an ‘obesogenic’ environment that has facilitated positive calorie imbalance³⁰. It has even been insinuated that, without timely prevention, and additional government-lead regulation and investment, our social and healthcare systems may eventually be crippled by the burdens associated with this global obesity ‘pandemic’³⁰.

For the first time during an influenza pandemic, it became evident in 2009 that obesity was independently associated with multiple markers of poor outcome upon infection³¹. Several investigators have subsequently investigated this relationship (Table 1-5), with the overwhelming majority of studies showing that obesity ($\text{BMI} \geq 30 \text{ kg/m}^2$) is associated with increased risk of hospitalisation, intensive care admission and death.

Three systematic reviews, each with unique limitations, appraising the relationship between obesity and adverse outcomes post-influenza infection have been published³²⁻³⁴. The first, a study from Fezeu *et al*, assessed the association between obesity and the risk of ICU admission and death among patients hospitalised for pH1N1/09 infection³²; a total of 3059 subjects, from six cross-sectional studies, hospitalized for pH1N1/09 infection, were included in the meta-analysis. Obesity was associated with a twofold, non-significant, increased likelihood risk of ICU admission or death (odds ratio (OR): 2.14, 95% CI: 0.92–4.99, $P < 0.07$) ($I^2 = 81.8\%$, P for heterogeneity < 0.001) whereas morbid obesity ($\text{BMI} \geq 40 \text{ kg/m}^2$) significantly increased the risk of ICU admission or death (OR: 2.01, 95% CI: 1.29–

3.14, $P < 0.002$), with no heterogeneity among the included studies ($I^2 = 0.0\%$, $P = 0.48$). The conclusions from this systematic review were, however, considerably limited by the fact that only published literature up to January 2010 (eight months before the pandemic ended) was included and significant heterogeneity existed for the pooled summary estimate for obesity.

The second systematic review, authored by Phung *et al*, evaluated the specific relationship between BMI and the risk of developing influenza-related pneumonia (IRP)³³. The relative risk (RR) of IRP for obese individuals was 1.3 (95% CI 1.05 - 1.63) whilst a stronger association existed for morbid obesity (RR 4.6, 95% CI 2.2 - 9.8). In other words, obese individuals had more than 30% higher risk of developing IRP, whilst morbidly obese individuals had more than 300% higher risk of developing IRP compared with normal weight comparators. Again, significant heterogeneity between the studies was noted with ‘overweight’ (Cochran’s Q 44.8; $P < 0.01$), ‘obesity’ (Q 77.1; $P < 0.01$) and ‘morbid obesity’ (Q 41.51; $P < 0.01$), signifying inconsistent results between the ORs/RRs of selected studies. Moreover, this systematic review did not distinguish between studies concerning seasonal influenza and pandemic influenza.

Finally, and as discussed previously (section 1.2.5), the most recent systematic review & meta-analysis by Mertz *et al*, which assessed 59 pandemic influenza-obesity outcome observational studies concluded that obesity not only increased the risk of death (OR 2.74, 1.56 to 4.80, $I^2 = 92\%$), but was significantly associated with the need for hospital admission, ICU admission, as well as ventilator support³⁴.

The evidence for an association between obesity and adverse outcomes following *seasonal* influenza is less convincing; Kwong *et al* demonstrated an independent association between obesity and respiratory hospitalisations during seasonal influenza epidemics³⁵. Using a cohort study design, 82,545 respondents to a cross-sectional public health survey in Ontario were followed over 12 influenza seasons (348,350 person-seasons); analysis by logistic regression was employed to assess the association between self-reported BMI and respiratory hospitalisations. The authors concluded that elevated BMI was associated with greater odds of respiratory hospitalisation during influenza seasons (OR 1.45-2.2) and, for morbidly obese individuals (BMI >

35 kg/m²), the association was present (OR 5.1, 95% CI 2.53-10.24) irrespective of the presence of previously identified risk factors for severe disease (diabetes mellitus, respiratory disease, heart disease, cancers, immunodeficiency). Mertz *et al* identified only one observational study, with a small event rate, concerning obesity as a risk factor for severe outcomes following seasonal influenza; obesity significantly increased risk of death, albeit with an extremely wide confidence interval (OR 30.1, 1.17 - 773.12)³⁴.

In summary, randomised controlled trials on risk factors are unfeasible and therefore high quality evidence according to ‘Grading of Recommendations Assessment, Development and Evaluation’ (GRADE) methodology is unlikely ever to be achieved however, the quantity and conclusions of the existing literature provide a persuasive case to justify further scrutiny of the link between obesity and adverse outcomes following pandemic influenza.

Table 1-5 Summary of the epidemiological data associating pH1N1/09 infection with adverse outcomes
Presented in chronological order (adapted from Mertz *et al*²⁶).

1 st Author	Year	Study Design	PCR confirmed diagnosis	Study period	Country	n	BMI	Outcome Measure	OR/RR	95% CI
Napolitano ³⁶	2009	Case series	Yes	May 09 - Jun 09	USA	10	≥30	ICU mortality	0.8	0.04 - 14.64
Cullen ³⁷	2009	Case-control	Yes	Apr 09 -Oct 09	Ireland	180	>30	ICU admission following hospitalisation	9.35	1.24 - 70.7
Cui ³⁸	2009	Case-control	Yes	Nov 09 - Dec 09	China	68	>30	Hospital mortality	23.06	1.29 - 409.8
Morgan ³¹	2010	Case-cohort	Yes	Apr 09 - Jul 09	USA	196,684	≥30	Hospitalisation Death	1.6 3.1	0.7-3.7 1.5-6.6
							≥40	Hospitalisation Death	4.7 7.6	1.3-17.2 2.1-27.9
Buda ³⁹	2010	Case-control	Yes	May 09 - May 10	Germany	170,856	≥30	Hospitalisation Death	6.28 25.49	4.48-8.82 12.57-51.67
Choi W ⁴⁰	2010	Case series	Yes	Aug 09 - Oct 09	South Korea	17	≥30	Hospital mortality	1.93	0.06 - 62.17
Cutter ⁴¹	2010	Case-control	Yes	May 09 – Jul 09	Singapore	1301	>30	ICU mortality	1.39	0.14 - 14.23
Flint ⁴²	2010	Case-control	Yes	Jun 09 - Aug 09	Australia	918	>30	ICU admission	2.4	Not given
Gomez-Gomez ⁴³	2010	Case-control	Yes	Apr 09 – May 09	Mexico	50	>30	In hospital mortality	4.24	0.79 - 22.85
Miller ⁴⁴	2010	Case-control	Yes	May 09 - Jun 09	USA	47	≥30 ≥40	ICU admission ICU admission	2.4 15.8	1.3-3.5 8.3-23.4
Koegelenberg ⁴⁵	2010	Case series	Yes	Aug 09 – Sep 09	South Africa	19	>30	ICU mortality	1.5	0.12 - 18.36
Nicolay ⁴⁶	2010	Case-control	Yes	Jul 09 - May 10	Ireland	77	≥35	Death following ICU admission	1.01	0.3-3.39
Zykova ⁴⁷	2010	Case-control	Yes	Nov 09 – Feb 10	Czech Republic	13	≥30	Death following ICU admission	0.49	0.02 - 12.93
Sovig-Gmizi ⁴⁸	2010	Retrospective cohort	Not stated	Nov 09 – Feb 10	Serbia	61	≥30	Death following ICU admission	3.68	0.87 - 15.56
Subramony ⁴⁹	2010	Case-control	Yes	Jul 09 - Sep 09	Singapore	1348	≥30	ICU admission or death	7.06	2.00-24.95
Siau ⁵⁰	2010	Case series	Yes	Jul 09 - Sep 09	Singapore	12	≥30	ICU mortality	1	0.05 - 20.83
Viasus ⁵¹	2010	Prospective cohort	Yes	Jun 09 - Nov 09	Spain	585	≥40	ICU admission or death	6.7	2.25-20.19
Peralta ⁵²	2010	Case-control	Yes	Apr 09 - Dec 09	Spain	3025	≥40	ICU admission or death	2.01	1.38-2.94

1 st Author	Year	Study Design	PCR confirmed diagnosis	Study period	Country	n	BMI	Outcome Measure	OR/RR	95% CI
Fuhrman ⁵³	2010	Case-control	Partial (98%)	July 09 - Nov 09	France	758	≥30	ICU admission or death	9.1	4.4-18.7
Tutuncu ⁵⁴	2010	Case-cohort	Yes	Octo 09 – Dec 09	Turkey	74	>30	In hospital mortality	6.11	1.47 - 25.43
Blakemore ⁵⁵	2010	Case series	Yes	Aug 09 - Sep 09	S Africa	7	>30	ICU mortality	3	0.09 - 102.05
Chacko ⁵⁶	2010	Case-control	Yes	Aug 09 - Oct 09	India	31	>30	ICU mortality	4.89	0.9 - 26.7
Chien ⁵⁷	2010	Case-control	Yes	Jul 09 - Aug 09	Taiwan	149	≥30	Respiratory failure	1.67	0.05 - 53.92
Yang ⁵⁸	2010	Case-control	Yes	Jan 09 - Jan 10	China	475	≥30	Severe illness following hospitalisation	0.5	0.2 - 1.1
Vasoo ⁵⁹	2010	Case-control	Yes	Apr 09 - Jun 09	USA	83	≥30	ICU admission	1.1	0.26-4.2
Poggensee ⁶⁰	2010	Case-control	Yes	Apr 09 - Oct 09	Germany	53968	>30	Hospitalisation	4	2.5-6.5
								Influenza-related pneumonia	13.8	6.9 - 27.5
Van't Klooster ⁶¹	2010	Case-control	Yes	Jun 09 - Dec 09	Holland	1108		ICU mortality	10.55	4.79 - 23.22
Estenssoro ⁶²	2010	Case-control	Yes	Jun 09 - Aug 09	Argentina	337	≥30	ICU mortality	1.3	0.79-2.15
Parcevaux ⁶³	2010	Prospective cohort	Yes	Aug 09 – Sep 09	Reunion island	13	≥30	ICU mortality	0.49	0.02 - 12.93
Nguyen-Van-Tam ⁶⁴	2010	Case-control	Yes	Apr 09 - Sep 09	UK	631	≥30	Hospital mortality	1.71	0.38 - 7.59
								ICU admission or death	6.08	1.45 - 25.37
Fezeu ⁶⁵	2011	Systematic review and meta-analysis	Partial	Jan 09 - Jan 10	Several	3059	≥30	ICU admission/death	2.14	0.92-4.99
							≥40	ICU admission	8.69	4.44-17.02
								Death	1.78	1.13-2.80
								ICU admission/death	2.01	1.29-3.14
Van Kerkhove ⁶⁶	2011	Pooled analysis	Yes	Apr 09 - Jan 10	Several	70,000	≥30	Death	1.5	0.9-2.8
							>40	Hospital mortality	2.9	1.3-6.6
								Death	36.3	22.4-50.1
								Hospitalisation	15	9.5-20.4
Louie ⁶⁷	2011	Case-control	Yes	Apr 09 - Aug 09	USA	534	≥30	Death	1.1	0.5-2.3
							≥35	Death	1.1	0.4-2.5
							≥40	Death	2.8	1.4-5.9
							≥45	Death	4.2	1.9-9.4
Yokota ⁶⁸	2011	Case-control	Yes	Jul 09 - Jan 10	Brazil	157	≥40	Hospitalisation	6.2	1.3-29.2
Diaz ⁶⁹	2011	Case-control	Yes	Jan 09 - Jan 10	Spain	872	>30	ICU mortality	1.1	0.69 - 1.75

1 st Author	Year	Study Design	PCR confirmed diagnosis	Study period	Country	n	BMI	Outcome Measure	OR/RR	95% CI
Hung ⁷⁰	2011	Retrospective cohort	Yes	Sep 09 – Jun 10	Hong Kong	93	>27	ICU mortality	0.84	0.28 - 2.49
Riquelme ⁷¹	2011	Retrospective cohort	Yes	Apr 09 – Oct 09	Several	250	>30	In hospital mortality	2.23	1.09 - 4.57
Yu ⁷²	2011	Case-control	Yes	Sep 09 - Feb 10	China	9966	≥30	ICU admission or death	1.34 1.91 0.68	1.10-1.63 1.57-2.31 0.37-1.25
Zolotusca ⁷³	2013	Case-control	Yes	Oct 09 - May 11	Romania	148	Visual	Death	2.9	1.6-31.2
Kok ⁷⁴	2013	Prospective cohort	Yes	Jun 09 - Sep 09	Australasia	173	≥30 ≥40 ≥30 ≥30	Pneumonitis Pneumonitis Hospital mortality ICU mortality	2.19 2.11 0.37 0.42	1.12-4.71 0.69-2.45 0.11-1.3 0.1-1.77
Zhang	2013	Case-control	Yes	Sep 09 - Dec 09	China	2151	≥30	Death	1.86	0.72-0.92
Ren ⁷⁵	2013	Case-control	Yes	May 09 - Mar 10	China	686	≥25 ≥30	Severe clinical manifestations Severe clinical manifestations	3.7 35.61	2.04-6.72 7.96-159.21
Cocoros ⁷⁶	2013	Case-control	No	Apr 09 - Dec 09	USA	8266	≥30	Hospitalisation	1.76	0.91 - 3.42

1.3 The innate immune response to influenza viruses

Having outlined the basic virology and epidemiology of influenza viruses, and presented the case for obesity as a risk factor for adverse outcomes following influenza infection, the postulated mechanism by which obesity predisposes to these severe outcomes must be described. However, in order to understand the justification for the hypotheses subsequently presented, the innate immune response to influenza viruses and the roles of SOCS proteins, in both influenza infection and obesity, must be reviewed.

1.3.1 Innate immune sensing

In humans, influenza infection is predominantly restricted to the respiratory tract. Upon infection, airway epithelial cells and alveolar macrophages (AMs) produce chemokines, interferons (IFNs) and pro-inflammatory cytokines that act in an autocrine and paracrine fashion to induce an antiviral state. Dendritic cells (DCs), located beneath the airway epithelium above the basal membrane, are potent producers of IFN and form an important connection between the innate and adaptive immune systems, migrating to local lymph nodes upon infection to present antigen to T cells⁷⁷.

In order to defend against viruses mammalian cells have evolved mechanisms in which pathogen-associated molecular patterns (PAMPs) are detected by innate cellular sensors, known as pattern recognition receptors (PRRs), to initiate signalling cascades resulting in the production of antiviral IFNs and pro-inflammatory cytokines (Figure 1-6). Three families of PRRs are relevant for the detection of influenza viruses: the toll-like receptors (TLRs), retinoic acid-inducible gene 1 (RIG-I) protein-like helicases (RLRs) and nucleotide-binding domain and leucine-rich-repeat-containing proteins (NLRs)⁷⁸.

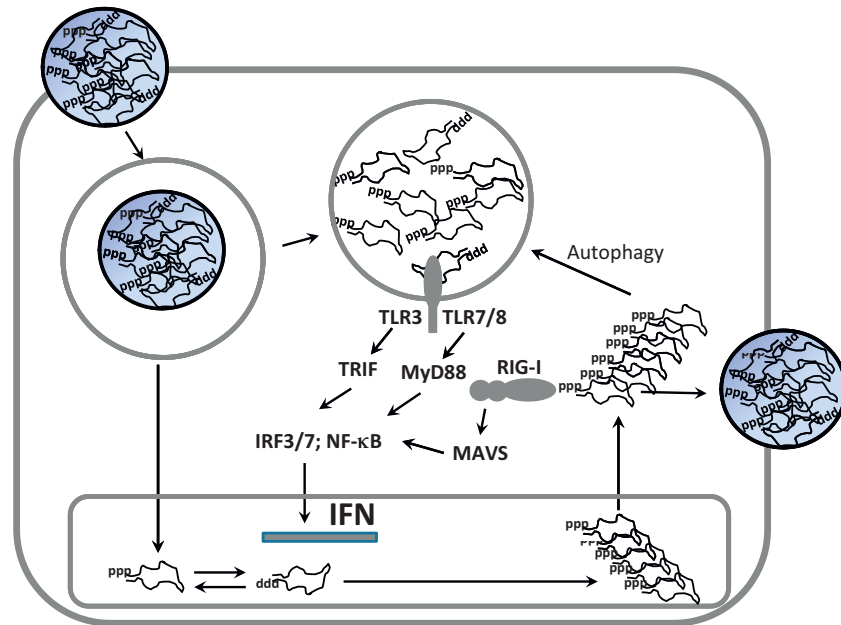


Figure 1-6 Innate immune sensing of influenza viruses and IFN induction

Following endocytosis of the influenza virus fusion of the viral envelope to that of the endosome releases the segmented genome of the virus into the cytoplasm. It is then transported to the nucleus where replication takes place. During this entry process, some virions may be left within the endosome and expose viral RNA to the TLRs 3, 7 & 8, which recognize double stranded and single stranded RNA. Later on in the infection, newly synthesized viral RNA segments move to the cytoplasm, where they encounter and trigger RIG-I before assembly of new virions takes place. TLRs signal by the adaptors TRIF or MyD88, and RIG-I by the adaptor MAVS to trigger a signal transduction cascade that results in activation of IRF3/7 and NF-κB. These transcription factors translocate to the nucleus where they promote the synthesis of type I and type III IFNs and other cytokines (adapted from Garcia-Sastre 2011⁷⁸).

1.3.2 PAMPs & PRRs

The most influenza-relevant TLRs (TLRs 3, 7 & 8) are located in cellular endosomes. TLR 3 recognizes double stranded RNA (dsRNA) whilst TLR 7 & TLR 8 recognise single stranded RNA (ssRNA)⁷⁸.

Influenza-infected cells are not thought to generate dsRNA and it is likely that TLR3 recognizes dsRNA that is present in dying influenza virus-infected cells that have been phagocytosed by macrophages⁷⁹. Upon recognition of dsRNA, the Toll-IL-1R (TIR) domain of TLR3 recruits the adaptor molecule TIR-domain-containing adapter inducing interferon (TRIF), resulting in the induction of nuclear factor-kappa B (NF-κB)-dependent pro-inflammatory cytokines and type I IFN & IFN stimulated genes (ISGs) downstream of IFN-regulatory factor 3 (IRF3)⁷⁸.

Plasmacytoid dendritic cells (pDCs) are potent type I IFN producers and constitutively express TLR7, which recognises the ssRNA genomes contained within the influenza virion that are taken up into the endosome. Upon recognition, the adapter molecule MyD88 induces nuclear translocation of the transcription factors, NF- κ B and IRF7 to induce pro-inflammatory cytokine and type I IFN expression⁷⁹ (Figure 1-6). TLR8 is expressed by human monocytes and macrophages; however, the significance of TLR 8 in influenza infection requires further clarification.

The cytosolic RIG-I plays a key role in viral detection & type I IFN production in infected epithelial cells, conventional DCs and AMs⁷⁹. RIG-I recognises the 5'triphosphate viral ssRNA (that is generated in the nucleus) following its transportation to the cytosol. The C- terminus of the RIG-I protein comprises a repressor domain (RD) that normally maintains two caspase-associated recruitment domains (CARDs), located at the N-terminus, in an inactive state. Upon binding of 5'triphosphate by the RD, the central RNA helicase of RIG-I binds to adenosine triphosphate (ATP), leading to a conformational change that results in exposure of the N-terminal CARDs. The latter are ubiquitinated by E3 ligases, including tripartite motif-containing protein 25 (TRIM25), promoting the association of RIG-I with mitochondrial antiviral signalling (MAVS) protein⁷⁹. MAVS subsequently activates the transcription factors IRF3 and NF- κ B, which undergo nuclear translocation to induce IFN (type I & III) and pro-inflammatory cytokine expression (e.g. IL-1 β & IL-18).

1.3.3 *Interferons*

IFNs are antiviral cytokines that act in an autocrine and paracrine fashion to induce an antimicrobial state within infected and neighbouring host cells. Three types of IFN (I, II and III) exist; regarding influenza infection, type I and III IFN responses are of paramount importance⁸⁰.

There are seventeen subtypes of type I IFN, of which IFN- α and IFN- β are most relevant to humans⁸¹. They bind the type I IFN receptor (IFNAR) that, upon receptor binding, activates a janus kinase/signal transducers and activators of transcription (JAK/STAT) signalling cascade that results in the translocation of a STAT1/STAT2/IRF9 complex (known as IFN stimulated gene factor (ISGF)-9) to the

nucleus and induction of ISG transcription upon binding to IFN stimulated response elements (ISREs)⁸⁰.

Identified in 2003, type III IFNs, comprising IFN λ 1 (IL-29), IFN λ 2 (IL-28A) and IFN λ 3 (IL-28B), share many common biological features with the type I IFNs. Upon binding the heterodimeric IFN- λ receptor, composed of two chains of transmembrane glycoproteins (IFN λ R1 and IL-10R2), signal transduction via the JAK-STAT culminates in the nuclear translocation of IFN-stimulated gene factor 3 (ISGF 3), which binds to ISREs in the promoters of various ISGs⁸².

1.3.4 *IFN stimulated genes*

The foremost downstream consequence of the JAK/STAT signalling cascade, initiated by IFN binding to the appropriate IFN receptor, is the transcriptional stimulation of ISGs, the main antiviral effectors that are able to prevent the translation of viral and cellular mRNAs to limit viral replication⁸³. Around 300 ISGs have been described to date⁸⁴; the functions of the ‘classical’ antiviral ISGs OAS, PKR and MxA and two of the more recently discovered ISGs, viperin and IFITM3 are briefly summarized herein.

Located in the cytoplasm on intracellular membranes, the orthomyxovirus resistance (Mx) proteins were the first ISGs found to restrict influenza virus replication⁷⁸. The human MxA protein has a dynamin-like N-terminal GTPase, central interacting domain (CID) and a C-terminal leucine zipper (LZ) domain. Upon upregulation by type I and III IFNs, MxA proteins accumulate as oligomers (formed by CID-LZ association) in the cytoplasm near the endoplasmic reticulum, trapping the PB2 and nucleocapsid proteins of the influenza virus polymerase and ultimately leading to their degradation⁸⁵.

One of the initial ISGs to be described was the double-stranded, RNA-dependent protein kinase R (PKR)⁷⁸. PKR is an IFN inducible protein kinase that becomes activated upon binding dsRNA, forming a homodimer that phosphorylates elongation initiation factor-2 subunit alpha (eIF2 α) preventing translation, yet also the protein kinase IKK β , promoting gene expression. eIF2 α is a critical translation co-factor and phosphorylation by PKR results in its irreversible binding to the nucleotide exchange factor eIF2B, preventing ribosomal translation of cellular and viral proteins, blocking

cellular viral replication. IKK β phosphorylation results in phosphorylation of the NF- κ B inhibitor I κ B, with resultant NF- κ B nuclear translocation and upregulation of pro-inflammatory cytokines⁸³.

Like PKR, oligoadenylate synthetase (OAS) is a cytosolic, IFN-inducible ISG that requires binding of dsRNA for enzymatic activation. Subsequent conversion of ATP to 2'-5' oligoadenylate activates RNaseL (a latent cytoplasmic RNase) that cleaves viral and cellular RNA, inhibiting protein synthesis and viral replication. Products of RNaseL cleavage also activate RIG-I, resulting in a positive-feedback scenario that further increases IFN production⁷⁸.

Two of the more recently identified ISGs are viperin and IFITM3. Viperin (virus inhibitory protein endoplasmic reticulum-associated IFN inducible), located on the cytosolic face of the endoplasmic reticulum and in cytosolic lipid droplets is induced in a variety of cell types by all three types of IFN and infection with a range of viruses⁸⁶. Viperin restricts viral replication by interacting with farnesyl diphosphate synthase (FPPS) leading to dispersal of lipid rafts, with subsequent reduction in membrane fluidity and inhibition of the budding of influenza A virions⁸⁷. Unlike viperin, IFITM3 (IFN inducible transmembrane 3) is located in endosomes and is thought to prevent release of viral contents into the cytoplasm by restricting virus-host cell membrane fusion pore formation following viral attachment and endocytosis⁸⁸.

1.3.5 *Innate immune response evasion strategies adopted by influenza viruses*

In order to circumvent the anti-viral host response, influenza viruses have evolved numerous innate immune evasion strategies. The influenza A NS1 protein has been most widely studied in this regard and antagonises the host innate immune response on several levels, ranging from inhibition of PRR signal transduction through to direct inhibition of ISGs⁷⁸.

In order to attenuate PRR signal transduction, and thus transcriptional activation of ISREs, the NS1 protein has evolved to prevent oligomerisation of the E3 ubiquitin ligase, TRIM25, thus inhibiting ubiquitination of RIG-I. Without this essential post-translational modification, the association between RIG-I and MAVS is disrupted, preventing the activation and nuclear translocation of the transcription factors IRF3

and NF- κ B⁸⁹. Furthermore, host cell protein synthesis is downregulated by NS1 binding ‘cleavage and polyadenylation specificity factor’ (CPSF30), thus preventing 3’ polyadenylation of host pre-mRNAs. The IAV polymerase complex (PB1, PB2 & PA) also downregulates host protein synthesis through cleavage of the 5’ end of host mRNAs (‘cap snatching’). To further avoid host detection of viral PAMPS influenza viruses replicate in the nucleus, thus limiting the exposure of viral RNAs to cytosolic PRRs.

Influenza viruses can also directly antagonise the antiviral ISGs PKR and OAS. IAV-induced dissociation of p58IPK, the inhibitor of PKR, from heat shock protein 40, causes augmented p58IPK activity, with resultant attenuation of PKR efficacy. Additionally, NS1 can bind directly to PKR to inhibit the conformational change that permits its antiviral activity⁸⁹. The antiviral activity of OAS may also be inhibited by NS1, as it binds and sequesters viral dsRNA, thus preventing binding by OAS.

Of most significance for the forthcoming study hypotheses, IFN signal transduction via JAK-STAT is subject to negative regulation through several proteins, including the SOCS proteins^{90,91}. Influenza viruses have evolved to take advantage of this negative regulation and are able to upregulate both SOCS1 and SOCS3 proteins upon infection (see below), thus attenuating IFN signal transduction. Notably, SOCS proteins are also upregulated in obesity and thus, as described later, could present a potential mechanism by which obesity contributes to the severe outcomes seen following influenza infection.

1.3.6 *SOCS proteins*

Suppressor of cytokine signalling proteins are key regulators of cytokine signalling pathways and may be of particular relevance with regards to the obesity-associated increased susceptibility to severe outcomes following influenza infection⁹². There are eight members of the SOCS/cytokine-inducible SH2 domain-containing proteins (CISs), SOCS1 - SOCS7 and CIS, each of which comprises a central Src homology 2 (SH2) domain, a C-terminal 40-amino acid ‘SOCS box’ and a N-terminal of variable length and sequence that contains a kinase inhibitory region (KIR)⁹³.

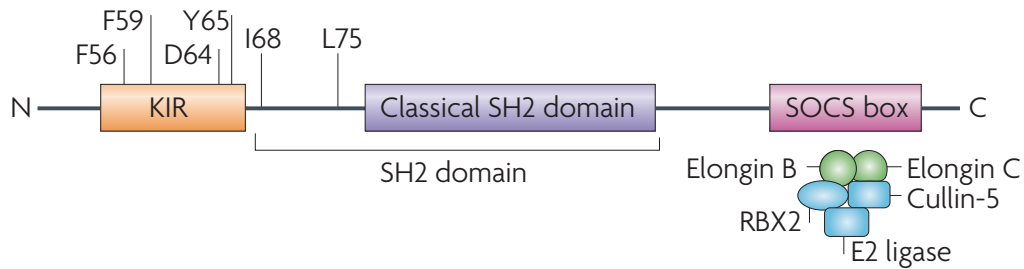


Figure 1-7 Structure of SOCS proteins

Each member of the SOCS family comprises a central Src homology 2 (SH2) domain, a C-terminal 40-amino acid ‘SOCS box’ and a N-terminal of variable length and sequence that contains a kinase inhibitory region (KIR). (adapted from Yoshimura et al 2007⁹³)

Whilst the central SH2 domain determines the target of SOCS protein, the KIR can directly inhibit JAK tyrosine kinase activity by functioning as a pseudosubstrate. The SOCS box interacts with a complex containing elongin B, elongin C, cullin-5, RING-box-2 (RBX2) and E2 ligase and most likely functions as an E3 ubiquitin ligase to mediate the proteosomal degradation of proteins that are associated with these family members through their N-terminal regions⁹³.

1.3.7 *SOCS and influenza*

Pothlichet *et al* first investigated the expression and roles of the SOCS family members during influenza infection in 2008⁹¹. Utilizing a strain of H3N2 (A/Scotland/20/74) and the BEAS-2B human respiratory epithelial cell line, the authors demonstrated that IAVs were capable of inducing both SOCS1 and SOCS3 mRNA protein expression upon infection in a RIG-I, MAVS and IFNAR1 dependent, but TLR3 independent manner (Table 1-6). By using vectors overexpressing SOCS1 and SOCS3 they also showed attenuation of IFN β signal transduction, potentially via IRF3 for SOCS1 and via NF- κ B for SOCS3.

To further evaluate the mechanism and consequences of SOCS induction, Pauli *et al* transfected RNA from IAV-infected A549 cells into uninfected A549 cells, resulting in early strong induction of SOCS3, weak induction of SOCS1 and diminished type I IFN-induced STAT phosphorylation⁹⁰. Transfection after treatment of the RNA from the infected cells with a phosphatase to remove the 5'-triphosphate termini resulted in much weaker SOCS induction, implying that the major trigger for induction of SOCS

is viral 5'-triphosphate RNA, the major PAMP for RIG-I. To confirm dependence of SOCS induction on NF- κ B, Pauli transfected both A549 cells expressing dominant-negative forms of IKK2 and A549s exposed to BAY 11-7085 (a NF- κ B specific inhibitor) with RNA from A549 cells infected with influenza A/PR8. Levels of SOCS3 mRNA were significantly reduced, suggesting a critical role for NF- κ B in SOCS3 mRNA induction however; NF- κ B was not sufficient *per se* to induce SOCS3 induction (data from Pothlichet *et al* suggests IRF3 is also required).

Whilst 5'-triphosphate appears to be the major stimulus for RIG-I induced SOCS gene transcription, Jia *et al* showed that NS1 protein *per se* may also result in SOCS induction⁹⁴. By transfecting HeLa cells (a cervical cancer cell line) with the NS1 from an H5N1 virus, they demonstrated significant induction of both SOCS1 and 3 mRNA, although the mechanism has yet to be elucidated.

Ramirez-Martinez *et al* recently demonstrated strain and cell-specific SOCS induction patterns by infecting A549 cells and monocyte-derived macrophages (MDMs) with either PR8 or pH1N1/09 and measuring changes in whole-genome expression⁹⁵. SOCS1 mRNA was upregulated by PR8, but not by pH1N1 in MDMs whilst SOCS3 mRNA was upregulated by both viruses. In A549 cells, SOCS1 and SOCS3 mRNA was upregulated by both PR8 and pH1N1.

Subsequently, Wei *et al* have assessed the effects of IAV-induced SOCS expression on IFN production, finding that attenuation of JAK-STAT signalling by SOCS1 may result in an NF- κ B-mediated increased production in IFN λ which, in turn, further upregulates SOCS1 and IFN λ , resulting in a harmful 'IFN λ storm'⁹⁶. Similar to Pauli *et al*, IFN-induced STAT phosphorylation (in this case IFN λ rather than type I IFN) was shown to be dramatically inhibited in IAV-infected cells, in association with increased SOCS1 expression. Interestingly, they found that IFN λ expression was reduced in IAV-infected A549 cells expressing shRNAs targeting SOCS1, implying that, in the context of IAV-infection, SOCS1 expression promotes IFN λ production. This finding was supported by experiments in SOCS1 knockdown transgenic mice in which IFN λ was significantly decreased following IAV infection. Hence, whilst influenza induces SOCS proteins in an attempt to circumvent the host antiviral IFN

response, they may induce adaptive increase in IFN λ production, resulting in a vicious cycle of type III IFN and SOCS production.

Little is known about the roles of the different SOCS proteins in influenza infection, outside of the ability of SOCS1 and SOCS3 to regulate specific cytokine receptor complexes. A novel human challenge study from Huang *et al*, in which 17 healthy adults were inoculated with H3N2/Wisconsin and then changes in host peripheral blood gene expression followed at 16 time points over the following 132 hours, demonstrated distinct SOCS expression profiles dependent on whether the subject was symptomatic or asymptomatic⁹⁷. Expression of SOCS1 and SOCS3 was shown to decline at early time points in asymptomatic individuals but strongly increase amongst the symptomatic, whilst SOCS2 and SOCS5 showed a marked increase in the asymptomatic, but diminished expression in the symptomatic. These findings warrant further investigation. Data from Kedzierski *et al* have recently suggested a protective role for SOCS4 during influenza infection although the mechanism is unclear⁹⁸. Transgenic SOCS4 knockout mice were shown to exhibit greater weight loss, mortality and viral load following infection with A/PR/8/34 and A/HK/x31 relative to wild type controls in association with higher pulmonary pro-inflammatory cytokine levels.

In summary, influenza viruses have evolved to take advantage of the negative regulation of IFN signal transduction in an attempt to circumvent the innate immune response. The 5'-triphosphate of influenza viral RNA appears to be the major PAMP that induces SOCS1 and SOCS3 expression in a RIG-I, MAVS dependent manner with resultant attenuation of type I & III IFN signalling. Whilst the NS1 of H5N1 has also been shown to induce SOCS expression, the mechanism is unclear. The pattern of SOCS expression appears to be both cell and virus-strain specific and there is some evidence to suggest that attenuated type III IFN signalling due to the inhibitory effect of SOCS1 may result in an adaptive increase in IFN λ expression by the host to protect cells against IAV infection, resulting in potentially harmful hypercytokinaemia. Further investigation into the roles of SOCS2, SOCS4 and SOCS5 is warranted.

Table 1-6 Summary of the literature concerning SOCS & influenza

Author	Methods	Cell type	Virus	Key Findings
Pothlichet 2008⁹¹	Infection of respiratory epithelial cell lines Transfection of SOCS1 & SOCS3 vectors	BEAS-2B	A/Scotland/20/74 (H3N2)	SOCS1 to SOCS7, but not CIS are constitutively expressed by BEAS-2B cells SOCS1 and SOCS3 only are induced by IAV infection, in a TLR3 independent fashion SOCS induction is RIG-I, MAVS & IFNAR1 dependent SOCS1 & SOCS3 over expression attenuates IFN beta signal transduction, via IRF3 for SOCS1 and NFkB for SOCS3
Pauli 2008⁹⁰	Infection of epithelial cell lines Interferon/cytokine cell stimulations Cell stimulation with viral/cellular RNA siRNA mediated knock down of SOCS3	A549 HEK293 HUVECs	A/PR/8/34 (H1N1) A/Victoria/3/75 (H3N2)	Type I IFN-induced STAT phosphorylation is inhibited in IAV infected cells IAV infection induces SOCS3 mRNA expression Early induction of SOCS3 gene transcription is not indirectly mediated by IFN beta Viral 5'triphosphate is the major inducer of SOCS3 gene transcription and causes reduced STAT1 phosphorylation SOCS3 gene transcription involves the NF-kB signalling pathway SOCS3 knockout enhances constitutive STAT1 phosphorylation and virus-induced expression of ISGs SOCS3 knockout reduces viral titres post-infection, SOCS3 over expression results in elevated viral titres
Jia 2010⁹⁴	Transfections with the H5N1 NS1 plasmid	HeLa	A/Vietnam/3046/04 (H5N1) A/HK/483/97 (H5N1) A/HK/54/98 (pH1N1/09) A/Ca/04/09 (pH1N1)	H5N1 NS1 expression inhibits IFN-inducible STAT phosphorylation H5N1 NS1 induces both SOCS1 and SOCS3 gene transcription H5N1 NS1 induces SOCS1, but not SOCS3 protein expression
Huang 2011⁹⁷	Human challenge study with peripheral blood transcriptomics		A/Wis/67/2005 (H3N2)	SOCS1 and SOCS3 mRNA strongly increased in symptomatic individuals but diminished in asymptomatic individuals SOCS2 and SOCS5 mRNA markedly increased in asymptomatic individuals, diminished in the symptomatic
Nelli 2012⁹⁹	<i>Ex vivo</i> infection of human and pig MDMs and epithelial cells Transfection of human MDMs with SOCS3 plasmid	BECs MDMs	A/Turkey/1/05 (H5N1) A/USSR/77 (H1N1)	Human cells mount vigorous cytokine responses to H5N1 & H1N1 vs weak cytokine responses in pigs SOCS3 is constitutively highly expressed and induced in H5N1 infected pig in contrast to human cells Over expression of SOCS3 in infected human MDMs dampens TNF alpha induction High constitutive SOCS3 in pig cells associated reduced output of viable virus and attenuated pro-inflammatory responses following H5N1 infection
Ramirez-Martinez 2013⁹⁵	In vitro infection of macrophages & epithelial cells Microarray gene expression analysis Cytokine/chemokine quantitation in culture supernatants	MDMs A549	A/PR/8/3 (H1N1) pH1N1/09	MDMs: SOCS1 mRNA upregulated by A/PR8, but not pH1N1/09. SOCS3 mRNA upregulated by A/PR8 and pH1N1/09 A549s: Both SOCS1 and SOCS3 mRNA upregulated by both A/PR8 and pH1N1/09 Lack of SOCS1 induction by pH1N1/09 in MDMs associated with higher pro-inflammatory cytokine responses
Wei 2014⁹⁶	Infection and transfection of epithelial cell lines Transgenic SOCS1 knockout mouse infection	A549	A/WSN/33 (H1N1)	Influenza-induced SOCS1 inhibits STAT1 phosphorylation SOCS1 k/o mice have diminished IFN lambda production following infection SOCS1 enhances IFN lambda production following influenza infection via NFkB
Kedzierski 2014⁹⁸	Transgenic SOCS4 knockout mouse infection	N/A	A/PR/8/34 (H1N1) A/HK/x31(H3N2)	SOCS4 k/o mice fare worse following influenza infection (greater weight loss, mortality & viral load) SOCS4 k/o mice exhibit elevated pulmonary pro-inflammatory cytokine levels early in infection

1.4 Animal models of obesity and influenza infection

Mouse models of diet-induced obesity (DIO) support the premise that the innate immune response to influenza is dysregulated in the obese state. DIO, induced in mice by feeding them a high-fat/high-sucrose diet for around twenty weeks¹⁰⁰, more closely mimics human obesity than genetic models (in which mice exhibit deficiency in either the satiety hormone leptin (*ob/ob*) or the leptin receptor (*db/db*))¹⁰¹. In 2007 Smith *et al* infected DIO and lean C57BL/6J mice intranasally with the mouse adapted strain of influenza, A/Puerto Rico/8/34 (A/PR/8/34)¹⁰⁰. Mortality was 6.6-fold greater in the DIO mice (42%) compared to lean (5.5%) and lung pathology was significantly increased. Concurrently, despite similar viral titres in the lung, DIO mice exhibited diminished lung IFN α and IFN β mRNA expression at 3 days post infection (dpi). Lung and splenic natural killer (NK) cell cytotoxicity was also reduced in DIO mice in association with decreased IL-18 mRNA expression (IL-18 is known to enhance NK cell cytotoxicity). Pro-inflammatory cytokine (IL-6, TNF α and IL-1 β) and chemokine (MCP-1 and RANTES) mRNA expression was also significantly lower in the lungs of DIO mice at 3dpi.

Using the same DIO model, Smith *et al* went on to evaluate pulmonary inflammatory cell recruitment during influenza infection, again using A/PR/8/34¹⁰². Flow cytometric analysis of total lung cells demonstrated delayed monocyte infiltration in DIO mice up to 6dpi, with DC numbers particularly low throughout infection. DC antigen uptake and lymph node trafficking remained intact; however, obesity did impair splenic DC antigen presentation to T lymphocytes, possibly secondary to increased IL-6 and decreased IL-2 and IL-12 expression. Despite overall fewer CD8+ T cells infiltrating the lungs of DIO mice, the frequency of influenza-antigen specific cells was comparable to that of lean mice.

Karlsson *et al* subsequently conjectured that the poorer outcomes seen in DIO mice were due to impaired memory T cell-mediated defenses against heterosubtypic influenza infection¹⁰³. To explore this theory, DIO and lean mice were infected with X-31 (a mouse-adapted recombinant strain of influenza A comprising the HA and NA from A/Aichi/2/68 (H3N2) and the internal proteins of PR8 (H1N1)) and subsequently challenged 31 days later with A/PR/8 at a dose that is normally lethal in naive mice.

Both groups of mice lost weight; however, by 7dpi 25% of the DIO mice had died with no loss of lean controls. Increased lung pathology and viral titres were observed in the DIO mice and, reinforcing the findings of Smith *et al*, DIO mice exhibited diminished IFN α and IFN β mRNA expression in the lungs after infection. Additionally, memory CD8⁺ T cells from DIO mice showed a >50% reduction in IFN γ production when stimulated with influenza-pulsed DCs from lean mice.

To determine whether the increased morbidity and mortality following secondary influenza infection was due to a failure to maintain functional, influenza-specific CD8⁺ memory T cells Karlsson *et al* infected DIO and lean mice with X-31 and monitored memory T cell population development, demonstrating a 10% reduction in memory T cell numbers in DIO mice compared to lean controls¹⁰⁴.

Easterbrook *et al* assessed the effects on obesity on infection outcome using more biologically relevant strains in the same DIO mouse model¹⁰⁵. As with the earlier A/PR/8 experiments, following inoculation with CA/09 (a pandemic H1N1 2009 strain) DIO mice had higher mortality (80%) than lean control mice (0%) and lost more weight despite similar lung viral titres. Interestingly, obesity did not affect morbidity and mortality following infection with NY312 (a recent seasonal H1N1) and Sw31 (a close relative to the 1918 pandemic). IFN β and TNF α protein responses were lower in the lungs of obese mice compared to lean following CA/09 infection. Viral antigen distribution was assessed by immunohistochemistry and revealed that CA/09 localized more in the alveolar cells of the lungs of the DIO mice compared to controls, suggesting increased spread to the alveolar epithelium in obesity may contribute to disease severity.

O'Brien *et al* infected both genetically obese (*ob/ob*) mice lacking leptin and DIO mice with pH1N1/09 and a seasonal H3N2 virus¹⁰⁶. Similar to the previous studies, the obese mice had increased mortality and lung pathology and similar pulmonary viral titres. However, epithelial proliferation, measured using the proliferation marker Ki-67, demonstrated a notable reduction in airway re-epithelialization at day 14 post-infection. Additionally, BAL protein and albumin levels were significantly elevated over control in the obese mice, implying a defect in barrier permeability potentially leading to the oedema. Taken together, they concluded that the increased

pathogenicity in virus infected obese mice was independent of enhanced viral replication, but a consequence of impaired wound healing. Finally, but importantly, O'Brien also demonstrated that treatment with oseltamivir afforded complete protection to the obese animals.

To further elucidate the potential mechanism by which obesity impairs the immune response to influenza, Milner *et al* evaluated the dynamic changes in the metabolic profiles of various tissues and biofluids during influenza infection¹⁰⁷. Following infection of lean and DIO mice with a mild dose of A/PR/8/34 (H1N1) virus, urine, faeces, lung tissue, BALF, mediastinal lymph nodes, serum, mesenteric WAT and liver were harvested for 1H-NMR based metabolic profiling. Indeed, the metabolic profile of both urine and faeces was altered by infection, allowing discrimination between the infected and uninfected state in both obese and lean mice. Supporting the association between the metabolic perturbations and the immune response, several immune-metabolic correlations were identified following a correlation analysis conducted between T cell population numbers and 1H NMR data for lean and obese statuses.

Furthermore, Zhang *et al* implicated circulating leptin in the pathogenesis of severe influenza A infection following pH1N1/09 infection by demonstrating improved mortality in DIO mice that had been administered a neutralising anti-leptin antibody following infection with a mouse-adapted strain of pH1N1/09¹⁰⁸. Notably, administration of the antibody did not affect pulmonary viral titres, but did decrease the level of pulmonary pro-inflammatory cytokines leading the authors to propose that leptin could be used as a potential therapeutic target and that the pre-existing elevated leptin levels seen in obesity may sensitise alveolar macrophages and other respiratory cell types to a harmful pro-inflammatory mode.

Radigan *et al* subsequently showed that genetically obese (*db/db*) mice, lacking the leptin receptor globally, have increased mortality, impaired viral clearance and diminished IFN α levels upon infection relative to lean, wild-type controls¹⁰⁹. To determine whether absent leptin receptor signalling in the lung was responsible for these adverse outcomes non-obese, SpC-Cre/LepR^{fl/fl} (lacking leptin receptors specifically in the lung epithelium) and LysM-Cre/LepR mice (lacking leptin receptors in type II pneumocytes and macrophages only) were infected and exhibited improved

survival and viral clearance relative to controls, leading to the conclusion that the worse outcomes observed in *db/db* mice were independent of leptin signalling in the lung epithelium and macrophages. The authors hypothesise that the worse outcomes seen in the *db/db* mice could potentially be due to either impaired leptin signalling in some other tissue compartments e.g. NK cells or T cells, or secondary to the metabolic syndrome associated with obesity.

1.5 The potential mechanism by which obesity predisposes to severe outcomes following pH1N1/09 infection

1.5.1 Obesity is a state of chronic low-grade inflammation

Over the past two decades it has become evident that obesity gives rise to a heightened state of inflammation¹¹⁰. The link between obesity and inflammation was first revealed by Hotamisligil *et al* who demonstrated a positive correlation between adipose tissue mass and the expression of TNF α ¹¹¹. Subsequently, increased plasma levels of several pro-inflammatory cytokines e.g. IL-6 and acute phase proteins e.g. C-reactive protein (CRP) have been described in obesity; many of which appear to originate from adipose tissue^{112,113}. These observations have led to the view that obesity is a state of chronic low-grade inflammation that is initiated by changes within adipose tissue.

1.5.2 Adipose tissue as an 'endocrine organ'

In addition to serving as an energy-storage depot, adipose tissue has endocrine functions, producing 'adipokines' that exert immunomodulatory effects. The most extensively studied adipokine found in the lung (expressed by AMs, bronchial epithelial cells and type II pneumocytes) is leptin¹¹⁴. Identified in 1997, leptin is a 16kDa pro-inflammatory adipokine produced predominantly by white adipose tissue that increases, in serum and bronchoalveolar lavage fluid¹¹⁵, in proportion to body adiposity and whose primary function is appetite control via the hypothalamus¹¹⁶.

Besides leptin and TNF α , adipose tissue produces a range of other adipokines with well-described effects on metabolism and systemic inflammation, including adiponectin, resistin, visfatin and adipon¹¹⁷; however, the relationship between obesity and the pulmonary expression of these latter adipokines has not yet been fully investigated.

Resistin, also known as ‘found in inflammatory zone 3’ (FIZZ3) is encoded by the *RETN* gene and is predominantly produced by peripheral blood mononuclear cells (PBMCs)¹¹⁸. Although the receptor is currently unidentified, resistin is thought to promote both insulin resistance and inflammation¹¹⁹. To date, the presence of resistin has not been demonstrated in bronchoalveolar lavage (BAL) fluid and its role in respiratory infection has not been studied. Of relevance for the main study hypothesis stated below, resistin has been shown to upregulate SOCS3 in adipocytes and human endothelial cells¹¹⁹. It is uncertain whether it has the same effect on human epithelial cells.

Visfatin, or nicotinamide phosphoriboxyltransferase (NAMPT), is also pro-inflammatory¹²⁰ and produced by adipocytes and macrophages. It has been shown to be present in BAL during acute lung injury¹²¹; however, its role in the lung has not been fully elucidated. Adipsin, alternatively known as complement factor D, is a member of the complement system and cleaves factor B¹²². It is also pro-inflammatory and produced primarily by adipocytes and monocytes/macrophages¹²³. It is uncertain again whether it is expressed in the lung, although plasma levels have been shown to correlate with certain occupational lung diseases^{124,125}.

Unlike leptin and resistin, adiponectin, produced primarily by adipocytes, is anti-inflammatory¹²⁶. It is present in BAL and the adiponectin 1 receptor is expressed by lung epithelial cells^{127,128}. Whilst adiponectin has been shown to abrogate TNF α production by macrophages¹²⁹ and inhibit TLR-mediated NF- κ B activation in macrophages¹³⁰, very little is known about the role of adiponectin in the lung.

Whilst obesity, particularly abdominal obesity, may compromise respiratory mechanics by reducing compliance and functional residual capacity^{131,132} little is known about the pulmonary effects of obesity-associated inflammation¹¹⁷. Plasma levels of adipokines and pro-inflammatory cytokines in obesity have been extensively studied; however, the characterization of adipokines within the lung has been minimal aside from a few studies demonstrating elevated leptin levels and decreased adiponectin levels in BAL fluid from obese individuals^{115,128,133}. There is growing interest in the biological interaction between obesity and asthma^{115,134}, however, little is known about the inflammatory state of the ‘healthy’ obese airways. Understanding

the immunology of a pathological condition without full comprehension of ‘healthy’ immune physiology is problematic; one of the aims of this study is therefore to further characterize the inflammatory milieu within obese, co-morbidity-free airways.

1.5.3 *Leptin receptor signal transduction & negative feedback*

There are six isoforms of the leptin receptor (ObR) of which only the long isoform (ObRb or LepRb) is functionally active¹³⁵. In the resting state, ObRb exists as a homodimer at the plasma membrane. Upon binding of leptin to the extracellular ligand binding domain, the two receptor subunits undergo a conformational change resulting in transphosphorylation and transactivation of Janus kinase 2 (JAK2), leading to phosphorylation of three tyrosine residues on the intracellular domain of ObRb (Tyr 985, 1077, and 1138) (Figure 1-8). These phosphorylated residues are in turn recognized by signal transducer and activator of transcription 3 (STAT3), STAT5 and Src homology phosphatase 2 (SHP2) which initiate downstream signalling cascades resulting in expression of genes important in metabolism and/or inflammation e.g. pro-opiomelanocortin (POMC) and IL-6. Of particular relevance for the forthcoming hypotheses, pSTAT3 induces expression of SOCS3 that in turn, negatively feeds back on the ObRb (by binding Tyr 985), attenuating STAT3 activation and leptin signal transduction¹³⁶.

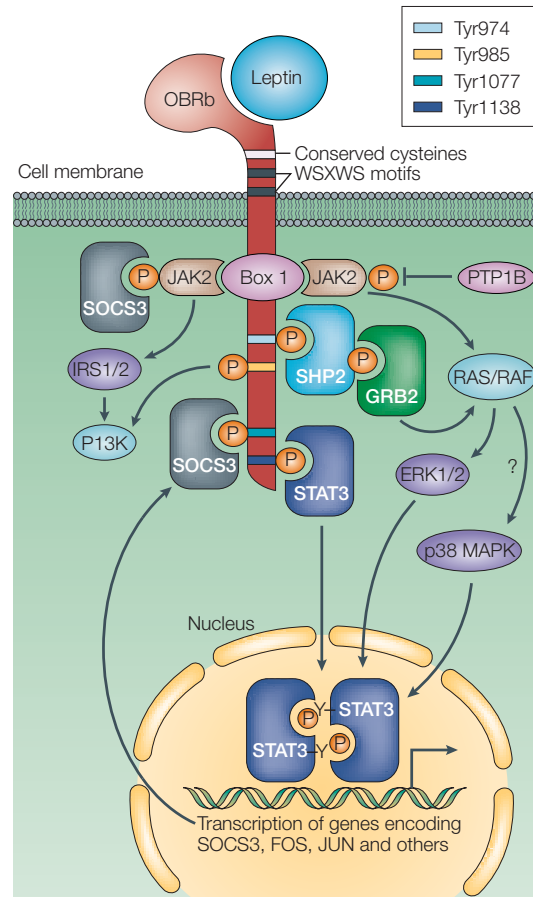


Figure 1-8 Leptin induces SOCS3 via a JAK-STAT signal transduction pathway

Upon binding of leptin to the extracellular domain of the long isoform of the leptin receptor (ObRb) the two receptor subunits undergo a conformational change resulting in transphosphorylation and transactivation of Janus kinase 2 (JAK2), leading to phosphorylation of three tyrosine residues on the intracellular domain of ObRb. These phosphorylated residues are in turn recognized by signal transducer and activator of transcription 3 (STAT3) and Src homology phosphatase 2 (SHP2) which initiate downstream signalling cascades resulting in expression of genes important in metabolism and/or inflammation e.g. pro-opiomelanocortin (POMC) and IL-6. pSTAT3 induces expression of suppressor of cytokine signalling 3 (SOCS3) that in turn, negatively feeds back on the ObRb, attenuating STAT3 activation and leptin signal (adapted from La Cava 2004¹³⁶)

1.5.4 *Leptin receptor expression*

The ObRb receptor is expressed in numerous cell types, including neurons, intestinal epithelial cells and immune cells, such as macrophages, T cells, NK cells and PMNs¹³⁵ (Table 1-7). The presence of ObRb in human bronchial epithelial cells (hBECs) obtained from bronchial biopsies and lobectomy samples has been demonstrated in several studies by RT-qPCR¹³⁷, flow cytometry¹³⁸ and immunohistochemistry¹³⁹. Functionality of the ObRb in immortalized BEC cell lines has also been demonstrated by showing an increase in pSTAT3 in response to leptin exposure *in vitro* in A549 cells¹³⁹ and an increase in cell proliferation in 16HBE cells¹³⁸. Functional ObRb has

also been shown to be present in human PBMCs¹⁴⁰, AMs¹⁴¹ and DCs both at mRNA and protein level, again with increased STAT3 phosphorylation or pro-inflammatory cytokine induction in response to leptin exposure^{142,143}.

Table 1-7 Summary table of the evidence for leptin receptor expression in human respiratory and immune cells

Cell Type	Method	References
Bronchial epithelial cells (hBECs)	RT-PCR Flow cytometry Immunohistochemistry	Tsuchiya 1999 ¹³⁷ Bruno 2009 ¹³⁸ Vernooy 2009 ¹³⁹
Dendritic cells (DCs)	RT-PCR & Western	Al-Hassi 2013 ¹⁴² Mattioli 2005 ¹⁴³ La Cava 2004
Peripheral blood mononuclear cells (PBMCs)	RT-PCR	Lord 1998 ¹⁴⁰ La Cava 2004 Tsiotra 2000 ¹⁴⁴
Alveolar Macrophages (AMs)	RT-PCR	Lugogo 2012 ¹⁴¹

1.5.5 *Leptin resistance and SOCS3*

The primary function of leptin is to regulate food intake via the hypothalamus. Leptin levels increase physiologically in proportion to body adiposity; however, this negative feedback system is dysregulated in obesity as obese individuals exhibit high circulating levels of leptin without decreasing food intake. This implies a situation of ‘leptin resistance’, which evidence suggests may be mediated by upregulation of SOCS3¹⁴⁵ (Figure 1-9). Leptin resistance has been demonstrated in NK cells¹⁴⁶, T cells¹⁴⁷ and peripheral blood monocytes¹⁴⁴.

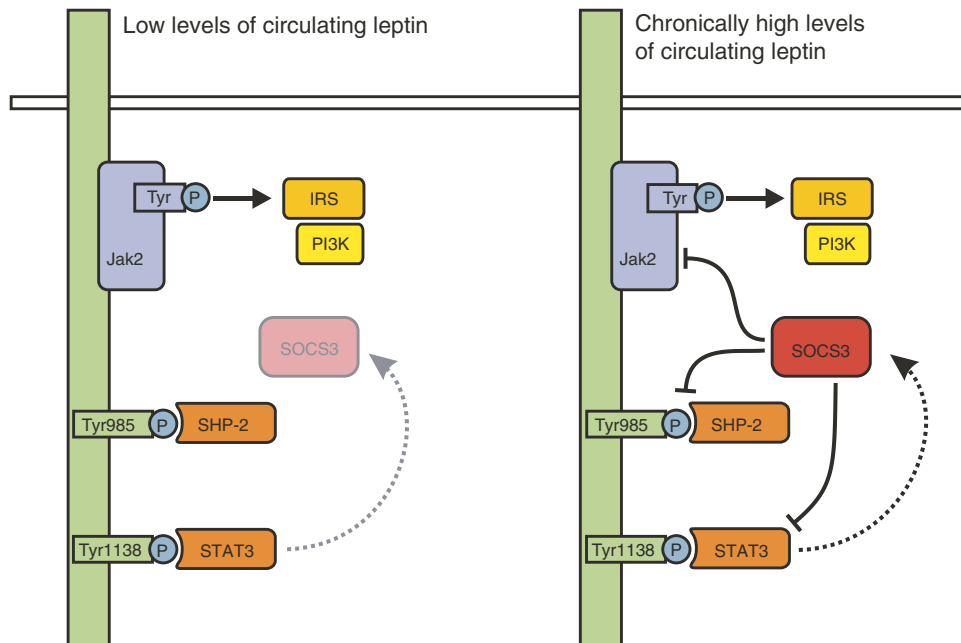


Figure 1-9 Leptin resistance is mediated by SOCS3.

Upon leptin binding to the ObRb, STAT3 induces the transcription of SOCS3, which attenuates ObRb signal transduction (left panel). At high levels of leptin, as seen in obesity, increased baseline STAT3 activation would result in increased expression of SOCS3, resulting in attenuation of ObRb signal transduction and a state of leptin resistance (right panel) (adapted from Münzberg *et al* 2005¹⁴⁸).

Plasma leptin concentration correlates with body fat content and is therefore increased in obesity¹⁴⁹⁻¹⁵¹. Additionally, leptin has been shown to be present in BAL fluid^{128,141} and to decrease in BAL after bariatric surgery¹¹⁵. *In vitro* experiments using CHO cells transfected with the leptin receptor and subsequently stimulated with leptin have demonstrated increases in both SOCS3 mRNA and protein¹⁵²; however, leptin-induced increases in SOCS3 in pulmonary cells have not yet been investigated. Leptin has however, been shown to signal in respiratory epithelial cells (A549), resulting in a dose dependent increase in STAT3 phosphorylation¹³⁹ and alveolar macrophages, resulting in increased TNF α secretion¹⁴¹. A summary of published *in vitro* leptin stimulation studies is presented in Table 1-8.

Table 1-8 Summary of *in vitro* leptin stimulation studies

Authors	Cell type	Outcomes	Leptin dose	Duration of leptin exposure	Timepoints	Results
Gainsford ¹⁵³	1996 Murine peritoneal macrophages	GM-CSF, G-CSF protein expression	50ng	3 hours	3 hours	Leptin induces GM-CSF and G-CSF production
Bjørnbæk ¹⁵²	1999 CHO cells transfected with leptin receptor	SOCS3 mRNA and protein expression	100nM	0 - 20 hours	0, 1, 2, 4 hours	Transient increase in SOCS3 mRNA at 1 hour, protein expression peaks at 4 hours, gradually decreases to 20 hours
Santos-Alvarez ¹⁵⁴	1999 Human PBMCs	Flow detection of IL-6 and TNF alpha synthesis	0.01nM - 100nM	6 hours	6 hours	3-28% increase in percentage of monocytes producing IL-6 and TNF alpha
Gabay C ¹⁵⁵	2001 Human PBMCs, THP-1 cells	IL-1Ra mRNA & protein expression	10 - 625nM	24 hours	8, 19, 24 hours	12 fold increase in IL-1Ra protein, significant mRNA induction at 8 and 19 hours
Martín-Romero ¹⁵⁶	2001 Human PBMCs	STAT-3 phosphorylation	0.1 - 100nM	5 minutes	5 minutes	Leptin activates PI3K, MAPK pathways in human PBMCs
Sanchez-Margalef ¹⁵⁷	2001 Human PBMCs	STAT-3 phosphorylation	10nM	0, 5, 10, 20 minutes	0, 5, 10, 20 minutes	Leptin results in JAK2, STAT3 phosphorylation and nuclear translocation
Dreyer MG ¹⁵⁸	2003 HepG2 hepatoma cell line, THP-1 cells	NFkB reporter activity	625nM	24 hours	24 hours	Leptin activates NFkB
Zhao Y	2003 NK-92 (NK cells), YT cells	IL2, perforin mRNA expression	0 - 200ng/ml	72 hours	72 hours	Significant increase in perforin and IL-2 mRNA at 72 hours

Authors	Cell type	Outcomes	Leptin dose	Duration of leptin exposure	Timepoints	Results
Mancuso P ¹⁵⁹	2004 A-23187 stimulated Murine Alveolar Macrophages	LTB4, cysteinil leukotrienes synthesis	0 - 500ng/ml	16 hours	30 minutes	Leptin promotes alveolar macrophage leukotriene synthesis
Shen Y ¹⁶⁰	2009 A549, H157	ObRb receptor expression, VEGF, IL-6, PGE2 protein expression (ELISA)	100ng/ml	24 hours	24 hours	1.5 to 2 fold increase in VEGF, IL-6 and PGE2
Vernooy J ¹⁵⁹	2009 A549, NCL-H292	STAT-3 Phosphorylation	20, 100, 500ng/ml	0.5, 1, 2 hours	0, 0.5, 1, 2 hours	Leptin results in early STAT3 phosphorylation in a dose dependent manner
Kiguchi N ¹⁶¹	2009 J774A.1 cells (murine macrophages)	CCL3, CCL4, CCL5 mRNA expression	0.001-1uM	1, 3, 6, 12, 24 hours	1, 3, 6, 12, 24 hours	Significant upregulation of CCL3, 4 & 5 from 3 hours, greatest effect seen with 1uM dose of leptin
Taildeman ¹⁶²	2009 Human mast cells	Immuno-staining for leptin receptor expression	N/A	N/A	N/A	Leptin receptors present on human mast cells
Bruno A ¹³⁸	2009 16HBEs, NHBEs, hBECs	TGF-beta 1 protein expression	0.5umol/L	24, 48 hours	24, 48 hours	Leptin decreases spontaneous TGF beta 1 release and increases cell proliferation
Gomez R ¹⁶³	2011 Human cultured chondrocytes	IL-8 mRNA & protein expression	800nM	48 hours	48h	40 fold increase in IL-8 mRNA
Bruno A ¹⁶⁴	2011 A549	A549 colony number	0.5uM	24, 48, 72 hours	24, 48, 72 hours	Leptin significantly increases colony number
Handy J ¹⁶⁵	2011 Rat HSCs (hepatic stellate cells)	JAK2 phosphorylation	100ng/ml	30 minutes	30 mins	Adiponectin blocks leptin-mediated activation of JAK-2

1.5.6 Potential attenuation of IFN signal transduction by leptin-induced upregulation of SOCS3

Given that both leptin and IFN signal transduction are JAK-STAT dependent and subject to negative regulation by SOCS3 we propose that obesity-associated, chronic, leptin-induced upregulation of SOCS3 attenuates type I and III IFN signal transduction, thus impairing the innate antiviral immune response and predisposing to severe outcomes following pH1N1/09 infection in obesity (Figure 1-10).

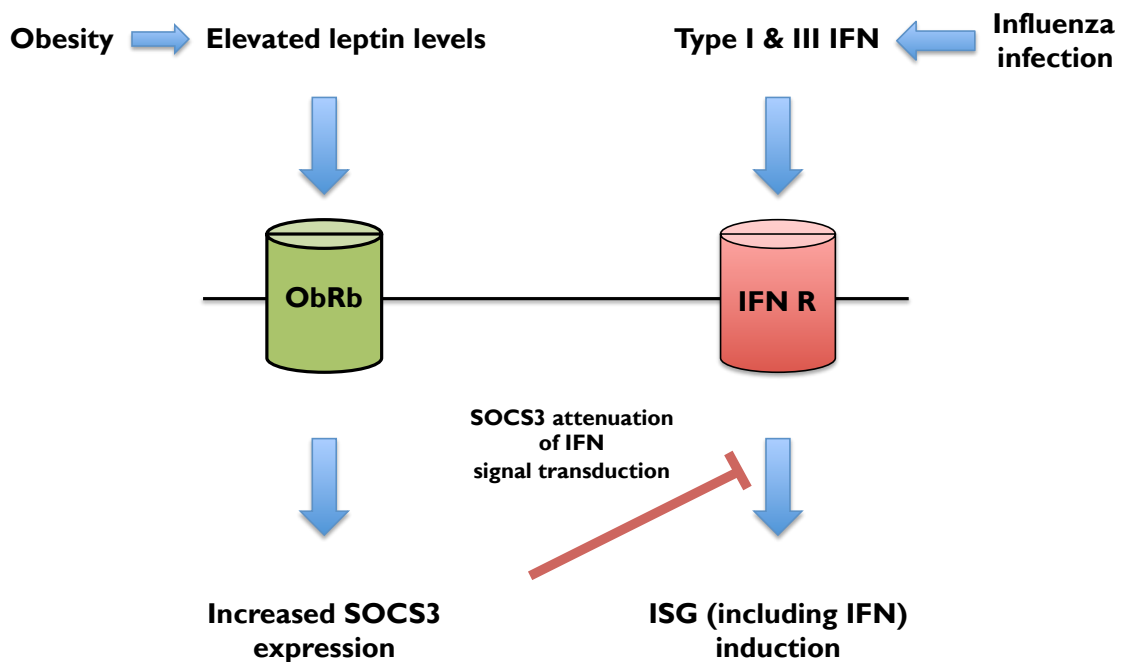


Figure 1-10 Simplified model outlining the potential mechanism by which obesity may contribute to the severe outcomes seen following pH1N1/09 infection

Obesity is a leptin-resistant state in which leptin levels are chronically elevated due to increased body adiposity. The functional isoform of the leptin receptor, ObRb, which signals via a JAK-STAT signalling cascade, is expressed on key respiratory and immune cells. Leptin binds these ObRb receptors inducing SOCS3 expression, which negatively feeds back on ObRb signal transduction. Leptin-induced SOCS3 also attenuates influenza-induced IFN signal transduction on the same cells, thus resulting in diminished ISG (including interferon) expression. IFN R = IFN receptor.

Two recently published *ex vivo* studies in human PMBCs from Teran-Cabanillas *et al* support this hypothesis^{166,167}. In their initial study, PBMCs were isolated from 30 obese (BMI ≥ 30 kg/m²) and 30 age-matched, non-obese (BMI ≤ 25 kg/m²) individuals (confounding co-morbidities not specified) and stimulated with TLR ligands. Following TLR-3 stimulation with poly I:C, PBMCs from obese individuals exhibited diminished

levels of IFN α and β mRNA relative to controls in association with elevated basal levels of the negative regulator SOCS3, but not SOCS1¹⁶⁶.

In their subsequent study, PBMCs from obese and lean individuals with and without confirmed pH1N1/09 infection were again stimulated with TLR ligands¹⁶⁷. The PBMCs from obese subjects infected with influenza A/H1N1 showed a diminished ability to produce type I IFN in response to poly I:C and again, PBMCs from obese influenza-free subjects showed an increased basal expression of SOCS3, but not SOCS1. However, any conclusions from this latter study should be interpreted with gross caution given several major limitations; firstly, no demographic data concerning the subjects was provided hence any observed differences may have been due to confounding factors other than obesity e.g. age. Secondly, the time at which the PBMCs were harvested during the pH1N1/09 was not specified (nor the method of diagnosis) hence any differences observed between infected obese and lean individuals could have been merely due to differences in the stage of infection.

1.5.7 IFN deficient states

Relative IFN deficient states, in which anti-viral IFN responses are attenuated, have previously been described in the literature (Table 1-9); individuals with atopic asthma have been shown to have increased susceptibility to severe outcomes following rhinovirus infection compared to healthy controls, exhibiting more frequent lower respiratory tract infections and suffering from more severe, and longer lasting symptoms¹⁶⁸. Recent studies by Johnston *et al* have identified deficiencies in IFN α/β and IFN λ induction by rhinovirus infection in primary hBECs^{168,169} and lung macrophages¹⁶⁸ isolated from asthmatics.

Table 1-9 Table summarizing the evidence for IFN deficient states

Condition	Cell types	Stimulus	Innate deficiency	References
Asthma	hBECs	Rhinovirus 16	IFN β	Wark 2005 ¹⁶⁹
	BAL cells	Rhinovirus 16	IFN λ IFN α , IFN β	Contoli 2006 ¹⁶⁸ Sykes 2012 ¹⁷⁰
COPD	BAL cells	Rhinovirus 16	IFN β	Mallia 2011 ¹⁷¹
Pregnancy	PBMCs	A/Auckland/3/2009	IFN α , IFN λ , PKR	Forbes 2012 ¹⁷²
Cystic Fibrosis	IB3 (Δ 508) epithelial cells	<i>P.aeruginosa</i>	IFN β	Parker 2012 ¹⁷³
Obesity	PBMCs	Poly I:C	IFN α , IFN β , IL6, IL10, IL1 β	Teran-Cabanillas 2013 ¹⁶⁶ Teran-Cabanillas 2014 ¹⁶⁷

Indeed, deficient type I and III IFN production following stimulation of PBMCs with pH1N1/09 has been demonstrated in pregnant women, an ‘at risk’ group for severe influenza infection. Forbes *et al* took PBMCs from 28 healthy pregnant women (in the second and third trimesters) and 26 healthy, non-pregnant women and infected them with A/Auckland/3/2009 (MOI 0.1), collecting supernatants for measurement of IFN α , γ and λ at 24, 48, 72 and 96 hours¹⁷². Significantly less IFN α and λ were produced by the PBMCs and the ISG, PKR was also significantly reduced in the pregnant state despite similar viral replication.

The evidence for IFN deficiency in chronic obstructive pulmonary disease (COPD) is conflicting. *Ex vivo* rhinovirus stimulations of BAL cells from COPD patients have demonstrated deficient induction of type I and III IFN, associated with deficiency of the ISG, CXCL10¹⁷¹. Comparable findings have been observed in a mouse model in which deficient induction of type I & II IFN were reported in COPD mice compared to controls¹⁷⁴. However, no differences in IFN production were observed in a study comprising *in vitro* RV infection of hBECs from COPD patients and healthy controls¹⁷⁵.

IFN deficiency has been suggested in cystic fibrosis (CF) although the evidence is limited; Parker *et al* stimulated CF epithelial cell lines with *pseudomonas aeruginosa* and LPS and demonstrated a significant early (2 & 4 hours) reduction in IFN β and λ mRNA induction (and STAT1 phosphorylation) relative to control cell lines. Endogenous levels of PKR and Mx were also diminished in the CF cell line¹⁷³.

Given these data, and combined with corroborative evidence from mouse models of obesity and influenza infection, it is clear that IFN deficiency may be a plausible explanation for the severe outcomes seen in obesity following pH1N1/09 infection.

1.5.8 *Non-immune factors contributing to obesity-associated adverse outcomes*

Several non-immune factors may contribute to obesity-associated adverse medical outcomes following influenza infection¹⁷⁶ (Table 1-10); however, no mechanistic influenza-specific literature exists. Obesity, although not associated with airflow obstruction *per se*, is known to adversely affects pulmonary mechanics (and thus ventilation) by reducing lung tidal volume, functional residual capacity, airway calibre and increasing airway resistance and airway hyperreactivity¹¹⁵. To complicate medical management further, obesity alters the volume of distribution of certain antimicrobial medications e.g. aminoglycosides; however, evidence suggests that dose adjustment is not required for the neuraminidase inhibitor, oseltamivir, in obesity¹⁷⁷. Morbid obesity also impairs mobility, thus complicating nursing care, and increasing the risk of pressure sores and venous thromboembolism.

Furthermore, obesity-related co-morbidities such as obstructive sleep apnoea (OSA) and diabetes mellitus may contribute to adverse outcomes. OSA is commonly seen in bariatric subjects¹⁷⁸; however, no studies linking OSA with adverse outcomes following influenza infection have been published. Some evidence exists though to suggest that innate immunity may be altered by the presence of OSA; NF- κ B activation may be increased in monocytes from individuals with OSA¹⁷⁹ and TLR 2 and 4 expression higher¹⁸⁰; however, in both these studies, the observed changes in innate immunity were reversed with the use of CPAP. OSA may also result in increased activation of alveolar

macrophages, as evidenced by decreased PPAR- γ activity and gene expression in BAL cells from OSA patients¹⁸¹.

Individuals with diabetes mellitus (DM) are at increased risk for influenza and influenza-related complications and this is reflected by current vaccination policy²⁵. The WHO-commissioned systematic review and meta-analysis by Mertz *et al*³⁴ found that DM increases risk of hospitalisation following seasonal influenza (OR 9.91, 95% CI 5.46 to 17.99) and, following pandemic influenza infection, increases risk of hospitalisation (4.26, 95% CI 3.14 to 5.77), intensive care admission (1.60, 95% CI 1.32 to 1.94) and death (2.21, 95% CI 1.37 to 3.57).

As discussed earlier, a large body of epidemiological evidence supports the association between obesity and adverse influenza-associated outcomes (Table 1-5); however, no studies recounting the actual pathology in the severe obese cases have been published. Indeed, no human data describing the viral loads, cytokine levels and pathology in the severe obese cases compared to the severe lean cases are available. Thus, one can only speculate on the actual pathophysiological mechanism by which the obese are rendered more susceptible to severe outcomes. The hypotheses proposed herein outline an obesity-associated innate immune deficiency; we are not proposing that an innate immune deficiency is the sole reason why obese individuals experience severe outcomes; as detailed in Table 1-10 there are many other non-immune factors that may also contribute although the relative contributions of each are uncertain.

Table 1-10 Non-immune obesity-associated factors that may contribute to adverse medical outcomes

(adapted from Huttunen et al¹⁷⁶)

Non-immune contributory factors	Examples
Respiratory	Pulmonary restriction Ventilation-perfusion mismatching Difficult mechanical ventilation Increased risk of pulmonary emboli Obstructive sleep apnoea Difficult interpretation of medical imaging
Obesity related co-morbidities	Type 2 diabetes mellitus Atherosclerosis Hypertension Osteoarthritis Decreased mobility
Medical care	Difficulty moving and washing patient Increased risk of pressure sores Discrimination/medical prejudice
Pharmacological	Altered volume of distribution of antimicrobials
Psychological	Depression

1.6 Study hypotheses

Evidence from epidemiological, animal and human laboratory studies suggest that obesity may be associated with attenuated IFN responses following influenza infection. We propose that SOCS3 expression will be higher in obesity secondary to elevated leptin levels. Given that IFN signal transduction is JAK-STAT dependent and therefore subject to negative regulation by SOCS3 we postulate that attenuated type I and III IFN signal transduction will be observed in obesity, thus impairing the innate antiviral immune response and predisposing to severe outcomes (Figure 1-10).

Prior to delineating a potential mechanism, it is essential to establish first whether attenuated IFN responses are indeed present in obesity upon influenza infection. The primary hypothesis reflects this necessity and the comparison of the influenza-induced immune responses of primary hBECs, BAL cells, PBMCs and peripheral blood DCs from obese and lean individuals is presented in chapter 4.

1.6.1 Primary hypothesis

Morbid obesity ($\text{BMI} \geq 35 \text{ kg/m}^2$) impairs the innate IFN (α , β and λ), ISG (MxA) and pro-inflammatory cytokine (IL-6, IL-8 and TNF α) responses of primary hBECs, BAL cells, PBMCs and peripheral blood DCs to pandemic influenza A and seasonal influenza A and B.

1.6.2 Sub-hypotheses:

In addition to establishing whether an obesity-associated innate immune deficiency is evident following influenza infection we also aimed to reproduce the IFN deficiency observed by Teran-Cabanillas *et al* upon stimulation of PBMCs from obese and lean individuals with TLR ligands¹⁶⁶. The sub-hypothesis tested by this experiment is outlined below (as this is also an *ex vivo* stimulation experiment the results are presented in chapter 4 alongside the influenza infection data).

- The innate IFN (α , β and λ) and pro-inflammatory cytokine (IL-6, IL-8 and TNF α) responses of PBMCs to the TLRs: CPG-A, R848, poly (I:C), LPS and LTA will be attenuated in the obese state.

The remaining sub-hypotheses are essentially the pre-requisites for the theoretical model outlined in Figure 1-10. All are tested and presented in chapter 5 aside from the final sub-hypothesis, which is presented in chapter 4 alongside the other *ex vivo* experiment results.

- Obesity is a pro-inflammatory state in which leptin, acute phase protein and pro-inflammatory cytokine protein levels will be elevated, and anti-inflammatory adipokine levels diminished, in the airways and serum of obese individuals relative to those of healthy weight controls.
- SOCS3 over-expression will attenuate IFN induction following infection with pH1N1/09.
- Constitutive SOCS3 expression will be higher at baseline (i.e. prior to infection) in primary hBECs, BAL cells, PBMCs and DCs from obese subjects relative to normal weight comparators.
- Exogenous leptin will upregulate baseline SOCS3 mRNA expression in primary hBECs *ex vivo*.
- Pre-exposure of hBECs from lean controls to exogenous leptin will attenuate the innate IFN (α , β and λ) and pro-inflammatory cytokine (IL-6, IL-8 and TNF α) responses to pandemic influenza A and seasonal influenza A and B.

1.7 Study aim and objectives

1.7.1 Aim

To investigate the effect of obesity on the host innate immune response to influenza infection

1.7.2 Primary Objective

To determine whether morbid obesity ($\text{BMI} \geq 35 \text{ kg/m}^2$) impairs the innate IFN (α , β and λ), ISG (MxA) and pro-inflammatory cytokine (IL-6, IL-8 and TNF α) responses of primary hBECs, BAL cells, PBMCs and peripheral blood DCs to pandemic influenza A and seasonal influenza A and B infection.

1.7.3 Secondary Objectives

- To determine whether the innate IFN (α , β and λ) and pro-inflammatory cytokine (IL-6, IL-8 and TNF α) responses of PBMCs to the TLRs: CPG-A, R848, poly (I:C), LPS and LTA are attenuated in the obese state.
- To characterise the adipokine, acute phase protein and cytokine/chemokine milieu within healthy, obese airways from the nose to the alveoli (NLF, BLF, BAL fluid and serum).
- To determine the modulatory effects of SOCS expression on IFN induction following influenza infection.
- To determine whether constitutive SOCS3 expression is elevated in primary hBECs, BAL cells, PBMCs and DCs from obese subjects relative to normal weight comparators.
- To determine the effect of exogenous leptin on SOCS3 expression in primary hBECs
- To determine whether pre-exposure of hBECs from lean controls to exogenous leptin attenuates the innate IFN (α , β and λ) and pro-inflammatory cytokine (IL-6, IL-8 and TNF α) responses to pandemic influenza A and seasonal influenza A and B.

Chapter 2 Materials and Methods

To appropriately address the proposed hypotheses a prospective, case-control study design was adopted. Ethical approval for the study was granted by the Surrey Borders Research Ethics Committee (ref: 12/LO/1812) and informed consent was obtained from all participants in accordance with Good Clinical Practice guidelines. 15 morbidly obese ($\text{BMI} \geq 35 \text{ kg/m}^2$), bariatric surgery candidates (cases) and 15 healthy, normal weight ($\text{BMI} 20\text{-}25 \text{ kg/m}^2$) individuals (controls) were recruited between 13th June 2013 and 6th January 2015. Following anthropometric characterisation nasal, pulmonary and blood samples were obtained for either *ex vivo* infection with pandemic or seasonal influenza viruses or for measurement of adipokines/cytokines and mRNA expression. In this chapter, the materials and methods used throughout the ObFlu study are described. Regarding nomenclature, the healthy-weight volunteers are referred to as either ‘normal weight’ or ‘lean’ controls.

2.1 Materials

2.1.1 Buffers and reagents

Table 2-1 Buffers and reagents used throughout the study

Name	Composition	Application	Supplier
β -Mercaptoethanol	14.3M $C_2H_6O_5$	RNA isolation	Sigma Aldrich
Bovine Serum Albumin Powder (BSA)	1% BSA in 0.15M NaCl pH 7.0	Flow cytometry buffer	Sigma Aldrich
Dulbecco's PBS (x1)	Phosphate buffered saline	Cell culture	Invitrogen
Histopaque-1077	Polysucrose and sodium diatrizoate, adjusted to a density of 1.077 g/mL	PBMC isolation	Sigma Aldrich
LightCycler 480 Probes Master	FastStart Taq DNA polymerase, reaction buffer, dNTP mix, 6,4mM $MgCl_2$	RT-qPCR	Roche
Lipofectamine 2000	Not available	Transfections	Invitrogen
4% Paraformaldehyde	4g in 100mls PBS	Bronchial biopsy processing	Sigma Aldrich
Random primers	Random hexamers (0.5 μ g/ml)	cDNA synthesis	Promega
RLT Lysis Buffer	1% Beta-Mercaptoethanol in RLT lysis buffer	Primary cell lysis	Qiagen
Recombinant human leptin	1mg recombinant human leptin	<i>Ex vivo</i> cell stimulation	Invitrogen
Tween 20 (polyoxyethylene-sorbitan monlaurate)	20 ethylene oxide units, 1 sorbitol unit, 1 lauric acid unit	MSD	Sigma Aldrich
Trypan blue	0.4% trypan blue in 0.85% saline solution	Cell viability	Invitrogen

2.1.2 Media and media supplements

Table 2-2 Media and media supplements used throughout the study

Media/Supplement	Composition	Application	Supplier
BEBM Basal medium	Refer to manufacturer's media formulation	Primary hBEC culture	Lonza
BEGM Single quot additives	Bovine Pituitary Extract, Hydrocortisone, human Epidermal Growth Factor, Epinephrine, Transferrin, Insulin, Retinoic Acid, Triiodothyronine, Gentamicin/Amphotericin-B, and Bovine Serum Albumin – Fatty Acid Free	Primary hBEC culture	Lonza
BEGM Complete medium	BEBM with BEGM single quot additives	Primary hBEC culture	Lonza
Dulbeccos modified eagles medium (DMEM)	Refer to manufacturer's media formulation	A549 & MDCK culture	Invitrogen
DMEM complete medium	DMEM plus 10% FBS, 12ml HEPES buffer	A549 culture	Invitrogen
Fetal bovine serum (FBS)	Heat inactivated foetal bovine serum	PBMC culture	Invitrogen
HEPES buffer	C8H18N2O4S 1M solution 4-(2-hydroxyethyl)piperazine-1-thanesulfonic acid solution	PBMC culture	Sigma-Aldrich

Optimem-1 medium	Manufacturer's formula – no other information given	Transfection	Invitrogen
Recombinant human like collagen	>95% human collagen from E.Coli	Primary hBEC culture	Biovision
RPMI Medium	Refer to manufacturer's media formulation	PBMC culture	Invitrogen
RPMI Complete	RPMI medium plus 10% FBS, 12ml HEPES buffer	PBMC culture	Invitrogen
ReagentPack™ Subculture Reagents	Trypsin/EDTA Trypsin neutralizing solution HEPES Buffered saline	Passage of primary hBECs	Lonza
Stimulation media	RPMI-1640 with L-glutamine, 2% HEPES, 1% sodium bicarbonate, 1% penicillin/streptomycin and 10% foetal bovine serum	PBMC infection	Invitrogen
Sodium bicarbonate	7.5% sodium bicarbonate	BAL cell and PBMC culture	Invitrogen

2.1.3 Toll like receptor (TLR) ligands

Table 2-3 TLR ligands used in throughout the study for PBMC stimulation

Ligand	Receptor	Dose	Source
Polyinosinic-Polycytidylic Acid (Poly (I:C))	TLR 3	100µg/ml	Sigma Aldrich
Lipopolysaccharide (LPS)	TLR 4	10µg/ml	Sigma Aldrich
R848 (Resiquimod)	TLR 7	1µg/M	Eurogentec
Lipoteichoic acid (LTA)	TLR 2	25µg/ml	Eurogentec
CpG-A-ODN	TLR 9	1µM	Eurogentec

2.1.4 Commercially available kits

Table 2-4 Commercially available kits used in the study

Name	Components	Application	Supplier
Blood Dendritic Cell Isolation kit II	Non-DC Depletion Cocktail, human: Cocktail of monoclonal biotin-conjugated antibodies against human CD1c (BDCA-1) (clone: AD5-8E7; isotype: mouse IgG2a), and MicroBeads conjugated to monoclonal antibodies against human CD14 (isotype: mouse IgG2a) and human CD19 (isotype: mouse IgG1). DC Enrichment Cocktail: MicroBeads conjugated to monoclonal antibodies against human CD304 (BDCA-4/Neuropilin-1) (isotype: mouse IgG1), human CD141 (BDCA-3) (isotype: mouse IgG1), and Biotin (isotype: mouse IgG1)	Isolation of DCs from PBMCs	Mitenyi Biotec
Multiplex IL-6, IL-8, TNF α MSD	Cytokine plate (96-well) Diluent 100 Read buffer T 4x Sulfo-Tag anti-human IL-6, IL-8, TNF α antibodies	IL-6, IL-8, TNF α protein quantification	Meso Scale Discovery
Human IFN- α MSD	Cytokine panel 13 plate (96-well) Diluent 100 Read buffer T 4x Sulfo-Tag anti-human IFN- α 2 antibody	IFN alpha protein quantification	Meso Scale Discovery
Human IFN β MSD	hIFN- β Capture Antibody SULFO-TAG Anti-human IFN- β Antibody MULTI-ARRAY 96-well Avidin Plate Diluent 100 Read buffer T 4x	IFN β protein quantification	Meso Scale Discovery
Human IFN λ MSD	MULTI-ARRAY 96-well plate IL-29 capture antibody IL-29 detection antibody Recombinant IL-29 standard Streptavidin-HRP	IFN λ protein quantification	Meso Scale Discovery
Bio-Plex Pro human acute phase multiplex assays	1 x 96-well plate, coupled beads, detection antibodies, standards, controls, reagents and diluents for detecting 4 human acute phase biomarkers	Acute phase protein quantification	Bio-Rad
Bio-Plex Pro human cytokine 27-plex assay	1 x 96-well plate, coupled magnetic beads, detection antibodies, standards, reagents, and diluents for detecting 27 human cytokines	Cytokine and chemokine quantification	Bio-Rad
Bio-Plex Pro human diabetes 6 plex assay	1 x 96-well plate, conjugated magnetic beads, detection antibodies, standards, buffers, diluents, streptavidin-PE, filter plate, for the detection of 6 human adipokines	Adipokine protein quantification	Bio-Rad
RNeasy Mini kit	Buffer RLT, Buffer RW1, Buffer RPE, RNase free water, RNeasy mini spin columns, collection tubes	Total RNA isolation	Qiagen
RNase free DNase set	1500 IU RNase free DNase 1, RNase free buffer RDD	Total RNA isolation	Qiagen
Omniscript RT (reverse transcriptase) kit	dATP, dCTP, dGTP, dTTP (10mM each) in water, 10x buffer RT, omniscript reverse transcriptase, RNase free water	cDNA synthesis	Promega

2.1.5 *qPCR primer and probe sequences*

All qPCR primers were designed in-house and purchased from Invitrogen (Paisley, UK). All qPCR probes were purchased from EuroFins. Primers and probes were reconstituted using sterile TE buffer to create a 100µM stock solution. Working stocks (5µM) were created by diluting the original stock with nuclease free water (1:20). Stocks were stored at -20°C.

Table 2-5 RT-qPCR primers and probes and their optimal concentration

Name	Sequence (5'-3')
18S Forward (300nM)	CGCCGCTAGAGGTGAAATTCT
18S Reverse (300nM)	CATTCTTGGCAAATGCTTTTCG
18S Probe (100nM)	FAM-ACCGGCGCAAGACGGACCAGA-TAMRA
IAV M gene Forward (300nM)	AAGACAAGACCAATYCTGTCACCTCT
IAV M gene Reverse (300nM)	TCTACGYTGCAGTCCYCGCT
IAV M gene Probe (100nM)	FAM-TYACGCTCACCGTGCCCAGTG-TAMRA
IBV M gene Forward	CCATCTGCATTTCCTGCTT
IBV M gene Reverse	ATCACATTCACACAGGGCTCATA
IBV M gene Probe	FAM-CTCCAGGCACTGAAGATCTCGCTGC-TAMRA
MxA Forward (900nM)	CAGCACCTGATGGCCTATCAC
MxA Reverse (900nM)	CATGAACTGGATGATCAAAGG
MxA Probe (100nM)	FAM-AGGCCAGCAAGCGCATCTCCAG-TAMRA
SOCS1 Forward (900nM)	CCCTGGTTGTTGTAGCAGCTT
SOCS1 Reverse (900nM)	GGTTTGTGCAAAGATACTGGGTATAT
SOCS1 Probe (100nM)	FAM-AGGTAGGAGGTGCGAGTTCAGGTCCTG-TAMRA
SOCS3 Forward (900nM)	TGGGACGATAGCAACCACAA
SOCS3 Reverse (900nM)	CGAAGTGTCCCCTGTTTGGA
SOCS3 Probe (100nM)	FAM-TGGATTCTCCTTCAATTCCTCAGCTTCCC-TAMRA

2.1.6 Flow cytometry antibodies

Table 2-6 Antibodies used for flow cytometry staining of DCs and their optimal dilutions

Antibody	Fluorochrome	Dilution	Supplier
Lineage cocktail	FITC	1 in 50	eBioscience
FCeRI	PerCP	1 in 100	Biolegend
HLA-DR	QDOT605	1 in 200	Invitrogen
CD11c	AlexaFluor 700	1 in 12.5	eBioscience
CD123	PE	1 in 50	eBioscience
BDCA1	PerCP eFluor 710	1 in 25	eBioscience
BDCA2	PE-Cy7	1 in 50	eBioscience
BDCA3 (CD141)	VioBlue	1 in 5	Miltenyi
BDCA4 (CD304)	APC	1 in 20	Miltenyi

2.1.7 Laboratory instruments

Table 2-7 Laboratory instruments used throughout the study

Instrument	Application	Company
LightCycler 480 Instrument II	RT-qPCR	Roche
Sector Imager 1200 plate reader	Multiplex protein quantification	Meso Scale Discovery
Bio-Plex® 3D Suspension Array System	Multiplex protein quantification	Bio-Rad
BD LSRFortessa	Flow cytometry	BD Biosciences

2.1.8 Clinical instruments

Table 2-8 Clinical instruments used throughout the study

Name	Composition	Application	Supplier
Disposable cytology brush	5mm cytology brush	Bronchoscopy	Olympus
Endo-jaw disposable biopsy forcep	Endobronchial biopsy forceps	Bronchoscopy	Olympus
Bronchosorption device	Synthetic Absorptive Matrix	Bronchosorption	Hunt
Bronchoscope BFT260	Fibre-optic bronchoscopy	Bronchoscopy	Olympus
Spirometer	Electronic spirometer	Spirometry	Micro medical
Vacutainer blood tubes 9NC SST	Vacutainer tubes	Blood sampling	BD
Body Fat Calipers	Skin fold calipers	Skin fold measurement	Slim Guide
ALK Lancet	Disposable micro-lancet	Skin prick tests	Boge Alle
Allergen solutions	Allergen	Skin Prick tests	Modelo Harwell

2.1.9 Computer software

Table 2-9 Computer software used throughout the study

Name	Application	Company
Prism 6 for Mac OS X (v6.0g)	Data presentation & statistical analysis	GraphPad Software, California, USA
LightCycler 480 Software (v1.5)	Analysis of RT-qPCR data	Roche Diagnostics, Indiana, USA
FlowJo for Mac OS X (v10)	Analysis of flow cytometry data	FlowJo Data Analysis Software, USA
Discovery Workbench (v4.0)	Analysis of supernatant protein data	Meso Scale Diagnostics, USA

2.2 Clinical Methods

2.2.1 Study design

A prospective, case-control study design was adopted to address the study hypotheses. 15 morbidly obese bariatric surgery candidates and 15 healthy, normal weight controls were recruited between 13th June 2013 and 6th January 2015. Following anthropometric characterization nasal, pulmonary and blood samples were obtained for either *ex vivo* infection with influenza viruses or for measurement of adipokines/cytokines or RNA expression. An overview of the ObFlu study design is provided in Figure 2-1 and a list of the samples procured in Table 2-10.

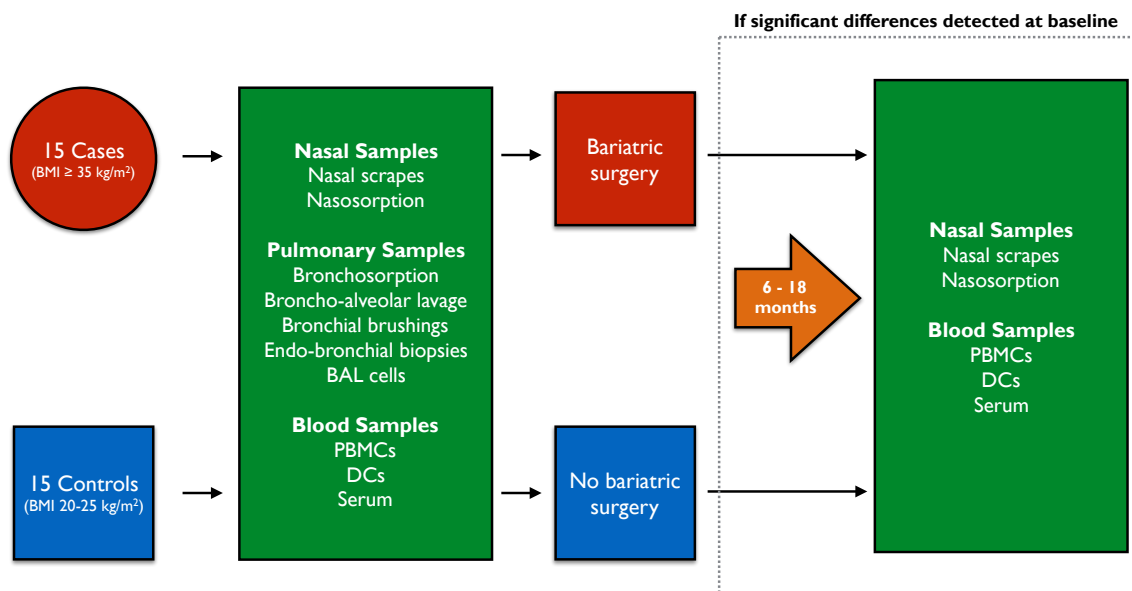


Figure 2-1 Overview of the ObFlu Study

Samples of nasal lining fluid (NLF) were obtained by nasosorption for quantification of adipokine, cytokine and chemokine protein levels by the Luminex® immunoassay platform. Additionally, nasal scrapes were performed using rhinoprobes to obtain nasal epithelium for mRNA gene expression studies.

Pulmonary samples were procured by bronchoscopy. Similarly to the nose, bronchial lining fluid (BLF) was obtained by bronchosorption for quantification of adipokine, cytokine and chemokine protein levels by the Luminex® platform. BAL was performed to facilitate measurement of adipokine, cytokine and chemokine protein levels in the BAL

fluid and to obtain BAL cells for *ex vivo* infection. Bronchial brushings were then undertaken for culture of hBECs and subsequent *ex vivo* infection. Finally, endobronchial biopsies were obtained for histology and immunohistochemistry.

Following venesection, PBMCs were isolated for *ex vivo* infection with influenza viruses and stimulation by TLR ligands. Remaining PBMCs underwent magnetic activated cell sorting (MACS) using a commercially available kit to isolate total peripheral blood DCs for *ex vivo* infection.

Table 2-10 Summary of the samples obtained in the ObFlu study

Compartment	Sampling method	Resultant samples
Nose	Nasosorption Nasal curettage	Nasal lining fluid (NLF) Nasal Epithelium
Lung	Bronchosorption Bronchial brushing Endo-bronchial biopsies Broncho-alveolar lavage (BAL)	Bronchial Lining Fluid (BLF) Bronchial epithelial cells (hBECs) Endobronchial tissue Broncho-alveolar lavage fluid (BALF) BAL cells
Blood	Venesection	Peripheral blood mononuclear cells (PBMCs) Dendritic cells (DCs) Serum

PBMCs, DCs, hBECs and BAL cells were infected with three strains of influenza; pandemic H1N1/09, seasonal H3N2 and influenza B at a multiplicity of infection (MOI) of three. Supernatants were harvested during the subsequent time courses for quantification of IFN α , β , λ , IL-6, IL-8 and TNF α by the MSD platform. mRNA expression in the cellular lysates was measured by RT-qPCR.

Following sample procurement, the 15 cases immediately underwent bariatric ('weight loss') surgery via gastric bypass, gastric band or sleeve gastrectomy. To quantify the effect of weight loss on the influenza-induced immune response we planned to invite the bariatric subjects back to St. Mary's hospital, six to eighteen months post-surgery, for re-sampling. However, due to financial and time restrictions, repeat bronchoscopies were

not planned for the return visits. Furthermore, these repeat visits were dependent upon the identification of significant intergroup differences in the baseline samples.

2.2.2 Recruitment

Controls were recruited via advertisements in local newspapers and posters around the Imperial College Campus. Potential controls that contacted the study research nurse via telephone or e-mail were then invited to the Imperial College Respiratory Research Unit (ICRRU) for an initial screening visit to evaluate eligibility. Cases were recruited at bariatric surgery clinics and bariatric surgery education seminars held at Imperial College NHS Trust. Again, potential cases that expressed interest in the study were invited to ICRRU for screening.

2.2.3 Inclusion Criteria

Table 2-11 ObFlu study inclusion criteria

Inclusion Criteria (Cases)	Inclusion Criteria (Controls)
Age 18-60 years	Age 18-60 years
BMI ≥ 35 kg/m ²	BMI 20-25 kg/m ²
Accepted for bariatric surgery	Absence of current or previous history of significant respiratory disease
	Absence of significant systemic disease

Cases and controls were matched as best as practicable for age, gender and ethnicity. As discussed in the introduction, the strength of the association between obesity and adverse outcome increases with the degree of obesity^{26,33,65}; as this was the first human study to assess the effect of obesity on the innate immune response to influenza a high BMI cut-off i.e. ≥ 35 kg/m² was specified for the cases to optimize the likelihood of detecting a difference between the two groups.

2.2.4 Exclusion Criteria

Table 2-12 ObFlu study exclusion criteria

Exclusion Criteria (Cases)	Exclusion Criteria (Controls)
BMI <35 kg/m ²	BMI <20 or >25 kg/m ²
History of significant respiratory disease (including asthma and COPD)	History of significant respiratory disease (including asthma and COPD)
Evidence of obstructive airways disease on spirometry	Evidence of obstructive airways disease on spirometry
Use of thiazolidinediones or statins	Physician diagnosed diabetes mellitus
History of allergic rhinitis or eczema within the previous year	Physician diagnosed obstructive sleep apnoea
Atopic on skin prick testing	History of allergic rhinitis or eczema within the previous year
Pregnant or nursing women	Atopic on skin prick testing
Use of inhaled medications within the previous year	Pregnant or nursing women
Current smoker, smoking within the last 6 months or smoking history >20 pack years	Use of inhaled medications within the previous year
Any clinically relevant abnormality on screening or significant systemic disease	Current smoker, smoking within the last 6 months or smoking history >20 pack years
	Any clinically relevant abnormality on screening or significant systemic disease

As the primary hypothesis proposed that IFN induction would be attenuated in the obese state, study candidates with potentially confounding IFN deficient states were excluded. Atopy, the genetic predilection to produce specific Immunoglobulin E (IgE) following exposure to allergens has been associated with deficient induction of IFN β and λ in hBECs following rhinovirus-16 (RV-16) challenge¹⁸², thus potentially atopic individuals were excluded. For the purposes of this study, atopy was defined as ≥ 1 positive reaction (wheal 3 mm greater than the negative control) on skin prick testing to a panel of nine common aeroallergens.

Pregnant and nursing women were excluded as PBMCs from pregnant women have been shown to have deficient IFN α , γ and λ responses to pH1N1/09 relative to healthy, non-pregnant women¹⁷². Pregnancy has also been associated with deficient induction of IFN α and λ in PBMCs following human rhinovirus (HRV43 and HRV1B) challenge, a deficiency that persists 6-8 months post-partum¹⁸³.

Individuals with obstructive airways diseases, including asthma and COPD were excluded on the basis of clinical history (including a significant smoking history), medications, physician diagnoses and obstructive spirometry.

Both thiazolidinediones¹⁸⁴ (selective ligands of peroxisome-proliferator-activated receptor- γ increasingly used in the treatment of type 2 diabetes) and statins¹⁸⁵ (HMG CoA reductase inhibitors) have been shown to have immunomodulatory and anti-inflammatory properties; individuals on these medications were excluded from the study.

2.2.5 Study Visits

Three study visits were planned for each study subject: an initial screening visit to confirm study eligibility, a 'sampling' visit (including the bronchoscopy) and then a final, third review six to eighteen months after the initial visit. The final review was dependent upon the identification of significant intergroup differences in the baseline samples.

Study visit one

Following an initial recruitment consultation by the study doctor to assess eligibility, potential participants were invited to ICRRU for a screening visit, to confirm subject eligibility and obtain consent for the study. After explaining the study protocol in full and answering any questions, informed consent was obtained and a full medical history and examination performed by the study doctor.

Weight and height were measured and BMI, a surrogate marker of body adiposity, calculated by dividing the subject's weight (in kg) by height (in metres) squared. Using a tape measure, neck circumference, mid-upper arm circumference (MUAC), hip circumference and waist circumference were also measured by the study doctor. Skin fold calipers were then used to measure triceps, biceps, suprailiac and subscapular skin fold thicknesses.

Spirometry, to assess baseline lung function, was performed sitting for all study subjects. The subject was asked to breathe in fully (to total lung capacity) and forcibly

exhale into the spirometer, blowing as hard as possible and continuing until residual volume. The best of three attempts was recorded. The predicted or reference values for lung function measurements were those recommended by the Report Working Party for the European Community for Coal and Steel¹⁸⁶. Following spirometry skin prick testing, to a range of 9 common aeroallergens (mixed grass pollen, cat dander, *aspergillus fumigatus*, *alternaria alternate*, house dust mite, dog hair, *cladosporidium herbarum*, birch pollen, three trees), was performed by the research nurse to determine atopic status. Positive histamine / negative diluent controls were included. ≥ 1 positive reaction (wheal 3 mm greater than the negative control) was considered diagnostic of atopy. All potentially eligible patients had a single chest radiograph performed prior to their first bronchoscopy as per local research bronchoscopy guidelines. Screening visit blood tests (to exclude systemic disease) were obtained for full blood count, renal function, liver function tests, glucose, clotting, CRP and total IgE. These were processed in the haematology and chemical pathology laboratories of St Mary's Hospital and reviewed by the study doctor.

Study Visit Two

The procurement of the clinical samples took place at the second study visit, which took place within one month of the initial screening visit. Study subjects arrived at the hospital at 0730 and, following confirmation of consent, proceeded to venesection as per section 2.3.3. Nasosorption and nasal scrape samples were then obtained as described in section 2.3.1. Following nasal sampling, the subjects were taken for bronchoscopy as described in 2.3.2.

Study Visit Three

The final study visit was dependent upon the identification of significant intergroup differences in the samples obtained at baseline. The purpose of this visit was to reassess the innate immune response of the bariatric subjects six-months following their bariatric surgery, when the candidates would be expected to have shed a significant amount of excess body weight. The expected excess weight loss following bariatric surgery is dependent upon the type of procedure that is performed; expected excess weight loss at two years is 70% following Roux-en-Y gastric bypass¹⁸⁷, 60% following sleeve gastrectomy¹⁸⁸ and 50-60% following gastric banding¹⁸⁹. Due to

financial and time constraints bronchoscopies would not be repeated on these return visits. Subjects would have BMI and anthropometric measures re-evaluated, as per the initial screening visit, and blood drawn for repeat PBMC stimulation and serum measurement of adipokines and cytokines. Nasosorption and nasal scrapes would also be repeated.

2.3 Clinical Sample Procurement and Processing

2.3.1 Nasal samples

Nasosorption

Prior to subject sampling, 250µl of pre-chilled assay buffer (phosphate buffered saline, bovine serum albumin (1%); Tween[®]-20 (0.05%); sodium azide (0.05%)) was aliquoted into each of two Spin-X polypropylene centrifuge tubes with filter cups (CLS8161, Sigma-Aldrich) and kept on ice. A strip of synthetic absorptive matrix (SAM) was subsequently placed in each of the participant's nostrils for two minutes to obtain samples of NLF prior to the nasal scrape. A nose clip was applied to ensure consistent contact of the SAM strips with the nasal epithelium. Each SAM strip was then removed, placed in a single labeled Spin-X centrifuge tube filter cup, sealed, and transported to the laboratory, on ice, for centrifugation at 16,000g (5 minutes at 4°C). The resultant filtrate was then frozen at -80°C in 2 x 250µl aliquots in 0.65ml labeled eppendorf tubes.

Nasal Scrapes

A single nasal scrape was performed following nasosorption to obtain epithelial cells for mRNA gene expression studies. Following a careful examination of the nose to identify the nasal mucosa on the inferior turbinate a plastic nasal curette (Rhino-probe[™], 80087-274, Arlington Scientific) was advanced into the nostril until the tip was placed on the surface of the inferior turbinate. Using a gentle scraping motion a tissue sample was collected and subsequently placed in an aliquot of RLT lysis buffer (Qiagen) with beta mercaptoethanol (β-ME)(10µl/ml). Following transfer to the laboratory the aliquot was vortexed for 15 seconds before being stored at -80°C.

2.3.2 Lung samples

Bronchoscopy

All bronchoscopies were performed according to British Thoracic Society (BTS) guidelines¹⁹⁰ at St. Mary's hospital, Paddington. The healthy control subject bronchoscopies were carried out in the hospital's specialist bronchoscopy unit, whilst the obese subject bronchoscopies were performed in an anaesthetic suite in the presence of a consultant bariatric anaesthetist. The obese subjects underwent bronchoscopy via an endotracheal tube under general anaesthesia as they were scheduled to undergo bariatric surgery immediately post-procedure. All the control bronchoscopies were performed via oral intubation, under light sedation with up to 10mg of midazolam.

Bronchosorption

Prior to subject sampling, 200µl of pre-chilled assay buffer (PBS, bovine serum albumin (1%); Tween®-20 (0.05%); sodium azide (0.05%)) was aliquoted into each of four Spin-X centrifuge tubes with filter cups (CLS8161, Sigma-Aldrich) and kept on ice. Bronchosorption (Mucosal Diagnostics, Hunt Developments, UK) is a technique to sample bronchial mucosal lining fluid, thus avoiding the significant analyte dilution associated with bronchoalveolar lavage¹⁹¹. The bronchosorption device was passed down the operating port of the bronchoscope. The distal end of the inner probe incorporates a folded strip of polyester synthetic absorptive matrix measuring 1.8mm x 30mm which was placed on the bronchial mucosa for a period of 30 seconds. Following sampling, the bronchosorption device was withdrawn back into its sheath and the complete device removed from the bronchoscope. The sampling end of the probe was then cut off and treated in an identical way to the nasosorption strips of absorptive matrix.

Bronchoalveolar Lavage (BAL)

Following bronchosorption, the bronchoscope was wedged in the right middle lobe of the lung and 210 – 240ml of sterile normal saline (at room temperature) instilled under direct visualization in 30-60ml aliquots, aiming for a recovery of at least 100mls. BAL was performed prior to bronchial brushing and biopsy to avoid contamination with

erythrocytes. BAL fluid (BALF) was collected into a single plastic bronchoalveolar lavage trap and transported on ice to the laboratory for processing. On receipt in the laboratory, the BALF was passed through a 100µl filter into two 50ml Falcons tubes that were subsequently centrifuged at 1300rpm, 4°C for 10 minutes. The supernatant was poured off, aliquoted into 2ml eppendorfs and stored at -80°C until later analysis. The pellets were resuspended in stimulation medium and the cells quantified using a manual haemocytometer. The resuspended cells were then washed for a second time by centrifugation (1300rpm, 4°C, 10 minutes) in stimulation medium and resuspended to give a final concentration of 2×10^6 cells/ml. These were plated in 96 well bottom plates at 200,000 cells per well and incubated at 37°C/5%CO₂ ready for infection with influenza viruses.

Bronchial brushing

Five bronchial brushings were obtained for hBEC culture using 5mm single-use cytology brushes (BC-202D-2010, Olympus). Under direct visualization, a brush was extended from its sheath into a right lower lobe sub-segment and rubbed against the bronchial wall for approximately 20 strokes. The brush was then retracted into its sheath, withdrawn from the bronchoscope and shaken into warm BEGM within a sterile 50ml tube to dislodge the epithelial cells. Brushes (within their sheathes) were then cut and transported in the BEGM to the laboratory for immediate processing, with the aim of transferring the cells to the incubator for culture within 30 minutes of collection. Subsequent hBEC culture is described in section 2.4.1.

Endobronchial biopsies

Three to six (depending upon size) endobronchial biopsies were taken from the left upper lobe and lingula sub-segments using fenestrated biopsy forceps (FB-211D, Olympus). These biopsies were fixed in 4% paraformaldehyde and transferred to the department of lung pathology at the Royal Brompton Hospital for histological and immunohistochemical analysis.

2.3.3 Blood samples

Peripheral blood mononuclear cell isolation

80mls of blood were drawn from each subject (after being nil by mouth for at least 8 hours) into a vacutainerTM containing sodium heparin and transported to the laboratory. The blood was then diluted 1 in 2 in pre-warmed (37°C) RPMI-1640 medium (R8758, Sigma-Aldrich). The blood/RPMI mix was then carefully layered onto 15ml of Ficoll-Paque PLUS (17-1440-03, GE Healthcare) in a 50ml Falcon tube and centrifuged at 2500rpm for 30 minutes at 21°C with no brake. A sterile pasteur pipette was then used to collect the resultant buffy coat layer into a 50ml Falcon containing 5ml pre-warmed RPMI-1640. The PBMCs were then washed twice in RPMI-1640 by centrifugation (1500rpm, 10 minutes, 21°C) and resuspended in 'stimulation' medium (RPMI-1640 with L-glutamine, 2% HEPES, 1% sodium bicarbonate, 1% penicillin/streptomycin and 10% foetal bovine serum). Isolated PBMCs were plated at 200,000 cells/well on a 96 well round bottom plate and incubated at 37°C/5% CO₂ ready for influenza infection.

Dendritic cell isolation

Plasmacytoid (pDCs) and conventional dendritic cells (cDCs) were isolated concurrently from the PBMCs using a commercially available human DC isolation kit (130-091-379, Miltenyi Biotec). The two-step manufacturer's protocol was followed. Initially, PBMCs were incubated for 15 minutes (2-8°C) with a Non-DC Depletion Cocktail, comprising CD14 and CD19 MicroBeads for magnetic labeling of monocytes and B cells, as well as a biotin-conjugated antibody against CD1c (BDCA-1) for biotin-labeling of CD1c (BDCA-1)+cDCs. Upon subsequent manual magnetic separation of the cells over LD MACS® Columns (130-042-901, Miltenyi Biotec), the magnetically labeled monocytes and B cells were depleted. The flow-through fraction (containing the pre-enriched DCs) was then incubated (15 minutes, 2-8°C). with a DC Enrichment Cocktail, comprising CD304 (BDCA-4/Neuropilin-1) and CD141 (BDCA-3) MicroBeads for magnetic labeling of pDCs and CD141 (BDCA-3)+cDCs as well as Anti-Biotin MicroBeads for magnetic labeling of biotin-labeled CD1c (BDCA-1)+cDCs. Upon subsequent manual magnetic separation (MS columns (130-042-201, Miltenyi Biotec)), the magnetically labeled DCs were retained on the column

and eluted after removal of the column from the magnetic field. The eluted DCs were then counted and plated in 96-well plates (in biological duplicate) at 20,000 cells per well ready for infection. The purity of this positively selected cell fraction, containing the enriched DCs, was subsequently assessed by flow cytometry.

Serum and plasma isolation

Blood was drawn into two 5ml SST vacutainers (367954, BD Sciences), at the same time as collection of the blood for PBMC isolation, for serum isolation. Immediately after collection, the tubes were gently inverted a minimum of 5 times to thoroughly mix the blood with the clotting agent. The blood was allowed to clot for at least 30 minutes, but no longer than 60 minutes, at 21°C. The samples were then centrifuged (15 minutes, 1500g, 21°C) and serum supernatants removed to a 15ml polypropylene tube and mixed gently to ensure sample homogeneity. 1ml aliquots of serum were stored in cryotubes and stored at -80 °C until analysis by Luminex[®].

2.4 General Laboratory Methods

2.4.1 Tissue culture

MDCK & A549 cell culture

Madin-Darby Canine Kidney (MDCK) and A549 cells were cultured in DMEM with 10% heat inactivated foetal bovine serum, 0.01% penicillin and streptomycin. Medium was warmed to 37°C prior to use on the cells. The cells were cultured in 25cm² flasks (Nunc) and split when confluent (every 2-3 days). In order to split the cells diluted (1:2) trypsin-EDTA was administered to the cells after medium removal. The cells were then incubated at 37°C/5% CO₂ for approximately 15 minutes until they became detached from the base of the flask. The cells were then resuspended in 20ml of 10% DMEM and 2ml transferred to a new T175 flask for further culture.

Primary hBEC culture

Primary hBECs were cultured in BEGM (Lonza). After receipt of the bronchial brushes in BEGM from the bronchoscopy the brushes were passed repeatedly down the end of a cut, sterile 500µl pipette tip submerged in BEGM to dislodge the cells from the brushes. The cells were then counted and placed in a T25 culture flask and

incubated at 37°C/5% CO₂. Every two days subsequently the cells were examined for contamination and the BEGM medium replenished. The hBECs were passaged once 95% confluency had been achieved. To passage the cells, the medium was removed and the cells washed twice with HEPES buffered saline (Lonza). Following washing 2ml of Trypsin/EDTA (Lonza) was added and the cells incubated for up to two minutes. After 85% cell detachment, they were removed to a 15ml Falcon containing BEGM and trypsin neutralizing solution added to the remaining 15% of cells in the flask. Fresh BEGM was added to these cells, which were then incubated again at 37 °C /5% CO₂. The cells within the 15ml were washed by centrifugation at 1150rpm for 6 minutes at room temperature and the pellet resuspended in BEGM. These cells were then placed in a T75 flask, incubated at 37 °C /5% CO₂ and passaged for a second time when 95% confluency was achieved. Once 95% confluency was achieved following the second passage the cells were seeded onto 24 well plates ready for infection. It typically took two to three weeks to culture the hBECs from bronchial brushing to infection.

A549 transfections

All A549 transfections were performed in designated 'clean' category II tissue culture hood. The plasmids used included SOCS1-pORF, SOCS3-pORF, pORF and the reporters Renilla luciferase (control), IFN β luciferase and IFN λ 1 luciferase. All stock plasmids were at 1 μ g/ μ L. Confluent A549 monolayers in 12 well plates (Nunc) were transfected with plasmid DNA (1 μ g per well). A master mix of plasmid DNA, diluted in Optimem-1 medium, was prepared at a volume of 100 μ L per well. Ratios of the various plasmids used are shown in Table 2-13. Lipofectamine 2000 was diluted in Optimem-1 medium to 10 μ g/mL and vortexed to mix following incubation at room temperature for 5 minutes. Diluted lipofectamine 2000 and DNA were combined at a 1:1 ratio, vortexed and incubated at room temperature for 20 minutes to allow lipid-DNA complexes to form. Medium was then removed from the cells and replaced by fresh 10% DMEM. 200 μ L of complexes were added to each well and tilted by hand to mix. Transfections were incubated for 5 hours at 37°C/5%CO₂. Cells were then washed twice with PBS and supplied with fresh 2% DMEM. Following overnight incubation (16 hours at 37°C/5%CO₂), medium was removed and the cells infected, in biological duplicate, with A/Eng/195 in 200 μ L SF-DMEM at an MOI of 3. After one

hour of incubation at 37°C/5%CO₂ the inoculum was removed, the cells washed with PBS and then 500µL of fresh 2% DMEM added to each well. The cells were incubated for 24 hours at 37°C/5%CO₂. The A549s were then lysed for the luciferase assay. Transfection efficiency was measured by Renilla firefly activity.

Table 2-13 Ratios of plasmid and reporter

Component	Amount/well
Plasmid	650ng
Report	250ng
Luciferase	100ng

Cell quantification

To quantify cell number following resuspension, cells were manually counted with a haemocytometer. Cells were diluted either 1:2 or 1:10 in 0.1% trypan blue and 10µl applied to a haemocytometer. Viable cells were counted and cells/ml calculated accordingly.

2.4.2 Virus culture

Growth of viral stocks

In order to produce large volumes of high-titre influenza stocks for the *ex vivo* cell stimulations, 2µl of thawed influenza virus was added to 5ml of SF-DMEM and mixed briefly by vortex, this was repeated twelve times to produce twelve 5ml vials containing virus. Medium was then removed from 12 fully confluent T175 flasks of MDCK cells and the cells washed twice with 10mls PBS. After decanting the PBS, one vial of virus-containing medium was added to each T175 flask of MDCK cells. The cells were then incubated for one hour at 37°C/5% CO₂ when tilting of the flasks every 15 minutes. The inoculum was then removed and 20mls of SF-DMEM (containing PenStrep and NEAA) and 1.5µl/ml of flu trypsin (30µl total) added.

The cells were inspected on a daily basis and the virus harvested when the cells demonstrated 50% CPE (approximately 48 hours). To harvest the virus, the medium was removed from each T175 flask and centrifuged at 2000rpm for 5 minutes in 50ml Falcon tubes (30ml medium per tube). The resultant supernatants were decanted

(without the cell debris) and combined into a T175 flask. The virus was then distributed into pre-labelled screw capped eppendorfs in 0.5ml aliquots and frozen at -80°C.

Viral titration by plaque assay

Plaque assays were performed in MDCK cells to determine the viral titre of each stock (in PFU/ml). Two random vials of each viral stock were selected (to assess consistency of titre throughout the stock), thawed and serially diluted (in triplicate) in SF-DMEM to a dilution of 1×10^{-6} . Medium was removed from four twelve well plates of confluent MDCK cells (two plates per plaque assay) and the cells washed with 1ml PBS. 100µl of virus was then added to each well (each dilution assessed in triplicate) and the cells incubated on a plate shaker at 37°C/5% CO₂ for one hour. The inoculum was then removed from the wells and 1ml of overlay and oxoid agar added to each well. The plates were then inverted and placed in the incubator for three days. The plaques were then counted pre and post-staining with crystal violet to determine the viral titre of each stock. An average of the two vials per stock was taken as the final titre.

2.4.3 Viral infections

Ex vivo primary hBEC infection

Once 90% hBEC confluency had been achieved (typically after two to three weeks), the BEGM was removed and the cells serum starved by placing in BEBM for 16 hours overnight. The cells were infected with A/Eng/195, B/Florida or A/Eng/691/2010 at a multiplicity of infection (MOI) of 3 to ensure that all cells were infected. Following removal of the PBS, 200 µl of virus-containing BEBM was added to each well (in duplicate) and the cells incubated on a plate shaker (low-speed) at 37°C/5% CO₂ for one hour. The inoculum was then discarded (in Virkon) and replaced with 500 µl of BEBM. The cells were incubated at 37°C/5% CO₂ until harvesting of both supernatants and cell lysates at 2, 4, 8, 24 and 48 hours. Uninfected BEBM medium controls were also harvested at these time-points. The supernatants and lysates were stored at -80°C until MSD analysis or RNA extraction respectively.

Ex vivo PBMC infection

PBMCs were plated in 96 well round bottom tissue culture plates at 200,000 cells per well. Immediately prior to infection the plates were centrifuged at 2000rpm for three minutes at 4°C to pellet the cells. The medium was then removed and the cells dispersed again by placing the plate on a plate shaker at 750rpm for ten seconds. 200µl of serum free RPMI containing either A/Eng/195, A/Eng/691/10 or B/Florida was then added to the wells in biological duplicate at a MOI of 3 and the cells returned to the incubator (37°C/5% CO₂) on a plate shaker for one hour. After an hour of incubation with the virus the plates were centrifuged at 2000rpm for three minutes at 4°C to pellet the cells. The viral inoculum was removed and 200µl of fresh stimulation medium (RPMI-1640 with L-glutamine, 2% HEPES, 1% sodium bicarbonate, 1% penicillin/streptomycin and 10% foetal bovine serum) added. The cells were returned to the incubator and the cells harvested at 4, 8, 24 and 48 hours. Harvesting comprised centrifuging the plates at 2000rpm for three minutes at 4°C to pellet the cells followed by removal of the supernatant and storage at -80°C for subsequent protein quantification and lysis of the cells in 350µl of RLT/ME lysis buffer for subsequent RNA extraction. Supernatants were pooled and cell lysates stored in biological duplicate. Stimulation medium alone was used as a negative control.

Ex vivo PBMC TLR ligand stimulation

In a similar fashion to viral PBMC infection the plates were centrifuged at 2000rpm for three minutes at 4°C to pellet the cells immediately prior to stimulation. The medium was then removed and the cells dispersed by placing the plate on a plate shaker at 750rpm for ten seconds. TLR ligands were then added to the cells in biological duplicate at the concentrations listed in Table 2-14. Unlike the PBMC viral infections, the TLR ligands were not removed after one hour of incubation. Stimulation medium alone was used as a negative control. The cells were harvested at the same time points and in the same manner as for the PBMC viral infections.

Table 2-14 TLR ligand concentrations

TLR ligand	TLR stimulated	Concentration
R848	TLR 7/8	1µg/M
Poly I:C	TLR 3	100µg/ml
CpG-A	TLR 9	1µM
Lipopolysaccharide (LPS)	TLR 4	10µg/ml
Lipoteichoic acid (LTA)	TLR 2	25µg/ml

Ex vivo BAL cell infection

BAL cells were plated in 96 well bottom plates at 200,000 cells per well and infected in the same manner as the PBMCs. Unlike the PBMCs however, cell yield limited the number of time points hence cells were harvested at 4, 24 and 48 hours only.

Ex vivo primary peripheral blood DC infection

Following MACS isolation using a commercially available kit peripheral blood dendritic cells were plated in 96 well round bottom tissue culture plates at 20,000 cells per well in 100µl of stimulation medium. Following an hour of incubation at 37°C/5% CO₂, influenza viruses (A/Eng/195, A/Eng/691/10, B/Florida) were added (in biological duplicate) to the wells at an MOI of 3 in 200µl of serum free RPMI. Serum free RPMI was used as a negative control. Due to the small cell number, the viral inoculum was not removed after an hour of incubation (cf. hBECs and PBMCs). At four and 24 hours post-infection, the plates were centrifuged at 1800rpm for 10 minutes, the supernatants removed and the cells lysed using 350µl of RLT lysis buffer with β-Mercaptoethanol. The supernatants and lysates were stored at -80°C until MSD analysis or RNA extraction respectively.

*2.4.4 Protein quantification**IFN alpha*

IFN α2a was quantified using a Meso Scale Discovery (MSD) tissue culture kit (K151ACB-4, MSD). The manufacturer's protocol was adhered to. A standard curve (ranging from 0 pg/ml to 10,000 pg/ml) was generated by serial dilution (1 in 4) of an IFN α2a calibrator stock. Following the addition of 25µl of calibrator or sample in technical duplicate the plates were sealed and incubated at room temperature on a plate

shaker (set at 750 rpm) for two hours. Following this initial incubation the plates were washed thrice with 0.05% Tween-20/PBS and 25µl of human IFN α 2a detection antibody (diluted to a working concentration of 1x in diluent 100, MSD) added to each well. The plates were resealed and incubated at room temperature for a further 1.5 hours on the plate shaker (750 rpm). After the final incubation, the plates were again washed thrice with 0.05% Tween-20/PBS and 150µl of 2x read buffer T (MSD) added to each well by reverse pipetting. The plates were then read immediately using an MSD SECTOR Imager 2400. Data were analysed using Discovery Workbench v4.0 (MSD). The lower limit of detection was 3.3 pg/ml.

IFN beta

IFN β was quantified using a MSD tissue culture kit (K151ADB-4, MSD). The manufacturer's protocol was adhered to. After washing the multi-array 96-well avidin plates with PBS three times, 25µl of anti-hIFN β biotinylated capture antibody (diluted to a working stock of 1x in diluent 100, MSD) was added to each well. The plates were then sealed and incubated at room temperature on a plate shaker (set at 750 rpm) for one hour. Serial 1 in 4 dilutions of the IFN- β calibrator stock were made to produce a standard curve ranging from 0 pg/ml to 100,000 pg/ml. Following the initial incubation the plates were again washed thrice with PBS. 25µl of human IFN β detection antibody (also diluted to a working concentration of 1x in diluent 100, MSD) was subsequently added to each well, followed by 50µl of sample or calibrator in technical duplicate. The plates were resealed and incubated at room temperature for a further two hours on the plate shaker (750 rpm). After this final incubation, the plates were again washed thrice with PBS and 150µl of 1x read buffer T (MSD) added to each well by reverse pipetting. The plates were then read immediately using an MSD SECTOR Imager 2400. Data were analysed using Discovery Workbench v4.0 (MSD). The lower limit of detection was 12.5 pg/ml.

IFN lambda-1 (IL-29)

Interferon λ 1 was quantified using MSD prototype multi-spot 96-well 4 spot plates that had been pre-coated with human IL-29 capture antibody (DY1598B, R&D systems). The manufacturer's protocol was adhered to. A standard curve (ranging from 0 pg/ml to 10,000 pg/ml) was generated by serial dilution (1 in 4) of an IFN- λ 1

calibrator stock. Following the addition of 25µl of calibrator or sample in technical duplicate the plates were sealed and incubated at room temperature on a plate shaker (set at 750 rpm) for two hours. Following this initial incubation the plates were washed thrice with 0.05% Tween-20/PBS and 25µl of human IFN λ 1 detection antibody (0.5µg/ml, R&D systems)/SULFO-TAG labelled streptavidin (1µg/ml, MSD) added to each well. The plates were resealed and incubated at room temperature for a further 1.5 hours on the plate shaker (750 rpm). After the final incubation, the plates were again washed thrice with 0.05% Tween-20/PBS and 150µl of 2x read buffer T (MSD) added to each well by reverse pipetting. The plates were then read immediately using an MSD SECTOR Imager 2400. Data were analysed using Discovery Workbench v4.0 (MSD). The lower limit of detection was 45.8 pg/ml.

Pro-inflammatory cytokines (IL-6, IL-8, TNF α)

IL-6, IL-8 and TNF α were quantified using a custom Meso Scale Discovery (MSD) tissue culture kit (N45IB-1, MSD). The manufacturer's protocol was adhered to. A standard curve (ranging from 0 pg/ml to 10,000 pg/ml) was generated by serial dilution (1 in 4) of IL-6, IL-8 and TNF α calibrator stocks. Following the addition of 25µl of calibrator or sample in technical duplicate the plates were sealed and incubated at room temperature on a plate shaker (set at 750 rpm) for two hours. Following this initial incubation the plates were washed thrice with 0.05% Tween-20/PBS and 25µl of human IL-6, IL-8 and TNF α detection antibody solution (diluted to a working concentration of 1x in diluent 100, MSD) added to each well. The plates were resealed and incubated at room temperature for a further 1.5 hours on the plate shaker (750 rpm). After the final incubation, the plates were again washed thrice with 0.05% Tween-20/PBS and 150µl of 2x read buffer T (MSD) added to each well by reverse pipetting. The plates were then read immediately using an MSD SECTOR Imager 2400. Data were analysed using Discovery Workbench v4.0 (MSD). The lower limits of detection were 6.6 pg/ml, 8.6 pg/ml and 20.4 pg/ml for IL-6, IL-8 and TNF α respectively.

Quantification of adipokines, cytokines and chemokines using the Luminex[®] platform

Adipokines, acute phase proteins, cytokines and chemokines were quantified in NLF, BLF, BAL and serum samples using three commercially available Luminex[®] kits; the manufacturer's protocol was adhered to. The mediators quantified are listed in Table 2-15.

Table 2-15 Mediators quantified in this study using the Luminex system

Adipokines	Acute phase proteins	Cytokines & Chemokines	
Leptin	C reactive protein (CRP)	FGF basic	IL-2, IL-4, IL-5
Resistin	Haptoglobin	Eotaxin	IL-6, IL-7, IL-8
Visfatin	α -2 macroglobulin	G-CSF	IL-9, IL-10, IL-12
Adipsin	Serum amyloid P	GM-CSF	IL-13, IL-15, IL-17
Adiponectin		IFN γ	IP-10, MCP-1, VEGF
		IL-1 β , IL-1RA	PDGF-BB, RANTES, TNF α
			MIP-1 α , MIP-1 β

Luminex assays are essentially immunoassays formatted on magnetic beads; the principle is similar to that of a sandwich ELISA (Figure 2-2). Capture antibodies directed against the desired biomarker are covalently coupled to magnetic beads that react with the sample containing the biomarker of interest. After a series of washes to remove unbound protein, a biotinylated detection antibody is added to create a sandwich complex. The final detection complex is formed with the addition of streptavidin-phycoerythrin (SA-PE) conjugate. Phycoerythrin serves as a fluorescent indicator or reporter¹⁹².

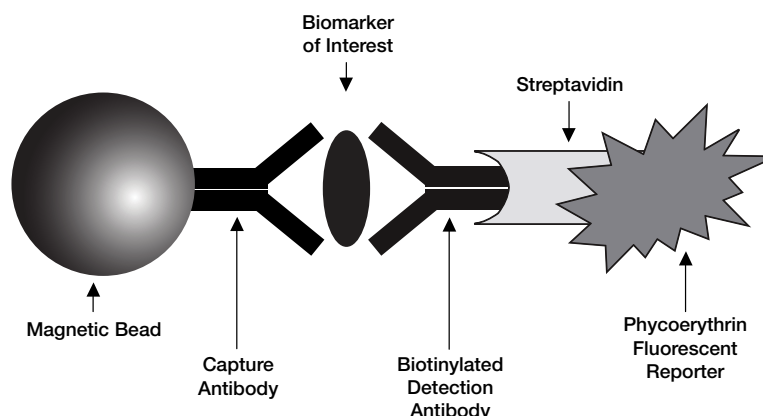


Figure 2-2 Bio-plex sandwich immunoassay
(adapted from the Bio-Rad Bio-plex ProTM instruction manual¹⁹²)

After thawing the samples and bringing the kit reagents to room temperature the wells of the 96 well Luminex plate were pre-wetted with assay buffer. Lyophilized calibrators were then reconstituted in 500µl of standard diluent and serially diluted 1:4 to generate a standard curve (to allow median fluorescence intensity (MFI) to be related to analyte concentration (pg/ml)). The coupled magnetic beads were then vortexed for 30 seconds and 50µl subsequently added to each well. After washing the plate twice with 100µl of wash buffer the samples, standards and controls were vortexed for five seconds and 50µl added to the plate. The plate was then covered with a sheet of sealing tape, protected from light with aluminum foil and incubated on a plate shaker at 850 ± 50 rpm at room temperature for 30 – 60 minutes (depending on the assay). After washing the plate three times with wash buffer, diluted detection antibodies were vortexed and 25µl added to each well. The plate was then incubated in the dark on a shaker at 850 ± 50 rpm for 30 min at room temperature. Following this step, streptavidin-PE was diluted 1 in 100, 50µl added to each well after washing the plate three times with wash buffer and, again, incubated in the dark for 10 minutes on a plate shaker. After a further three washes with 100µl wash buffer 125µl of assay buffer was added to resuspend the beads for plate reading. The Bio-Plex Manager software presented the data as median fluorescence intensity (MFI) and concentration (pg/ml); the concentration of analyte bound to each bead was proportional to the MFI of reporter signal.

2.4.5 RNA quantification

RNA extraction

Cells were lysed using 350µl of RLT buffer containing 10µl/ml of 2-β mercaptoethanol (Sigma Aldrich) and stored at -80°C until required for RNA extraction. RNA was subsequently extracted using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions.

The cell lysates were thawed and 350µl of 70% ethanol added. The ethanol/lysate was then mixed by pipette and transferred to an RNeasy mini-spin column and centrifuged at 10,000 rpm for 1 minute. The flow through was discarded and 350µl of RW1 buffer added to the columns, which were centrifuged again for 1 minute at 10,000 rpm. Following removal of the flow through, 80µl of DNase 1 (Qiagen) was added to the spin columns, which were then left at room temperature for 15 minutes. A further 350µl of RW1 buffer was added and then centrifuged for 1 minute at 10,000rpm. 500µl of RPE buffer was then added and the spin columns centrifuged at 10000rpm for 1 minute. A second RPE wash was then performed for two minutes, after which the spin columns were transferred to new collection tubes and underwent a dry spin at 10000rpm for 1 minute. After the dry spin the spin columns were transferred to 1.5ml collection tubes and RNA eluted using 30µl of nuclease free water (Promega) following a final spin at 10,000rpm for 1 minute.

cDNA synthesis

Following elution of the RNA, the collection tubes were stored on ice to prevent degradation. 20µl volumes of cDNA were then amplified using the components of the Omniscript reverse transcription kit:

Table 2-16 cDNA synthesis components

Component	Volume/reaction
Template RNA	13µl
Omniscript RT	1µl
dNTP mix	2µl
10x Buffer RT	2µl
Random primers	2µl

Following addition of all the kit components to the template RNA, the components were incubated on a heat block at 37°C for one hour. The resultant cDNA was then stored at -80°C until ready for processing.

Quantitative PCR

LightCycler 480 (Roche) machines were used to quantify mRNA for genes of interest. Primer and probe sequences for these genes were already established within the laboratory and designed to span exon-intron boundaries. 96 well optical PCR plates (Roche) were loaded with forward & reverse primers, specific probes and QuantiTect probe PCR mastermix (11.5µl) per well, plus 1µl of cDNA. Taqman standards also existed within the lab for each gene of interest and a set of standards was used on each PCR plate (aside from 18S). TaqMan data was analysed using 7500 Software v2.0.6 (Applied Biosystems) and genes of interest were normalised to 18S by dividing the copy number of the gene of interest by the copy number of 18S. Data are therefore presented as copy number/million copies of 18S.

2.4.6 Flow cytometry

A simple flow cytometric analysis of MACS-isolated dendritic cells was performed in several of the subjects to check the purity of the DCs that were subsequently infected *ex vivo* with influenza.

Flow cytometry staining

Dendritic cell suspensions containing 200,000 cells were transferred to polystyrene FACS tubes (BD Falcon) and centrifuged at 1300rpm for 8 minutes. Supernatants were discarded and pellets resuspended in 50µl 1:1000 human serum and incubated for 15min at 4°C. Antibody cocktails were made up at 2x concentration (Table 3) and 50 µl added to cells and incubated in the dark at 4°C for 30min. Cells were washed with 4ml PBS and pelleted at 1300rpm for 8 minutes. Supernatants were aspirated and pellets resuspended in 100µl 1:2000 Live/Dead® fixable Near infra-red (IR) dead cell stain (Life Technologies) prior to incubation at room temperature in the dark for 20 minutes. Cells were then washed and resuspended in 100ul 1% PFA (Sigma) to achieve fixation and incubated at room temperature for 15min. Finally,

cells were washed and resuspended in 150µl FACS buffer and kept at 4°C in the dark until analysis within 48 hours of collection.

DCs were acquired on a Becton Dickinson (BD) LSRFortessa equipped with 20mW 355nm, 50mW 405nm, 50mW 488nm, 50mW 561nm, 20mW 633nm lasers and a 1.0 neutral density filter in front of the forward scatter (FSC) photodiode. Photomultiplier tube (PMT) voltages were adjusted after standardized cytometer set-up and tracking (CST) checks, minimizing the spectral overlap to increase data precision.

Flow cytometric assessment of DC purity

After gating for live/dead (excluding dead cells) and excluding doublets, the isolated cells were gated for CD11c and CD123 positivity; indeed, two distinct populations of cells were present, representative of conventional DCs (cDCs) and plasmacytoid DCs (pDCs). CD11c is the surface antigen specific for cDCs, whilst CD123 is specific for pDCs.

2.4.7 Statistics

We were unable to perform a power calculation as this is the first study of its kind; however, previous publications have demonstrated significant intergroup differences in IFN production with numbers fewer than 15 per study arm. For example, Contoli *et al* demonstrated an asthma-associated type III IFN deficiency in BAL cells and hBECs using nine cases and nine controls¹⁶⁸. Additionally, Mallia *et al* demonstrated an IFN deficiency in COPD using 13 subjects and 13 controls¹⁷¹. Given the resources available for the study, 15 study subjects per arm was also a practical and achievable number.

As subject numbers were relatively small (15 per group), data were assumed to have a non-parametric distribution. To test for differences between two groups, the Mann-Whitney test was applied for unpaired data and the Wilcoxon matched-pairs signed rank test for paired data. For comparison of three or more groups the Kruskal-Wallis test with Dunn's correction for multiple comparisons was applied. However, the sensitivity of the Kruskal-Wallis test with Dunn's correction to detect statistically significant differences between two salient groups within four groups is reduced. Therefore, when trends approaching significance were identified the non-parametric Mann Whitney test was applied to the two salient groups following application of the Kruskal-Wallis test.

The experiments presented in the study involved many different stimuli and the evaluation of numerous different outcomes. It is therefore possible, that with a p value of 0.05, 1 in 20 of results may have been found to be significant simply due to chance. Isolated significant results were therefore interpreted with caution and discussed when they arose. However, as shall be seen in the results chapters herein, isolated significant results were rare and most of the significant results were supported by other significant results obtained using different assays; thus supporting the validity of the reported findings.

For the correlations presented in chapter five, in which analyte concentration was plotted against BMI, the parametric Pearson product-moment correlation coefficient was calculated ($n = 30$). It was appropriate to analyse the group as a whole as BMI is a continuous variable; however, as the inclusion criteria for the healthy control subjects

limited the BMI to between 20-25 kg/m² any correlations involving all the subjects could have potentially been skewed due to an ‘anchoring’ effect of the control subjects. Therefore, correlations were also performed on the obese subjects alone; as there were only 15 obese subjects, the non-parametric Spearman’s correlation coefficient was calculated.

For clarity, data are presented graphically as means and standard error of the mean (SEM); however, the author appreciates that non-parametric data is more correctly presented as median and interquartile range.

All statistical analyses were performed using GraphPad Prism v6. Significance levels are outlined in Table 2-17 (two-tailed p values):

Table 2-17 Graphical representation of statistical significance

P value (two-tailed)	Graphical representation
P > 0.05	ns
P ≤ 0.05	*
P ≤ 0.01	**
P ≤ 0.001	***
P ≤ 0.0001	****

Chapter 3 Clinical, demographic and anthropometric characteristics of the ObFlu study participants

3.1 Introduction

The prevalence of obesity has more than doubled since 1980, such that it is now the commonest nutritional disorder worldwide²⁷. 2.8 million deaths annually, and a significant proportion of diabetes mellitus, ischaemic heart disease and malignancy have been attributed to being overweight¹⁹³. For the first time during an influenza pandemic, it became evident in 2009 that obesity was independently associated with multiple markers of poor outcome upon infection³¹. Several investigators have subsequently investigated this relationship (Table 1-5), with the overwhelming majority of studies showing that obesity ($\text{BMI} \geq 30 \text{ kg/m}^2$) is associated with increased risk of hospitalisation, intensive care admission and death.

The hypotheses presented in this study propose that obesity is associated with an impairment of the innate immune response to the pH1N1/09 virus. Testing of this hypothesis required the recruitment of two populations: a healthy weight group (controls) and an obese group (cases). Over a period of 60 weeks, 15 control subjects were recruited via advertisement and 15 cases through attendance, by the author, at bariatric surgery clinics, group seminars and multidisciplinary team (MDT) meetings at St. Mary's Hospital, Paddington. To avoid potential confounding factors, subjects were matched as best as practicable for age, sex, ethnicity and co-morbidity.

This chapter reports the demographic, clinical and anthropometric characteristics of the two study populations followed by a discussion of the appropriateness of the recruited cohort to address the study hypotheses. As the study involved invasive fiberoptic bronchoscopy immediately prior to bariatric surgery, the safety profile of the study is also presented and discussed.

3.2 Aim

To recruit, and safely procure samples from, an age, gender and ethnicity-matched study population comprising fifteen healthy, normal weight (BMI 20-25 kg/m²) individuals and fifteen obese (BMI $\geq$ 35 kg/m²) bariatric surgery candidates to enable appropriate testing of the study hypotheses.

3.3 Results

3.3.1 Recruitment

Subject screening for the ObFlu study commenced on 13th June 2013 and ceased on 6th January 2015, following recruitment of the final obese subject. Cases were identified throughout the recruitment period by attendance of the study doctor at weekly bariatric surgery clinics, bariatric patient group seminars and MDT meetings held at Imperial College NHS Trust (Figure 3-1). Approximately 2400 bariatric surgery candidates were provisionally assessed for study eligibility; this estimate is based upon 60 weeks of active recruitment with ten patients assessed per clinic (by medical note review), twenty patients per group seminar (by questionnaire) and ten patients per MDT (by medical note review). From this initial screening, 32 bariatric surgery candidates were identified and invited for formal screening at the Imperial College Respiratory Research Unit (ICRRU). The main reasons for exclusion prior to formal screening at the ICRRU were: consumption of statins, asthma, allergic rhinitis, smoking, age and incompatible operation dates. Of the 32 potential cases screened in ICRRU, 16 were excluded; seven due to positive skin prick tests, four due to elevated IgE levels, two due to potentially confounding co-morbidities, two due to incompatible operation dates and one due to age above the inclusion criteria. Sixteen bariatric cases underwent bronchoscopy and bariatric surgery; however, one case was excluded due to evidence of infection (asymptomatic) on bronchoscopic visualisation of the airways thus, 15 bariatric cases were included in the final analysis.

Control subjects were identified through advertisement in local newspapers and on the Imperial College website. 21 healthy weight controls were invited to ICRRU for formal screening and 15 proceeded to bronchoscopy. Prior to bronchoscopy, two controls were excluded due to positive skin prick tests and a further two due to elevated IgE levels. One control withdrew due to family issues and a final control was excluded

due to inability to commit to the follow up period. No significant intergroup (obese vs. lean) difference in BAL return volume, nor BAL cell number was identified (data not shown).

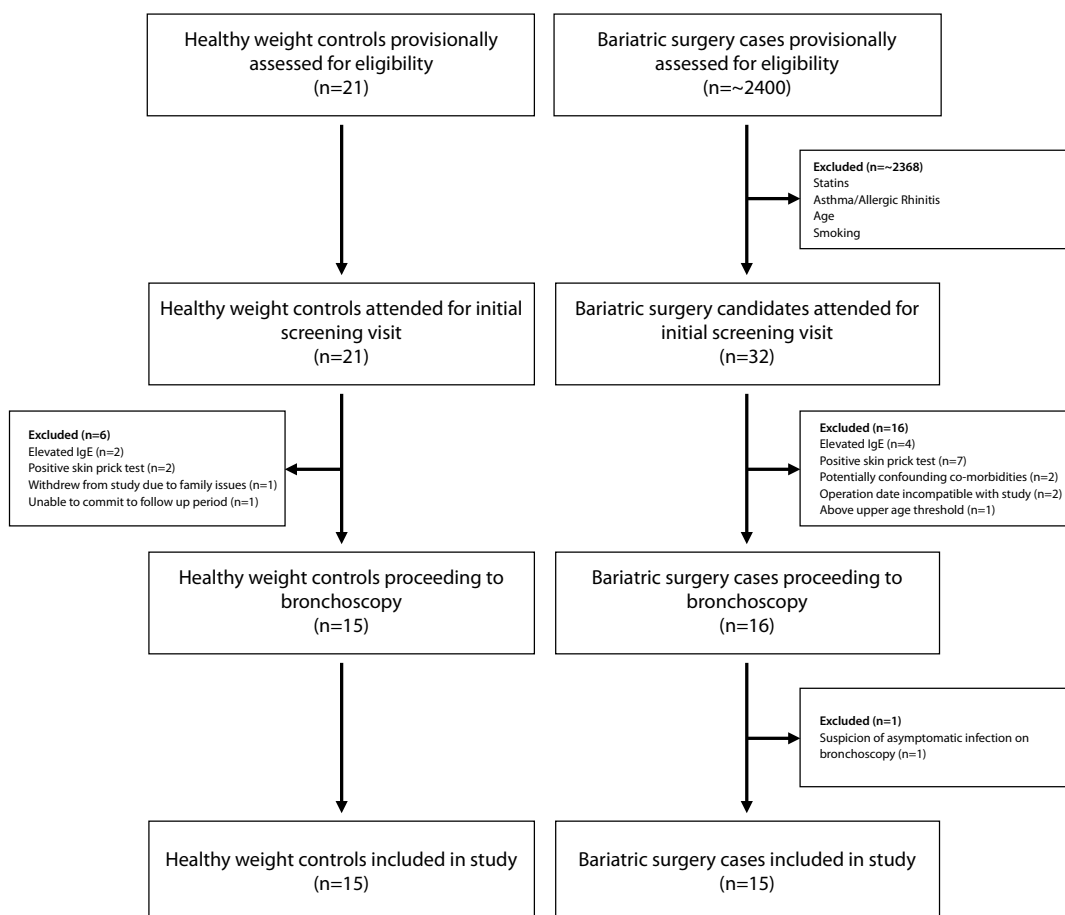


Figure 3-1 Assessment and enrolment of ObFlu study subjects

Healthy weight controls were recruited through advertisement whilst obese cases were recruited at bariatric surgery clinics, group seminars and multi-disciplinary team meetings. Following provisional assessment for eligibility, potential candidates attended the Imperial College Respiratory Research Unit (ICRRU) for formal screening. If eligible, the subjects proceeded to undergo bronchoscopy. 15 subjects per group were included in the final analysis.

3.3.2 Study subject demographics

The obese and lean groups (n=15 per group) were matched for age, sex and ethnicity (Figures 3-2 & 3-3).

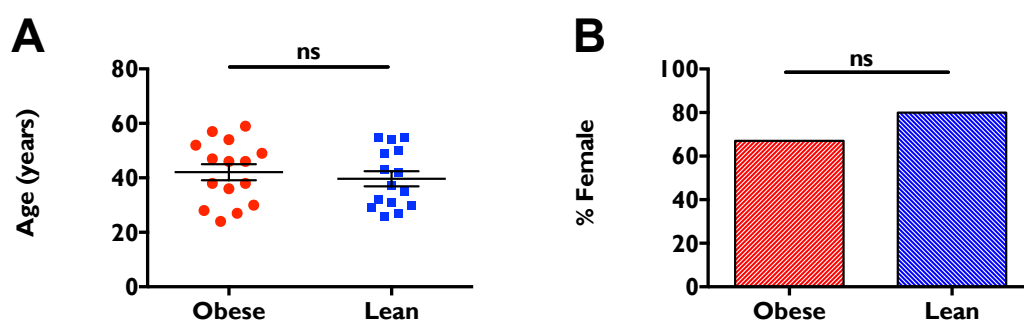


Figure 3-2 ObFlu subjects were matched for age and sex

Age [A] and sex [B] were recorded in 15 obese and 15 healthy weight ('lean') subjects. Data presented as mean plus SEM. Statistics: The Mann-Whitney test was applied to age and Fisher's exact test to sex. Statistical significance achieved when $p < 0.05$, ns = non-significant.

There were no significant intergroup differences in median age (obese 46 (range 24-59) vs. lean 37 (range 26-55) years, $p=0.61$), sex (67 vs 80% female, $p=0.68$) or ethnicity (73.3 vs 53.3% White British, $p=0.45$). However, a female, White British predominance existed in both groups (Figure 3-3).

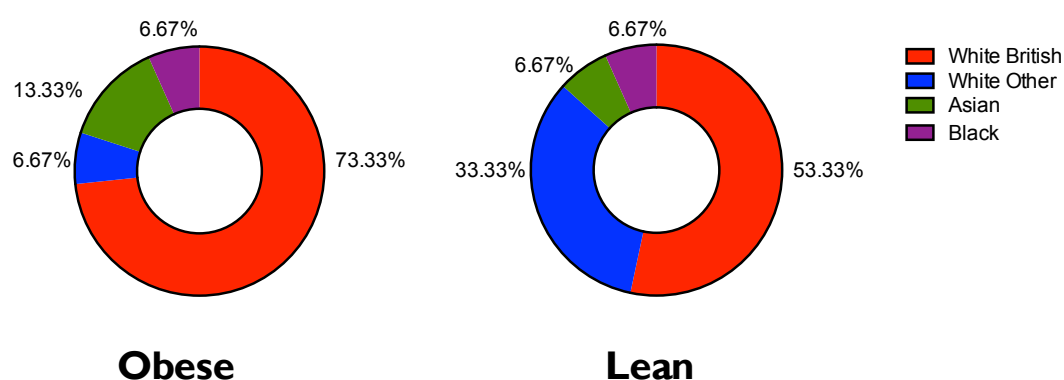


Figure 3-3 The obese and lean groups were matched for ethnicity

Ethnicity was recorded in the two recruited groups. No significant inter-group differences were observed. Statistics: Fisher's exact test (n=15 per group).

3.3.3 Anthropometric characteristics

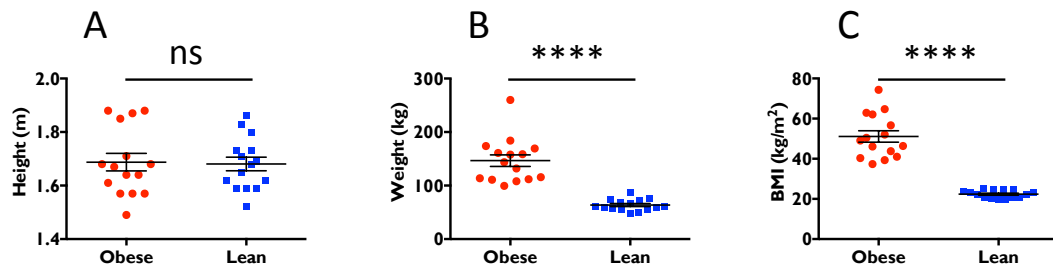


Figure 3-4 Weight and BMI were significantly greater in the obese subjects.

Study subject height [A], weight [B] and body mass index [C] at the initial screening visit. There was no significant difference in height between the obese (n=15) and lean (n=15) subjects. Weight and body mass index were significantly greater in the obese subjects (****p<0.0001, ns = non-significant, Mann-Whitney test). Data presented as mean and SEM.

To determine each subject's BMI, the only surrogate measure of obesity that has been epidemiologically associated with adverse outcome following influenza infection, height and body mass were assessed at the initial screening visit. BMI was calculated by dividing the body mass (in kilograms) by the square of the body height (in metres).

No significant difference in height was observed between the obese and lean participants (Figure 3-4). Unsurprisingly, the body mass and BMI of the obese participants were significantly elevated in the obese group (p<0.0001). The median weight of the obese participants was 143.6 kg v 61 kg in the lean group. The median BMI of the obese group was 49.11 kg/m² versus 22.15 kg/m² in the lean group.

To provide corroborative evidence that the elevated BMI observed in the obese population was indeed due to increase body adiposity rather than muscle mass, skin fold thickness and body circumference were assessed at four separate sites. Skinfold thickness, a surrogate marker of body adiposity, was significantly greater in the obese participants when evaluated at the biceps, triceps, suprailiac and subscapular skinfolds (p<0.0001)(Figure 3-5). Additionally, body circumferences were significantly increased at four sites (neck, mid-upper arm, hip and waist) in the obese population (p<0.0001)(Figure 3-6).

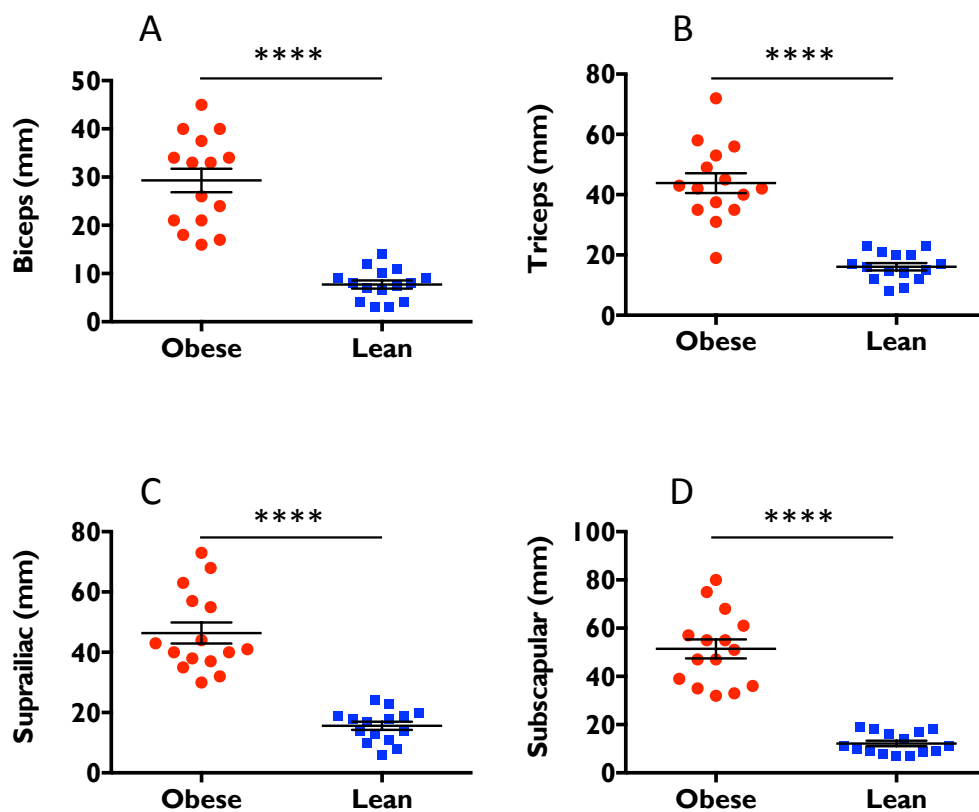


Figure 3-5 Skinfold thickness was significantly greater in the obese participants.

Skinfold thickness was evaluated by a single operator in 29/30 subjects using skinfold calipers at four sites: biceps [A], triceps [B], suprailiac [C] and subscapular [D]. Skinfold thickness was significantly greater in the obese participants at all four sites (****p<0.0001, Mann-Whitney test). Data presented as mean and SEM.

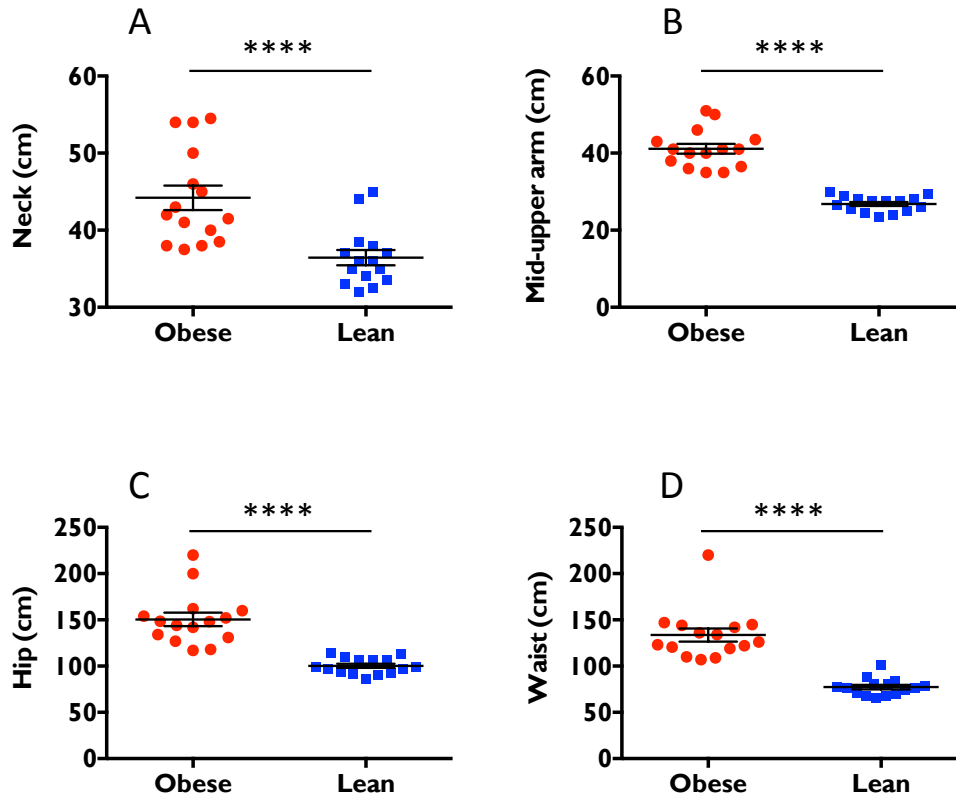


Figure 3-6 Body circumference was significantly greater in the obese subjects.

Body circumference was evaluated by a single operator in 29/30 subjects using a tape measure at four sites: neck [A], mid-upper arm [B], hip [C] and waist [D]. Body circumference at these sites was significantly greater in the obese participants (**** $p < 0.0001$, Mann-Whitney test). Data presented as mean and SEM.

3.3.4 Baseline spirometry and assessment of atopy

To assist with the exclusion of potentially confounding obstructive lung disorders such as asthma and COPD, baseline spirometry was measured at the initial screening visit. No intergroup differences in FEV1, FVC nor FEV1/FVC ratio were observed (Figure 3-7).

To exclude atopy, skin prick testing to a panel of nine common aeroallergens was performed in all subjects with a single wheal 3mm greater than the negative control resulting in study exclusion. All subjects had negative skin prick tests. Total serum IgE levels were also evaluated (Figure 3-8); all subjects had normal total serum IgE levels ($< 160 \text{ iU/ml}^{194}$) and no intergroup difference was observed.

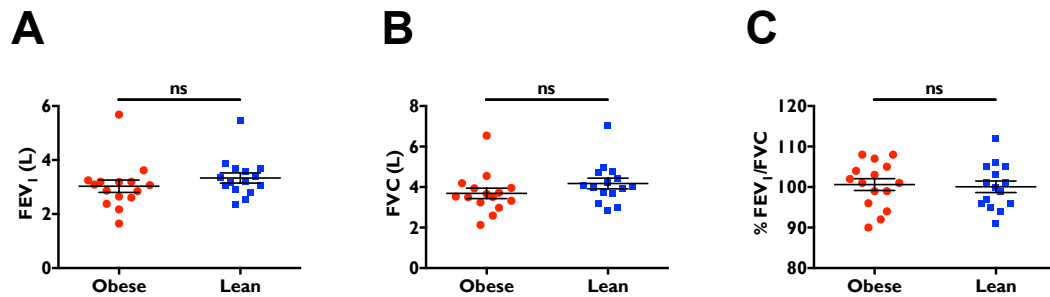


Figure 3-7 No intergroup differences in baseline spirometry.

Forced expiratory volume in one second (FEV1) [A], forced vital capacity (FVC) [B] and FEV1/FVC [C] were assessed at the screening visit by spirometry. No significant intergroup differences in baseline spirometry were observed. Mann-Whitney test (n=15 per group). Data presented as mean and SEM.(ns = non-significant).

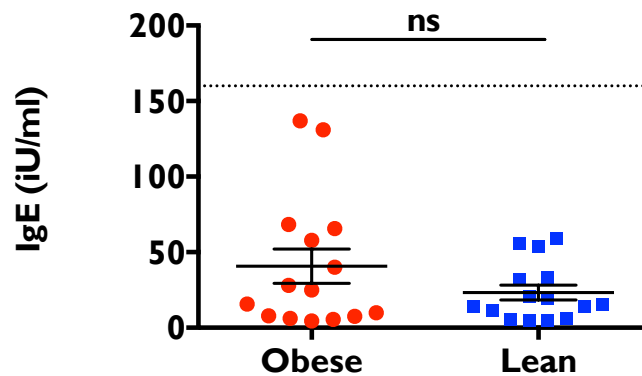


Figure 3-8 All subjects had total serum IgE levels within normal limits.

Total serum immunoglobulin E (IgE) levels were measured in the hospital laboratory at the screening visit. No intergroup difference was observed and all values were within the normal range (<160 iU/ml, as indicated by the dotted line). Mann Whitney test. Data presented as mean and SEM.

3.3.5 Co-morbidities of the recruited bariatric subjects

15 bariatric surgery candidates (median BMI 49.11 kg/m²) were recruited for the study (Table 3-1); median age was 46 years, 67% were female and 73.3% were White British. 4/15 (27%) did not report any co-morbidity, whilst hypertension was the commonest co-morbidity, affecting 6/15 (40%). Surprisingly, only 2/15 (13%) reported type 2 diabetes mellitus and 4/15 (27%) obstructive sleep apnoea (all treated and controlled with nocturnal continuous positive airway pressure (CPAP)). One subject (7%) was on medication for depression and another (7%) was taking medication for epilepsy, although had been seizure-free for over five years. Subject MA028 was taking daily penicillin as prophylaxis against developing cellulitis.

Table 3-1 Co-morbidities, medications and bariatric surgery type of the obese cases

Summary of the age, sex, ethnicity, BMI, co-morbidities, medication and operative history of the 15 recruited obese cases in addition to the type of bariatric surgery they subsequently underwent. T2DM = Type 2 Diabetes Mellitus, OSA = Obstructive Sleep Apnoea

Subject	Surgery	Age	Sex	Ethnic Origin	BMI (kg/m ²)	Current medical issues	CPAP	Prior-bariatric surgery	Medications
MA002	Balloon	38	Male	White British	74.4	Hypertension	No	No	Nil
MA006	Band	52	Female	White British	62.1	Osteoarthritis	No	No	Nil
MA022	Bypass	27	Female	White British	46.1	Chronic knee pain	No	No	Co-codamol pm
MA024	Bypass	57	Female	Asian (India)	50.4	Hypertension, T2DM, OSA	Yes	No	Losartan, Metformin, Gliclazide, Vitamin D
MA027	Bypass	46	Female	White British	64.7	Nil	No	No	Nil
MA028	Bypass	54	Female	White British	62.9	Hypertension, OSA	Yes	No	Penicillin, Ramipril, Spironolactone
MA029	Band	49	Female	White British	40.4	Epilepsy (seizure free for 5 years)	No	No	Sodium valproate, Omeprazole
MA038	Sleeve	36	Male	White Other	46.3	Nil	No	No	Nil
MA047	Sleeve	47	Female	Black Other	41.0	Hypertension	No	No	Amlodipine
MA048	Sleeve	46	Female	White British	49.1	Nil	No	No	Vitamin D
MA050	Bypass	24	Female	White British	43.8	Depression, OSA	Yes	No	Fluoxetine
MA058	Sleeve	30	Female	White British	56.7	Polycystic Ovarian Syndrome	No	No	Vitamin, Progesterone
MA062	Bypass	38	Male	White British	52.1	Hypertension	No	Yes (Sleeve gastrectomy)	Lansoprazole
MA063	Sleeve	59	Male	White British	39.3	Hypertension, T2DM, OSA	Yes	No	Metformin, Losartan, Doxazosin, Rampril
MA067	Sleeve	28	Male	British Asian	37.3	Nil	No	No	Nil

6/15 (40%) of the subjects underwent gastric bypass and 6/15 (40%) underwent sleeve gastrectomy immediately post-bronchoscopy. Two patients (13%) underwent gastric banding, whilst one patient (7%) underwent gastric balloon insertion.

3.3.6 *Safety profile of the bariatric bronchoscopies*

The author reviewed all the bariatric surgery patients twice following their surgery to check for complications related to their fiberoptic bronchoscopy. The initial review took place on the day of surgery, six to eight hours following the procedure. The second review took place approximately 24 hours later. Each review comprised evaluation of symptoms, clinical examination and review of the patient's observation chart (Table 3-2).

Table 3-2 Bronchoscopy in the bariatric surgery patients was uncomplicated

The subjects that underwent bariatric surgery were reviewed twice following their procedures to check for complications related to their bronchoscopy. None of the subjects experienced bronchoscopy related complications.

Complications	Review 1 (6-8 hours)	Review 2 (24 hours)
O2 Saturations (%)	>96%	>96%
Respiratory Rate	16	14
Cough	0/15	0/15
Dyspnoea	0/15	0/15
Haemoptysis	0/15	0/15
Pyrexia (>38°C)	0/15	0/15
Pneumothorax	0/15	0/15

3.4 Discussion

The two recruited study groups, obese and lean, were matched in terms of age, gender and ethnicity and were as free from confounding co-morbidities as practically achievable given the study timescale.

The median age of the recruited cohort was 40 years and no significant intergroup (obese vs. lean) difference was present. Whereas most cases of severe seasonal influenza occur in those aged over 65 years, the relative sparing of this age group during the 2009 pandemic was thought to be due to cross immunity from previous exposure to antigenically-similar H1N1 strains prior to 1957¹⁹⁵. The recruited study cohort accurately reflected the age group most at risk for severe outcomes following pH1N1/09 infection¹⁹⁶.

Sex refers to the genetic and biological characteristics that define males and females, whilst *gender* refers to the roles and behaviours that individual societies consider appropriate for men and women¹⁹⁷. No significant intergroup difference in sex was observed in the study population thus eliminating it as a confounding factor however, a female predominance was (unintentionally) present and, whilst this may potentially limit the generalisability of the study results, it may also more accurately reflect the epidemiology of the 2009 pandemic as there is a body of literature to suggest that females were more susceptible to severe outcomes upon infection^{196,197}. Additionally, at the time of study conception, there was a suggestion that a female predominance within the study population may in fact optimise the chances of detecting a difference in the immune response between the obese and lean groups; however, the paper upon which this suggestion was based has subsequently been retracted secondary to data reliability issues¹⁴¹. Of the three systematic reviews^{26,33,65} that evaluated the association between obesity and adverse outcomes, only one³³ looked at whether female sex affected the relative risk of adverse outcomes; however, inclusion as an additional co-variate made no change to the magnitude of risk of influenza-related pneumonia.

Due to the geographical location of the study centre the recruited population was predominantly 'White British'. Again, no significant intergroup difference in ethnicity

was present. Although not a confounder in this study, there is a limited body of literature evaluating the effect of ethnicity on influenza outcome; for example, Zhao *et al* have shown that, in the United Kingdom, those of non-White ethnicity experienced an increased mortality risk compared to White populations during the 2009 pandemic, with the highest risk in those of Pakistani ethnicity¹⁹⁸. Similarly to sex, there are no data from the three obesity-influenza systematic reviews suggesting that ethnicity alters the strength of the association between obesity and influenza-related adverse outcomes.

Given that all the obese subjects were scheduled for bariatric surgery, the median BMI of this group was unsurprisingly significantly greater than that of the control group (49.11 kg/m² versus 22.15 kg/m²). The relatively expansive range of BMI in the obese group was due to the nature of the inclusion criteria; the criteria for the obese subjects did not specify an upper limit whereas the control group was limited to a BMI between 20 and 25. As this was the first human study to address the effect of obesity on the innate immune response to viral infection the large differential between BMI inclusion criteria (minimum 10 kg/m²) was specifically chosen to optimise the probability of identifying a significant difference in immune response between the obese and lean groups. Whereas the majority of epidemiological studies showed that class I obesity (BMI $\geq$ 30 kg/m²) was associated with increased risk of adverse outcome the strength of the association increased with BMI hence the lower limit of inclusion in the obese group being set at BMI $\geq$ 35 kg/m². The systematic review by Phung *et al* identified a 300% higher risk of developing influenza-related pneumonia if morbidly obese (BMI $\geq$ 40 kg/m²)³³ whilst that of Fezeu *et al* identified an odds ratio of 2.01 (95% CI: 1.29–3.14, $P < 0.002$) for ICU admission or death if morbidly obese³². Thus, the median BMI of 49.11 kg/m² seen in the obese cases optimised the probability of detecting a significant intergroup difference in immune response.

BMI is the only epidemiological marker of body adiposity to be associated with adverse outcome following influenza infection and although BMI generally correlates highly with adiposity, it can sometimes misrepresent total body fat resulting in the misclassification of some individuals as obese. For example, athletes who are muscular have a high BMI, due to muscle weighing more than fat, and will therefore tend to be incorrectly identified as obese if BMI is the sole measure of adiposity. The

skinfold and body circumference data presented in this chapter provide corroborative evidence that the increased BMI observed in the cases was, in fact, due to increased adiposity rather than increased muscularity. The gold standard measurement of body composition utilises air displacement plethysmography technology (BodPod®) and initial measures were taken on two of the control subjects; however, the author abandoned this method upon realisation that many of the bariatric patients were unable to fit into the plethysmography box.

Relative IFN deficient states, including asthma, atopy and COPD, in which anti-viral IFN responses are attenuated, have been described in the literature^{168,169,171,199,200} and were important to exclude in the recruited cohort. Atopy, the genetic predisposition to develop allergic disease, was defined by skin prick testing to a panel of common aeroallergens; all the study participants had negative skin prick tests and did not exhibit symptoms of allergic rhinitis nor atopic dermatitis. Additionally, all subjects had total serum IgE levels within normal limits ($<160\text{iU/ml}^{194}$). None of the study participants had symptoms of asthma (wheeze, cough, dyspnoea) or were using inhaled medications. There was no evidence of obstructive lung function defects on spirometry in any of the subjects; indeed, there were no intergroup differences in baseline spirometry. A histamine or methacholine provocation test would have more confidently excluded asthma however, on the basis of normal spirometry, absence of asthma diagnosis, symptoms or inhaler usage, this was not deemed necessary. Exclusion of COPD was based upon absence of symptoms, normal chest radiography, normal spirometry and absence of a significant smoking history. Excluding these common respiratory co-morbidities facilitates interpretation of the data presented herein; if any significant intergroup differences in influenza-induced innate immune responses are detected we can be confident that they are not secondary to these respiratory conditions that are also associated with innate immune deficiencies.

Given the BMI inclusion criteria for the obese group, it was inevitable that some degree of co-morbidity would be present. Indeed, the commonest co-morbidity was hypertension; however, this is not a confounder as no studies have been published linking hypertension with adverse outcome following influenza infection aside from a single observation that in 165 fatal cases of pH1N1/09 in Thailand, 43% had concurrent hypertension²⁰¹.

Obstructive sleep apnoea was present in 4/15 (27%) of the bariatric subjects; again, no studies have linked OSA with adverse outcome following influenza infection. However, there is some evidence to suggest that innate immunity may be altered by the presence of OSA; NF- κ B activation may be increased in monocytes from individuals with OSA¹⁷⁹ and TLR 2 and 4 expression higher¹⁸⁰; however, in both these studies, the observed changes in innate immunity were reversed with the use of CPAP and all the subjects with OSA in the ObFlu study were controlled on CPAP. OSA may also result in increased activation of alveolar macrophages, as evidenced by decreased PPAR- γ activity and gene expression in BAL cells from OSA patients¹⁸¹.

Individuals with diabetes mellitus (DM) are at increased risk for influenza and influenza-related complications and this is reflected by current vaccination policy²⁵. The WHO-commissioned systematic review and meta-analysis by Mertz *et al*³⁴ found that DM increases risk of hospitalisation following seasonal influenza (OR 9.91, 95% CI 5.46 to 17.99) and, following pandemic influenza infection, increases risk of hospitalisation (4.26, 95% CI 3.14 to 5.77), intensive care admission (1.60, 95% CI 1.32 to 1.94) and death (2.21, 95% CI 1.37 to 3.57). 2/15 (13%) of the obese subjects included in the ObFlu study had diabetes mellitus.

Ideally, an obese cohort entirely free of co-morbidities would have been recruited to enable hypothesis testing free from confounders. Indeed, all efforts were made to achieve this; however, the practicalities of recruitment given the study timescale meant that some compromises had to be made, hence the inclusion of individuals with OSA and DM. It could be argued though that, with the inclusion of obese individuals with these co-morbidities, the recruited cohort more accurately reflects a ‘real-world’ snapshot of the obese population.

Fibreoptic bronchoscopy is a well-tolerated procedure that can be performed safely on an outpatient basis¹⁹⁰; however, pulmonary physicians are hesitant to perform bronchoscopy in the morbidly obese secondary to concerns regarding difficult airway access/management and potential complications, despite no formal publications addressing the effect of obesity on bronchoscopic outcomes. The bariatric subjects in the study underwent a 20-minute long, procedure-intensive (BAL, biopsies, bronchosorptions & brushes) bronchoscopy immediately prior to their bariatric

surgery and safety was therefore of paramount importance. No significant complications were observed following the bronchoscopies, confirming that bronchoscopy under GA in the conditions observed in this study was safe.

3.5 Summary of key points

- The obese and lean groups recruited were matched for age, gender and ethnicity and appropriately reflect the demographic most affected by severe outcomes during the 2009 pandemic.
- BMI and other surrogate markers of body adiposity were significantly increased in the obese group.
- The differential in BMI between the obese and lean groups was large to optimise the probability of detecting a difference in the primary study outcome measure, namely the innate immune response to pandemic influenza.
- All of the study subjects were non-atopic, non-asthmatic, non-smokers with normal chest radiographs.
- Invasive research bronchoscopy in this study population was without complication.

Chapter 4 The effect of obesity on the IFN and pro-inflammatory cytokine responses of primary respiratory epithelial and immune cells to influenza infection

4.1 Introduction

In March 2009, a novel strain of influenza A virus (pH1N1/09) emerged in Mexico, rapidly spreading around the world to cause the first influenza pandemic of the 21st century. As described in chapter one, obesity was convincingly identified as a novel, independent risk factor for multiple markers of disease severity, including hospitalisation, intensive care unit admission and death following pH1N1/09 infection^{26,33,65}. However, despite the widespread prevalence of both obesity and influenza infection, surprisingly little is known about the mechanism by which obesity increases host susceptibility to these severe outcomes.

Mouse models of DIO support the concept of an obesity-attenuated innate immune response to influenza infection (section 1.4). Furthermore, *ex vivo* TLR ligand stimulation of PBMCs taken from obese individuals exhibit diminished IFN responses relative to those observed in lean individuals¹⁶⁶. Combining these observations with existing knowledge regarding the physiology of leptin lead to the hypothesis that obesity-associated elevated leptin levels may attenuate the host innate immune response by reducing IFN signal transduction, thus potentially contributing to the observed adverse outcomes following pH1N1/09 infection.

Obesity is a leptin-resistant state in which leptin levels are chronically elevated due to increased body adiposity¹⁴⁸. The functional isoform of the leptin receptor, ObRb, which signals via a JAK-STAT signalling cascade¹³⁶, is expressed on key respiratory and immune cells including hBECs, AMs, DCs and PBMCs (Table 1-7). We hypothesized that leptin binds these ObRb receptors, thus inducing SOCS3 expression, which negatively feeds back on ObRb signal transduction¹⁵². Notably, leptin-induced SOCS3 also attenuates influenza-induced IFN signal transduction on the same cells⁹¹, thus resulting in diminished ISG (including IFN) expression⁹⁰. As ISGs are the main antiviral effectors, we proposed that obesity-associated attenuation of the host innate immune response might contribute to the adverse outcomes seen following influenza infection.

Relative IFN deficient states, including asthma, pregnancy and COPD, in which anti-viral IFN responses are attenuated, have previously been described in the literature (section 1.5.7). Given these data, and combined with corroborative evidence from the DIO mouse models, it is clear that IFN deficiency may be a plausible explanation for the severe outcomes seen in obesity following pH1N1/09 infection. It must be emphasized though that we are not proposing that an innate immune deficiency is the *sole* reason why obese individuals experience severe outcomes; there are several other non-immune factors that may also contribute (Table 1-10).

The results in this chapter are presented in three sections. Section one outlines the effect of obesity on the IFN and pro-inflammatory cytokine responses of the primary immune and respiratory epithelial cell types involved in influenza infection. BAL cells, hBECs, DCs and PBMCs were isolated, cultured and stimulated *ex vivo*. Three contemporaneous and clinically relevant influenza viruses were selected as test viruses in these experiments: A/Eng/195 (pandemic H1N1/09), A/Eng/691/10 (seasonal H3N2) and B/Florida (influenza B). The supernatants from the stimulated cells were subsequently harvested at various time points up to 48 hours post-infection (h.p.i.). Protein levels of IFN α , β and λ and the pro-inflammatory cytokines IL-6, IL-8 and TNF α were quantified using the MSD platform to allow comparison between the obese and lean states. As this was the first human study to assess the effect of obesity on the innate immune response to influenza a high BMI cut-off ($\geq 35 \text{ kg/m}^2$) was specified for the cases to optimize the likelihood of detecting a difference between the two groups.

Section two evaluates the specific effects of leptin on the IFN and pro-inflammatory cytokine responses of the respiratory epithelium; hBECs from the lean controls were pre-exposed to high-dose leptin²⁰² for 16 hours prior to infection and throughout the subsequent time course in an attempt to mimic the obese state. The IFN and pro-inflammatory cytokine responses post-influenza infection were then compared with the responses of hBECs from lean individuals infected in medium alone.

Finally, in an attempt to replicate the work of Teran-Cabanillas *et al*, who had described attenuated IFN responses in PBMCs from obese individuals stimulated with TLR ligands¹⁶⁶, PBMCs from both groups were stimulated with the TLR agonists

CPG-A, R848, poly (I:C), LPS and LTA and the IFN and pro-inflammatory cytokine responses compared.

It should be noted that the primary purpose of this chapter was to screen for the presence of deficiencies in the IFN or pro-inflammatory cytokine responses of the respiratory epithelial and innate immune cell types involved during influenza infection. Where intergroup i.e. obese vs. lean differences were identified, viral stimulation (quantified by viral M gene RT-qPCR) and ISG expression were subsequently assessed. Data concerning leptin and SOCS expression are presented and discussed separately in chapter five.

4.2 Hypotheses

- Morbid obesity ($\text{BMI} \geq 35 \text{ kg/m}^2$) impairs the innate IFN (α , β and λ), ISG (MxA) and pro-inflammatory cytokine (IL-6, IL-8 and TNF α) responses of primary hBECs, BAL cells, PBMCs and peripheral blood DCs to pandemic influenza A and seasonal influenza A and B.
- Pre-exposure of hBECs from lean controls to exogenous leptin will attenuate the innate IFN (α , β and λ) and pro-inflammatory cytokine (IL-6, IL-8 and TNF α) responses to pandemic influenza A and seasonal influenza A and B.
- The innate IFN (α , β and λ) and pro-inflammatory cytokine (IL-6, IL-8 and TNF α) responses of PBMCs to the TLRs: CPG-A, R848, poly (I:C), LPS and LTA will be attenuated in the obese state.

4.3 Aims

To determine whether:

- hBECs, PBMCs, DCs and BAL cells from morbidly obese individuals exhibit diminished antiviral IFN (α , β and λ), ISG (MxA), and pro-inflammatory cytokine (IL-6, IL-8 and TNF α) responses following pH1N1/09, sH3N2 and influenza B infection relative to those of normal weight controls.
- pre-exposure of hBECs from lean controls to exogenous leptin attenuates the innate IFN (α , β and λ) and pro-inflammatory cytokine (IL-6, IL-8 and TNF α) responses to pandemic influenza A and seasonal influenza A and B.

- the innate IFN (α , β and λ) and pro-inflammatory cytokine (IL-6, IL-8 and TNF α) responses of PBMCs to the TLRs: CPG-A, R848, poly (I:C), LPS and LTA are attenuated in the obese state.

4.4 Results

4.4.1 *Obesity did not alter type I & III IFN or pro-inflammatory cytokine expression in hBECs following pandemic or seasonal influenza infection*

To assess the effect of obesity on the immune response of the primary target cells of influenza viruses, namely the respiratory epithelial cells²⁰³, hBECs were procured from 15 bariatric surgery candidates and 15 lean subjects. However, despite initially successful hBEC culture from all participants, several cultures were lost sporadically to contamination over the subsequent 2-3 week culture period. Thus, hBECs from 10 obese and 13 lean subjects were eventually infected with pH1N1/09, sH3N2 and influenza B in biological duplicate at an MOI of 3.

Supernatants and cellular lysates were harvested at 2, 4, 8, 24 and 48 hours. IFN (Figure 4-1) and pro-inflammatory cytokine (IL-6, IL-8, TNF α)(Figure 4-2) levels were quantified in the supernatant obtained at 24 and 48 hours using the MSD platform.

No intergroup differences in hBEC IFN expression were observed

No significant ($p < 0.05$) intergroup (i.e. obese vs. lean) differences in type I (α , β) or type III (λ) IFN protein expression were observed in the supernatants of hBECs infected with pH1N1/09, sH3N2 or influenza B 24 or 48 hours following infection (Figure 4-1).

No significant expression of IFN α was observed following infection of the hBECs with any of the three strains of influenza [A-C, J-L]. Furthermore, at 24 hours, only the sH3N2 virus induced significant IFN expression (IFN β and IFN λ), with equivalent levels of expression in both the obese and lean groups [E, H]. 48 h.p.i the sH3N2 virus again resulted in the most significant IFN β and λ expression [N, Q]; however, the pH1N1/09 virus also induced significant, albeit lower levels, of IFN β and IFN λ in the lean controls only [M, P].

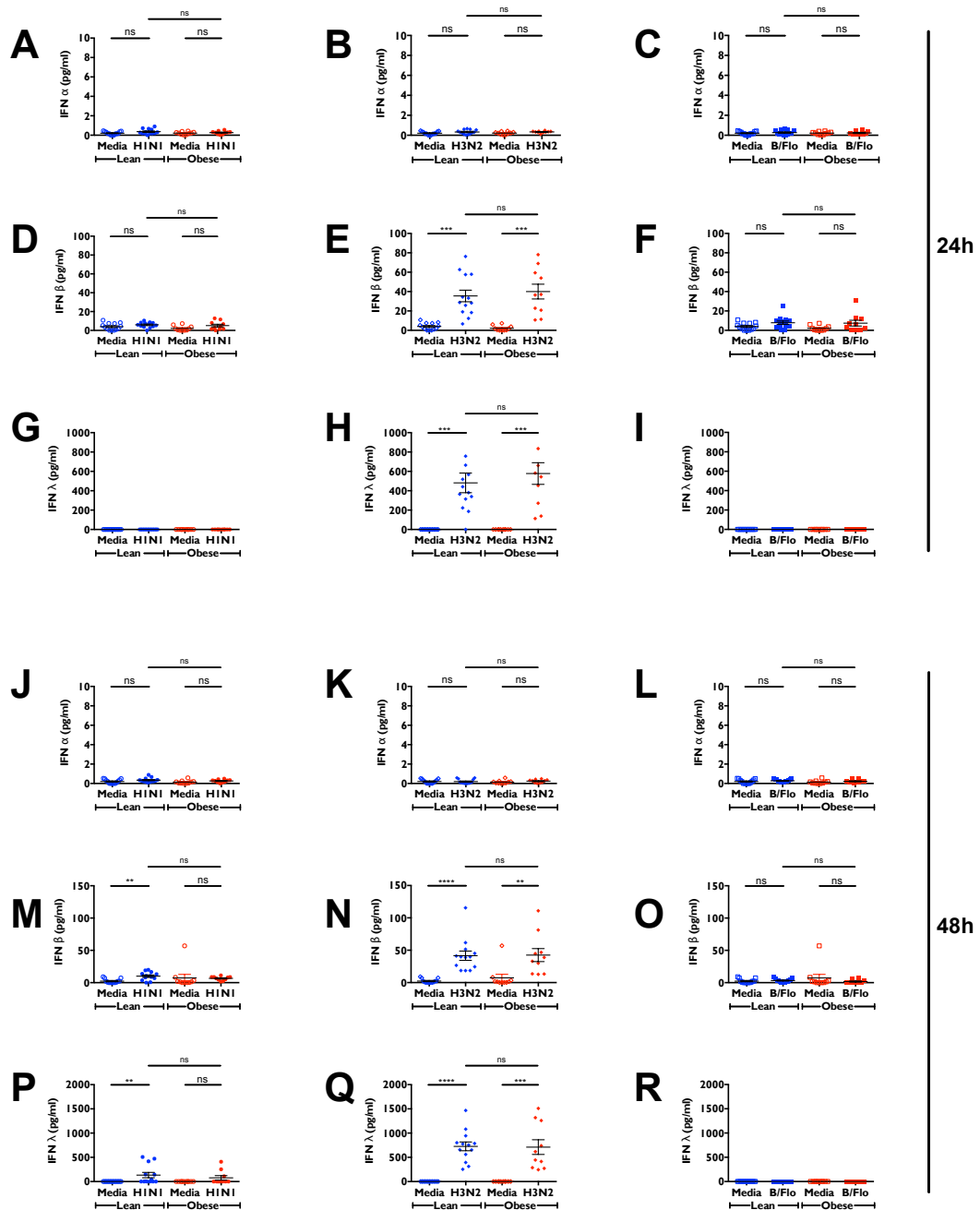


Figure 4-1 Obesity did not alter the IFN response of hBECs to pandemic or seasonal influenza infection

IFN α , β and λ protein levels were quantified by MSD in hBEC supernatants 24 [A – I] and 48 hours [J – R] post-infection with pH1N1/09, sH3N2 and influenza B (MOI 3). No significant intergroup (obese vs. lean) differences were observed. None of the three influenza strains induced IFN α expression [A-C, J-L]. sH3N2 resulted in significant induction of IFN β and λ at both 24 [E,H] and 48 hours [N,Q]; however, no significant intergroup differences were noted. 48 post-infection pH1N1/09 resulted in significant induction of IFN β [M] and IFN λ [P] in the lean subjects only. Each data point represents the average of two independent biological duplicates, each measured in technical replicate by MSD. (Statistics: Kruskal Wallis with Dunn's Correction, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.) ($n = 10$ obese vs. 13 lean)

No intergroup differences in hBEC pro-inflammatory cytokine expression were observed

No significant intergroup differences in IL-6, IL-8 or TNF α protein expression were observed at either 24 or 48 hours following influenza infection (Figure 4-2).

24 h.p.i. pH1N1/09 did not significantly induce IL-6, IL-8 or TNF α [A, D, G], whereas sH3N2 resulted in significant expression of all three cytokines in both the obese and lean subjects [B, E, H]. B/Florida infection resulted in significant expression of IL-8 and TNF α in both groups 24 h.p.i. [F, I]; however, significant IL-6 expression was observed in the lean controls only [C].

48 h.p.i. pH1N1/09 induced significant TNF α expression in both the obese and lean groups [P], IL-8 in the lean group only [M] but no statistically significant expression of IL-6 in either group [J]. sH3N2 infection resulted in significant expression of IL-6, IL-8 and TNF α in the lean group only [K, N, Q]. B/Florida infection induced significant IL-8 and TNF α expression in both groups at 48 hours [O, R]. Significant IL-6 expression following B/Florida infection was observed in the lean group only [L].

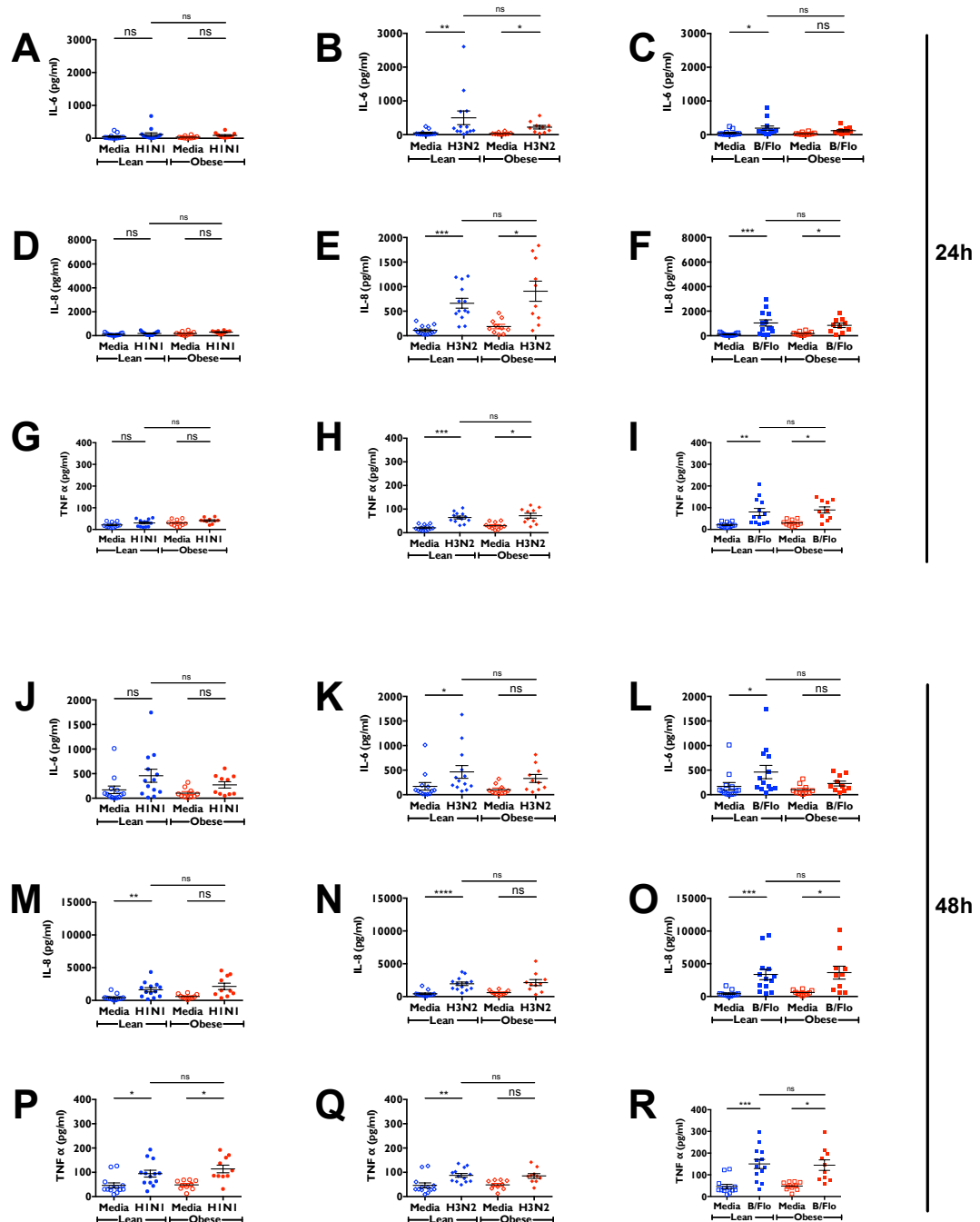


Figure 4-2 Obesity did not alter the pro-inflammatory cytokine response of hBECs to pandemic or seasonal influenza infection

IL-6, IL-8 and TNF α protein levels were quantified by MSD in hBEC supernatants 24 [A – I] and 48 hours [J – R] post-infection with pH1N1/09, sH3N2 and influenza B (MOI 3). No significant ($p < 0.05$) intergroup (obese vs. lean) differences were observed. 24 h.p.i. sH3N2 [B,E,H] and influenza B [C,F,I] resulted in significant pro-inflammatory cytokine induction whereas no significant induction was observed upon pH1N1/09 infection [A,D,G]. 48 h.p.i. all three influenza strains resulted in significant pro-inflammatory cytokine induction [J – R]. Each data point represents the average of two independent biological duplicates, each measured in technical replicate by MSD. Red = obese, blue = lean. (Statistics: Kruskal Wallis with Dunn's Correction, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.) ($n = 10$ obese vs. 13 lean)

4.4.2 *Obesity did not alter type I & III IFN or pro-inflammatory cytokine expression in PBMCs following pandemic or seasonal influenza infection*

As Teran-Cabanillas *et al* had published evidence suggesting that PBMCs from obese individuals have deficient IFN responses to TLR agonists¹⁶⁶, the effect of obesity on the influenza-associated immune response within the peripheral blood was assessed. PBMCs, isolated by density gradient centrifugation (n = 15 per group), were stimulated *ex vivo* with pH1N1/09, sH3N2 and influenza B (MOI 3). Supernatants and cellular lysates were harvested at 4, 8, 24 and 48 hours and IFN α , β , λ , IL-6, IL-8 and TNF α levels quantified in the 24 and 48h supernatants by MSD.

Obesity did not alter PBMC IFN responses to influenza infection

No intergroup differences in IFN expression were identified (Figure 4-3). Both pH1N1/09 and sH3N2 resulted in significant expression of IFN α and β at 24 [A, B] and 48 hours [J, K]. None of the influenza strains induced IFN λ at either time point [G – I, P – R]. B/Florida infection induced IFN α at 24 and 48 h.p.i only [C, L].

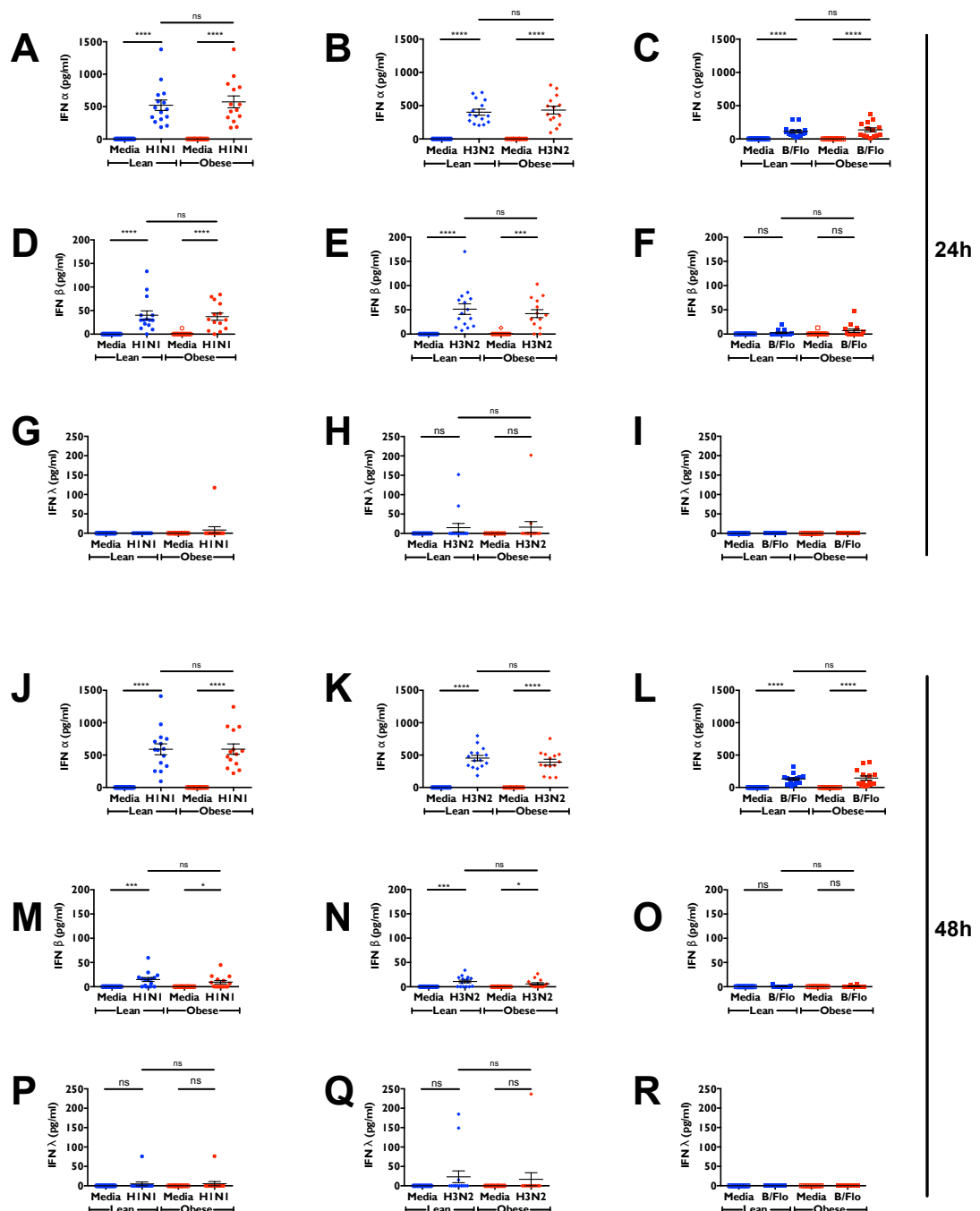


Figure 4-3 Obesity did not alter the IFN response of PBMCs to pandemic or seasonal influenza infection

IFN α , β and λ protein levels were quantified by MSD in PBMC supernatants 24 [A – I] and 48 hours [J – R] post-infection with pH1N1/09, sH3N2 and influenza B (MOI 3). No significant intergroup (obese vs. lean) differences were observed. IFN α was induced by all three strains of influenza [A-C, J-L] whereas no IFN λ expression was observed [G-I, P-R]. IFN β was induced at both time points by pH1N1/09 and sH3N2, but not by B/Florida [D-F, M-O]. Each data point represents the average of two pooled biological duplicates, each assessed in technical replicate by MSD. (Statistics: Kruskal Wallis with Dunn's Correction, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Where error bars are absent, no statistical test was necessary) ($n=15$ per group)

Obesity did not alter PBMC pro-inflammatory cytokine responses to influenza infection

No intergroup differences in pro-inflammatory cytokine expression by PBMCs were observed (Figure 4-4). pH1N1/09 induced significant expression of IL-6 and TNF α in both the obese and lean groups, with no significant intergroup differences, at 24 and 48 h.p.i. [A, G, J, P]. pH1N1/09 did not induce IL-8 expression at either time point [D, M]. sH3N2 resulted in significant expression of IL-6 and TNF α 24 h.p.i., again with no observable intergroup difference [B, H]. Interestingly, IL-8 expression was diminished relative to medium in both groups; however, a statistically significant diminution was only observed in the obese group [E]. 48 h.p.i sH3N2 significantly induced IL-6 in both groups [K] and expression of TNF α , although statistical significance was only seen in the lean group [Q]. Again, IL-8 expression was significantly attenuated in the obese group relative to medium [N]. B/Florida significantly induced TNF α in both groups 24 h.p.i. [I], but not IL-6 [C]. Expression of IL-8 post-B/Florida infection was diminished relative to medium in both groups, but only achieved statistical significance in the obese group [F]. 48 h.p.i. the IL-8 response remained significantly diminished in the obese group [O]; however, expression of IL-6 and TNF α was significant in the lean group only [L, R].

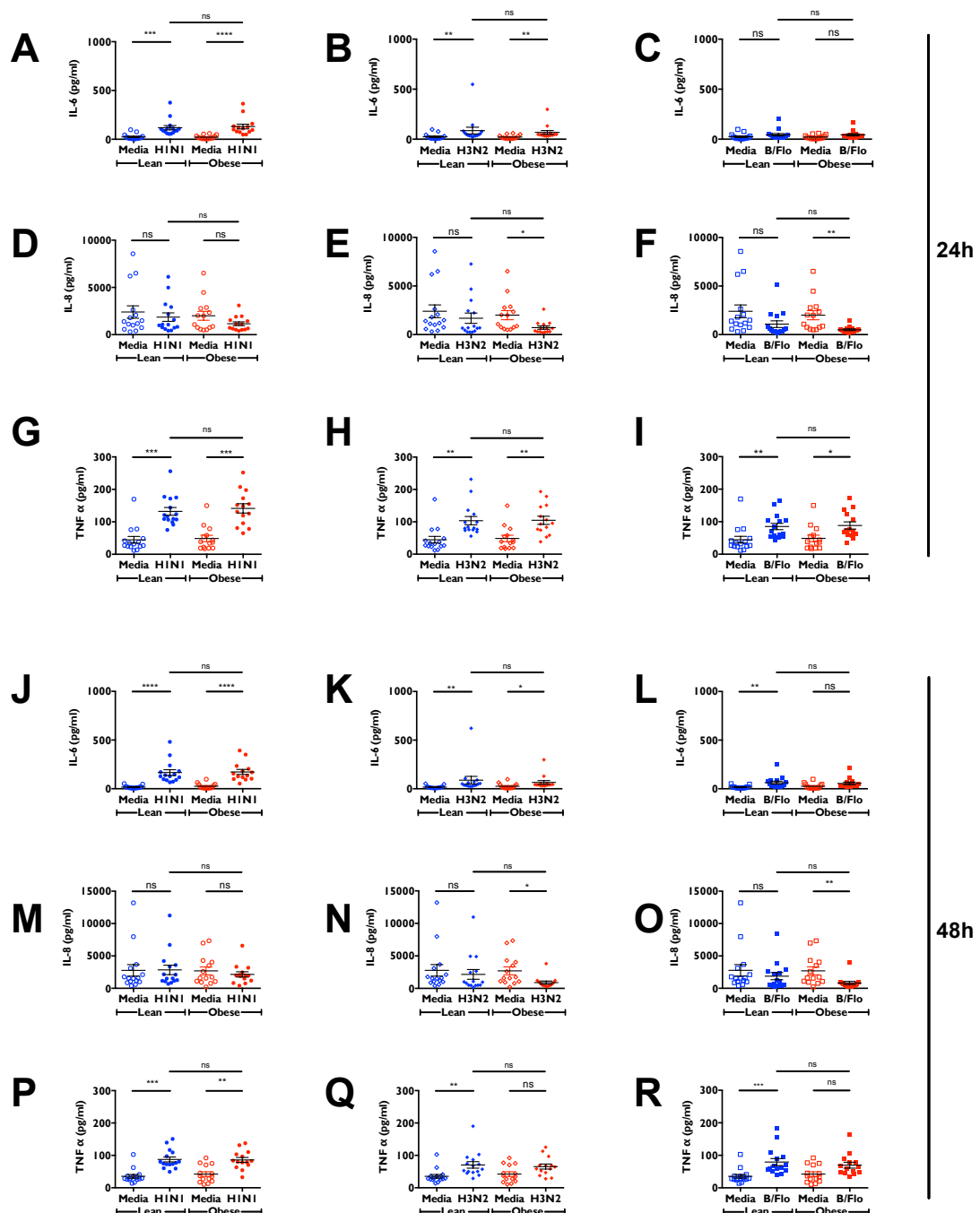


Figure 4-4 Obesity did not alter the pro-inflammatory cytokine response of PBMCs to pandemic or seasonal influenza infection

IL-6, IL-8 and TNF α protein levels were quantified by MSD in PBMC supernatants 24 [A – I] and 48 hours [J – R] post-infection with pH1N1/09, sH3N2 and influenza B (MOI 3). No significant intergroup (obese vs. lean) differences were observed. pH1N1/09 significantly induced IL-6 and TNF α in both the obese and lean groups at 24 and 48 h.p.i. [A, G, J, P]. sH3N2 induced IL-6 in both groups 24 & 48 h.p.i. [B, K], but TNF α in the lean group only at 48 hours [B, Q]. IL-8 expression post-infection was diminished relative to media control at both time points for all three strains [D-F, M-O]. B/Florida induced IL-6 only 24 h.p.i. [C] and IL-6 & TNF α in the lean group only 48 h.p.i. [L, R]. Each data point represents the average of two pooled biological duplicates, each assessed in technical replicate by MSD. (Statistics: Kruskal Wallis with Dunn's Correction, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.) ($n = 15$ per group).

4.4.3 *Obesity did not alter type I & III IFN or pro-inflammatory cytokine expression in DCs following stimulation with pandemic and seasonal influenza*

To further evaluate the effect of obesity on the immune response within the peripheral blood, dendritic cells (DCs), potent IFN-producing cells²⁰⁴, were isolated from PBMCs using a commercially available kit and infected *ex vivo* with pH1N1/09, sH3N2 and influenza B (MOI 3). The purity of the isolated DCs was assessed in a sample of the subjects (11 obese and 9 lean) by flow cytometry (Figure 4-6). After gating for live/dead (excluding dead cells) and excluding doublets, the isolated cells were gated for CD11c and CD123 positivity; indeed, two distinct populations of cells were present, representative of conventional DCs (cDCs) and plasmacytoid DCs (pDCs); CD11c is the surface antigen specific for cDCs, whilst CD123 is specific for pDCs.

The panel in Figure 4-5 is taken from one of the lean subjects and representative of typical purity results. No significant intergroup difference in DC purity was identified; approximately 55% of the live cells stained following MACS isolation were DCs. 69.6% of these total DCs were cDCs (CD11c⁺) and 30.4% pDCs (CD123⁺). Supernatants and cellular lysates were harvested at 4, 24 and 48 hours. IFN α , β , λ , IL-6, IL-8 and TNF α levels were quantified in the 24 and 48h supernatants by MSD.

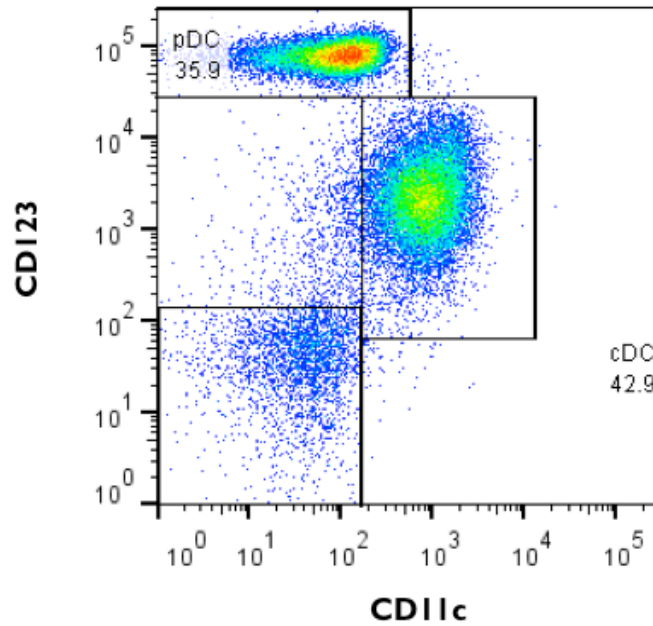


Figure 4-5 Flow cytometric analysis of DCs isolated by MACS using a human DC isolation kit.

Viable cells were gated according to a live/dead marker and doublets excluded. Single live cells were then gated according to CD123 and CD11c to distinguish pDCs and cDCs.

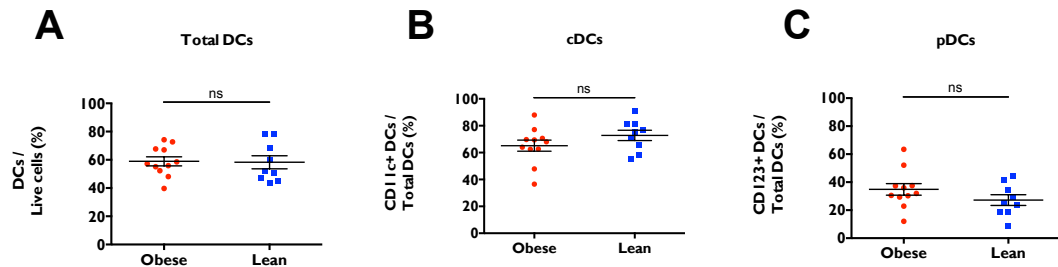


Figure 4-6 No intergroup difference in the purity of the MACS isolated DCs was observed

DC purity was assessed by flow cytometry in a sample of subjects (n = 11 obese and 9 lean) following MACS isolation from PBMCs using a commercially available kit. After gating for live/dead and excluding doublets, the isolated cells were gated for CD11c and CD123 positivity. No intergroup differences were observed for total DCs [A], cDCs [B] or pDCs [C] Data presented as mean $\pm$ SEM. (Statistics: Mann Whitney, * $p < 0.05$)

Obesity did not alter DC IFN responses

No intergroup differences in absolute IFN levels were observed at either 24 or 48 hours post-infection (Figure 4-7). pH1N1/09 infection significantly induced IFN α , β and λ in both groups at 24 and 48 hours [A, D, G, J, M, P]. sH3N2 infection resulted in IFN α expression in both groups at 24 and 48 hours, IFN β expression in both groups at 24 hours [B] and the obese group only at 48 hours [N]. IFN λ was induced in DCs following sH3N2 infection in the obese group only at 24 and 48 hours [H, Q]. B/Florida infection induced IFN α and β expression in both groups at 24 and 48 hours [C, F, L, O]. IFN λ was expressed following B/Florida infection in the lean group only at 24 hours and in both groups at 48 hours [I, R].

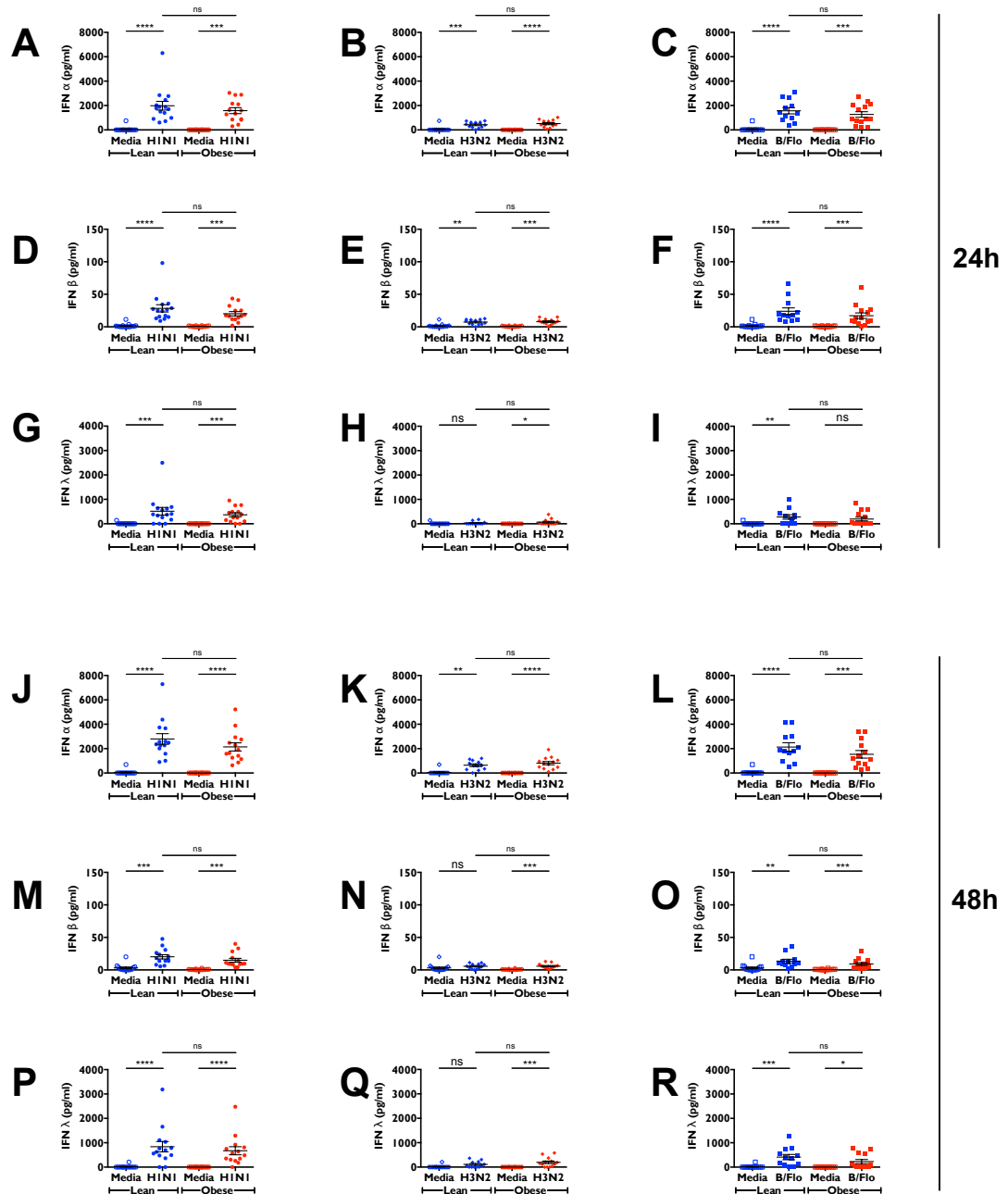


Figure 4-7 Obesity did not alter the IFN response of DCs to pandemic or seasonal influenza infection

IFN α , β and λ protein levels were quantified by MSD in DC supernatants 24 [A – I] and 48 hours [J – R] post-infection with pH1N1/09, sH3N2 and influenza B (MOI 3). No significant intergroup (obese vs. lean) differences were observed. pH1N1/09 resulted in the strongest IFN response [A,D,G,J,M,P], followed by influenza B [C,F,I,L,R], which was, in turn, greater than sH3N2 [B,E,H,K,N,Q]. Each data point represents the average of two pooled biological duplicates, each assessed in technical replicate by MSD. (Statistics: Kruskal Wallis with Dunn's Correction, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.)

Obesity did not alter DC pro-inflammatory cytokine responses

pH1N1/09 and B/Florida resulted in significant induction of IL-6, IL-8 and TNF α in both groups at 24 [Figure 4-8 A, D, G, C, F, I] and 48 hours [J, M, P, L, O, R]; no intergroup differences in absolute IFN levels were observed. sH3N2 resulted in significant IL-6 induction at 24 hours in both groups [B] and significant induction of IL-8 in the obese group only at 48 hours [K]. No significant expression of IL-8 was observed following sH3N2 infection in either group at 24 or 48 hours [E, N].

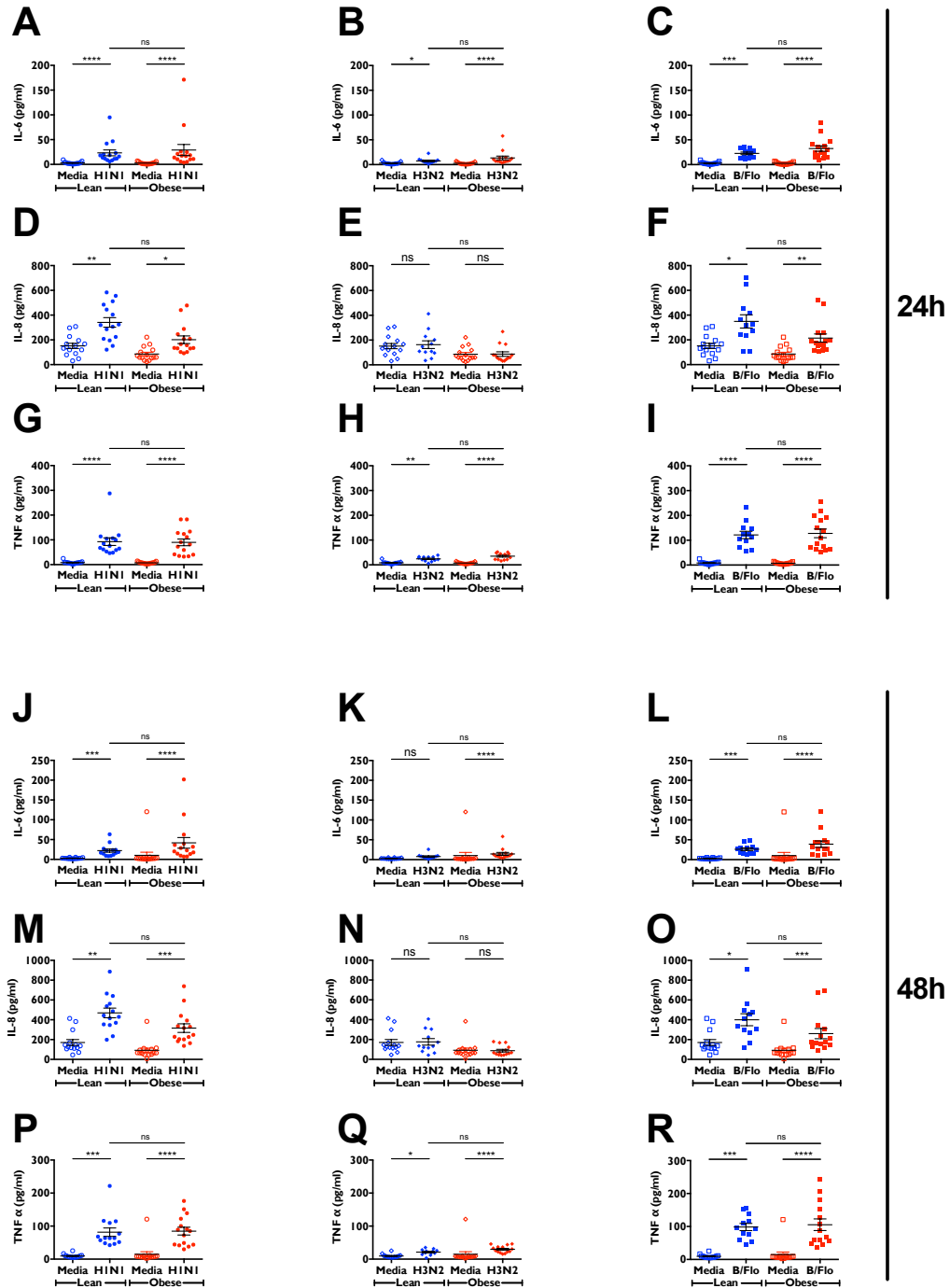


Figure 4-8 Obesity did not alter the pro-inflammatory cytokine response of DCs to pandemic or seasonal influenza infection

IL-6, IL-8 and TNF α protein levels were quantified by MSD in DC supernatants 24 [A – I] and 48 hours [J – R] post-infection with pH1N1/09, sH3N2 and influenza B (MOI 3). No significant intergroup (obese vs. lean) differences were observed. pH1N1/09 and B/Florida resulted in significant induction of IL-6, IL-8 and TNF α in both groups at 24 [A, D, G, C, F, I] and 48 hours [J, M, P, L, O, R]. sH3N2 induced IL-6 at 24 hours in both groups [B] and in the obese group only at 48 hours [K]. No induction of IL-8 was observed following sH3N2 infection in either group at 24 or 48 hours [E, N]. Each data point represents the average of two pooled biological duplicates, each assessed in technical replicate by MSD. (Statistics: Kruskal Wallis with Dunn's Correction, * $p < 0.05$ ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.)

4.4.4 Obesity significantly attenuated the type I and III IFN and pro-inflammatory cytokine responses of BAL cells to pandemic and seasonal influenza viruses

Alveolar macrophages are key players in the host defence system of the respiratory tract²⁰⁵; bronchoalveolar lavage (BAL) cells were therefore isolated by centrifugation from BAL fluid obtained at bronchoscopy and infected *ex vivo* with pH1N1/09, sH3N2 and influenza B (MOI 3). Supernatants and cellular lysates were harvested at 4, 24 and 48 hours. IFN α , β , λ , IL-6, IL-8 and TNF α levels were quantified in the 24 and 48h supernatants by MSD. As BAL fluid return was variable BAL cell yield did not always allow all conditions to be tested at all time points. However, no significant intergroup (obese vs. lean) difference in median BAL return volume, nor median BAL cell number was present (data not shown).

Obesity significantly attenuated the type I & III IFN responses of BAL cells

24 h.p.i. significant expression of IFN α occurred in both groups with all three influenza strains although a trend for attenuated responses in the obese was noted [Figure 4-9 A, B, C]. Significant IFN β induction occurred following sH3N2 and B/Florida infection in both groups [E, F] however, upon pH1N1/09 infection significant IFN β responses were observed in the lean group only [D]. Significant IFN λ induction occurred at 24 hours only in the lean group following infection with all strains [G, H, I] and, notably, a significant intergroup difference was present.

48 h.p.i. significant expression of IFN α was observed with all viruses and, again, a trend towards attenuation in the obese was present [J, K, L]. Significant IFN β induction occurred upon sH3N2 and B/Florida infection in the lean groups only [N, O]. No significant IFN β expression was observed in either group following pH1N1/09 infection [M]. Significant IFN λ expression was observed in the lean group only at 48 hours for all three viruses [P, Q, R] with statistically significant intergroup differences for pH1N1/09 and sH3N2 [P, Q].

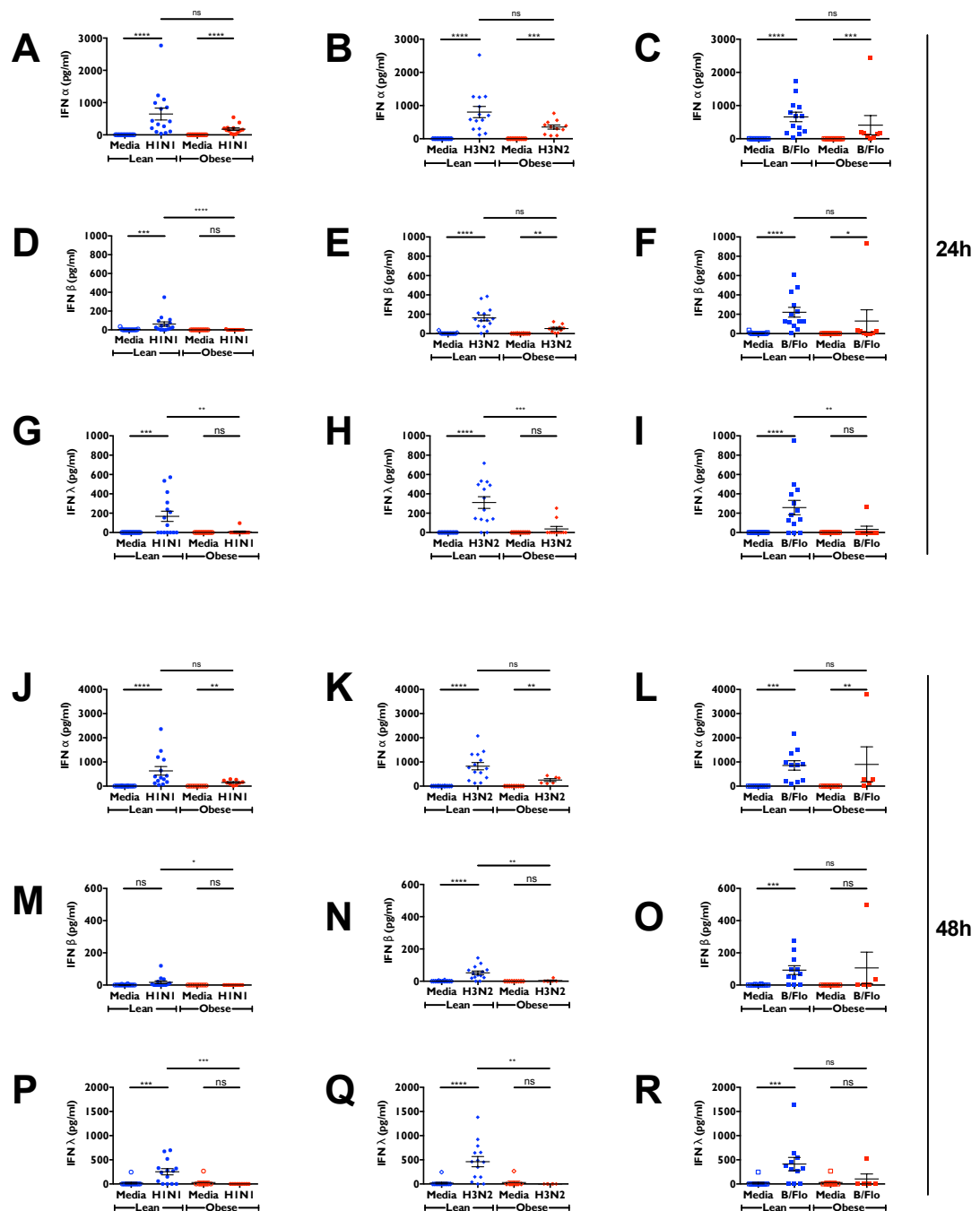


Figure 4-9 Obesity significantly attenuated the type I and III IFN responses of BAL cells to pandemic and seasonal influenza infection

IFN α , β and λ protein levels were quantified by MSD in BAL cell supernatants 24 and 48 h.p.i. with pH1N1/09, sH3N2 and influenza B (MOI 3). 24 h.p.i. induction of IFN α occurred in both groups with all three influenza strains; a trend for attenuated responses in the obese was noted [A, B, C]. pH1N1/09 infection induced significant IFN β responses in the lean group only at 24h [D]. Significant IFN λ induction occurred 24 h.p.i. in the lean group only following infection with all strains [G, H, I]. 48 h.p.i. induction of IFN α was observed with all viruses; a trend towards attenuation in the obese was present [J, K, L]. IFN β induction occurred upon sH3N2 and B/Florida infection in the lean groups only [N, O]. Significant IFN λ expression was seen in the lean group only at 48 hours for all three viruses [P,Q,R]. Each data point represents the average of two pooled biological duplicates, each assessed in technical replicate by MSD. (Kruskal Wallis with Dunn's Correction, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

The Kruskal Wallis test with Dunn's correction was the appropriate test for statistical significance for three or more groups of unpaired, non-parametric data, hence its use in the analyses presented in this chapter. However, by comparing four groups (obese-media, obese-virus, lean-media, lean-virus), the sensitivity of the test to detect a statistically significant difference between two salient groups, namely 'obese infected' vs. 'lean infected' was reduced. Therefore, the immune responses of the two infected groups were analysed *per se* using the non-parametric, unpaired Mann Whitney test (Figure 4-10). Indeed, statistically significant attenuation of the type I & III IFN responses following infection with pH1N1/09, sH3N2 and B/Florida was observed 24 h.p.i. [Figure 4-10 A-I] and post-influenza A infection at 48 hours [J-K, M-N, P-Q]. A trend for attenuation in the IFN response in the obese group was observed following influenza B infection at 48 hours but did not reach statistical significance [L, O, R].

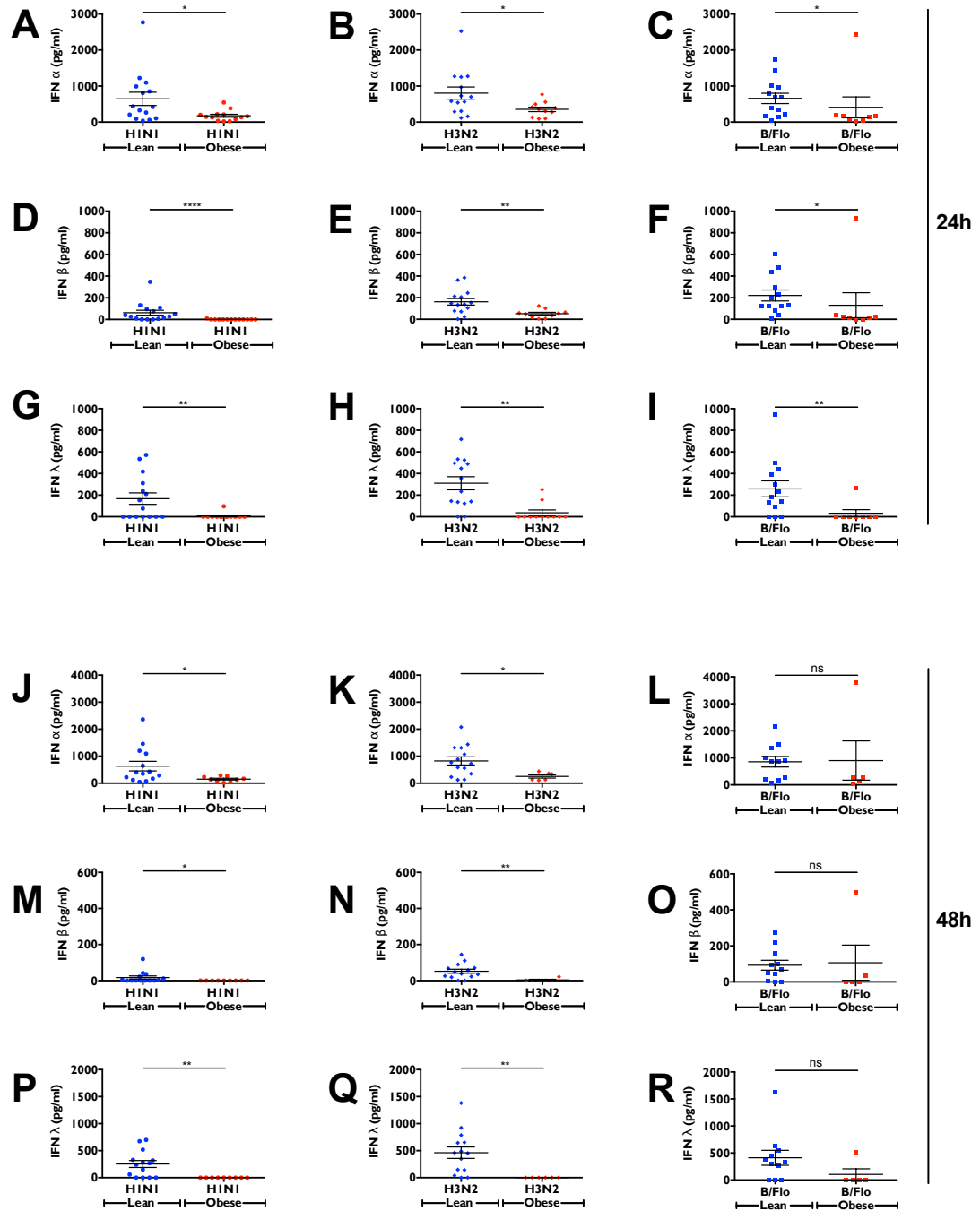


Figure 4-10 Obesity significantly attenuated the type I and III IFN responses of BAL cells to pandemic and seasonal influenza infection

IFN α , β and λ protein levels were quantified by MSD in BAL cell supernatants 24 and 48 h.p.i. with pH1N1/09, sH3N2 and influenza B (MOI 3). The Mann Whitney statistical test was applied to compare the responses of the 'obese infected' and 'lean infected' groups only. At 24 h.p.i. the IFN α , β and λ responses of the BAL cells from the obese group were significantly attenuated upon infection with each influenza strain relative to the lean group [A-I]. At 48 hours, the IFN α , β and λ responses were significantly attenuated in the obese group following infection with pH1N1/09 [J, M, P] and sH3N2 [K, N, Q] only. Each data point represents the average of two pooled biological duplicates, each assessed in technical replicate by MSD. (Mann Whitney test, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

Obesity significantly attenuated BAL cell pro-inflammatory cytokine responses

No significant induction of IL-6, IL-8 or TNF α relative to media was observed in the lean group post-infection with pH1N1/09, sH3N2 or B/Florida at 24 or 48 hours (Figure 4-11); however, baseline medium expression was significantly higher than that seen in the obese group [A-R]. IL-8 levels in lean, infected BAL cells appeared to be suppressed relative to media, although statistical significance was not achieved (using the Kruskal Wallis test) [D-F, M-O].

The expression of all the evaluated pro-inflammatory cytokines was significantly lower in the obese group relative to lean [A-R]. However, unlike in the lean group, a trend for IL-6 and TNF α induction was observed in the obese group following infection at both time points. Similar to the lean group, a trend for IL-8 suppression was noted [A-R].

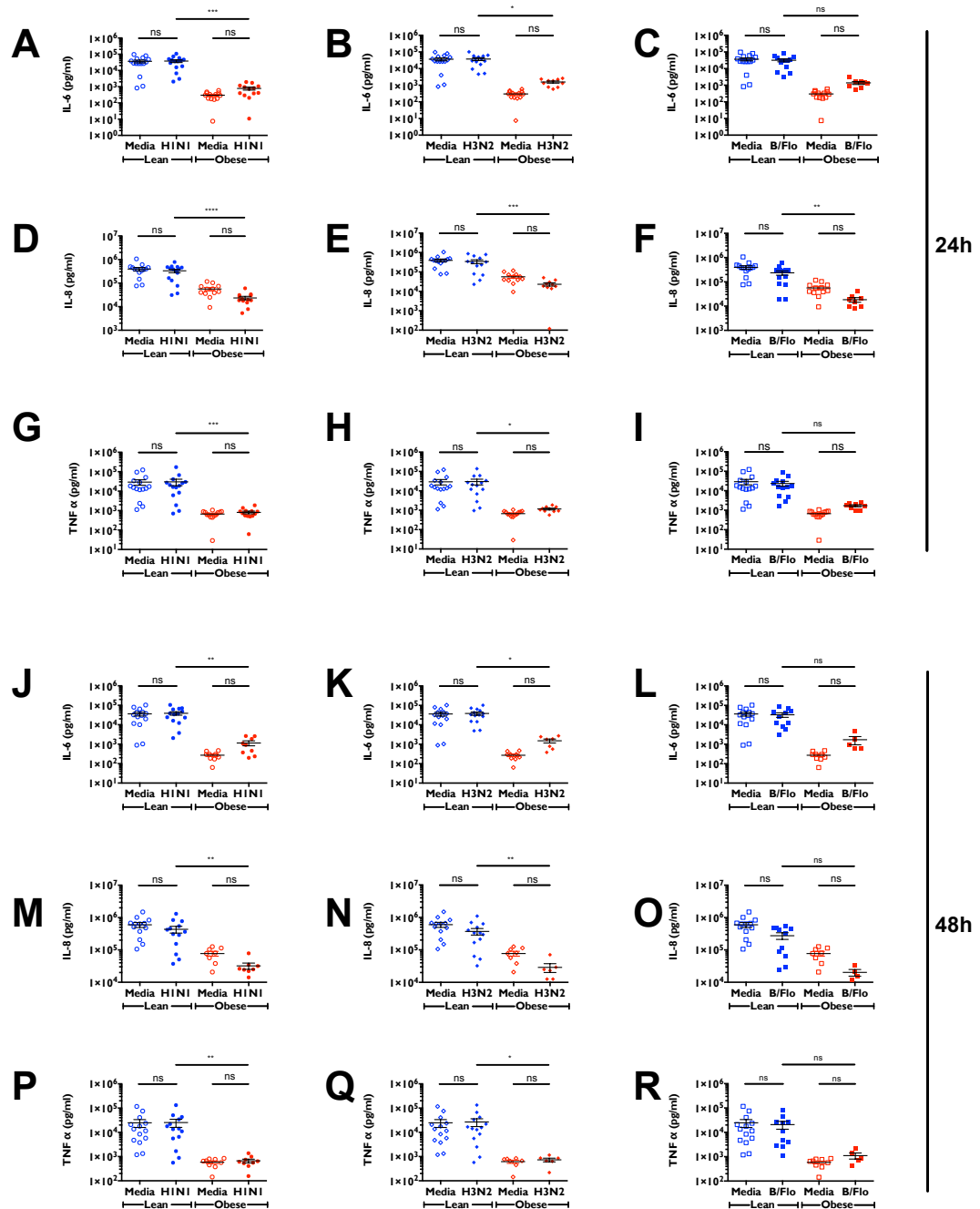


Figure 4-11 Obesity significantly attenuated pro-inflammatory cytokine expression in BAL cells

IL-6, IL-8 and TNF α protein levels were quantified by MSD in BAL cell supernatants 24 and 48 h.p.i. with pH1N1/09, sH3N2 and influenza B (MOI 3). No significant induction of IL-6, IL-8 or TNF α relative to media was observed in the lean group post-infection with pH1N1/09, sH3N2 or B/Florida at 24 or 48 hours; however, baseline media expression was significantly higher than that seen in the obese group [A-R]. A trend for IL-6 and TNF α induction was observed in the obese group following infection at both time points [A-R]. A trend for IL-8 suppression was noted in both groups at both timepoints [G-I, M-O]. Each data point represents the average of two pooled biological duplicates, each assessed in technical replicate by MSD. (Kruskal Wallis with Dunn's Correction, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

As it was important to demonstrate that the obese cells were still biological active, the Mann Whitney test was used to evaluate statistical significance between the immune responses of the 'obese infected' and 'obese media' groups only (Figure 4-12). At 24 and 48 h.p.i. statistically significant expression of IL-6 and significant suppression of IL-8 following infection with all three viruses was observed [A-C, J-L, D-F, M-O]. Regarding TNF α , significant induction following sH3N2 and B/Flo infection was seen at 24 h.p.i. with pH1N1/09 trending towards significance [G-I]. At 48 hours, none of the viruses resulted in significant induction [P-R].

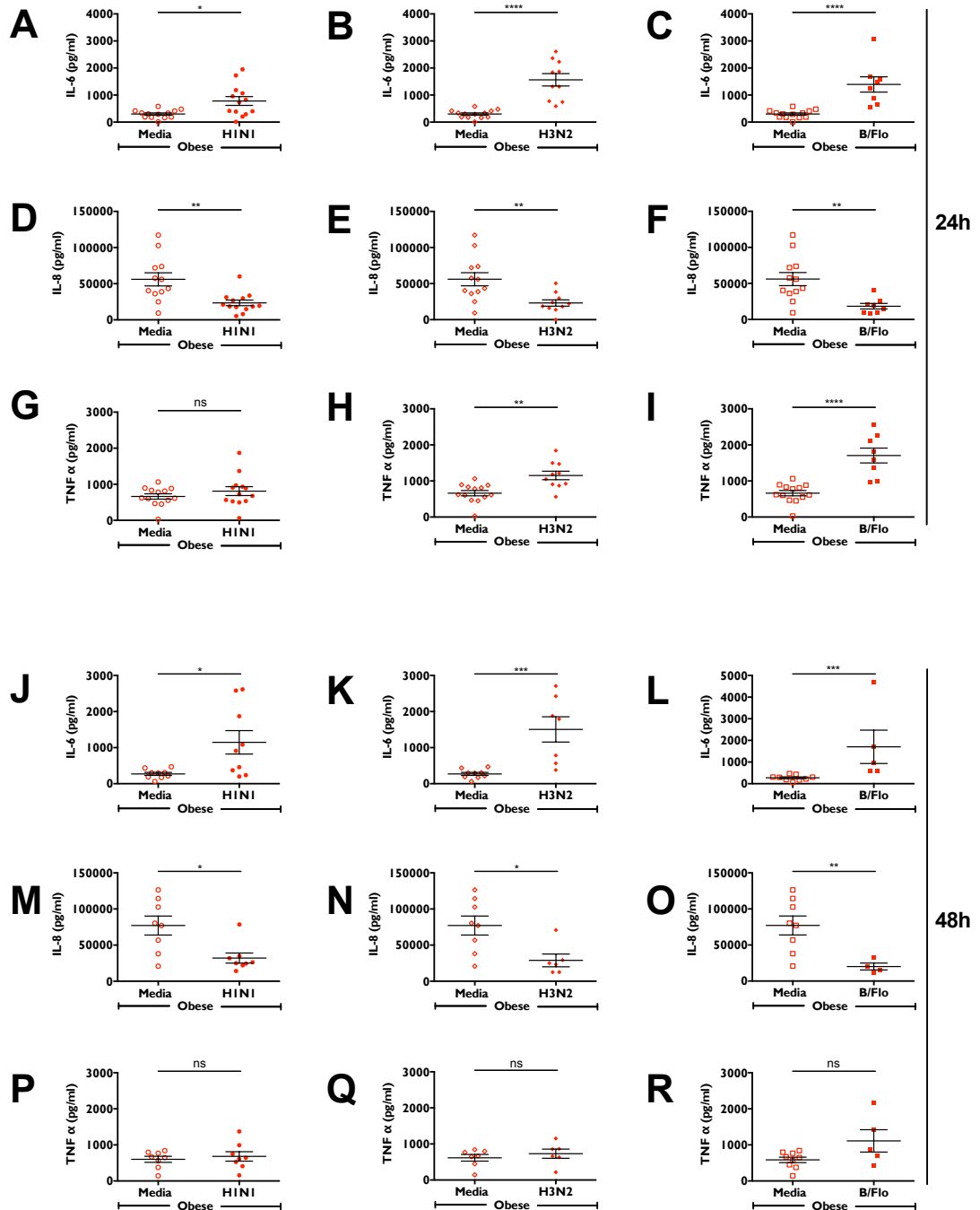


Figure 4-12 Significant induction of IL-6 and TNF α and significant suppression of IL-8 occurred in BAL cells from obese individuals infected with pandemic and seasonal influenza

IL-6, IL-8 and TNF α protein levels were quantified by MSD in BAL cell supernatants from the obese group only 24 and 48 h.p.i. with pH1N1/09, sH3N2 and influenza B (MOI 3). The Mann Whitney statistical test was applied to compare the responses of the 'obese infected' and 'obese uninfected' groups only. 24 h.p.i. IL-6 and TNF α were significantly induced by all three influenza strains; [A-I]. 48 h.p.i. IL-6 was significantly induced by all three strains; TNF α was also induced but did not achieve statistical significance. IL-8 was significantly suppressed by all viruses at both 24 and 48 h.p.i. (Mann Whitney test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

4.4.5 Influenza virus stimulation of BAL cells from the obese and lean groups was equal

To demonstrate that the differences observed in the IFN responses of the BAL cells were not due to differences in viral stimulation, influenza M gene copy number was evaluated in the cellular lysates at 24 and 48 hours by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) (Figure 4-13).

No difference was observed in corrected influenza A virus (IAV) M gene copy number between the obese and lean groups for pH1N1/09 or sH3N2 at either 24 or 48 hours following infection [A-D]. Additionally, no difference was observed in corrected influenza B virus (IBV) M gene copy number at either time point [E-F].

No significant increase in corrected M gene copy number was observed for any of the three influenza viruses between 24 and 48 hours [A-F].

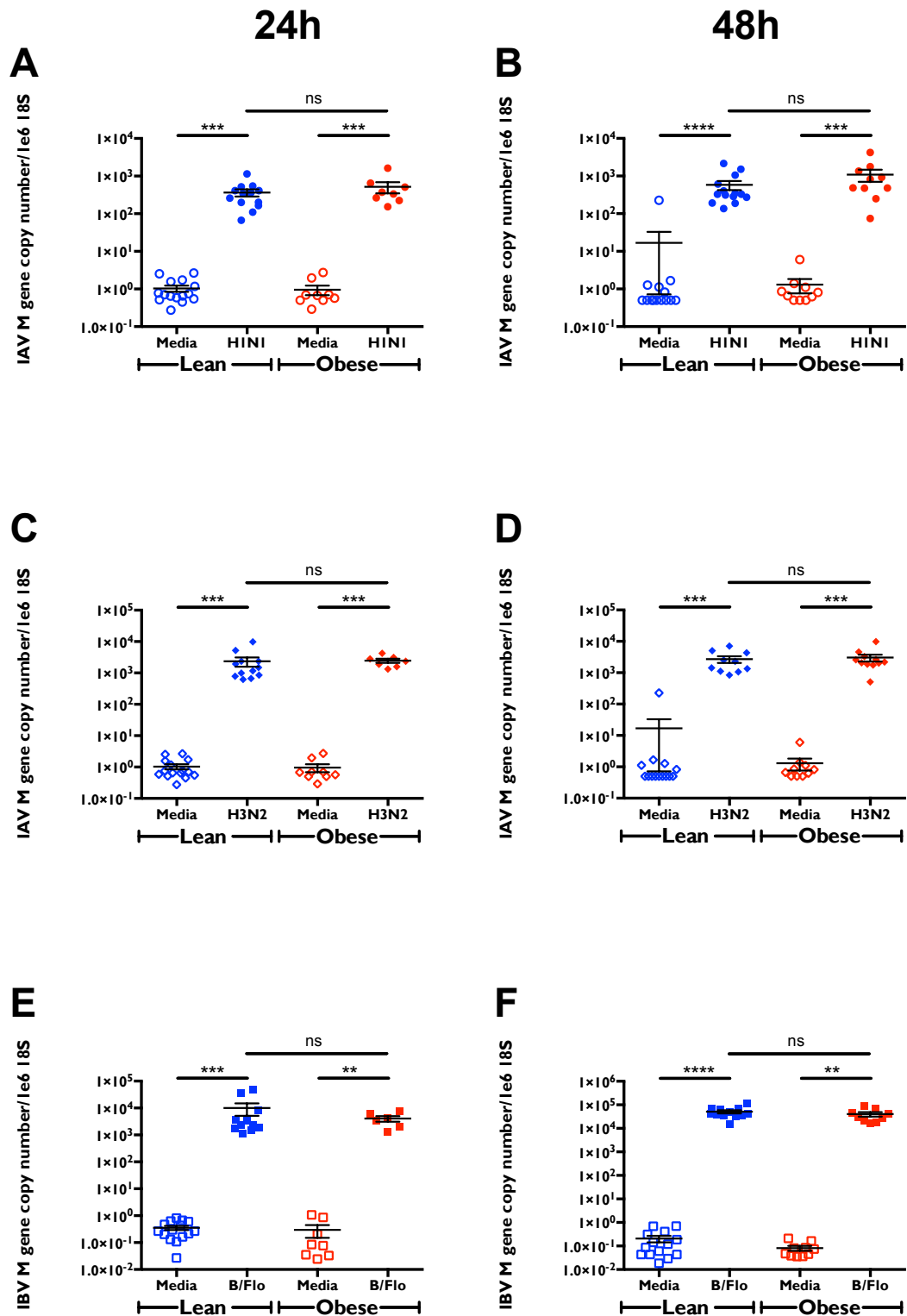


Figure 4-13 Viral stimulation of the BAL cells from the obese and lean groups was equal
 RT-qPCR was used to measure influenza M gene mRNA expression in BAL cells 24 and 48 h.p.i. Levels of influenza M gene were significantly greater in the infected cells relative to media, but no significant intergroup (obese vs. lean) differences in viral stimulation were observed [A-F]. M gene mRNA copy number was corrected for 18S. Each data point represents the average of two biological duplicates, each assessed in technical replicate by RT-qPCR. (Kruskal Wallis with Dunn's Correction, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

4.4.6 *MxA mRNA expression was induced by influenza A viruses in BAL cells from obese individuals*

The main anti-viral effectors induced by IFN are the ISGs such as MxA¹³. Given the observed attenuation of the IFN response in the BAL cells from the obese subjects MxA mRNA expression was quantified in the BAL cell lysates at 24 and 48 hours by RT-qPCR (Figure 4-14).

24 h.p.i. pH1N1/09 infection did not induce MxA expression in the BAL cells from either the obese or lean groups however, baseline MxA levels were higher in the lean group [A]. Significant MxA induction was observed in both groups at 48 hours [B]. Despite lower baseline levels, MxA mRNA induction was greater in the obese group, although no statistically significant difference in absolute levels was detected [B]. Regarding sH3N2, media MxA mRNA expression was again lower in the obese relative to lean at both time points. Infection significantly attenuated MxA mRNA expression relative to media in the lean group at 24 hours [C]; a trend towards suppression was observed in the obese group. At 48 hours, significant MxA induction was observed in the obese group only [D].

Whilst baseline media MxA expression was again lower in the obese group [E, F] no significant induction or suppression was observed at 24 or 48 hours following influenza B infection [E, F].

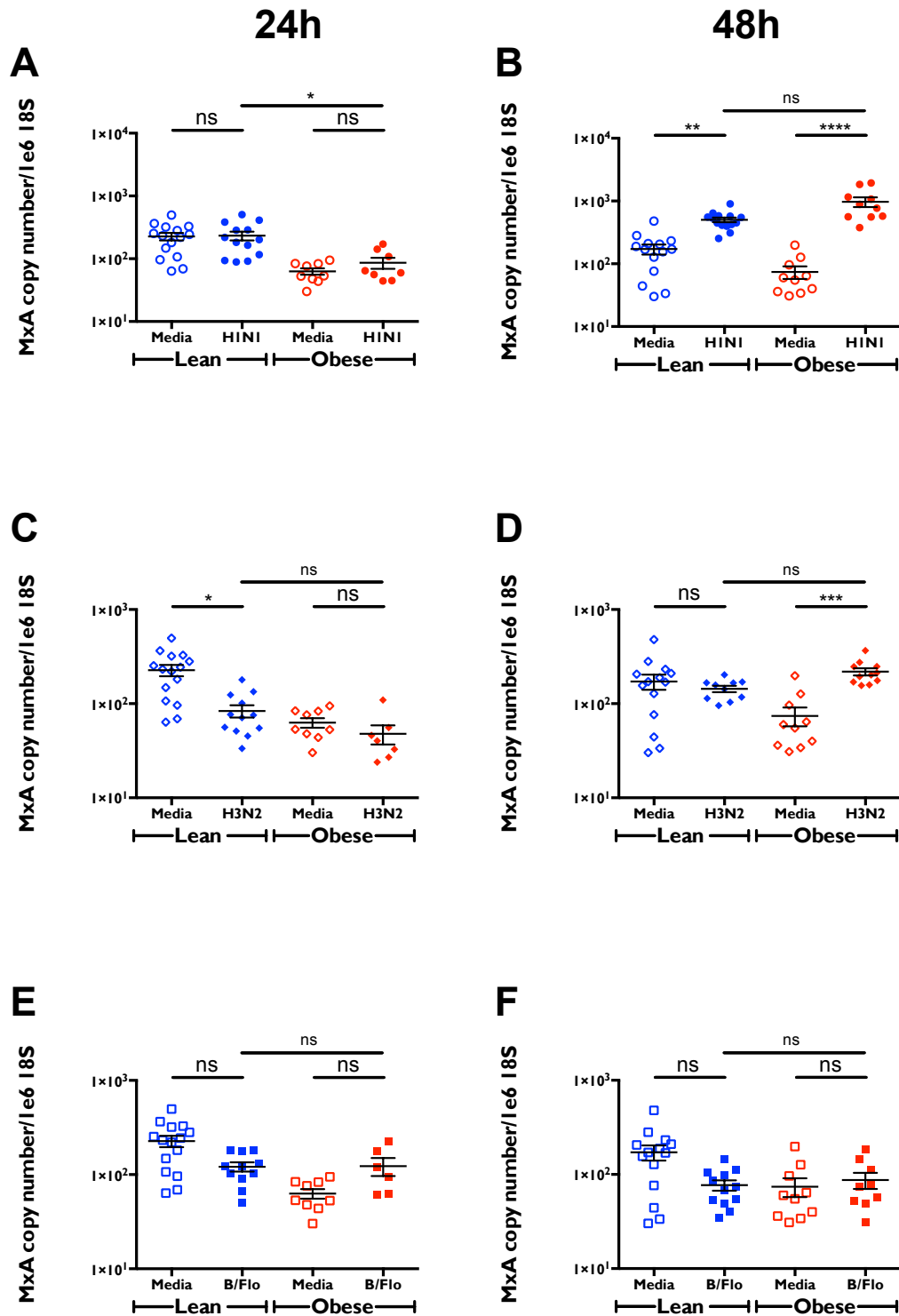


Figure 4-14 IAVs induced MxA mRNA expression in BAL cells from obese individuals
 RT-qPCR was used to measure MxA mRNA expression in BAL cells 24 and 48 h.p.i. Significant induction of MxA was observed only in the obese group 48 h.p.i. with IAVs [B, D]. Baseline media MxA expression was lower in the obese relative to lean [A-F]. MxA mRNA copy number was corrected for 18S. Each data point represents the average of two biological duplicates, each assessed in technical replicate by RT-qPCR. (Kruskal Wallis with Dunn's Correction, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

4.4.7 Exposure of hBECs to exogenous high-dose leptin did not alter the IFN or pro-inflammatory cytokine responses to pandemic or seasonal influenza infection

In an attempt to recreate the obese environment (i.e. high leptin concentration), hBECs from the lean controls only (n=13) were pre-exposed to high-dose exogenous leptin (500ng/ml²⁰²) for 16 hours prior to infection and throughout the subsequent infection timecourse. The IFN (Figure 4-15) and pro-inflammatory cytokine (Figure 4-16) responses were then compared with those observed in hBECs from the same lean subjects cultured in medium alone.

Exogenous leptin did not alter hBEC IFN expression

Exposure of the hBECs to exogenous leptin did not result in any observable differences in IFN expression (Figure 4-15). Only the sH3N2 virus resulted in significant expression of IFN β and IFN λ at 24 hours [E, H]. 48 h.p.i. sH3N2 virus again resulted in the greatest IFN β and IFN λ expression [N, Q]; however, the pH1N1/09 virus also induced IFN β and IFN λ expression [M, P].

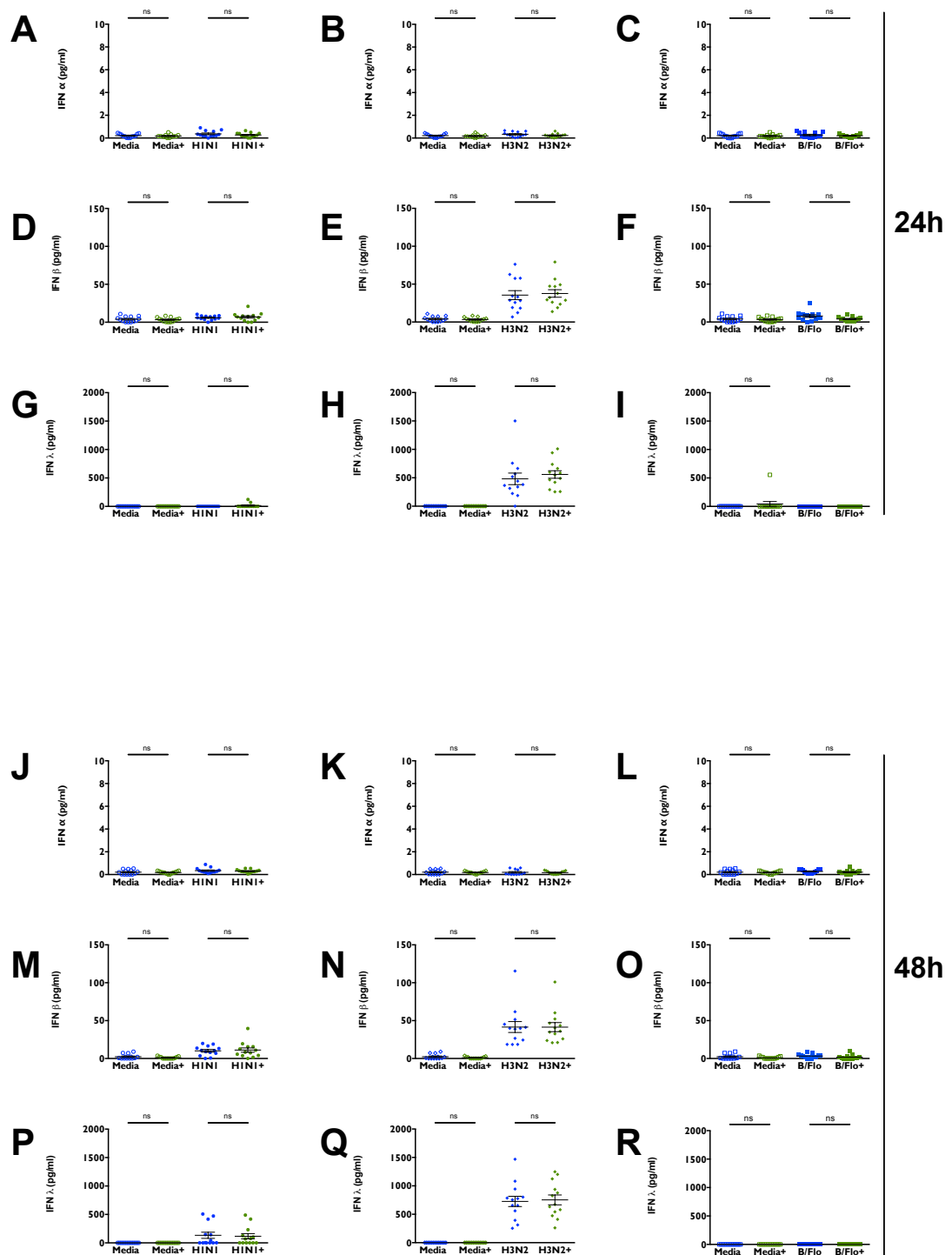


Figure 4-15 Exposure of hBECs from lean subjects to exogenous leptin did not alter the IFN response to pandemic or seasonal influenza infection

IFN α , β and λ protein levels were quantified by MSD in hBEC supernatants from lean subjects only 24 [A – I] and 48 hours [J – R] post-infection with pH1N1/09, sH3N2 and influenza B (MOI 3) either in the presence (as indicated by the '+' sign) or absence of exogenous leptin (500ng/ml). No significant intergroup (leptin vs. no leptin) differences were observed. Each data point represents the average of two independent biological duplicates, each measured in technical replicate by MSD. Blue = no leptin, green = leptin present. (Statistics: Kruskal Wallis with Dunn's Correction, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.) ($n = 10$ obese vs. 13 lean)

Exogenous leptin did not alter hBEC pro-inflammatory cytokine expression

Exogenous leptin did not alter the expression of IL-6, IL-8 or TNF α in hBECs following influenza infection at either 24 or 48 hours relative to that observed in the hBECs cultured in medium alone (Figure 4-16).

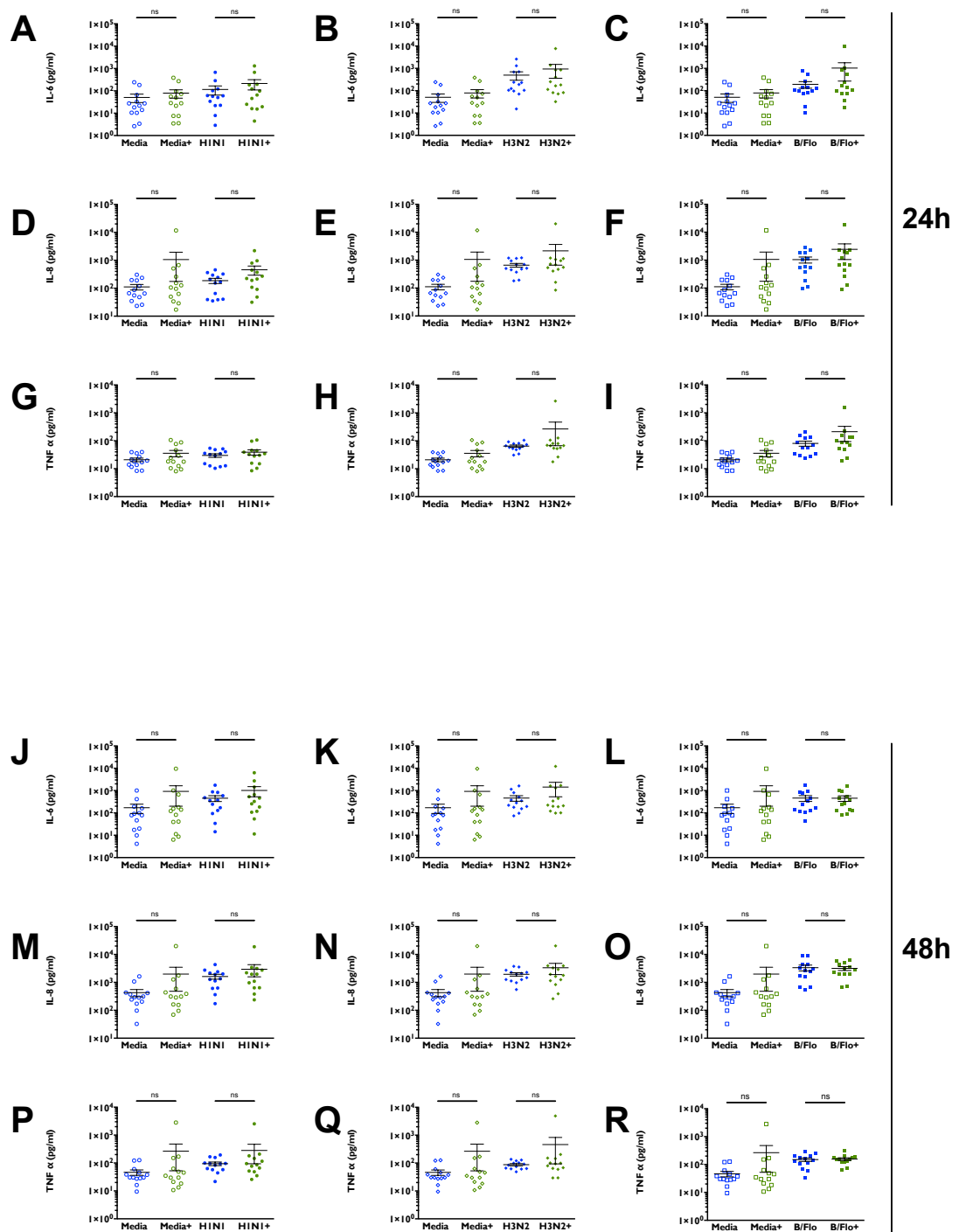


Figure 4-16 Exposure of hBECs from lean subjects to exogenous leptin did not alter the pro-inflammatory cytokine response to pandemic or seasonal influenza infection

IL-6, IL-8 and TNF α protein levels were quantified by MSD in hBEC supernatants from lean subjects only 24 [A – I] and 48 hours [J – R] post-infection with pH1N1/09, sH3N2 and influenza B (MOI 3) either in the presence (as indicated by the ‘+’ sign) or absence of exogenous leptin (500ng/ml). No significant intergroup (leptin vs. no leptin) differences were observed. Each data point represents the average of two independent biological duplicates, each measured in technical replicate by MSD. Blue = no leptin, green = leptin present. (Statistics: Kruskal Wallis with Dunn’s Correction, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.) ($n = 10$ obese vs. 13 lean)

4.4.8 *Obesity did not alter type I & III IFN or pro-inflammatory cytokine expression in PBMCs following stimulation with TLR agonists*

In an attempt to replicate the work of Teran-Cabanillas *et al*¹⁶⁶, PBMCs were stimulated with a panel of five TLR agonists (R848, Poly (I:C), LPS, LTA and CPG-A). IFNs and pro-inflammatory cytokines were subsequently quantified by MSD in the PBMC supernatants at 8 and 24 hours.

Obesity did not alter PBMC IFN responses to TLR ligands

No significant intergroup differences in IFN responses to TLR stimulation were observed. R848 and CPG-A resulted in significant expression of IFN α at 8 hours in both obese and lean groups [Figure 4-17 A, E]. LPS and poly (I:C) resulted in statistically significant, but physiologically irrelevant, expression of IFN α (levels <1.5pg/ml) in the obese group at 8 hours [B, C]. Also at 8 hours, R848 induced significant IFN β expression in both groups [F] whereas CPG-A, whilst inducing IFN β in both groups, statistical significance was only achieved in the lean group [J]. No significant IFN λ expression was observed with any of the TLR ligands at 8 hours [K-O].

24 hours post-stimulation (Figure 4-18), IFN α expression remained significantly elevated in both groups following R848 and CPG-A stimulation [A, E]. IFN β was induced upon R848 stimulation in both groups; however, statistical significance was only achieved in the obese group [F]. IFN β was also induced in both groups by CPG-A, however statistically significant expression was not observed in either group [J]. At 24 hours, IFN λ expression became evident in both groups following CPG-A stimulation; however, statistically significant expression was only observed in the lean group [O].

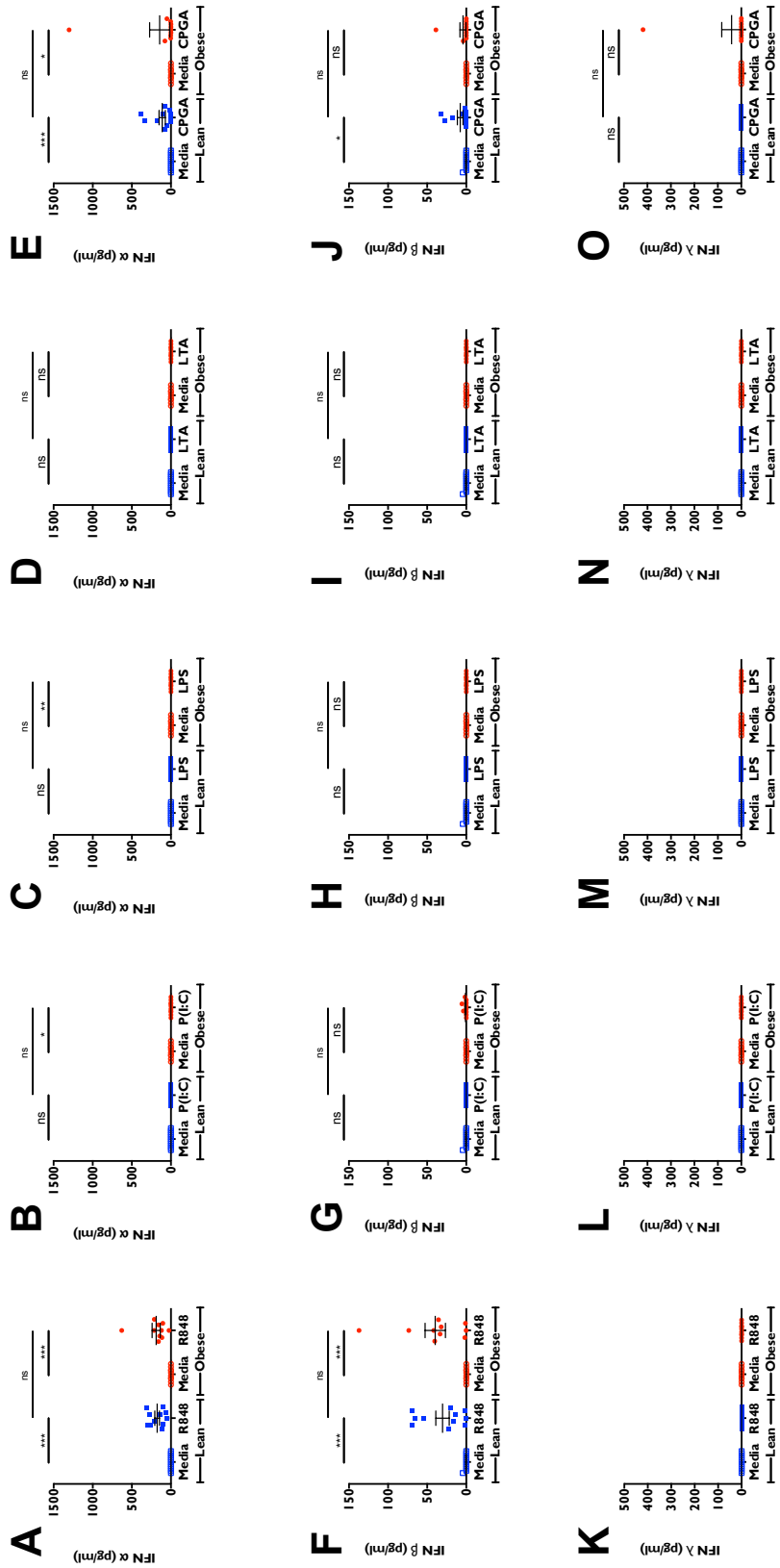


Figure 4-17 Obesity did not alter the IFN response of PBMCs to TLR agonists 8 hours post-stimulation

IFN α , β and λ protein levels were quantified by MSD in PBMC supernatants 8 hours post-stimulation with the TLR agonists R848, Poly (I:C), LPS, LTA and CPG-A. No significant intergroup (obese vs. lean) differences were observed. R848 and CPG-A resulted in significant expression of IFN α at 8 hours in both obese and lean groups [A, E]. R848 induced significant IFN β expression in both groups [F]. CPG-A induced IFN β in both groups; however, statistical significance was only achieved in the lean group [J]. No significant IFN λ expression was observed with any of the TLR ligands at 8 hours [K-O]. Each data point represents the average of two pooled biological replicates, each assessed in technical replicates by MSD. (Statistics: Kruskal Wallis with Dunn's Correction, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

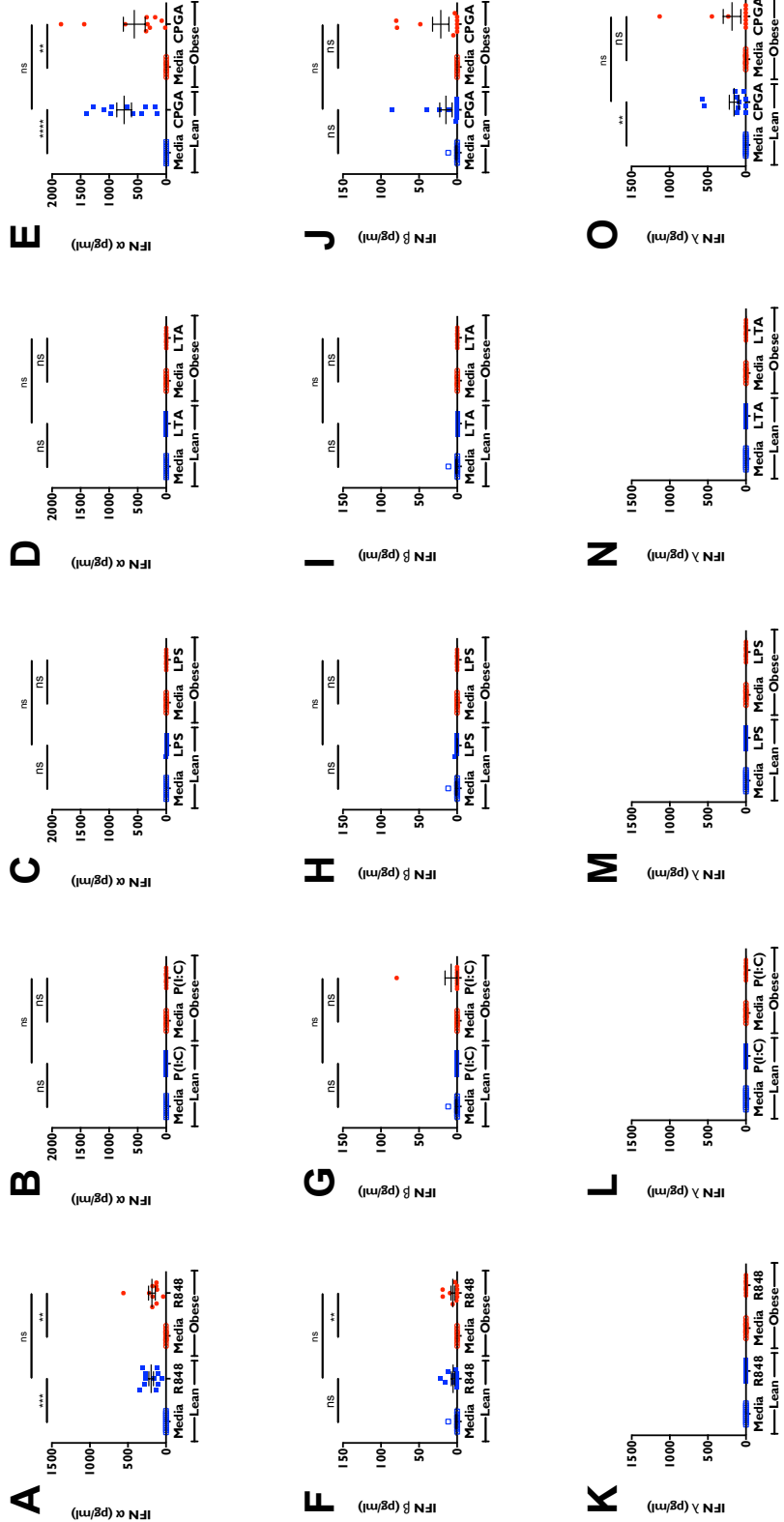


Figure 4-18 Obesity did not alter the IFN response of PBMCs to TLR agonists 24 hours post-stimulation

IFN α , β and λ protein levels were quantified by MSD in PBMC supernatants 24 hours post-stimulation with the TLR agonists R848, Poly (I:C), LPS, LTA and CPG-A. No significant intergroup (obese vs. lean) differences were observed. R848 and CPG-A resulted in significant expression of IFN α at 8 hours in both obese and lean groups [A, E]. R848 induced significant IFN β expression in the obese group only [F]. CPG-A induced IFN β in both groups; however, statistical significance was not achieved [J]. IFN λ was significantly induced in the lean group only following CPG-A stimulation [O]. Each data point represents the average of two pooled biological replicates, each assessed in technical replicates by MSD. (Statistics: Kruskal Wallis with Dunn's Correction, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

Obesity did not alter PBMC pro-inflammatory cytokine responses to TLR ligands

R848, Poly (I:C), LPS and LTA all resulted in significant expression of IL-6, IL-8 and TNF α at both 8 (Figure 4-19) and 24 hours (Figure 4-20) [A-D, F-I, K-N]; no intergroup differences were present. LPS stimulation resulted in the strongest induction of the pro-inflammatory cytokines [C, H, M]. Interestingly, despite inducing IFN, CPG-A stimulation did not result in IL-8 or TNF α expression in either group [J, O] and resulted only in limited induction of IL-6 in the lean group at 24 hours [Figure 4-20 E].

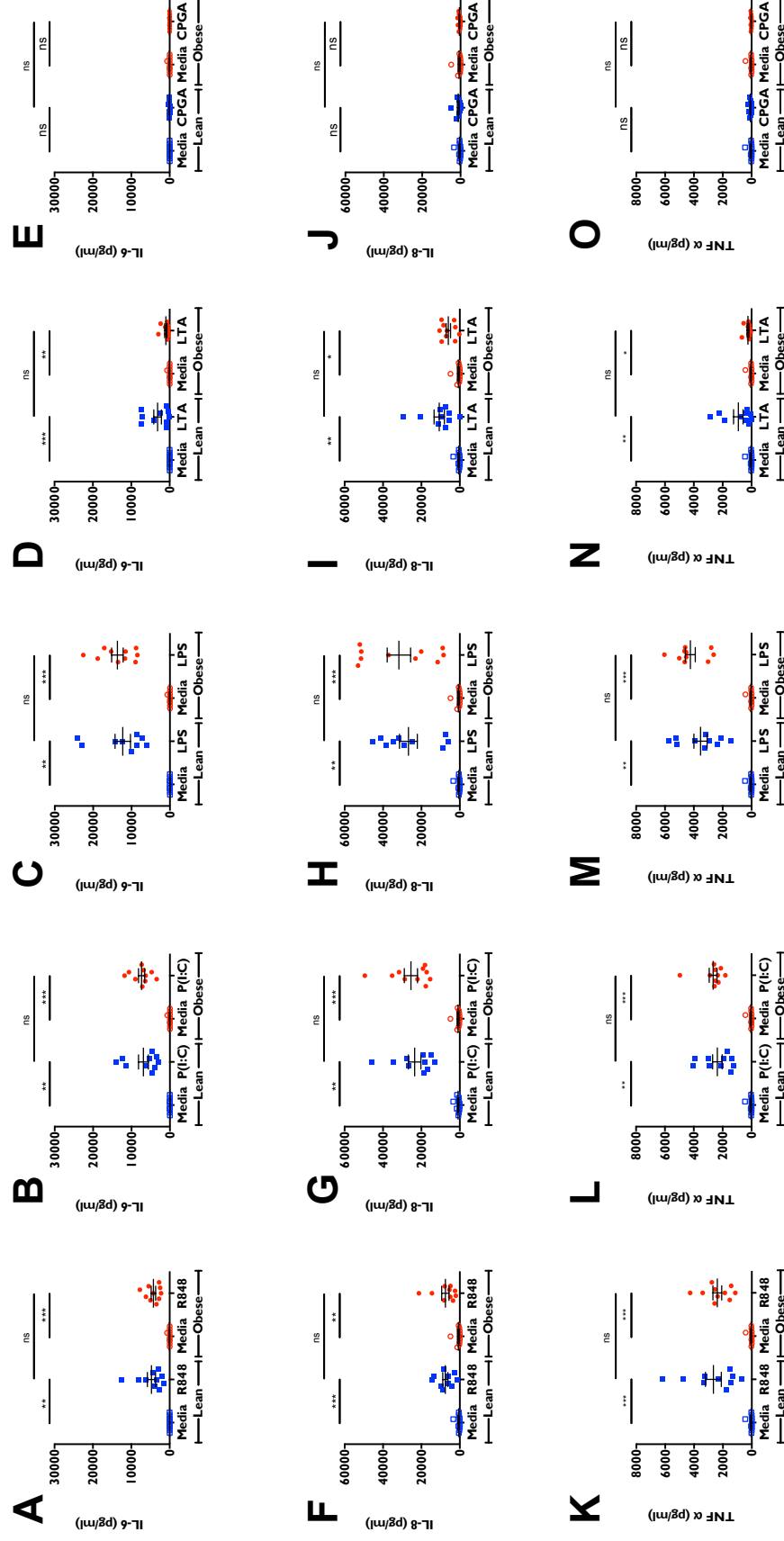


Figure 4-19 Obesity did not alter the pro-inflammatory cytokine response of PBMCs to TLR agonists 8 hours post-stimulation

IL-6, IL-8 and TNF α protein levels were quantified by MSD in PBMC supernatants 8 hours post-stimulation with the TLR agonists R848, Poly (I:C), LPS, LTA and CPG-A. No significant intergroup (obese vs. lean) differences were observed. R848, Poly (I:C), LPS and LTA all resulted in significant expression of IL-6, IL-8 and TNF α at 8 hours [A-D, F-I, K-N]. LPS stimulation resulted in the strongest induction of the pro-inflammatory cytokines [C, H, M]. CPG-A stimulation did not result in IL-6, IL-8 or TNF α expression in either group [E, J, O]. Each data point represents the average of two pooled biological replicates, each assessed in technical replicates by MSD. (Statistics: Kruskal Wallis with Dunn's Correction, * p < 0.05, ** p < 0.01, *** p < 0.001.

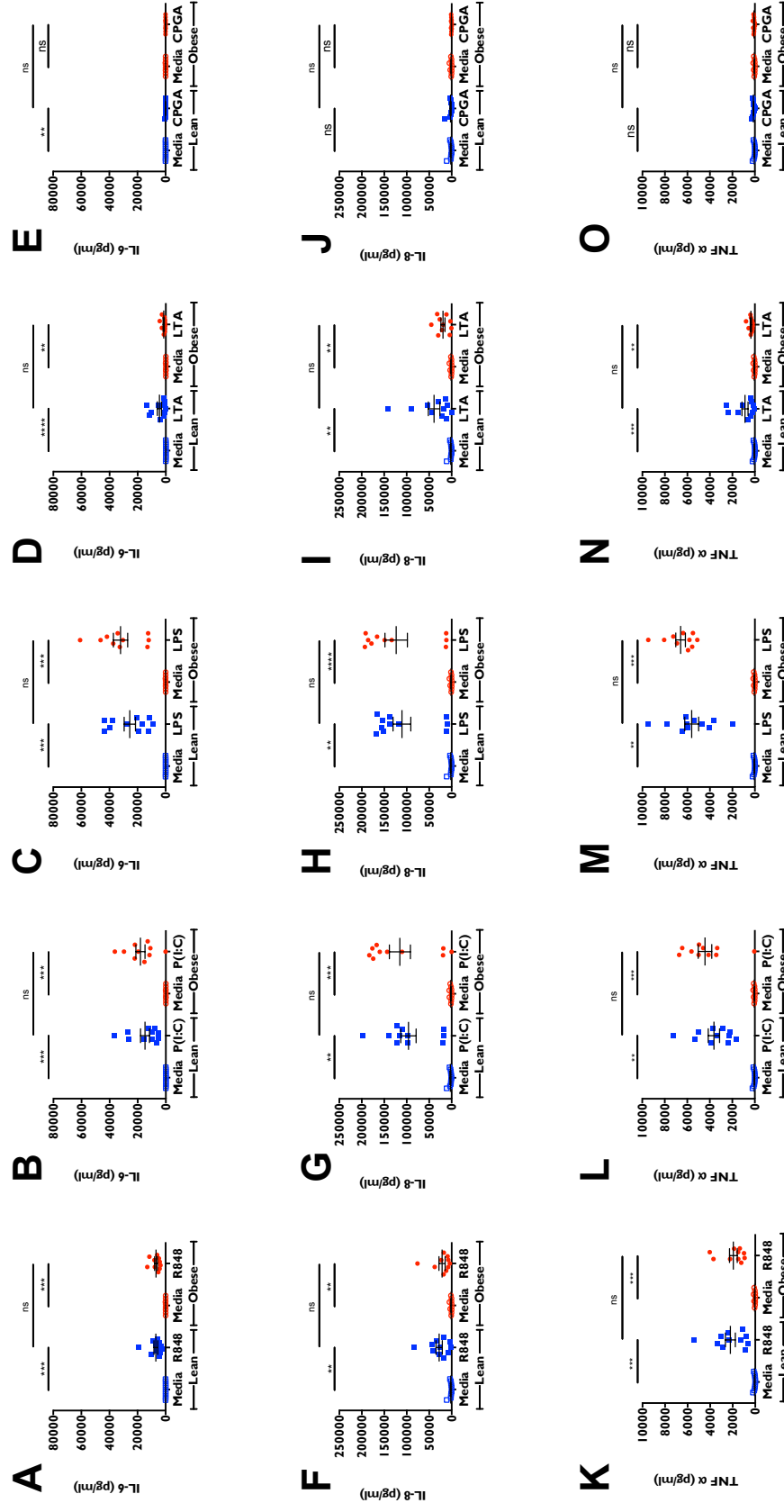


Figure 4-20 Obesity did not alter the pro-inflammatory cytokine response of PBMCs to TLR agonists 24 hours post-stimulation

IL-6, IL-8 and TNF α protein levels were quantified by MSD in PBMC supernatants 24 hours post-stimulation with the TLR agonists R848, Poly (I:C), LPS, LTA and CPG-A. No significant intergroup (obese vs. lean) differences were observed. R848, Poly (I:C), LPS and LTA all resulted in significant expression of IL-6, IL-8 and TNF α [A-D, F-I, K-N]. LPS stimulation resulted in the strongest induction of the pro-inflammatory cytokines [C, H, M]. CPG-A stimulation did not result in IL-8 or TNF α expression in either group [J, O] and resulted only in limited induction of IL-6 in the lean group at 24 hours [E]. Each data point represents the average of two pooled biological duplicates, each assessed in technical replicates by MSD. (Statistics: Kruskal Wallis with Dunn's Correction, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

4.5 Discussion

In this chapter hBECs, PBMCs, DCs and BAL cells were isolated from morbidly obese (n=15) and healthy weight (n=15) individuals and stimulated *ex vivo* with three contemporaneous, clinically relevant strains of influenza (pH1N1/09, sH3N2, influenza B). IFN and pro-inflammatory cytokine protein levels were quantified in the supernatants of the stimulated cells by MSD and viral M gene and MxA mRNA levels quantified by RT-qPCR where intergroup differences were identified. Furthermore, hBECs from lean individuals were infected in the presence of exogenous leptin in an attempt to mimic the obese state and PBMCs from both groups were stimulated with TLR ligands in an attempt to reproduce the results of Teran-Cabanillas *et al*¹⁶⁶.

The focus of the discussion in this chapter will be the comparison of the influenza-induced immune responses in the primary human respiratory epithelial and immune cells isolated from the obese and lean groups. Adipokine levels and SOCS expression data will be presented and discussed in chapter five.

4.5.1 *Obesity did not alter the IFN or pro-inflammatory cytokine responses of hBECs to influenza infection*

This is the first, and only study to date, to evaluate the effects of obesity on the innate immune response of primary hBECs to respiratory viral infection. The respiratory epithelium is the primary site for influenza replication^{1,206}; however, hBECs are also able to produce and, potentially more significantly, respond to IFN, especially types I and III^{207,208}. In addition to hBECs the respiratory tract harbours professional immune cells e.g. AMs that secrete much greater amounts of IFNs and pro-inflammatory cytokines; however, these likely do not contribute significantly to viral load. Although the *ex vivo* infection model used throughout this chapter lacked the complex interplay that would be present in an *in vivo* model, it offered the opportunity to evaluate the effect of obesity on the responses of each primary cell type in isolation, thus facilitating identification of the site of any potential immune deficiency.

Ultimately, obesity did not appear to affect the production of IFN or pro-inflammatory cytokines by hBECs upon infection with either influenza A or B. However, the response of hBECs to IFN, rather than the production by, is arguably more important;

thus, although no difference in IFN or pro-inflammatory cytokine response was observed in the hBECs *per se* it could be envisaged that if an obesity-associated IFN deficiency was evident in one of the other immune cells types present in the respiratory tract e.g. alveolar macrophages or DCs this could result in attenuated induction of ISGs within the hBECs. A co-culture model, in which hBECs are cultured with one of these immune cell types, would be best suited to address this issue.

Interestingly, sH3N2 infection resulted in the strongest IFN induction in hBECs, which is surprising given that the NS1 protein of other strains of sH3N2 has been shown to strongly suppress the host immune response^{209,210}. The observed induction of pro-inflammatory cytokines was however consistent with pre-existing literature; following influenza A infection, epithelial cells release IL-6 and IL-8²¹¹.

Potentially, the lack of observed intergroup difference in innate immune response may have been due to the hBECs losing their 'obese' phenotype over the 2-3 week period of cell culture. However, hBECs cultured from asthmatic individuals exhibit attenuated IFN responses following rhinovirus infection relative to hBECs from non-asthmatics, implying maintenance of the host phenotype *ex vivo* throughout the duration of cell culture¹⁶⁸.

There are several limitations to the hBEC data presented. Firstly, the level of differentiation of hBECs, which may have a profound effect on the expression of sialic acid receptors, virus replication competence and cytokine responses, including type I IFN²¹², was not evaluated in this study. However, each subject's hBECs were processed, cultured and seeded and infected in the same manner at the same passage. Secondly, each cell type was evaluated in isolation and therefore the relative contribution of the hBECs to overall pulmonary inflammation in the context of influenza infection is unclear.

4.5.2 Exogenous leptin administration did not alter the IFN or pro-inflammatory cytokine responses of hBECs from control subjects to influenza infection

In an attempt to mimic the obese state, hBECs from the lean controls were pre-exposed to high-dose leptin for 16 hours prior to infection and throughout the subsequent time course. The IFN and pro-inflammatory cytokine responses post-influenza infection were then compared with the responses of hBECs from lean individuals infected in medium alone; however, no observable differences in the IFN or pro-inflammatory cytokine expression profiles resulted.

Several groups have demonstrated the presence of the functional isoform of the leptin receptor on lung epithelial cells (Table 1-7) and shown a biological consequences of leptin exposure (Table 1-8); however, the effect of exogenous leptin on innate viral responses has not previously been evaluated. The lack of an effect in this study may have been due to lack of biological activity of the leptin used; with hindsight, a positive control to demonstrate that the exogenous leptin administered was biologically active should ideally have been included. Additionally, the cultured hBECs may not have expressed (or indeed may have downregulated) the functional form of the leptin receptor. Evaluation of the presence of ObRb by RT-qPCR, flow cytometry or immunohistochemistry would exclude this.

Exogenous leptin was applied to the hBECs only as lung epithelial cells have been shown to have relatively high constitutive SOCS expression (Table 1-6) and to respond to exogenous leptin¹³⁹. Furthermore, leptin pre-exposure experiments on the other cell types (PBMCs, DCs and BAL cells) were not feasible due to cell yield, financial and time constraints. With hindsight, the author would have performed leptin pre-exposure experiments on the BAL cells, as a relative IFN deficiency post-influenza infection was subsequently identified in these cells.

4.5.3 *Obesity did not alter the IFN or pro-inflammatory cytokine responses of PBMCs to influenza infection*

PBMCs comprise lymphocytes (T cells, B cells and NK cells), monocytes and dendritic cells; whilst PBMC functional assessment is not strictly a measure of the innate immune response it was included in the ObFlu study with the intention that, if any intergroup differences were identified, further dissection of the cell type responsible could be carried out.

Several groups have previously evaluated the responses of PBMCs from obese individuals to TLR agonists^{166,213-215}; Teran-Cabanillas *et al* took PBMCs from 30 obese (BMI ≥ 30 kg/m²) and 30 non-obese (BMI <25 kg/m²) individuals and stimulated them (1×10^6 cells/well) with poly (I:C), a TLR 3 agonist, and CL264, a TLR 7 agonist at a concentration of 5µg/ml¹⁶⁶. Supernatants were harvested at 20 hours and IFN α , IFN β , IL-6 and IL-1 β protein levels measured by ELISA. IFN α 2 and IFN α 6 mRNA expression was also assessed by RT-qPCR. The authors reported significant attenuation of IFN α protein expression in the PBMCs from obese individuals following TLR 7 stimulation and significant attenuation of IFN β protein expression following TLR 3 stimulation. IFN α 2 and IFN α 6 mRNA induction was also shown to be significantly diminished following both TLR 3 and TLR 7 stimulation. Notably, the IFN β protein expression data post-TLR 7 stimulation and the IFN α protein data post-TLR 3 stimulation were not reported, nor were any data regarding IFN β mRNA expression.

In light of this publication, PBMCs from the ObFlu study population were stimulated with a panel of TLR ligands, namely: R848 (a TLR 7/8 agonist), Poly (I:C) (a TLR 3 agonist, 100µg/ml), CPG-A (a TLR 9 agonist, 1µM), LPS (lipopolysaccharide, a TLR 4 agonist) and LTA (lipoteichoic acid, a TLR 2 agonist). The ObFlu protocol differed from that of Teran-Cabanillas *et al* in that 2×10^5 cells/well were stimulated, the supernatant was harvested at 8 and 24 hours and higher concentrations of TLR ligand used. Additionally, the ObFlu cohort included 15 subjects per group whereas the Teran-Cabanillas *et al* paper included 30 per group.

Notably, the IFN deficiency identified by Teran-Cabanillas *et al* was not reproduced. No significant induction of IFN α , β or λ was observed in the PBMCs following poly

(I:C) stimulation and no intergroup difference was detected; evidence that the poly (I:C) used was biologically active came from the pro-inflammatory cytokine data, which clearly showed induction of IL-6, IL-8 and TNF α . Indeed, this data also contradicted that of Teran-Cabanillas *et al* as no obesity-related deficiency in IL-6 induction was observed following poly (I:C) stimulation. Adding to the uncertainty as to whether obesity attenuates, augments or makes no difference to IFN/cytokine induction, Dicker *et al* stimulated PBMCs from obese and healthy-weight individuals with LPS (20ng/ml) for 24 hours and reported significantly higher TNF α and IFN γ levels in the supernatants from the obese group in addition to significantly less IL-10²¹³.

It could be suggested that the ObFlu study was underpowered to detect a difference in IFN expression, given that Teran-Cabanillas *et al* used twice as many subjects; however, to counter that suggestion, a subsequent paper published by the same group reported significant differences in IFN production between obese and lean populations with as few as four subjects per group¹⁶⁶. Additionally, Yen *et al* recently reported significant obesity-associated deficiencies in IFN γ , IL-6 and TNF α protein production in whole blood stimulated with TLR agonists with 16 lean and 21 obese subjects²¹⁴. Finally, Ahmad *et al* detected significant differences in unstimulated baseline PBMC mRNA expression of TNF α and IL-6 with only four subjects per group²¹⁵.

Whilst several studies have assessed the cytokine profile of PBMCs from obese individuals following TLR stimulation, only one methodologically sound paper has been published containing data generated following influenza virus stimulation. Paich *et al* performed cytokine analysis in the supernatants of PBMCs stimulated *ex vivo* with pH1N1/09 from 15 healthy weight, 14 overweight and 16 obese individuals 66 h.p.i (MOI 1)²¹⁶. Whilst they reported no differences in the levels of IL-12 and IL-7, they found that IL-5 levels were higher and IFN γ trended lower in the supernatants from obese individuals. Consistent with the main ObFlu hypothesis, they also reported impaired upregulation of TNF α and IL-6 following stimulation.

The observed pattern of pro-inflammatory cytokine induction, especially the suppression of IL-8 expression, was not unexpected; Sprenger *et al* previously infected

PBMCs with influenza A viruses and demonstrated upregulation of pro-inflammatory cytokines (including TNF α and IL-6) and mono-nuclear cell attracting chemokines but suppression of neutrophil chemokines (including IL-8 and GRO- α)²⁷³. The mechanism underpinning this suppression does however require further elucidation.

In short, the PBMC data presented in this chapter contrasts somewhat with the existing literature in that no intergroup difference in either IFN or pro-inflammatory cytokine induction upon influenza virus infection or TLR stimulation was evident. Whereas others have suggested that obesity-associated TLR impairment may explain the inadequate responses of obese individuals to influenza¹⁶⁶ this report does not support this concept. The absence of an intergroup difference may however have been due to methodological differences, for example, differences in TLR agonist dose, influenza virus strain and MOI, duration of stimulation and timing of supernatant harvesting.

4.5.4 Obesity did not alter the IFN or pro-inflammatory cytokine responses of DCs to influenza infection

Dendritic cells (DCs) are resident in the peripheral blood and lung and, through stimulation of naïve T cells, form the bridge between the innate and adaptive immune systems²¹⁷. There are two main subsets of DCs: plasmacytoid DCs (pDCs) and conventional DCs (cDCs). pDCs are potent producers of type I IFN and express TLRs 7-9 which, in turn, can be stimulated by the ssRNA of influenza viruses²⁰⁴. Conversely, cDCs do not produce large amounts of type I IFN, but are the primary cells responsible for presenting antigen to, and promoting the activation of, influenza-specific CD4+ and CD8+ T cells²¹⁸.

Paich *et al* first evaluated the specific effect of obesity on the activation and function of peripheral blood dendritic cells²¹⁶ following influenza infection; PBMCs from 28 healthy weight, 28 overweight and 27 obese individuals were stimulated *ex vivo* with pH1N1/09 (MOI 1) and markers of activation and function measured using flow cytometry at 72 h.p.i. Despite, unsurprisingly, observing increases in DC cell numbers upon infection, no difference in DC activation or function (measured by expression of CD80 and CD86), MHC II and IL-12) was reported.

In contrast, O'Shea *et al* reported that, in an obese cohort with a mean BMI of 51.7 kg/m² circulating DC numbers were significantly decreased relative to lean and that, following TLR stimulation, DCs from the obese cohort upregulated significantly less CD83 (a molecule important in the initiation of T cell responses) and produced twice as much IL-10 (an immunosuppressive cytokine). They concluded that obesity negatively impacted the ability of DCs to mature and elicit appropriate T-cell responses to general stimuli²¹⁹.

The DC data presented in this chapter adds significantly to the existing literature as the effect of obesity on the type I and III IFN and pro-inflammatory cytokine profiles of primary peripheral blood DCs upon influenza infection has not previously been reported. Whilst no intergroup differences in IFN or pro-inflammatory cytokine induction were observed, novel inter-viral differences were noted (see chapter six) with pH1N1/09 and influenza B resulting in the most potent responses. Whilst one of the strengths of this DC data is that it was generated from primary isolated human DCs, rather than monocyte-derived DCs, it is limited by the purity achieved through MACS isolation. FACS would have provided a purer population of DCs and allowed for further analysis of individual DC subpopulations and their relative contribution to IFN/pro-inflammatory cytokine induction; however, this was not feasible given the resources available for the study.

4.5.5 Obesity significantly attenuated the type I & III IFN responses of BAL cells to pandemic and seasonal influenza infection

To date, no studies evaluating the immune responses of BAL cells from obese individuals to respiratory viral infection have been published. Indeed, only two papers, one of which was subsequently retracted¹⁴¹, concerning obesity and BAL cells exist in the literature; Sharma *et al* found that constitutive IL-6 mRNA expression was significantly elevated in the AMs of obese individuals relative to lean¹⁸¹.

The data presented in this chapter show a significant attenuation in the type I and III IFN responses upon pandemic influenza A and seasonal influenza A and B infection. Additionally, constitutive pro-inflammatory cytokine expression was significantly lower in the unstimulated BAL cells from the obese subjects relative to lean. Combined with the attenuated IFN responses and SOCS expression data presented in

chapter five, these results suggest that an, as yet unidentified, obesity-associated negative regulator may be suppressing BAL cell transcriptional activity.

Alveolar macrophages play a key role in host defence²²⁰ and homeostasis of the inflammatory environment within the lung²⁰⁵, thus the attenuation of the innate immune responses demonstrated in this chapter may contribute to the adverse outcomes seen in the obese following influenza infection. As mentioned earlier, AMs are professional immune cells that are prolific inducers of IFN rather than major sites of viral replication²²¹. Whilst stimulation of the BAL cells in the two groups was equivalent (as measured by M gene copy number), no increase in copy number suggestive of replicative infection was observed with time. Nevertheless, significant IFN responses were observed in the BAL cells from the lean individuals and significant pro-inflammatory responses in the BAL cells from the obese cases. With hindsight, a dose-response experiment would have been performed initially using various MOIs to determine the optimum MOI to elicit the maximal IFN response; however, this was not feasible given the practicalities of obtaining primary BAL cells.

The lack of attenuation of the MxA response in the BAL cells from the obese subjects is surprising given the blunted IFN responses but not necessarily inconsistent with the overall hypothesis given that BAL cells are primarily IFN producers rather than responders. IFNs act in an autocrine and paracrine fashion so it is unexpected that the MxA responses are not attenuated in the BAL cells; nevertheless, in the overall context of influenza infection, the BAL cell ISG responses are arguably relatively unimportant compared to the ISG responses of the hBECs to IFN in controlling viral replication. It could thus be envisaged that the deficient BAL cell IFN responses result in deficient ISG responses in the hBECs, leading to increased viral replication. A co-culture model, in which both BAL cells and hBECs are present, would address the mechanism more appropriately.

BAL cells are comprised predominantly of macrophages (>90%); the remaining 10% of cells are lymphocytes, neutrophils and eosinophils¹²⁸. Indeed, data generated previously in our laboratory have shown that 95% of the cells present in the BAL fluid of healthy individuals are alveolar macrophages (AMs)¹⁷¹. Furthermore, AMs are the predominant type I & III IFN producing BAL cells²²². One caveat to the presented data

is that total BAL cells, rather than AMs *per se*, were stimulated and the exact cellular constitution of the BAL fluid was not determined. However, this approach, using total BAL cells, has previously been used to successfully demonstrate IFN deficiency in asthma¹⁶⁸. The BAL cells in this study were all isolated and manually quantified in the same manner using a haemocytometer; however, the exact cell differential was not quantified by cytocentrifugation due to time constraints on the bronchoscopy day. It is possible, although unlikely, that the constitution of the BAL cells may have differed between the obese and lean groups; Holguin *et al* have previously shown no significant differences in BAL cell differential between obese and lean non-asthmatic subjects¹²⁸. In future studies, cytocentrifugation of BAL fluid will be performed to determine the BAL fluid cell differential. The fact that the only significant intergroup differences in IFN expression were identified in the BAL cells suggests that some, as yet unidentified factor, localised to the environment from which the BAL cells were isolated was responsible. Given that the hBECs, which were cultured over a 2 to 3-week period did not exhibit the same attenuated IFN response, one could also speculate that the effect of this factor is relatively short-lived.

There was one confounding factor that must be addressed; the general anaesthesia (GA) that the obese subjects underwent. For safety and practicality all of the obese subjects underwent their bronchoscopy via an endotracheal tube whilst under GA immediately prior to their bariatric surgery. The induction element of the anaesthetic comprised a volatile inhalational agent (sevoflurane) and a parenteral element (propofol). None of the lean subjects underwent GA and therefore the global attenuation in IFN and inflammatory responses observed in the BAL cells could conceivably have been due to the GA.

Each bariatric bronchoscopy took a maximum of 25 minutes, with the BAL cells being procured after approximately six minutes. The cells were then taken to the laboratory, washed and processed immediately, so that the BAL cells were in the incubator, ready for infection within an hour of the start of the bronchoscopy. For the GA to be responsible for the observed attenuation, it would have to globally ‘shut down’ the BAL cells innate immune response within six minutes and maintain this ‘shut down’ state throughout the subsequent 48 hour *ex vivo* time course. The BAL cells from the

obese group were still able to mount IFN α and IL-6 responses to infection, along with the ISG, MxA providing evidence that the cells were still functional despite the GA.

There is a small body of literature concerning GA and the immune function of alveolar macrophages. Kotani *et al* evaluated human alveolar macrophage viability, phagocytic and microbicidal activity at 0, 2, 4 and 6 hours after anaesthetic induction with either propofol (n=30) or isoflurane (n=30) in patients undergoing orthopaedic surgery²²³. They reported that anaesthesia did eventually significantly decrease phagocytic and microbicidal ability (IFN nor cytokine production was assessed), but only after 4 hours of continuous anaesthesia; the BAL cells isolated in the ObFlu study were removed within 20 minutes of anaesthetic induction.

One approach to addressing the potential effect of the anaesthetic would be to expose the BAL cells directly to the anaesthetic agent *ex vivo*; this approach was adopted by Steurer *et al* who applied sevoflurane to rat AMs and measured inflammatory mediator expression upon LPS stimulation²²⁴. They reported attenuated TNF α , CINC-1 and MCP-1 induction following LPS, but only again after a minimum of 2 hours exposure to the anaesthetic agent.

In summary, the attenuation of the immune response observed in the BAL cells from the obese individuals could potentially have been due to the effect of the general anaesthesia however, given the duration of exposure to the anaesthetic agent i.e. <25 minutes, it is unlikely. To address this issue more formally, the BAL cells from the obese individuals should be re-sampled under GA after the subjects have lost a significant amount of weight post-bariatric surgery. If the IFN/pro-inflammatory cytokine response is restored then it can be reasonably assumed that the deficiency was due to obesity rather than the effects of GA.

4.6 Conclusions

This is the first report of defective type I and III IFN protein production following pandemic and seasonal influenza A and B infection in BAL cells from obese individuals. Furthermore, this is the first study in humans to assess the effects of obesity on the host innate immune response to respiratory viruses. Although the reason why obese individuals experience more severe outcomes post-influenza

infection is undoubtedly multifactorial, impaired BAL cell innate immune responses may contribute.

As alluded to earlier, it is important to consider which cells types are the main producers of, or responders to, the influenza-induced IFNs and cytokines. The primary site for influenza replication is the respiratory epithelium, which can also produce and respond to IFN. Although no intergroup differences in IFN or pro-inflammatory cytokine production were observed in the hBECs *per se*, the hBEC cytokine responses to virus are likely not the key factor in determining outcome; their responses to IFNs and cytokines produced by the professional, lung-resident immune cells are probably more important. Unfortunately, our model was limited as it lacked intercellular interaction; one could potentially envisage though that, due to the attenuated production of type I & III IFN by BAL cells, the induction of ISGs in the hBECs would be suboptimal, resulting in increased viral load and predisposing to worse outcomes.

Given that obesity is considered a systemic disease characterised by chronic, low-grade inflammation it is surprising that the obesity-associated IFN deficiency identified in this study was found only in the BAL cells, whereas paradoxically in asthma, which is considered an inflammatory disease of the airways, IFN deficiency has been described in hBECs, BAL cells and peripheral blood cells [Gehlhar K 2006].

Unfortunately, one of the major problems with interpretation of the data generated by this study, and that of the obesity-influenza field as a whole, is that no information concerning the actual pathogenesis of the influenza-associated adverse outcomes in the obese has been published. Numerous observational studies and systematic reviews outlining the epidemiological association between increasing BMI and adverse outcomes are available, yet no papers detailing *obesity-specific* pathological, autopsy, viral load, peripheral blood or pulmonary cytokine/IFN data are available. In future influenza pandemics, or indeed during seasonal influenza epidemics, collection and publication of obesity-specific pathological data would help put this study's findings into context.

The implications of the addition of obesity to the canon of potential IFN deficient states are two-fold: firstly, from a public health perspective the evidence from this

report, in conjunction with the epidemiological evidence supporting the association between increased BMI and adverse outcomes, provides a persuasive incentive for those individuals who are morbidly obese to normalize their BMI. Secondly, the observed obesity-related IFN deficiency in BAL cells implies that supplementary, inhaled IFN therapy may be beneficial in the treatment of morbidly obese individuals with severe influenza infection. Indeed, this is not a novel concept; trials of inhaled IFN β have shown beneficial effects for virus-induced exacerbations in individuals with difficult-to-treat asthma²²⁵. To explore this further, potential future work could include a placebo-controlled trial of inhaled IFN β in the DIO mouse-influenza infection model to determine whether it has any beneficial effect on lung injury and mortality.

4.7 Summary of key points

- Obesity significantly attenuated the type I & III IFN responses of BAL cells to infection with pH1N1/09, sH3N2 and influenza B
- Obesity did not alter the IFN or pro-inflammatory cytokine responses of hBECs, PBMCs or DCs to pandemic or seasonal influenza infection
- The application of exogenous high-dose leptin to primary hBECs did not alter the IFN or pro-inflammatory cytokine responses to pandemic or seasonal influenza infection
- Obesity did not alter the IFN or pro-inflammatory cytokine responses of PBMCs to TLR agonists

5 The effect of obesity on the inflammatory environment and negative regulator expression in the airways and blood

5.1 Introduction

Over the past two decades it has become evident that obesity gives rise to a heightened state of inflammation¹¹⁰. The link between obesity and inflammation was first revealed by Hotamisligil *et al* who demonstrated a positive correlation between adipose tissue mass and the expression of TNF α ¹¹¹. Subsequently, increased plasma levels of several pro-inflammatory cytokines e.g. IL-6 and acute phase proteins e.g. C-reactive protein (CRP) have been described in obesity; many of which appear to originate from adipose tissue^{112,113}. These observations have led to the general perception that obesity is a state of chronic low-grade inflammation that is initiated by changes within adipose tissue.

Consequently, adipose tissue is no longer considered to be simply an energy-storage depot; it has endocrine functions, producing ‘adipokines’ that exert immunomodulatory effects. The most extensively studied adipokine expressed in the lung (by AMs, hBECs and type II pneumocytes) is leptin¹¹⁴. Identified in 1997, leptin is a 16kDa pro-inflammatory adipokine produced predominantly by white adipose tissue that increases, in serum and BAL fluid¹¹⁵, in proportion to body adiposity and whose primary function is appetite control via the hypothalamus¹¹⁶. Besides leptin and TNF α , adipose tissue produces a range of other adipokines with well-described effects on metabolism and systemic inflammation, including adiponectin, resistin, visfatin and adipsin¹¹⁷; however, the relationship between obesity and the pulmonary expression of these latter adipokines remains to be fully elucidated.

Resistin, also known as ‘found in inflammatory zone 3’ (FIZZ3) is encoded by the *RETN* gene and is predominantly produced by PBMCs¹¹⁸. Although the receptor is currently unidentified, resistin is thought to promote both insulin resistance and inflammation¹¹⁹. To date, the presence of resistin has not been demonstrated in BAL fluid and its role in respiratory infection has not been studied. Of relevance for the mechanistic model proposed in chapter one (Figure 5-1), resistin has been shown to upregulate SOCS3 in adipocytes¹¹⁹ and human endothelial cells²²⁶. It is uncertain whether it has the same effect on human epithelial cells.

Visfatin, or nicotinamide phosphoriboxyltransferase (NAMPT), is also pro-inflammatory¹²⁰ and produced by adipocytes and macrophages. It has been shown to be present in BAL during acute lung injury¹²¹; however, its role in the lung has not been fully established. Adipsin, alternatively known as complement factor D, is a member of the complement system and cleaves factor B¹²². It is also pro-inflammatory and produced primarily by adipocytes and monocytes/macrophages¹²³. Again, it is uncertain whether it is expressed in the lung, although plasma levels have been shown to correlate with certain occupational lung diseases^{124,125}.

Unlike the adipokines mentioned above, adiponectin, produced primarily by adipocytes, is anti-inflammatory¹²⁶. It is present in BAL and the adiponectin 1 receptor is expressed by lung epithelial cells^{127,128}. Whilst adiponectin has been shown to abrogate TNF α production by macrophages¹²⁹ and inhibit TLR-mediated NF κ B activation in macrophages¹³⁰, very little is known about the role of adiponectin in the lung.

The theoretical model presented in chapter one to explain the increased susceptibility of obese individuals to severe outcomes following influenza infection postulated that obesity-associated hyper-leptinaemia would increase background levels of the negative regulator, SOCS3, in key structural and immune cells and that this, in turn, would attenuate IFN expression and signal transduction, thus diminishing ISG induction (Figure 5-1).

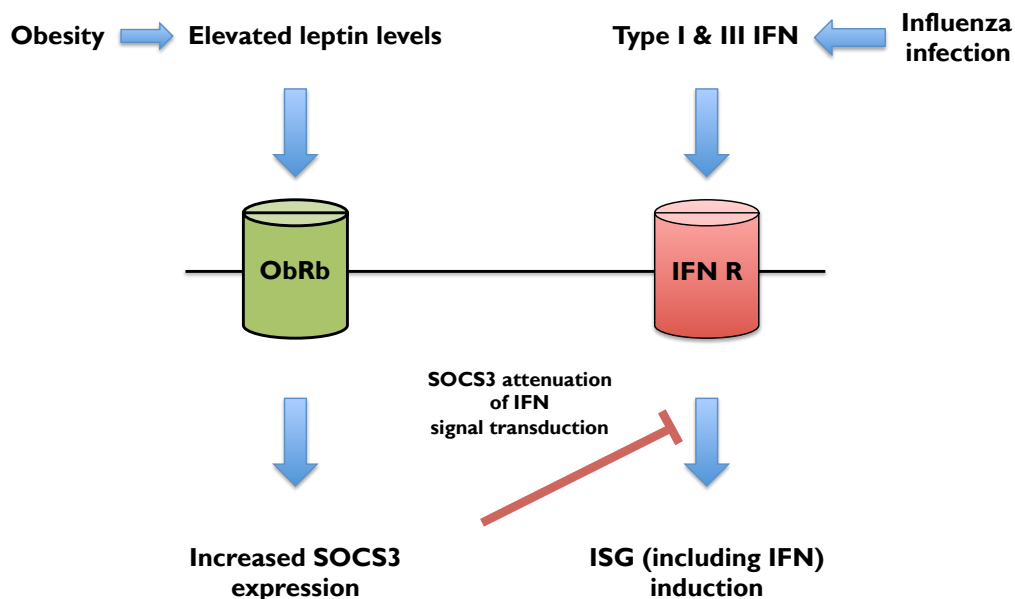


Figure 5-1 Theoretical model describing how obesity may attenuate IFN signal transduction, thus predisposing to severe influenza outcomes

Obesity is a leptin-resistant state in which leptin levels are chronically elevated due to increased body adiposity. The functional isoform of the leptin receptor, ObRb, which signals via a JAK-STAT signalling cascade, is expressed on key respiratory and immune cells. Leptin binds these ObRb receptors inducing SOCS3 expression, which negatively feeds back on ObRb signal transduction. Leptin-induced SOCS3 also attenuates influenza-induced IFN signal transduction on the same cells, thus resulting in diminished ISG (including IFN) expression.

Having recruited and characterised an appropriate study population in which to test this model (chapter 3) and demonstrated IFN deficiency post-influenza infection in BAL cells from obese subjects (chapter 4) the inflammatory constitution of the environment from which these cells were procured and the expression of SOCS3 are presented in this chapter.

For the model to hold true, four additional conditions need to be met. Firstly, the obese subjects should exhibit elevated baseline leptin levels. Secondly, leptin must be shown to increase SOCS3 expression in the cells of interest. Thirdly, increased constitutive expression of SOCS3 needs to be demonstrated in the obese subjects. Finally, SOCS3 must be shown to be capable of attenuating IFN signal transduction post-influenza infection. The results of the investigation of these latter four requirements are the primary focus of this chapter.

To test the hypothesis that leptin levels were elevated in the obese and associated with increased constitutive expression of SOCS3 samples of nasal epithelium, nasal lining

fluid (NLF) and peripheral blood were taken from both groups, obese and lean, immediately prior to bronchoscopy. During bronchoscopy, BAL and bronchial lining fluid (BLF) samples were obtained. Protein levels of the adipokines discussed above, acute phase proteins and cytokines/chemokines were subsequently quantified using the Luminex[®] immunoassay platform. Furthermore, mRNA expression of SOCS1 and SOCS3 was quantified (by RT-qPCR) in nasal epithelium, hBECs, PBMCs, BAL cells and DCs at baseline (i.e. prior to influenza infection).

To demonstrate the potential modulating effects of SOCS on IFN expression, A549 cells (an immortalised, adenocarcinoma human alveolar epithelial cell line) were transfected with plasmids over expressing SOCS1, or SOCS3 or empty vector control plasmid pORF and infected with pH1N1/09. IFN β and IFN λ 1 promoter activity was subsequently assessed using the luciferase reporter system. Finally, to evaluate the effects of leptin on SOCS3 expression, hBECs were exposed to exogenous high-dose leptin for 16 hours and SOCS3 mRNA expression, measured by RT-qPCR, compared with expression in hBECs cultured in BEBM alone.

Throughout this chapter, the evidence for each section of the model illustrated in Figure 5-1 will be presented. The adipokine protein levels in the various body compartments are presented initially, followed by the acute phase protein and cytokine/chemokine Luminex[®] data. The results of the SOCS transfection experiments are then outlined, followed by the constitutive SOCS1 and SOCS3 mRNA expression data for the hBECs, BAL cells, DCs and PBMCs. Finally, the results of the *ex vivo* experiments in which exogenous high-dose leptin was administered to hBECs from the lean controls, to determine the effects on SOCS3 mRNA expression, are presented.

5.2 Hypotheses

- Pro-inflammatory adipokine levels will be elevated and anti-inflammatory adipokine levels diminished in the airways and serum of obese individuals relative to those of healthy weight controls.
- Acute phase protein levels will be elevated in the airways and serum of obese individuals relative to those of healthy weight controls.

- Pro-inflammatory cytokine protein levels will be elevated in the airways and serum of obese individuals relative to those of healthy weight controls.
- Constitutive SOCS3 expression will be higher at baseline (i.e. prior to infection) in primary hBECs, BAL cells, PBMCs and DCs from obese subjects relative to normal weight comparators.
- SOCS1 and SOCS3 over expression in A549 cells will attenuate IFN induction following infection with pH1N1/09.
- Exogenous leptin will upregulate baseline SOCS3 mRNA expression in primary hBECs *ex vivo*.

5.3 Aims

- To characterise the adipokine, acute phase protein and cytokine/chemokine milieu within healthy, obese airways from the nose to the alveoli (NLF, BLF, BAL fluid and serum).
- To determine the modulatory effects of SOCS expression on IFN induction following influenza infection.
- To determine whether constitutive SOCS3 expression is elevated in primary hBECs, BAL cells, PBMCs and DCs from obese subjects relative to normal weight comparators.
- To determine the effect of exogenous leptin on SOCS3 expression in primary hBECs.

5.4 Results

5.4.1 *Leptin levels were elevated and adiponectin levels diminished in the obese subjects*

Leptin and adiponectin protein levels were quantified (using the Luminex[®] platform) in serum, BAL and for the first time, in NLF and BLF obtained at baseline i.e. immediately prior to (serum, NLF) and during (BAL, BLF) bronchoscopy.

Leptin levels were significantly greater in the serum, BAL, NLF and BLF from the obese subjects [Figure 5-2 A – D] relative to those from the lean subjects. Adiponectin levels were significantly diminished in the serum, NLF and BAL samples [E – G] from the obese subjects relative to those from the lean. A trend towards diminution of BLF adiponectin levels in the obese population was observed [H] but did not achieve statistical significance ($p = 0.14$).

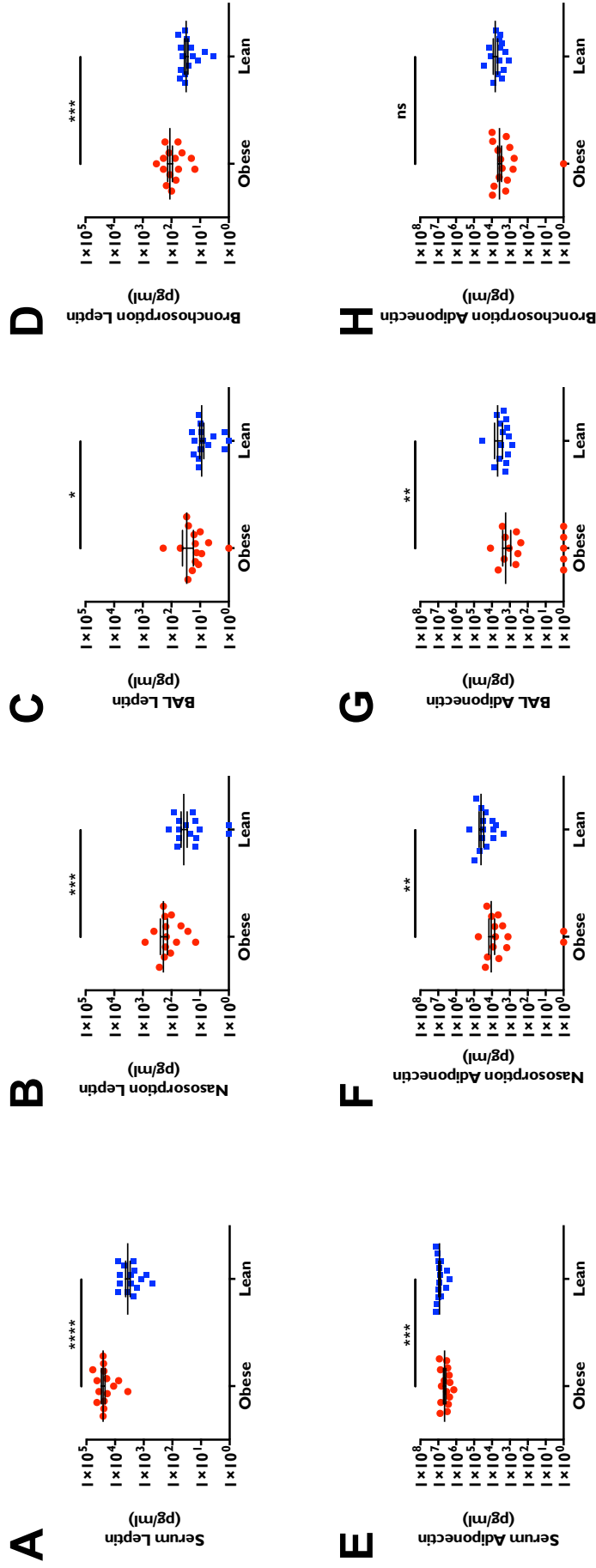


Figure 5-2 Leptin levels were significantly greater and adiponectin levels significantly diminished in the obese subjects.

Leptin [A – D] and adiponectin [E – H] protein levels were quantified (using the Luminex[®] platform) in serum [A, E], nasosorption [B, F], bronchoalveolar lavage [C, G] and bronchosorption [D, H] samples taken from the study subjects during the bronchoscopy visit. Leptin levels were significantly greater in all samples from the obese individuals [A – D]. Adiponectin levels were significantly diminished in serum, nasosorption and BAL samples [E – G] from the obese subjects. There was no significant difference in bronchosorption adiponectin levels [H] between the obese and lean subjects ($p=0.14$). (**** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$, Mann-Whitney U, $n=30$). To enable the plotting of all samples on the logarithmic scale, samples that were reported as zero by the Luminex[®] platform were assigned a value of 1 pg/ml.

Notably, the intergroup differences observed in leptin and adiponectin levels in BAL fluid were mirrored by those in the NLF. However, for both leptin and adiponectin, no significant correlation between NLF and BLF or BAL levels was observed (data not shown). NLF leptin levels were found to correlate with serum leptin levels ($r = 0.5$, $p = 0.0005$) (Figure 5-3 A) and NLF adiponectin levels correlated with serum adiponectin levels ($r = 0.41$, $p = 0.03$) (Figure 5-3 B). When the relationship was assessed in the obese group alone, no significant correlation was present between NLF and serum leptin ($r=0.007$, $p=0.98$, Spearman's correlation) nor NLF adiponectin and serum adiponectin ($r=-0.24$, $p=0.39$, Spearman's correlation).

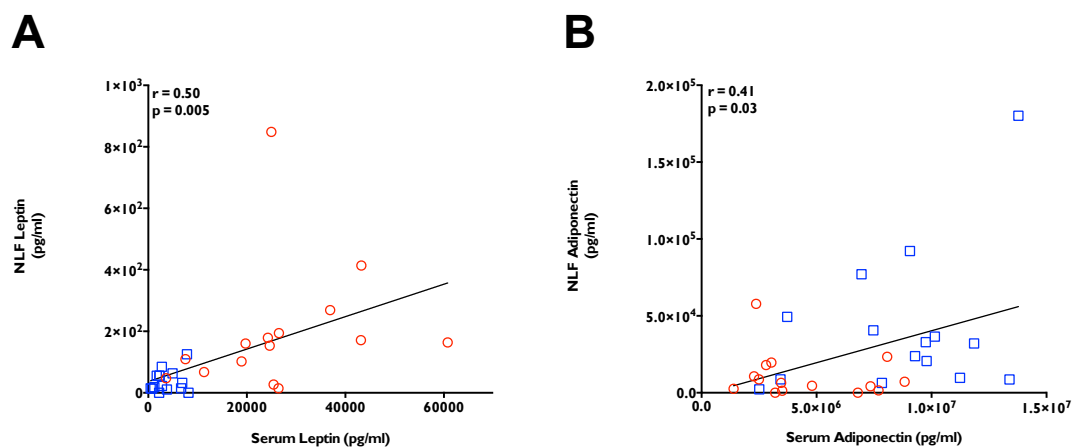


Figure 5-3 NLF leptin and adiponectin concentrations correlated with serum concentrations

Leptin [A] and adiponectin [B] protein concentrations were quantified in nasal lining fluid (NLF) and serum using the Luminex[®] platform. NLF levels were correlated with serum levels. The red circles indicate obese subjects, blue squares indicate healthy weight controls ($n = 15$ per group). NLF leptin levels significantly correlated with serum leptin levels ($r = 0.5$, $p = 0.0005$) [A]. NLF adiponectin levels significantly correlated with serum adiponectin levels ($r = 0.41$, $p = 0.03$) [B]. Statistics: Pearson's correlation coefficient. When the relationship was assessed in the obese group alone, no significant correlation was present between NLF and serum leptin ($r=0.007$, $p=0.98$, Spearman's correlation) nor NLF adiponectin and serum adiponectin ($r=-0.24$, $p=0.39$, Spearman's correlation).

5.4.2 *Pulmonary and peripheral blood leptin levels correlated positively with BMI*

To further establish the relationship between leptin levels and BMI, serum, BAL, NLF and BLF leptin concentrations were correlated with BMI (Figure 5-4). Serum leptin concentration [A] demonstrated a significant, strong positive linear relationship with BMI ($r = 0.87$, $p < 0.0001$), as did BLF leptin concentration ($r = 0.77$, $p < 0.0001$) [D]. BAL leptin concentration exhibited a significant, moderate positive linear relationship with BMI ($r = 0.53$, $p = 0.002$) [C] whilst NLF leptin concentration [B] demonstrated a weak, non-significant positive linear relationship with BMI ($r = 0.33$, $p = 0.07$).

When the correlation was performed in the obese group alone ($n=15$) a significant positive relationship existed between serum leptin and BMI ($r=0.56$, $p=0.03$, Spearman's correlation) [A] and BLF leptin and BMI [D] ($r=0.72$, $p=0.003$, Spearman's correlation). However, no significant relationship was evident between NLF leptin and BMI when the obese group was analysed alone [B] ($n=15$, $r=0.01$, $p=0.98$, Spearman's correlation) nor between BAL leptin and BMI [C] ($r=0.37$, $p=0.18$, Spearman's correlation).

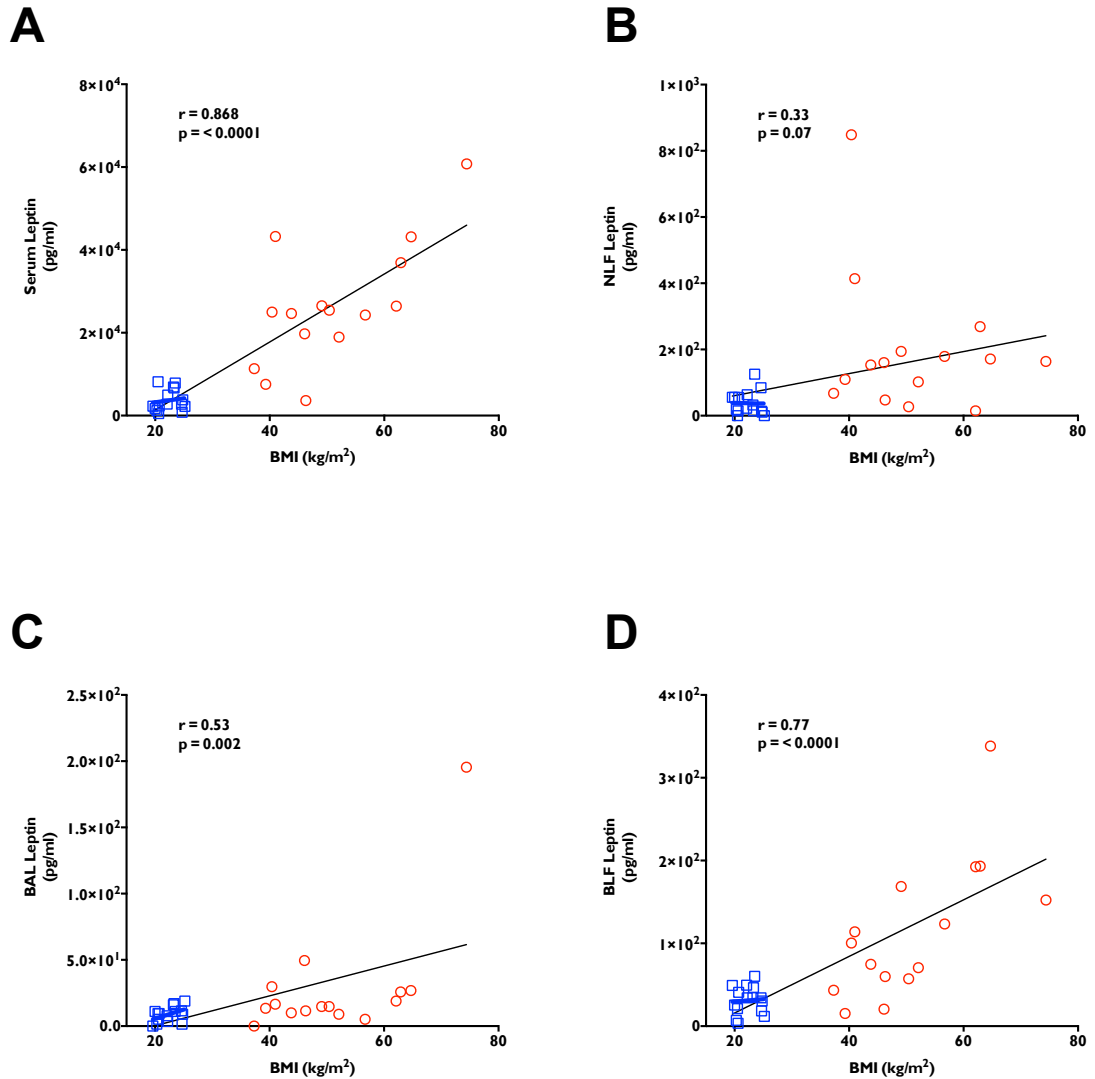


Figure 5-4 Pulmonary and peripheral blood leptin levels positively correlated with BMI

Serum [A], NLF [B], BAL [C] and BLF [D] concentrations of leptin were assessed by Luminex® and plotted against BMI. Red circles = obese subjects, blue squares = lean controls. Significant positive linear relationships were demonstrated for serum, BAL and BLF. NLF leptin concentrations exhibited a weak, non-significant relationship with BMI [B] ($n = 30$, Pearson's correlation coefficient). When the correlation was performed in the obese group alone ($n=15$) a significant positive relationship existed between serum leptin and BMI ($r=0.56$, $p=0.03$, Spearman's correlation) [A] and BLF leptin and BMI [D] ($r=0.72$, $p=0.003$, Spearman's correlation). However, no significant relationship was evident between NLF leptin and BMI when the obese group was analysed alone [B] ($n=15$, $r=0.01$, $p=0.98$, Spearman's correlation) nor between BAL leptin and BMI [C] ($r=0.37$, $p=0.18$, Spearman's correlation).

5.4.3 Nasal and serum adiponectin levels correlated negatively with BMI

To further investigate the relationship between BMI and adiponectin levels, serum, BAL, BLF and NLF concentrations of adiponectin were measured by Luminex[®] and correlated with BMI. No significant correlation was demonstrated in BAL ($r = -0.02$, $p = 0.92$) or BLF ($r = -0.15$, $p = 0.41$) (data not shown). Serum and NLF adiponectin levels exhibited a significant, moderate negative linear relationship with BMI (Figure 5-5).

However, a significant positive relationship existed between serum adiponectin and BMI within the obese group alone ($n=15$, $r=0.56$, $p=0.03$, Spearman's correlation). No significant relationship existed between NLF adiponectin and BMI in the obese group alone ($n=15$, $r=-0.46$, $p = 0.08$, Spearman's correlation)

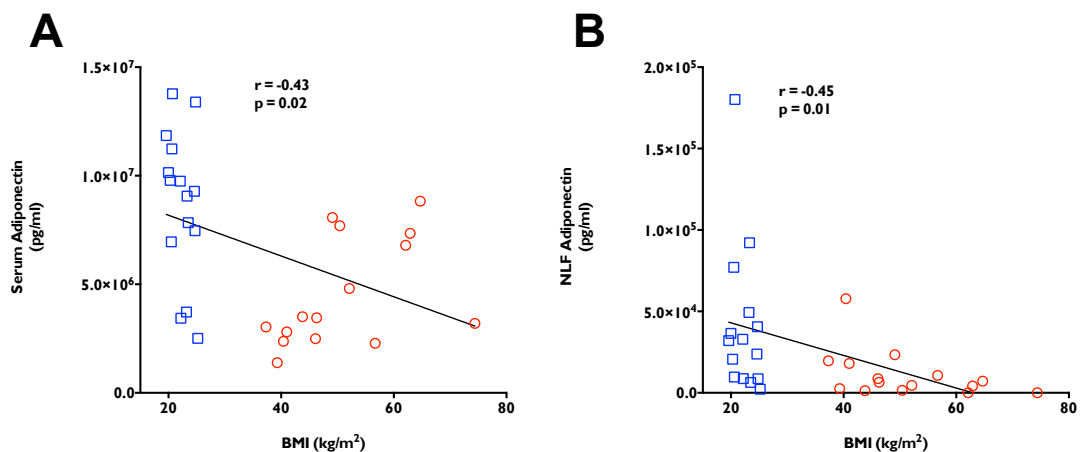


Figure 5-5 Serum and NLF adiponectin levels negatively correlated with BMI

Serum [A] and NLF [B] concentrations of adiponectin were assessed by Luminex[®] and plotted against BMI. Red circles = obese subjects, blue squares = lean controls. Significant negative linear relationships were demonstrated for serum, and NLF. BAL and BLF adiponectin concentrations did not significantly correlate with BMI (data not shown). $n = 30$. Statistics: Pearson's correlation coefficient. A significant positive relationship existed between serum adiponectin and BMI within the obese group alone ($n=15$, $r=0.56$, $p=0.03$, Spearman's correlation). No significant relationship existed between NLF adiponectin and BMI in the obese group alone ($n=15$, $r=-0.46$, $p = 0.08$, Spearman's correlation)

5.4.4 Serum resistin & adipsin levels were significantly greater and BAL visfatin levels significantly lower in the obese subjects

Resistin, visfatin and adipsin protein levels were quantified (using the Luminex[®] platform) in serum, BAL, NLF and BLF obtained immediately prior to and during bronchoscopy (Figures 5-6 & 5-7).

The range of the logarithmic scale used to present the adipokine data in Figure 5-6 has been preserved for each sample type to facilitate comparisons of relative levels of expression across the various samples. By adopting this approach, the ability to closely evaluate the significant differences may have been hindered hence, where significant differences were identified, the data was reproduced, at a more appropriate scale, in Figure 5-7.

Resistin [A] & adipsin [I] levels were significantly greater in the serum of the obese subjects relative to those from the lean group (Figure 5-6). Conversely, visfatin levels in BAL were significantly lower in the obese subjects [G]. No other significant differences in resistin, adipsin and visfatin levels were identified [B – F, H, J - L].

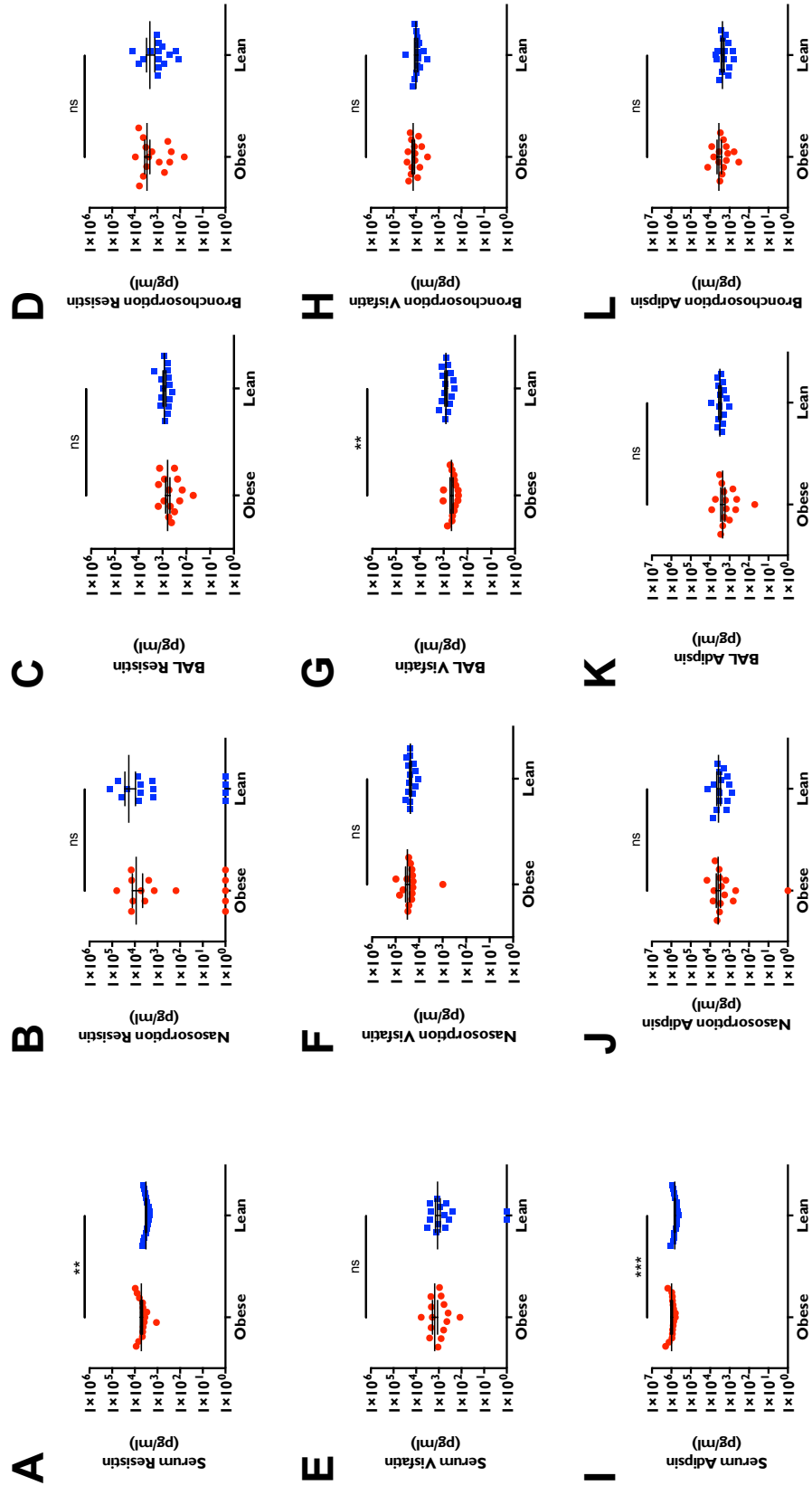


Figure 5-6 Serum resistin & adipsin levels were significantly greater and BAL visfatin levels significantly lower in the obese subjects
 Resistin [A – D], visfatin [E – H] and adipsin [I – L] protein levels were quantified (using the Luminex[®] platform) in serum [A, E, I], nasosorption [B, F, J], bronchoalveolar lavage [C, G, K] and bronchosorption [D, H, L] samples taken from the study subjects during the bronchoscopy visit. Resistin [A] & adipsin [I] levels were significantly greater in the serum of the obese subjects relative to those from the lean group. Conversely, visfatin levels in BAL were significantly lower in the obese subjects [G]. No other significant differences in resistin, adipsin and visfatin levels were identified. (Statistics: Mann-Whitney U, **** p<0.0001, *** p<0.001, ** p<0.01, *p<0.05. To enable plotting of all samples on the logarithmic scale, samples that were reported as zero by the Luminex[®] platform were assigned a value of 1 pg/ml)

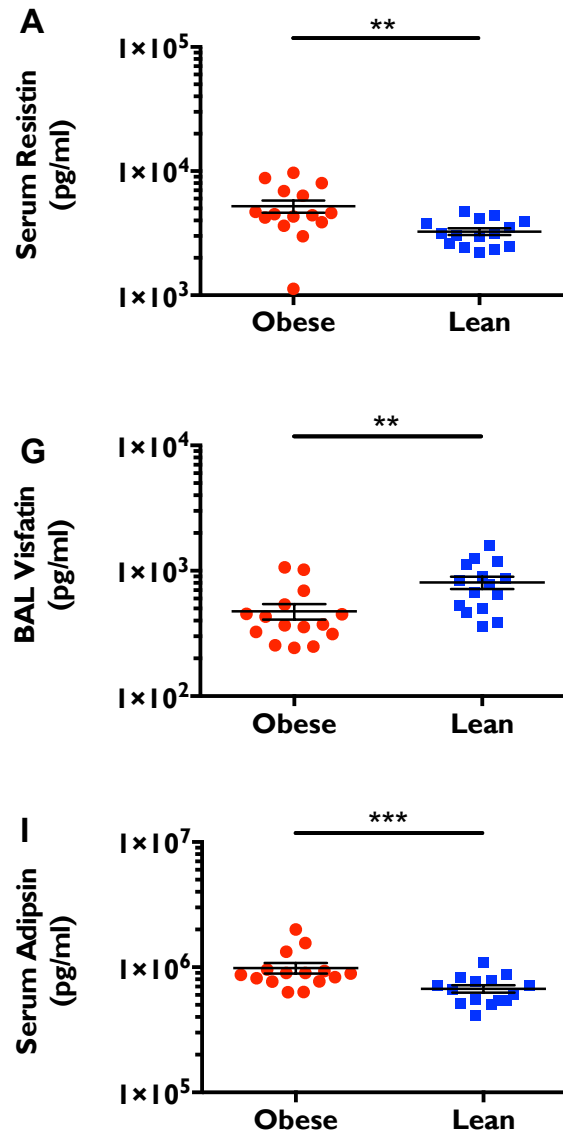


Figure 5-7 Serum resistin & adipsin levels were significantly greater and BAL visfatin levels significantly lower in the obese subjects

Resistin [A] & adipsin [G] levels were significantly greater in the serum of the obese subjects relative to those from the lean group. Conversely, visfatin levels in BAL were significantly lower in the obese subjects [I]. The data used to produce this figure is identical to that used in Figure 5-6 however the logarithmic scale has been optimised to highlight the significant differences. (Statistics: Mann-Whitney U, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

5.4.5 Serum resistin and adipsin levels correlated with BMI

To further evaluate the relationship between resistin and adipsin levels with BMI, serum resistin and adipsin levels were correlated with BMI (Figure 5-8). Both resistin [A] and adipsin [B] levels exhibited a significant, moderate positive linear relationship with BMI. When serum resistin was correlated with BMI in the obese group (n=15) only [A] no significant correlation was identified ($r=-0.08$, $p=0.79$, Spearman's correlation coefficient). However, when serum adipsin was correlated with BMI in the obese group (n=15) only [A] a significant correlation remained evident ($r=0.57$, $p=0.03$, Spearman's correlation coefficient).

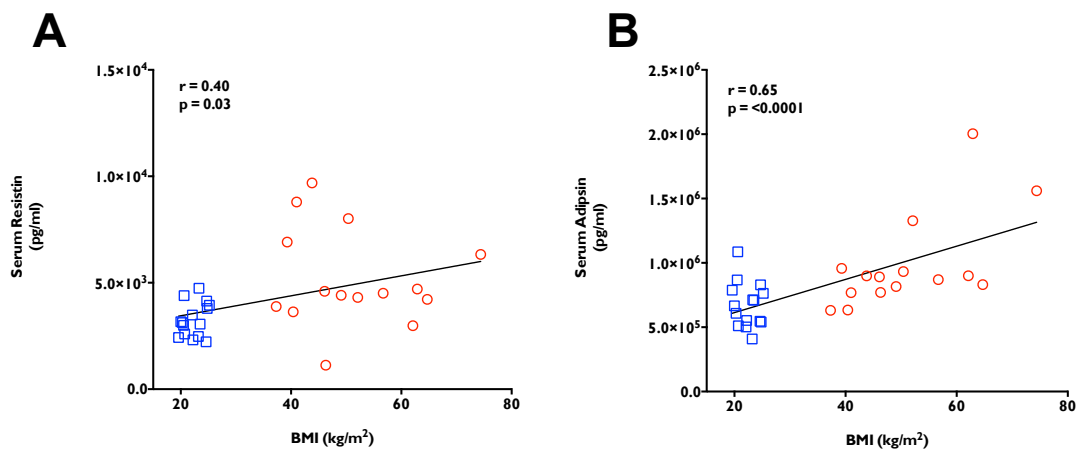


Figure 5-8 Serum resistin and adipsin levels positively correlated with BMI

Serum resistin [A] and adipsin [B] concentrations were assessed by Luminex[®] and plotted against BMI. Red circles = obese subjects, blue squares = lean controls. Significant positive linear relationships were demonstrated (n = 30, Pearson's correlation coefficient). When serum resistin was correlated with BMI in the obese group (n=15) only [A] no significant correlation was identified ($r=-0.08$, $p=0.79$, Spearman's correlation coefficient). When serum adipsin was correlated with BMI in the obese group (n=15) only [A] a significant correlation remained evident ($r=0.57$, $p=0.03$, Spearman's correlation coefficient).

5.4.6 Obesity altered the constitution of acute phase proteins within the bronchoalveolar lavage and nasal lining fluid

To further characterise the effect of obesity on systemic and pulmonary inflammation four acute phase proteins: C-reactive protein (CRP), haptoglobin, α -2 macroglobulin (α 2M) and serum amyloid P (SAP) were quantified, again using the Luminex[®] platform in the serum, BAL, NLF and BLF obtained at baseline.

CRP was significantly elevated in the BAL [B] and NLF [C] of the obese subjects (Figure 5-9). α 2M protein levels were significantly lower in the BAL and nasal lining fluid of the obese subjects [Figure 5-9 F, G]. No significant differences in serum [A, E] or BLF [D, H] levels of either CRP or α 2M were observed.

NLF CRP did not significantly correlate with BAL CRP, nor did NLF α 2M levels correlate with BAL α 2M levels (data not shown).

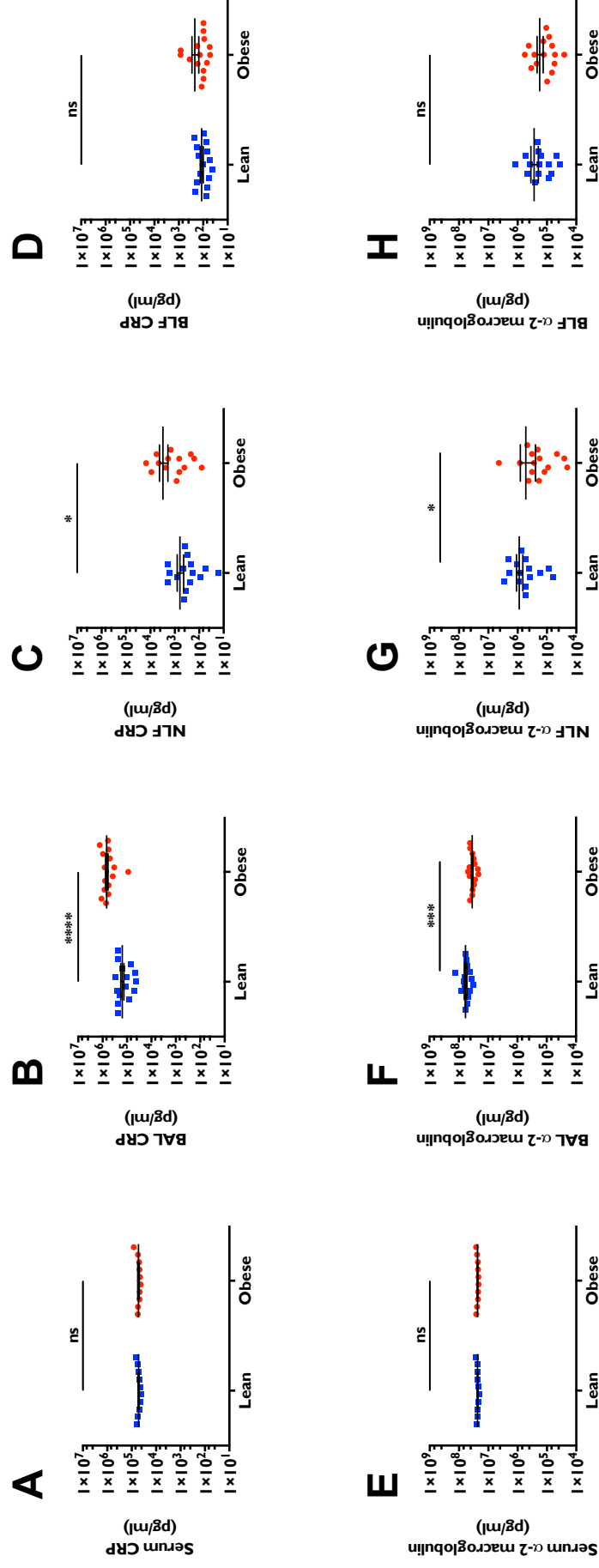


Figure 5-9 Obesity altered the constitution of acute phase proteins within the BAL and NLF.

C-reactive protein (CRP) and α 2M proteins were quantified in serum [A, E], BAL fluid [B, F], NLF [C, G] and BLF [D, H] using the Luminex[®] platform. CRP was significantly elevated in the BAL [B] and NLF [C] of the obese subjects relative to lean. α 2M protein levels were significantly lower in the BAL and nasal lining fluid of the obese subjects [F, G]. No significant differences in serum or BLF levels of either CRP or α 2M were observed. (Statistics: Mann-Whitney U, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

5.4.7 BAL CRP and α 2M levels correlated with BMI

To establish whether a correlation existed between BMI and acute phase protein levels, serum BAL, BLF and NLF CRP and α 2M levels were plotted against BMI (n=30). BAL CRP exhibited a significant, positive linear relationship with BMI while BAL α 2M exhibited a significant, negative linear relationship (Figure 5-10). However, these significant correlations were not evident when the obese group only was analysed (n=15). No acute phase protein/BMI correlations were observed in serum, BLF or NLF (data not shown).

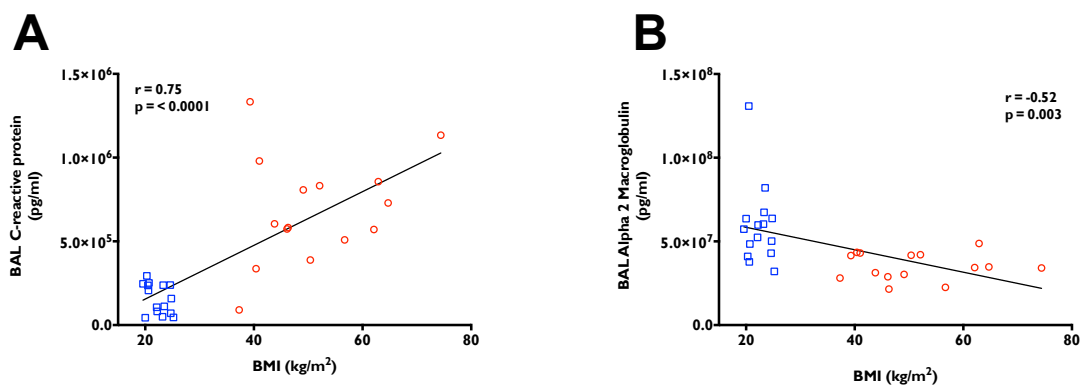


Figure 5-10 BAL CRP and α 2M levels correlated with BMI

BAL CRP [A] and α 2M [B] concentrations were assessed by Luminex® and plotted against BMI. Red circles = obese subjects, blue squares = lean controls. A Significant positive linear relationships was demonstrated for CRP and a significant negative relationship for α 2M. (n = 30, Pearson's correlation coefficient). When BMI was correlated with BAL CRP in the obese group (n=15) only [A] no significant correlation was identified (r=0.24, p=0.39, Spearman's correlation coefficient). When BMI was correlated with BAL α 2M in the obese group (n=15) only [B] no significant correlation was identified (r=0.08, p=0.79, Spearman's correlation coefficient).

5.4.8 Obesity did not alter haptoglobin or serum amyloid P expression

No significant differences in serum, NLF, BAL or BLF protein levels of either haptoglobin or SAP were observed [Figure 5-11 A – H].

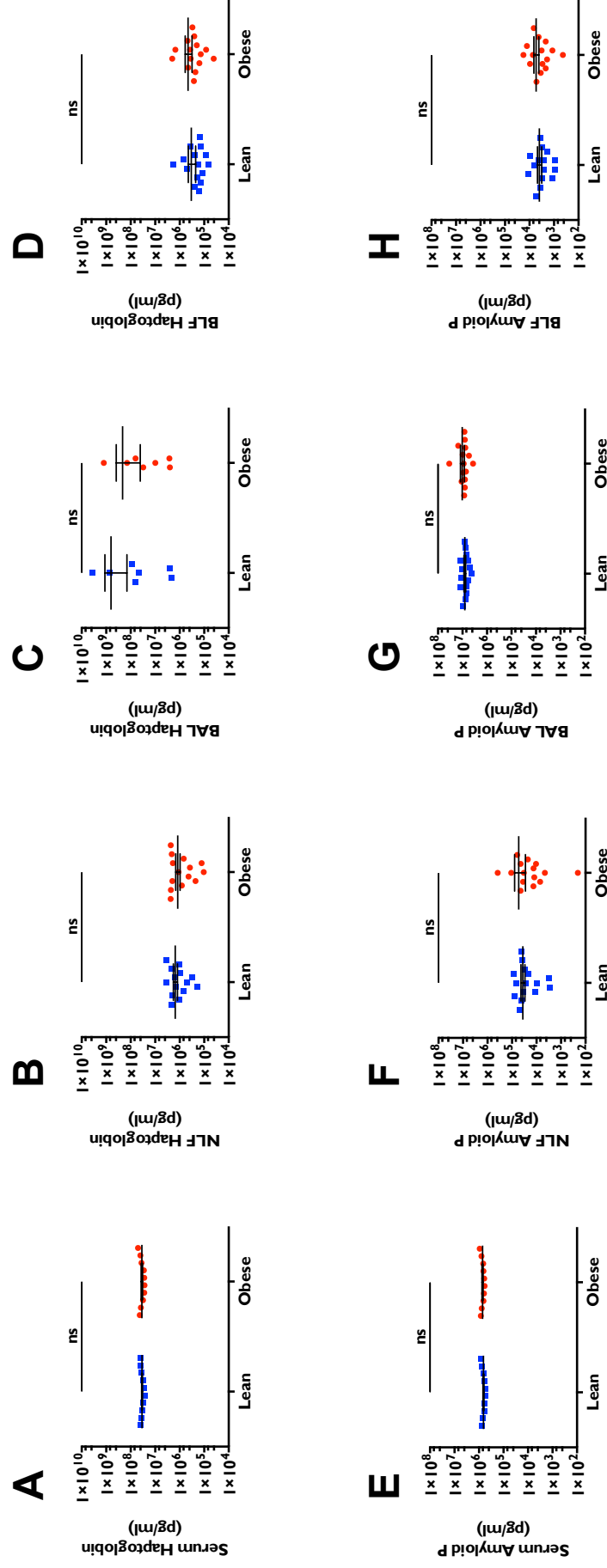


Figure 5-11 Obesity did not alter systemic or pulmonary haptoglobin or SAP protein levels

Haptoglobin and serum amyloid P (SAP) proteins were quantified in serum [A, E], BAL fluid [B, F], NLF [C, G] and BLF [D, H] using the Luminex[®] platform.. No significant differences in serum, NLF, BAL or BLF levels of either haptoglobin or SAP were observed [A – H]. (Statistics: Mann-Whitney U, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

5.4.9 Levels of IL-1 receptor antagonist were significantly diminished in the BLF of obese subjects

To characterise the cytokine and chemokine constitution of the peripheral blood and airways, 27 inflammatory cytokines and chemokines were quantified using the Luminex[®] platform. Unfortunately, due to a technical malfunction, valid readings were obtained only for the NLF and BLF samples. Of the 27 cytokines and chemokines measured only IL-1 β , IL-1RA, IL-8, IP-10, vascular endothelial growth factor (VEGF), 'Regulated on Activation, Normal T cell Expressed and Secreted' (RANTES) and macrophage inflammatory protein-1 β (MIP-1 β) fell within the detection limits of the assay standard curves. The remaining 20 cytokines and chemokines were below the lower limits of detection (LLD) and therefore excluded from analysis: FGF basic, eotaxin, G-CSF, GM-CSF, IFN γ , IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, MCP-1, MIP-1 α , PDGF-BB, TNF α .

With regards to pro-inflammatory cytokines, IL-6 and TNF α were below the LLD and no difference in the protein level of IL-1 β was identified in either NLF or BLF (Figure 5-12); however, BLF levels of IL-1RA were significantly diminished in the obese group [D].

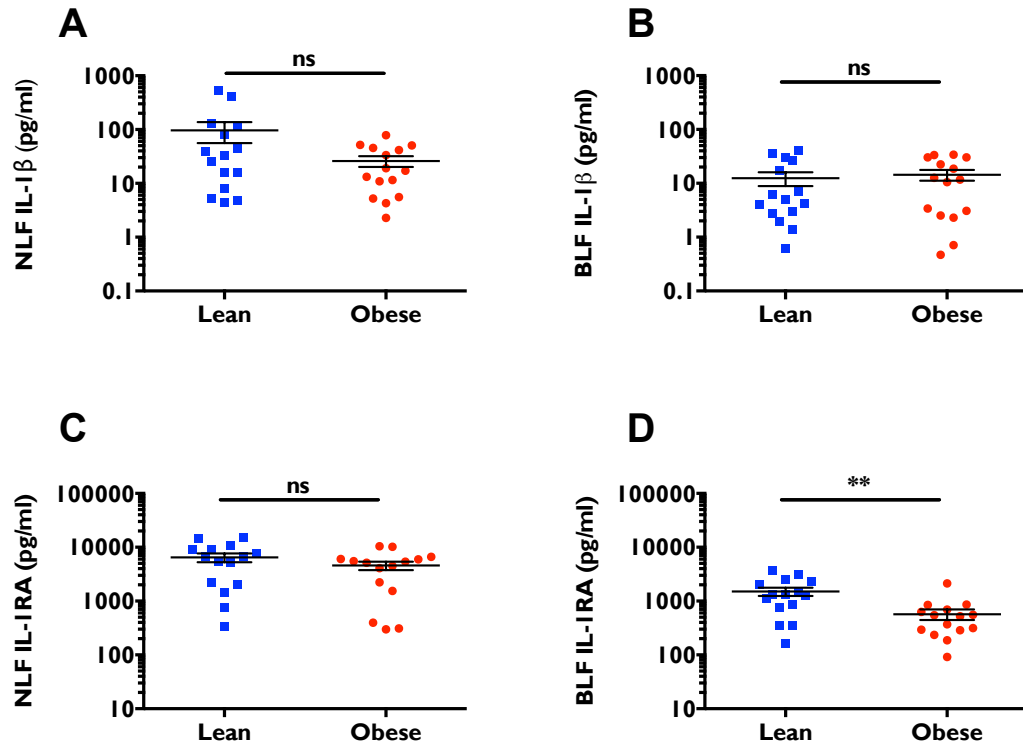


Figure 5-12 Levels of IL-1 receptor antagonist were significantly lower in the BLF of the obese subjects

IL-1 β and IL-1RA protein levels were quantified in nasal [A, C] and bronchial lining fluid [B, D] using the Luminex[®] platform. BLF IL-1RA levels were significantly lower in the obese group. (Statistics: Mann-Whitney U, **** p<0.0001, *** p<0.001, ** p<0.01, *p<0.05.)

5.4.10 Obesity did not alter baseline chemokine levels within nasal or bronchial lining fluid

Of the chemokines whose values were within the assay limits no intergroup differences in baseline IL-8, VEGF, RANTES, IP-10 and MIP-1 β protein levels were observed in the NLF or BLF [Figure 5-13 A – J].

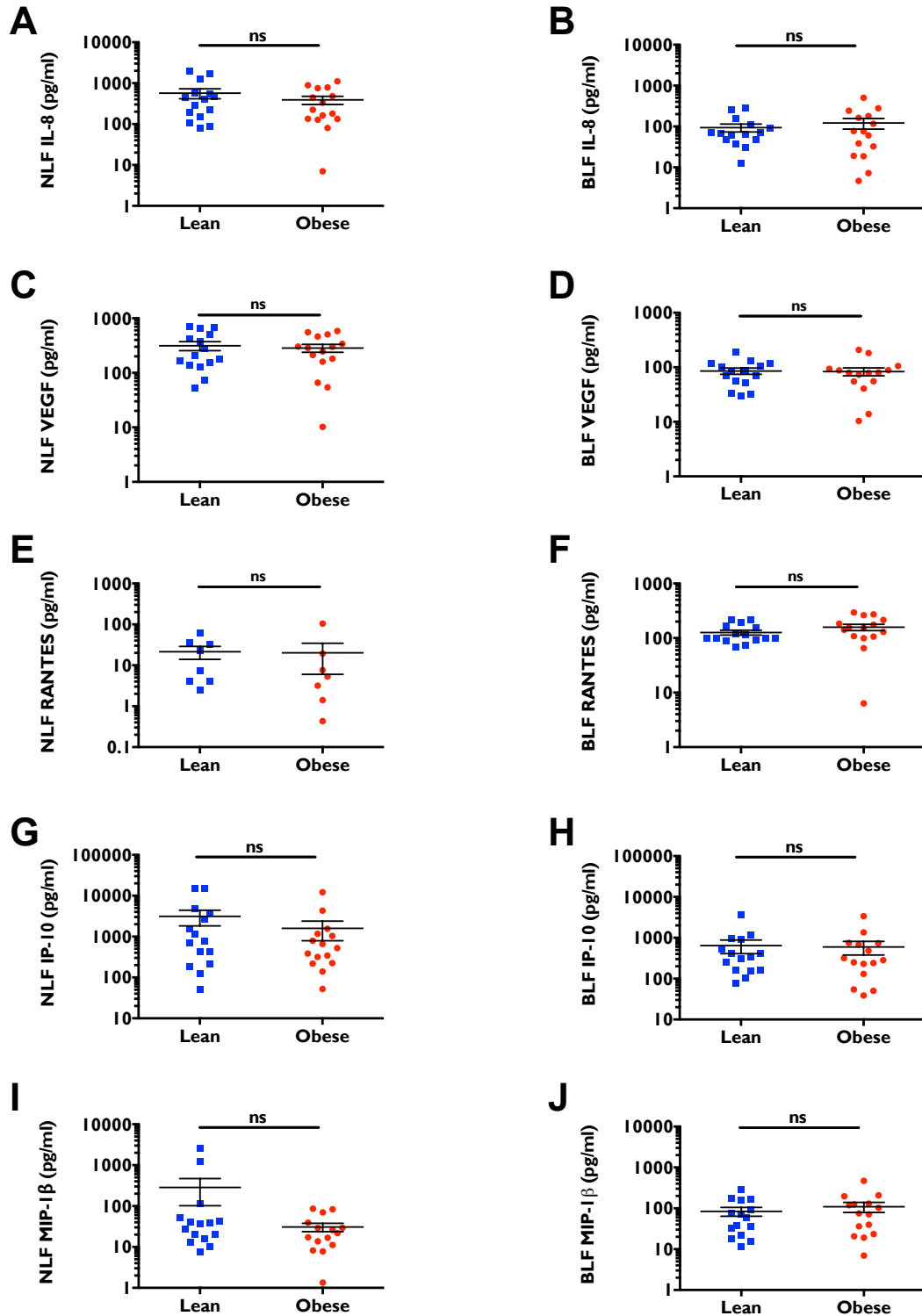


Figure 5-13 Obesity did not alter baseline chemokine levels within nasal or bronchial lining fluid

IL-8, VEGF, RANTES, IP-10 and MIP-1β protein levels were quantified in nasal [A, C, E, G, I] and bronchial lining fluid [B, D, F, H, J] using the Luminex® platform. No significant intergroup differences were observed. (Statistics: Mann-Whitney U, **** p<0.0001, *** p<0.001, ** p<0.01, *p<0.05.)

5.4.11 SOCS1 and SOCS3 over expression significantly attenuated IFN β and IFN $\lambda 1$ promoter activation in A549 cells

To investigate the modulating effect of SOCS expression on IFN induction, A549 cells were transfected with plasmids overexpressing either SOCS1 or SOCS3 and luciferase promoter reporter constructs driven by the outcomes of interest (IFN β , IFN $\lambda 1$ promoter). Following transfection, the A549 cells were infected with pH1N1/09 (MOI 3) (Figure 5-14).

24 h.p.i. pH1N1/09 induced significant IFN β [A] and IFN $\lambda 1$ [B] promoter activation in the cells transfected with the empty vector controls (pORF). IFN β promoter activation post-infection was significantly attenuated by over expression of both SOCS1 and SOCS3 [A]. IFN $\lambda 1$ promoter activation post-infection was also significantly attenuated by SOCS1 and SOCS3 over expression [B].

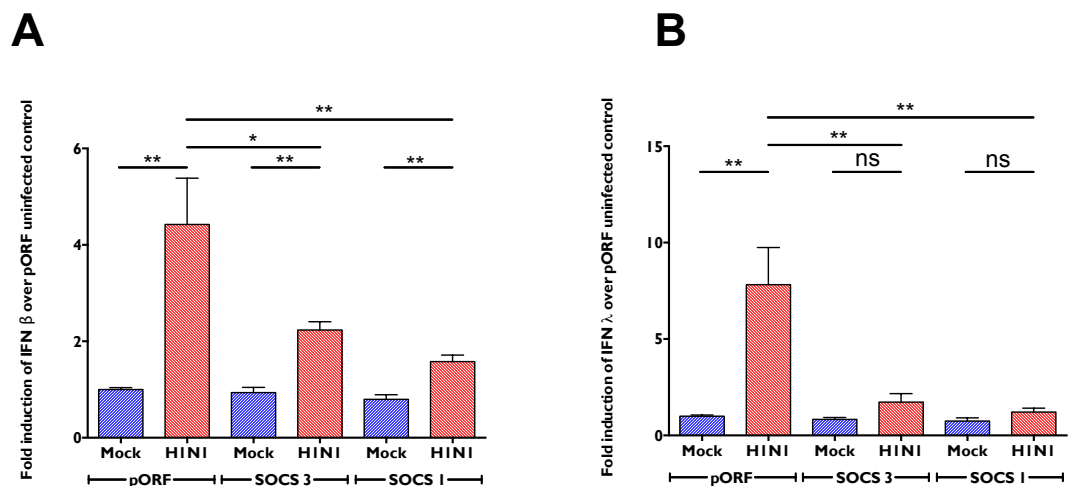


Figure 5-14 SOCS1 & 3 over expression significantly attenuated IFN β & IFN $\lambda 1$ promoter activation 24 h.p.i. with pandemic influenza A

A549 cells were transfected with either SOCS1, SOCS3 or pORF and subsequently infected with pH1N1/09 (MOI 3). Induction of IFN β [A] was significantly attenuated by over expression of both SOCS1 and SOCS3 relative to pORF. Induction of IFN λ [B] was also significantly attenuated by SOCS1 and SOCS3 over expression. Data generated from three independent experiments. (Statistics: Kruskal Wallis with post-test Mann Whitney * $p < 0.05$, ** $p < 0.01$.)

5.4.12 Baseline expression of SOCS1 and SOCS3 mRNA was diminished in BAL cells from the obese subjects

Having established that leptin levels were elevated in the obese and that SOCS3 over expression attenuated IFN expression, baseline mRNA expression of SOCS3 and SOCS1 was quantified in nasal epithelium, PBMCs, DCs, BAL cells and hBECs by RT-qPCR (Figure 5-15).

Expression of SOCS3 mRNA was greater than that of SOCS1 in all cell types [A – J]. No significant intergroup differences were observed except for the BAL cells, in which surprisingly, SOCS1 & SOCS3 expression were diminished in the obese subjects [E, F]. In DCs, SOCS levels were greater in the obese; however, this trend did not achieve statistical significance [G, H].

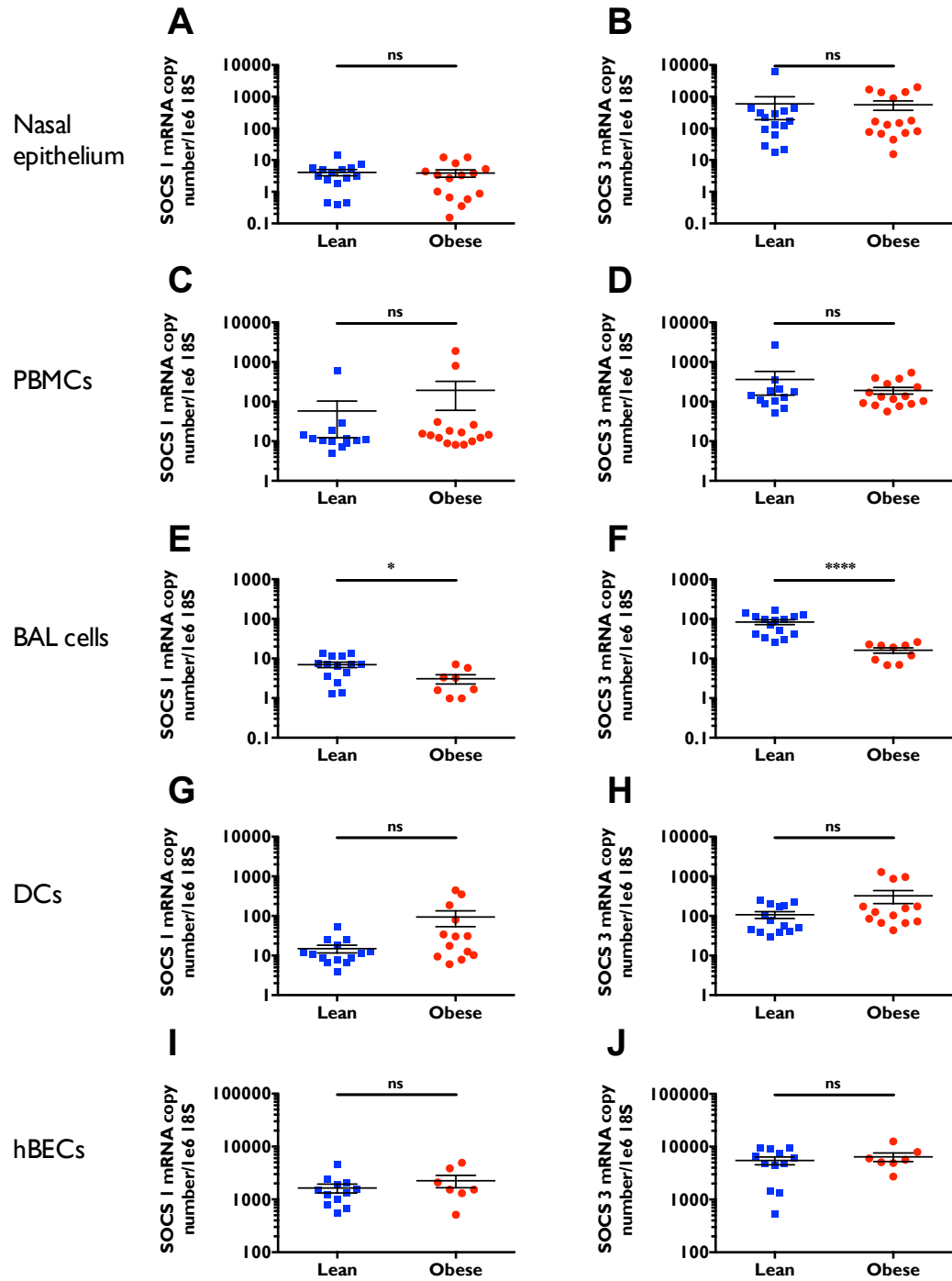


Figure 5-15 Baseline SOCS1 and SOCS3 mRNA expression was diminished in the BAL cells from the obese subjects

Baseline, uninfected SOCS1 and SOCS3 mRNA expression was quantified by RT-qPCR and corrected for the housekeeping gene 18S. SOCS1 and SOCS3 expression was significantly diminished in the BAL cells from the obese subjects [E, F]. No significant intergroup differences were identified in nasal epithelium, PBMCs, DCs or hBECs. (Statistics: Mann Whitney, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$)

5.4.13 Exogenous leptin did not alter basal SOCS3 mRNA expression in primary hBECs

To determine whether administration of exogenous leptin could augment constitutive SOCS3 expression, hBECs from six lean subjects were exposed to high-dose leptin (500ng/ml) for 16 hours. SOCS3 mRNA expression was subsequently quantified in the cell lysates by RT-qPCR; however, no significant intergroup differences were detected (Figure 5-16).

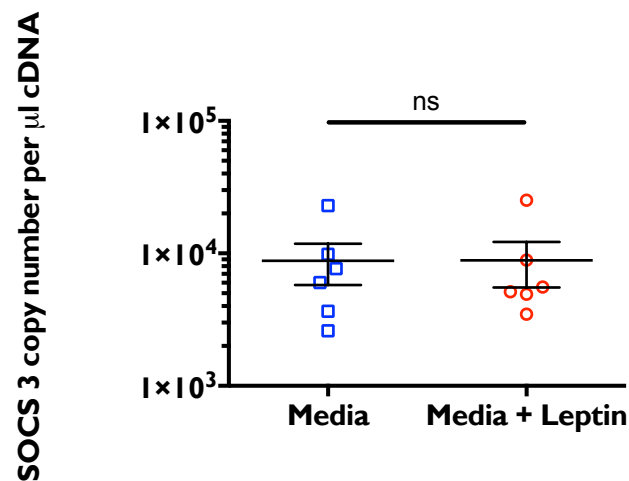


Figure 5-16 High-dose leptin did not alter basal SOCS 3 mRNA expression in hBECs from lean individuals

SOCS 3 mRNA expression was quantified by RT-qPCR in hBECs from lean individuals (n=6) following pre-exposure to high dose leptin (500ng/ml) for 16 hours. No significant difference in SOCS 3 expression was observed with the exogenous leptin (not corrected for 18S). Statistics: Mann Whitney, *p<0.05.

5.5 Discussion

The primary aims of this chapter, in accordance with the proposed model (Figure 5-1), were to determine whether leptin levels were elevated in the obese subjects, and if such levels were increased, to establish whether they were associated with increased constitutive SOCS3 expression. We also set out to characterise the adipokine, acute phase protein and cytokine/chemokine milieu within healthy, obese airways from the nose to the alveoli (NLF, BLF, BAL fluid and serum). We hypothesized that pro-inflammatory adipokine levels would be elevated and anti-inflammatory adipokine levels diminished in the obese subjects and that constitutive SOCS3 expression would be increased in the hBECs, PBMCs, DCs and BAL cells of the obese subjects. It should be noted that all the data presented in this chapter were obtained at baseline in the absence of infection.

5.5.1 *Obesity was associated with elevated leptin and diminished adiponectin levels in the airways and serum*

Leptin and adiponectin are the most widely studied adipokines; we hypothesised, in accordance with existing literature, that leptin levels would be elevated and adiponectin levels diminished in the obese group¹¹⁵. Indeed, this pattern was observed in all the extracellular fluids sampled in the study, supporting the proposed model (Figure 5-1).

Furthermore, and for the first time, we identified relatively high levels of the lesser-studied adipokines resistin, visfatin and adipsin in BAL, NLF and BLF; however, no significant intergroup differences in expression were identified aside from a higher level of visfatin in the BAL fluid of the control subjects. Indeed, it is possible that the significant difference identified in the BAL visfatin levels may be a false positive result secondary to multiple statistical testing. The relatively high expression of adipokines within the NLF was unexpected given the absence of a biological challenge. Indeed, given that the observed concentration of some of these adipokines was greater than those of leptin and adiponectin, the potential physiological roles of these adipokines in airway inflammation warrant further investigation.

Interestingly, the significant intergroup differences in leptin and adiponectin expression identified in the BAL and serum were also seen in the NLF. Nasosorption is simple, cheap and considerably less invasive (and therefore safer) than bronchoscopy. If it could be established that the concentration of adipokines in NLF reflected those seen in BAL, then it could be envisaged that nasosorption could replace the need for invasive bronchoscopy to assess pulmonary adipokine levels. However, whilst the overall, significant intergroup differences in adipokine expression in BAL fluid and serum were also observed in the NLF, no significant correlation between NLF and BAL adipokine levels was identified. Nevertheless, the identification of significant intergroup differences in NLF adipokine levels justifies further validation of this simple, non-invasive technique.

Moving further down the airways, bronchosorption is also a relatively novel sampling technique²²⁷ that has not previously been used to evaluate pulmonary adipokine levels; significant intergroup differences were identified, again mirroring the differences observed in BAL. Unexpectedly, given that bronchosorption did not necessitate the instillation of large volumes of normal saline, the concentration of adipokines in the BLF was found to be lower than that recorded in the BAL fluid. This may simply have been due to the site of sample collection being proximal to the site of adipokine production; if the key adipokine-expressing cells were located in the proximity of the alveoli, then a concentration gradient, originating from the distal airways, would exist. BAL technique recovers fluid from the distal airways whereas bronchosorption samples proximal BLF hence, the fact that BAL concentrations were higher than BLF despite dilution with normal saline suggests that adipokine concentrations were greater in the distal airways. Nevertheless, the observed intergroup differences in adipokine expression in the BLF reflected those in BAL, thus bronchosorption, like nasosorption, may be a feasible, less invasive research technique for quantifying and tracking changes in pulmonary adipokine levels.

We hypothesized that the airways of obese individuals would be relatively inflamed compared to those of normal weight comparators. Of the four pro-inflammatory adipokines evaluated, three were significantly elevated in at least one compartment and the sole anti-inflammatory adipokine assessed, adiponectin, was significantly reduced in all compartments in obese individuals. However, within the airways of the

obese subjects, while leptin was increased and adiponectin decreased, visfatin levels (a pro-inflammatory adipokine) were surprisingly decreased. These data suggest that a pre-dominantly pro-inflammatory adipokine milieu may exist within the airways of obese individuals; however, it must be noted that, although these are undoubtedly important adipokines, many others exist and the pattern seen in this small sample may not reflect the overall picture.

5.5.2 Obesity was associated with increased CRP levels in the upper and lower respiratory tract

To further assess the effect of obesity on the inflammatory environment, both systemically and locally within the airways, four acute phase proteins were quantified in the NLF, BLF, BAL and serum. Although no intergroup difference in the serum level of CRP was identified, upper (NLF) and lower (BAL) respiratory tract CRP levels were significantly higher in the obese subjects, supporting the notion of obesity-associated airway inflammation. Furthermore, BAL CRP significantly correlated with BMI. Although CRP is predominantly produced and secreted by hepatocytes, other cells, including lymphocytes, Kupffer cells and blood monocytes have been shown to synthesize the protein²²⁸. Little is known about the role of pulmonary CRP although its presence in human BAL fluid has previously been demonstrated²²⁹; rat AMs have also been shown to express CRP²²⁸. The data presented in this chapter are the first to demonstrate elevated CRP levels in the upper and lower airways of obese individuals. The fact that no intergroup difference in serum levels was observed suggests that local, intrapulmonary CRP production was somehow altered by obesity; however, the mechanism by which this may occur is currently unclear.

In contrast to the elevated CRP levels, α 2M levels were significantly lower in both the NLF and BAL of the obese individuals. Lower expression of α 2M in obesity has previously been reported in the literature²³⁰, although in serum only, which was not observed in this study. α 2M is a proteinase inhibitor, again produced predominantly by hepatocytes, but also locally by macrophages, fibroblasts and adrenocortical cells²³⁰. As mentioned above, no intergroup difference in serum α 2M was reported, implying a local intrapulmonary alteration in α 2M production; the mechanism of this requires further elucidation.

CRP and $\alpha 2M$ are both positive acute phase proteins i.e. their levels increase following exposure to inflammatory conditions. The reason why airway levels of $\alpha 2M$ were decreased whilst CRP levels were increased is unclear. Nevertheless, CRP is arguably the most clinically useful, and most commonly used, marker of systemic inflammation. Although the $\alpha 2M$ data appears to contradict the CRP data, the significant positive correlation between BMI and BAL CRP provides persuasive evidence to support the concept of obesity-associated airway inflammation. However, this correlation was not evident when the obese group alone was analysed.

5.5.3 *IL-1RA levels were diminished in BLF of the obese subjects*

Having reported elevated pro-inflammatory adipokine and CRP levels, in conjunction with diminished adiponectin expression, in the obese subjects we anticipated that the levels of pro-inflammatory cytokines and chemokines would also be increased^{231,232}. Unfortunately, the Luminex[®] assay evaluating the BAL and serum samples failed due to a technical fault with the Luminex plate reader; hence the only valid data generated were from the nasosorption and bronchosorption samples. Although, the majority of cytokines and chemokines analysed in these samples fell below the lower limits of detection (again, possibly due to the proximal bronchosorption sampling site) valid results for IL-1 β , IL-1RA, IL-8, IP-10, VEGF, RANTES and MIP-1 β were obtained. No significant intergroup differences in cytokine or chemokine expression were detected aside from a significantly lower level of IL-1 receptor antagonist (IL-1RA) protein in the BLF of the obese subjects. The (retracted) paper by Lugogo *et al* was the only publication to compare cytokine levels in BAL fluid from healthy lean and obese subjects (n =17 per group)¹⁴¹. They did not identify any significant intergroup differences in log-normalized levels of IFN γ , IL-10, IL-5, TNF α or MCP-1; however, they did observe a significant difference in VEGF, with the levels in the obese subjects approximately half those in the lean; this difference was not detected in the ObFlu study.

In summary, no significant intergroup differences were identified aside from lower IL-1RA levels in the BLF of the obese subjects. IL-1RA is the natural inhibitor of the pro-inflammatory cytokine IL-1 β , so the reduced levels observed in the obese subjects would be consistent with relatively increased airway inflammation. However, due to the failure of the Luminex[®] assay, only limited cytokine and chemokine data upon

which to base any conclusions were generated and thus caution should be exercised in their interpretation. Repeating the failed Luminex[®] assay would provide a more comprehensive insight into the effect of obesity on the cytokine and chemokine milieu.

5.5.4 *SOCS3 (and SOCS1) over expression attenuated influenza-induced type I & III IFN responses*

Prior to assessing the effects of the observed obesity-associated hyper-leptinaemia on basal SOCS expression, the ability of SOCS3 and SOCS1 to modulate influenza induction of IFNs in airway epithelial cells *in vitro* was evaluated by independently overexpressing SOCS3 and SOCS1 in the A549 cell line. The purpose of these experiments was to determine whether SOCS expression was capable of attenuating IFN expression and signal transduction following influenza virus infection, as proposed in the overall model. SOCS1, whilst not strictly relevant to the proposed model, was included in these experiments as it has been shown to be induced by influenza viruses⁹¹ and pro-inflammatory cytokines²³³. Notably, over expression of both negative regulators resulted in significant suppression of pH1N1/09-induced IFN β and IFN λ 1 promoter activation (Figure 5-14). Furthermore, demonstration of this SOCS-dependent attenuation of IFN induction provides a key mechanistic link in the proposed model of obesity-associated IFN deficiency following influenza infection. Our results were consistent with those of Pothlichet *et al* who demonstrated the attenuating effect of SOCS over expression on IFN β induction post-influenza²³⁴; however, the data presented in this chapter additionally confirmed the attenuating effect of SOCS on type III IFN induction. These results were also concordant with the work of Gielen *et al* who demonstrated suppression of rhinovirus-induced type I & III IFN with over expression of SOCS1 in BEAS-2B cells²³³.

5.5.5 *Constitutive SOCS3 mRNA expression was not increased in the obese subjects*

In chapter four we presented data suggesting that obesity was associated with deficient type I & III IFN responses in BAL cells following pandemic and seasonal influenza infection. As mentioned in the introduction to this chapter, four additional conditions needed to be met for the hypothesized model of the underlying mechanism to hold true. Having demonstrated that obesity was associated with elevated leptin levels and that SOCS3 attenuated influenza-induced IFN expression we assessed whether constitutive

expression of SOCS3 mRNA was elevated in the obese subjects and whether the administration of exogenous leptin upregulated SOCS3 mRNA expression.

Surprisingly, SOCS1 and SOCS3 expression was found to be decreased in the BAL cells from the obese subjects. Indeed, we were unable to demonstrate increased SOCS3 mRNA expression in any of the primary cell types in the obese subjects (Figure 5-15). The overall pattern of elevated leptin and diminished SOCS has previously been described (although not explained) in the literature²³⁵; in a study of obese individuals with osteoarthritis (OA), Vuolteenaho *et al* reported elevated leptin levels in synovial fluid, yet diminished SOCS3 expression in the cartilage of obese OA patients.

The mechanism underpinning the diminished BAL cell SOCS expression is uncertain and warrants further investigation. The observed lack of upregulation in SOCS3 expression in the other cell types may have been due to the removal of the cells from their (relatively inflamed) host environment. It is conceivable that, without constant stimulation from obesity-associated inflammatory mediators e.g. leptin, the levels of SOCS3 could return to those present in the cells of normal weight individuals. It is also a possibility that, despite the logical rationale behind the proposed model, SOCS3 may not play a role in the observed IFN deficiency. However, contrary to these explanations and, by adopting similar methods to those employed in this study, Teran-Cabanillas *et al* demonstrated increased constitutive SOCS3 mRNA expression in PBMCs from obese individuals relative to lean¹⁶⁶.

The fact that the only significant intergroup differences in SOCS expression were identified in the BAL cells suggests that some, as yet unidentified factor, localised to the environment from which the BAL cells were isolated was responsible. Furthermore, the findings of a recent paper imply that it may have been more relevant to measure SOCS protein levels in BAL fluid rather than mRNA expression in cellular lysates. Bourdonnay *et al* reported extracellular secretion of SOCS1 and SOCS3 in exosomes and microparticles from AMs, which are subsequently taken up by airway epithelial cells and lead to inhibition of STAT activation²³⁶. The BAL cell SOCS mRNA levels were diminished in the obese subjects from our study, which would be inconsistent with increased intracellular SOCS protein or BAL cell SOCS protein secretion.

Nevertheless, it would be interesting to measure and compare SOCS3 protein levels in the BAL fluid from the obese and lean groups to determine whether an intergroup difference exists on an extracellular protein level. Indeed, staining for intracellular SOCS proteins may also have been appropriate.

The diminished BAL cell expression of SOCS, combined with the attenuated IFN responses and reduced pro-inflammatory cytokine expression data presented in chapter four suggest that an, as yet unidentified, obesity-associated negative regulator may be suppressing BAL cell transcriptional activity. The fact that there was significant induction of pro-inflammatory cytokines and ISGs in the BAL cells reassuringly implied that the BAL cells were still biologically active. It is possible that the general anaesthetic (GA) may have suppressed the cells, but unlikely, given the duration of GA exposure (see chapter 4 discussion). Future transcriptomic analysis of the BAL cells may help identify an alternative obesity-associated negative regulator.

Despite the apparent disparity between the SOCS data and our proposed model, several aspects of the data generated agreed with pre-existing literature. For example, as per the Pothlichet *et al* paper²³⁴, constitutive SOCS3 mRNA expression was shown to be greater in the hBECs than that of SOCS1. Although the effect of obesity on SOCS expression within the nasal epithelium has not previously been described, SOCS expression in the noses of healthy individuals has been reported²³⁷; again, our data, showing the pattern of higher SOCS3 expression supports previous findings. SOCS proteins are important for regulating the homeostasis of DC development, physiology and replenishment²³⁸; whilst no human studies have previously looked at the effect of obesity on DCs, Smith *et al* found that, whilst obesity did not interfere with antigen uptake or migration in a DIO mouse model, it did impair antigen presentation¹⁰². Although SOCS1 and SOCS3 were expressed, no significant intergroup differences in peripheral blood DCs were observed in this study.

5.5.6 *Exogenous leptin administration did not upregulate SOCS3 mRNA expression by hBECs*

The leptin-induced upregulation of SOCS3 is thought to be one of the major contributing factors to leptin resistance²³⁹; to further investigate whether leptin upregulated SOCS3 expression in primary hBECs, hBECs from the lean subjects only

were exposed to high-dose recombinant human leptin (500ng/ml) for sixteen hours in the absence of infection and mRNA expression of SOCS3 quantified by RT-qPCR. The dose of leptin chosen was based up that shown by Vernooy *et al* to have a demonstrable effect on STAT-3 phosphorylation in A549 cells¹³⁹. However, no effects of exogenous leptin on SOCS3 mRNA expression were observed (Figure 5-16).

Several groups have however demonstrated effects of exogenous leptin on immortalised epithelial cell lines; Milner *et al* published an abstract²⁴⁰ (which notably has not progressed to publication by the time of writing) in 2010, stating that exogenous leptin upregulated SOCS3 and IL-6 expression in A549 cells²⁴⁰; numerous attempts were made in the ObFlu study to replicate this data (using various doses, durations and batches of leptin from different suppliers) but all were unsuccessful (data not shown). Feng *et al* took A549 cells and investigated the effect of leptin on cellular migration showing, using wound healing and transwell assays, that leptin enhances cell metastasis, upregulates TGF β and induces epithelial-mesenchymal transition²⁴¹. Woo *et al* used the mucin-producing NCI-H292 human pulmonary mucoepidermoid carcinoma cell line to investigate the effects of leptin on mucin production, showing upregulation of MUC5AC and MUC5B mRNA and protein upon leptin stimulation²⁴². More recently, Suzukawa *et al* performed *ex vivo* stimulations of primary hBECs derived from lung cancer biopsies, finding that leptin (10 μ M) upregulated ICAM-1 expression promoted hBEC migration towards leptin (using a transwell assay)²⁴³.

No data concerning the effect of leptin administration on SOCS expression in primary hBECs have previously published. There are several potential reasons why no effect was observed in this study: firstly, the leptin used may have been biologically inactive; inclusion of a positive control would have excluded this. Secondly, the process of culturing the hBECs *ex vivo* may potentially have changed the phenotype of the cells, down-regulating Ob-Rb expression, rendering the cells unresponsive to leptin; quantification of the Ob-Rb receptor by RT-qPCR would help clarify this. Finally, the time point at which the cells were harvested after the administration of leptin may not have been optimal, and a transient rise in SOCS3 mRNA expression may have been missed.

5.6 Conclusions

The primary aims of this chapter, in accordance with the proposed model, were to determine whether leptin levels were elevated in the obese subjects and, if confirmed, to establish whether they were associated with increased constitutive SOCS3 expression. Consistent with the model, leptin concentrations were demonstrated to be significantly greater than in healthy weight individuals for all of the extracellular compartments assessed. However, obesity was not associated with increased constitutive SOCS3 expression, indeed BAL cell expression of SOCS3 was decreased, and we were unable to show that leptin upregulated SOCS3 expression (Figure 5-17).

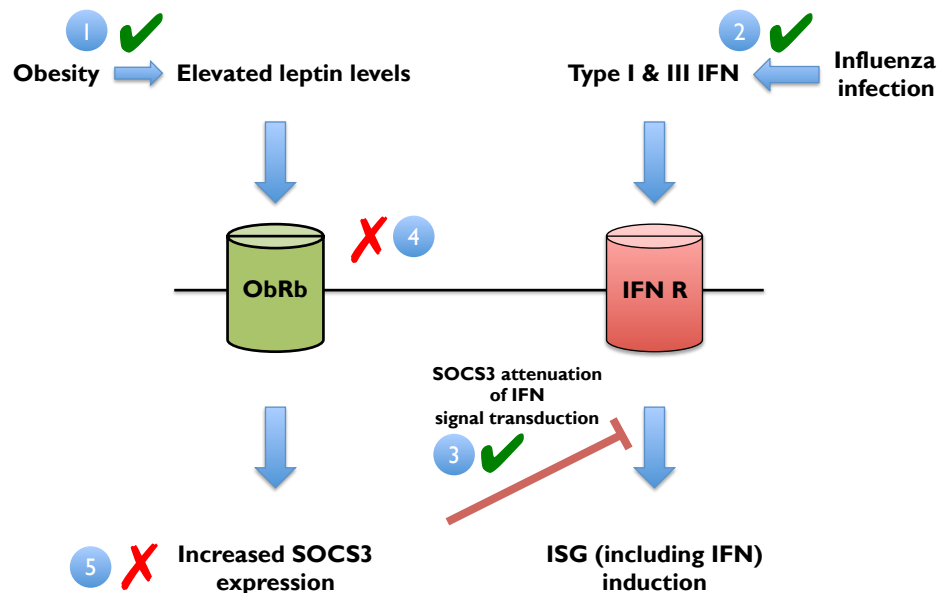


Figure 5-17 Simplified diagram to show which elements of the proposed model were supported by the data generated in the ObFlu study

[1] Obesity was shown to be associated with elevated leptin levels in serum, BAL, BLF and NLF [2] Pandemic and seasonal influenza infection induced type I & III IFN and ISG expression (chapter 4) [3] SOCS3 over expression attenuated IFN expression and signal transduction [4] Leptin did not upregulate SOCS3 in hBECs [5] Obesity was not associated with increased constitutive SOCS3 expression.

The secondary aim of the chapter was to characterise the adipokine and cytokine milieu within the blood and airways of obese individuals. Consistent with pre-existing literature, leptin levels were elevated and adiponectin levels diminished in the obese subjects. For the first time, we report the presence of relatively high levels of the lesser-studied adipokines resistin, visfatin and adipsin in BAL, NLF and BLF; the physiological roles of these adipokines in the lung warrant further investigation. Pulmonary CRP levels were found to be elevated in obesity and to significantly

correlate with BMI (when obese and lean groups were analysed concurrently); however $\alpha 2M$ levels were unexpectedly decreased. The cytokine and chemokine data generated were limited to NLF and BLF and no significant intergroup differences, aside from a reduction of IL-1RA in the obese BLF were noted.

If one were to focus solely on the most widely studied, and clinically relevant, inflammatory mediators pertinent to the field, namely leptin, adiponectin and CRP, then the data in this chapter would provide a compelling case for obesity-associated airway inflammation. However, given the fact that CRP data was contradicted by the $\alpha 2M$ data and that many other inflammatory mediators were not assessed in this study, no definite conclusions regarding the inflammatory status of the obese airways can be made.

5.7 Summary of key points

- Leptin levels were elevated and adiponectin levels decreased in the serum, BAL, BLF and NLF of obese subjects
- The lesser studied adipokines resistin, visfatin and adipsin were expressed at relatively high levels in the airways and their potential roles warrant further investigation
- Airway CRP levels are significantly increased in obesity and correlate with BMI
- Obesity is not associated with increased constitutive SOCS3 expression in hBECs, DCs nor PBMCs. Indeed, SOCS 1 and 3 expression is significantly decreased in BAL cells from obese subjects.
- Leptin does not upregulate SOCS3 mRNA expression in primary hBECs

6 Discussion & Future Directions

6.1 Key study findings

6.1.1 *Obesity was associated with significant attenuation of the type I and III IFN responses of BAL cells to pandemic and seasonal influenza virus infection*

This is the first report of an obesity-associated IFN deficiency in human BAL cells following infection with pandemic and seasonal influenza. Furthermore, this is the first human study to investigate the effects of obesity on the host innate immune response to respiratory viruses.

As discussed in chapter one, the reasons why obese individuals experience adverse outcomes following infection are complex and multifactorial. This report deliberately focused solely on the effect of obesity on the innate immune response, thus it cannot be concluded that the observed obesity-associated BAL cell IFN deficiency singularly explains the increased morbidity and mortality seen in the obese population following influenza infection. However, the reported findings enhance our understanding of the potential immunomodulatory effects of obesity and how they may contribute to the increased susceptibility to severe outcomes seen upon influenza infection. We initially proposed a theoretical model, deduced from the results of pre-existing literature, to explain the mechanism by which obesity may result in IFN deficiency. Several aspects of the model were verified, namely: elevated pulmonary and peripheral leptin levels were demonstrated in the obese subjects, SOCS3 overexpression was shown to attenuate pH1N1/09-induced type I & III IFN expression and deficient influenza-induced type I & III IFN responses were identified in the BAL cells from the obese cases. However, we were unable to demonstrate an association between obesity and increased constitutive SOCS3 expression, nor were we able to confirm leptin-induced upregulation of SOCS3. Hence, the exact mechanism by which obesity results in IFN deficiency remains unclear and warrants future investigation.

The major caveat to this reported IFN deficiency is the unresolved potential confounding effect of the GA that the bariatric candidates underwent. As discussed in chapter four, the GA in these subjects was unavoidable, given that the bronchoscopy

took place immediately prior to their bariatric surgery. Indeed, we have demonstrated, for the first time, that extensive, procedure-heavy research bronchoscopy in bariatric patients under GA immediately prior to major abdominal surgery was safe in our centre. For the GA to have been responsible for the observed attenuated IFN responses, it would have to have globally ‘shut down’ the BAL cells’ innate immune response within six minutes (the time taken for BAL cell procurement) and imprinted this ‘shut down’ state throughout the subsequent 48 hour *ex vivo* time course. Consistent with this, all of the responses measured in the BAL cells were impaired, notably IFN, but also pro-inflammatory cytokines and SOCS3. Conversely, the small body of literature concerning GA and the immune function of AMs shows that anaesthesia does *eventually* significantly decrease phagocytic and microbicidal ability *ex vivo*, but only after subjects are exposed to four hours of continuous general anaesthesia. Given the duration of exposure to the anaesthetic agents in our study, we believe that it is unlikely that the observed BAL cell IFN deficiency was due to the effects of GA. Nevertheless, it is important to address this issue more formally in future research. As a first step, the BAL cells from the obese subjects could be re-sampled under GA after the subjects have lost a significant amount of weight post-bariatric surgery (see future work below). If GA is confirmed to attenuate BAL cell innate immune responses this could still have major implications as GA-associated IFN deficiency could potentially increase susceptibility to post-operative pneumonia.

The implications of the addition of obesity to the canon of potential IFN deficient states are two-fold: firstly, from a public health perspective the evidence from this report, in conjunction with the epidemiological evidence supporting the association between increased BMI and adverse outcomes, provides a compelling incentive for those individuals who are morbidly obese to normalize their BMI. Secondly, the observed obesity-associated IFN deficiency in BAL cells implies that supplementary, inhaled IFN therapy may be beneficial in the treatment of morbidly obese individuals with severe influenza infection. Indeed, this is not a novel concept; trials of inhaled IFN β have shown beneficial effects for virus-induced exacerbations in individuals with difficult-to-treat asthma²²⁵. To explore this further, potential future work could

include a placebo-controlled trial of inhaled IFN β in the DIO mouse-influenza infection model to determine whether it has any positive effect on outcome.

While defective influenza-induced type I & III IFN expression was observed in BAL cells, IFN deficiency was not seen in hBECs, PBMCs or DCs isolated from the same individuals. This suggests that the influenza-induced IFN deficiency was cell type-specific within the lung environment, a feature that has also been described in asthma¹⁷⁰.

One of the problems with interpretation of the data generated by this study, in the context of the obesity-influenza field as a whole, is that no information concerning the actual clinical pathogenesis of the influenza-associated adverse outcomes in the obese has been published. Numerous observational studies and systematic reviews defining the epidemiological association between increasing BMI and adverse outcomes are available, yet no papers detailing *obesity-specific* pathological, autopsy, viral load, peripheral blood or pulmonary cytokine/IFN data are available. In future influenza pandemics, or indeed during seasonal epidemics, collection and publication of obesity-specific pathological data would help put this study's findings into context. Furthermore, a valuable future study could involve the recruitment and investigation of naturally infected obese individuals that become hospitalized during influenza epidemics/pandemics. Appropriate samples could be obtained from these patients, in a similar fashion to the ObFlu study, and immune responses and adipokine levels correlated with clinical outcomes.

6.1.2 Leptin and CRP levels were elevated and adiponectin levels diminished in the airways of co-morbidity-free obese individuals

This is the first report to extensively characterize the inflammatory environment within co-morbidity-free obese airways. Consistent with existing literature, leptin levels were increased and adiponectin levels decreased in serum and BAL fluid. However, this study adds considerably to our knowledge of pulmonary adipokine expression; for the first time, relatively high levels of the lesser-studied adipokines resistin, visfatin and

adipsin were recorded in BAL, NLF and BLF; the physiological roles of these latter adipokines in the airways warrant further investigation.

The reported acute phase protein data is the first to demonstrate elevated levels of CRP in the airways of obese individuals and a significant positive correlation with BMI. The fact that no intergroup difference in serum levels was observed suggests that local, intrapulmonary CRP production was somehow altered by obesity; however, the mechanism by which this may occur requires further elucidation. Aside from an observed diminution of constitutive IL-1RA expression in BLF from the obese subjects, no other significant intergroup differences in pro-inflammatory cytokine or chemokine expression were reported.

The relatively high expression of adipokines within the NLF was unexpected given the absence of a biological challenge. Interestingly, the significant intergroup differences in leptin and adiponectin expression identified in the BAL and serum were also seen in the NLF. Nasosorption is simple, cheap and considerably less invasive (and therefore safer) than bronchoscopy. Although the NLF adipokine levels did not significantly correlate with pulmonary levels the significant intergroup differences were reflected, warranting further validation of this simple technique.

As concluded in chapter five, if one were to focus solely on the most widely studied, and clinically relevant, inflammatory mediators pertinent to the field, namely leptin, adiponectin and CRP, then the data in this report would provide a persuasive case for obesity-associated airway inflammation. However, given the fact that CRP data was contradicted by the α 2M results and that many other inflammatory mediators were not assessed in this study, no definite conclusions regarding the inflammatory status of the obese airways can be reached. Nevertheless, the significant levels of adipokines shown to be present in the upper and lower airways of the study participants tempts one to speculate that adipokines may influence pulmonary immune responses to infection; further studies are clearly needed to further test this hypothesis.

6.1.3 Obesity was not associated with increased constitutive SOCS3 expression

This is the first report to evaluate the effect of obesity on the constitutive expression of SOCS in nasal epithelium, hBECs, BAL cells and DCs and the first to demonstrate that over expression of both SOCS1 and SOCS3 results in significant suppression of pandemic influenza-induced IFN β and IFN λ 1 promoter activation. We proposed that obesity-associated chronic hyper-leptinaemia would increase constitutive SOCS3 expression in all the cell types from the obese individuals. However, despite demonstrating obesity-associated BAL cell IFN deficiency, elevated leptin levels and the attenuating effects of SOCS over expression on IFN expression we were unable to demonstrate increased constitutive SOCS3 expression in the obese subjects. Furthermore, SOCS expression in the BAL cells from obese individuals was found to be decreased; the mechanism underpinning this decrease is uncertain and warrants further investigation.

The diminished BAL cell expression of SOCS, combined with the attenuated IFN responses and reduced pro-inflammatory cytokine expression data presented in chapter four suggest that an, as yet unidentified, obesity-associated negative regulator may be suppressing BAL cell transcriptional activity. The fact that there was significant induction of pro-inflammatory cytokines and ISGs in the BAL cells reassuringly implied that the BAL cells were still biologically active. It is possible that the GA may have suppressed the cells, but unlikely, given the duration of GA exposure. Future transcriptomic analysis of the BAL cells may help identify an alternative obesity-associated negative regulator. The complexity of the IFN system, and abundance of potential negative regulators, has recently been revealed through protein-protein interaction studies²⁴⁴. This information will be very useful to cross check differentially expressed genes in the BAL cells from the obese and healthy-weight volunteers recruited for the ObFlu study.

6.2 Future work

This study has raised several important questions that should be addressed in future work. The key question that currently remains unresolved is whether the observed

BAL cell IFN deficiency was secondary to obesity or the effects of GA. The most efficient way to address this would be to invite the bariatric surgery candidates back for repeat bronchoscopy under GA (using the same anaesthetic agents) several months following surgery, when they have lost a significant amount of weight. If the BAL cells exhibit a restoration of their IFN responses post-weight loss then it could be reasonably concluded that the IFN deficiency was secondary to obesity. If the BAL cell responses remain low, then this could be due to either the GA or an ‘imprinting’ effect of obesity on BAL cell innate responses²⁴⁵. In the event of consistently low BAL cell IFN responses, the healthy, normal weight candidates could be invited back for bronchoscopy under GA; if the BAL cell responses are attenuated in these individuals, whilst at the same BMI as the initial bronchoscopy, then it could be concluded that the GA has resulted in the deficiency.

Having assessed *ex vivo* immune responses the next logical step would be to perform a human challenge experiment in which obese and lean individuals would be infected with influenza and the immune response assessed at several stages during the infection by repeated bronchoscopy and venesection. One of the advantages of such an approach is that it would allow quantification of the effect of obesity on viral load *in vivo*; however, it is possible that the viral load may confound the observed immune responses. Due to association of obesity with adverse influenza-outcomes such a study would require extremely careful subject selection and monitoring, as a quarantined inpatient, throughout. Human influenza challenge studies have been performed before²⁴⁶; however, never in individuals with risk factors for severe outcomes; if such a study received ethical approval, it would provide a fascinating insight into host-virus interactions.

Although the epidemiological evidence supports an association between obesity and *influenza*-induced adverse outcomes only, it would be valuable to determine whether the obesity-related BAL-cell IFN deficiency extends to infection with other respiratory viruses, such as rhinovirus (RV). If obesity did impair RV-induced IFN responses it could have potential implications for the treatment of obese individuals with severe asthma. RV infection is the major cause of asthma exacerbations¹⁶⁸ and asthma is an

IFN deficient state. If obesity was shown to impair RV IFN responses it could form an additional justification for the trial of inhaled IFN in the treatment of virus-induced exacerbations in obese asthmatics.

Finally, the physiological roles of the lesser studied adipokines identified in the NLF, BLF and BAL of the study subjects, namely resistin, visfatin and adiponectin warrant further investigation.

6.3 Study Summary

For the first time during an influenza pandemic, obesity was convincingly identified in 2009 as a novel, independent risk factor for multiple markers of disease severity, including hospitalisation, intensive care unit admission and death following infection. This report demonstrates that BAL cells from morbidly obese individuals have deficient type I and III IFN responses to both pandemic and seasonal influenza virus infection. This IFN-deficiency was BAL cell specific and not observed following infection of primary hBECs, PBMCs or DCs. Although unlikely, it is possible that the observed IFN deficiency may have been due to the confounding effects of GA. Consistent with our proposed model of the underlying mechanism, elevated leptin levels were observed in the lung and serum of the obese subjects; however, baseline constitutive SOCS3 mRNA expression was not increased in the obese subjects and we were unable to demonstrate leptin-induced upregulation of SOCS3. Therefore, the exact mechanism by which obesity may result in BAL cell IFN-deficiency remains unclear. Nevertheless, this novel, obesity-associated BAL cell IFN deficiency may contribute to the adverse outcomes seen in the obese population upon influenza infection.

Bibliography

1. Palese P, Shaw M. Orthomyxoviridae: The Viruses and their replication. 5th ed: Wolters Kluwer.
2. Webster RG, Laver WG, Kilbourne ED. Reactions of antibodies with surface antigens of influenza virus. *The Journal of general virology* 1968; 3(3): 315-26.
3. Skehel JJ, Wiley DC. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annual review of biochemistry* 2000; 69: 531-69.
4. Horimoto T, Kawaoka Y. Influenza: lessons from past pandemics, warnings from current incidents. *Nature reviews Microbiology* 2005; 3(8): 591-600.
5. Hale BG, Albrecht RA, Garcia-Sastre A. Innate immune evasion strategies of influenza viruses. *Future Microbiology* 2010; 5(1): 23-41.
6. Noda T, Kawaoka Y. Structure of influenza virus ribonucleoprotein complexes and their packaging into virions. *Reviews in medical virology* 2010; 20: 380-91.
7. Eisfeld AJ, Neumann G, Kawaoka Y. At the centre: influenza A virus ribonucleoproteins. *Nature reviews Microbiology* 2015; 13(1): 28-41.
8. O'Neill RE, Talon J, Palese P. The influenza virus NEP (NS2 protein) mediates the nuclear export of viral ribonucleoproteins. *The EMBO journal* 1998; 17(1): 288-96.
9. Zamarin D, Ortigoza MB, Palese P. Influenza A virus PB1-F2 protein contributes to viral pathogenesis in mice. *Journal of virology* 2006; 80(16): 7976-83.

10. Trifonov V, Racaniello V, Rabadan R. The Contribution of the PB1-F2 Protein to the Fitness of Influenza A Viruses and its Recent Evolution in the 2009 Influenza A (H1N1) Pandemic Virus. *PLoS currents* 2009; 1: RRN1006.
11. Wu Y, Wu Y, Tefsen B, Shi Y, Gao GF. Bat-derived influenza-like viruses H17N10 and H18N11. *Trends in microbiology* 2014; 22(4): 183-91.
12. Matrosovich MN, Matrosovich TY, Gray T, Roberts NA, Klenk HD. Human and avian influenza viruses target different cell types in cultures of human airway epithelium. *Proc Natl Acad Sci U S A* 2004; 101(13): 4620-4.
13. Garcia-Sastre A. Induction and evasion of type I interferon responses by influenza viruses. *Virus research* 2011; 162(1-2): 12-8.
14. Taubenberger JK, Morens DM. 1918 Influenza: the mother of all pandemics. *Emerg Infect Dis* 2006; 12(1): 15-22.
15. Wright PF, Neumann G, Kawaoka Y. Influenza in Humans - Past pandemics and the H5N1 epidemic. *Field's Virology*. 5th ed: LWW; 2007: 1699.
16. Simonsen L, Spreeuwenberg P, Lustig R, et al. Global Mortality Estimates for the 2009 Influenza Pandemic from the GLaMOR Project: A Modeling Study. *PLoS medicine* 2013; 10(11): e1001558.
17. Dawood FS, Iuliano aD, Reed C, et al. Estimated global mortality associated with the first 12 months of 2009 pandemic influenza A H1N1 virus circulation: a modelling study. *The Lancet Infectious diseases* 2012; 12: 687-95.
18. Klenk HD, Garten W, Matrosovich M. Molecular mechanisms of interspecies transmission and pathogenicity of influenza viruses: Lessons from the 2009 pandemic. *Bioessays* 2011; 33(3): 180-8.

19. Elderfield R, Barclay W. Influenza pandemics. *Advances in experimental medicine and biology* 2011; 719: 81-103.
20. Peiris JSM, Cheung CY, Leung CYH, Nicholls JM. Innate immune responses to influenza A H5N1: friend or foe? *Trends in Immunology* 2009; 30(12): 574-84.
21. Sekkides O. Pandemic influenza—a timeline. *The Lancet Infectious Diseases* 2010; 10(10): 663.
22. Fineberg HV. Pandemic Preparedness and Response — Lessons from the H1N1 Influenza of 2009. *The New England journal of medicine* 2014; 370(14): 1335-42.
23. Mytton OT, Rutter PD, Donaldson LJ. Influenza A(H1N1)pdm09 in England, 2009 to 2011: a greater burden of severe illness in the year after the pandemic than in the pandemic year. *Eurosurveillance* 2012: 1-9.
24. Dunning J, Openshaw PJ. Impact of the 2009 influenza pandemic. *Thorax* 2010; 65(6): 471-2.
25. England PH. Influenza: the green book, chapter 19. 20th March 2013 ed; 2013.
26. Mertz D, Kim TH, Johnstone J, et al. Populations at risk for severe or complicated influenza illness: systematic review and meta-analysis. *BMJ (Clinical research ed)* 2013; 347: f5061.
27. Organization WH. Obesity and Overweight - Fact sheet No.311. 2015. <http://www.who.int/mediacentre/factsheets/fs311/en/2015>).
28. Schmatz M, Madan J, Marino T, Davis J. Maternal obesity: the interplay between inflammation, mother and fetus. *Journal of perinatology : official journal of the California Perinatal Association* 2010; 30(7): 441-6.

29. Wang YC, McPherson K, Marsh T, Gortmaker SL, Brown M. Health and economic burden of the projected obesity trends in the USA and the UK. *Lancet* 2011; 378(9793): 815-25.
30. MD PBAS, PhD GS, PhD KDH, et al. The global obesity pandemic: shaped by global drivers and local environments. *The Lancet* 2011; 378(9793): 804-14.
31. Morgan OW, Bramley A, Fowlkes A, et al. Morbid obesity as a risk factor for hospitalization and death due to 2009 pandemic influenza A(H1N1) disease. *PloS one* 2010; 5: e9694.
32. Fezeu L, Julia C, Henegar A, et al. Obesity is associated with higher risk of intensive care unit admission and death in influenza A (H1N1) patients: a systematic review and meta-analysis. *Obesity reviews : an official journal of the International Association for the Study of Obesity* 2011; 12(8): 653-9.
33. Phung DT, Wang Z, Rutherford S, Huang C, Chu C. Body mass index and risk of pneumonia: a systematic review and meta-analysis. *Obesity reviews : an official journal of the International Association for the Study of Obesity* 2013; 14(10): 839-57.
34. Mertz D, Kim TH, Johnstone J, et al. Populations at risk for severe or complicated influenza illness: systematic review and meta-analysis. *Bmj* 2013; 347: f5061.
35. Kwong JC, Campitelli Ma, Rosella LC. Obesity and respiratory hospitalizations during influenza seasons in Ontario, Canada: a cohort study. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2011; 53: 413-21.
36. Napolitano LM, Park P, KC S. Intensive-care patients with severe novel influenza A (H1N1) virus infection - Michigan, June 2009. *Morbidity and Mortality Weekly Report* 2009; 58(27): 749-52.

37. Cullen G, Martin J, O'Donnell J, et al. Surveillance of the first 205 confirmed hospitalised cases of pandemic H1N1 influenza in Ireland, 28 April - 3 October 2009. *Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin* 2009; 14(44).
38. Cui W, Zhao H, Lu X, et al. Factors associated with death in hospitalized pneumonia patients with 2009 H1N1 influenza in Shenyang, China. *BMC infectious diseases* 2010; 10(1): 145.
39. Buda S, Kopke K, Haas W. [Epidemiological characteristics of the influenza pandemic (H1N1) 2009 in Germany based on the mandatory notification of cases]. *Bundesgesundheitsblatt, Gesundheitsforschung, Gesundheitsschutz* 2010; 53(12): 1223-30.
40. Choi WJ, Kim WY, Kim SH, et al. Clinical characteristics of pneumonia in hospitalized patients with novel influenza A (H1N1) in Korea. *Scandinavian journal of infectious diseases* 2010; 42(4): 311-4.
41. Cutter JL. Outbreak of Pandemic Influenza A (H1N1-2009) in Singapore, May to September 2009 2010: 1-10.
42. Flint SM, Davis JS, Su JY, et al. Disproportionate impact of pandemic (H1N1) 2009 influenza on Indigenous people in the Top End of Australia's Northern Territory. *The Medical journal of Australia* 2010; 192(10): 617-22.
43. Gomez-Gomez A, Magana-Aquino M, Garcia-Sepulveda C, et al. Severe pneumonia associated with pandemic (H1N1) 2009 outbreak, San Luis Potosi, Mexico. *Emerg Infect Dis* 2010; 16(1): 27-34.
44. Miller RR, Markewitz Ba, Rolfs RT, et al. Clinical findings and demographic factors associated with ICU admission in Utah due to novel 2009 influenza A(H1N1) infection. *Chest* 2010; 137: 752-8.

45. Koegelenberg CF, Irusen EM, Cooper R, et al. High mortality from respiratory failure secondary to swine-origin influenza A (H1N1) in South Africa. *QJM* 2010; 103(5): 319-25.
46. Nicolay N, Callaghan MA, Domegan LM, et al. Epidemiology, clinical characteristics and resource implications of pandemic (H1N1) 2009 in intensive care units in Ireland. *Critical care and resuscitation : journal of the Australasian Academy of Critical Care Medicine* 2010; 12(4): 255-61.
47. Zykova I, Vancar P, Prattingerov J, Sedlk P, Krejzar Z, Remeta P. Analysis of the impact of pandemic influenza 2009 a H1N1 outbreak on intensive care in a tertiary care hospital. *Intensive Care Medicine* 2010; 36(S284).
48. Sovilj-Gmizic S, Obradovic D, Joves Sevic B, Batranovic U, Andrijevic I, Gavrilovic S. Obesity as a risk factor associated with death due to 2009 pandemic influenza a (H1N1). *Intensive Care Medicine* 2010; 36(S368).
49. Subramony H, Lai FY, Ang LW, Cutter JL, Lim PL, James L. An epidemiological study of 1348 cases of pandemic H1N1 influenza admitted to Singapore Hospitals from July to September 2009. *Annals of the Academy of Medicine, Singapore* 2010; 39(4): 283-8.
50. Siau C, Law J, Tee A, Poulouse V, Raghuram J. Severe refractory hypoxaemia in H1N1 (2009) intensive care patients : initial experience in an Asian regional hospital. 2010; 51: 490-5.
51. Viasus D, Paño - Pardo J. Factors associated with severe disease in hospitalized adults with pandemic (H1N1) 2009 in Spain. *Clinical Microbiology ...* 2011; 17: 738-46.
52. Peralta P, Cortes-García M, Vicente-Herrero M, Castrillo-Villamandos C. Risk factors for disease severity among hospitalised patients with 2009 pandemic influenza

A (H1N1) in Spain, April-December 2009. *Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin* 2010; 15(38).

53. Fuhrman C, Bonmarin I, Paty AC, et al. Severe hospitalised 2009 pandemic influenza A(H1N1) cases in France, 1 July-15 November 2009. *Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin* 2010; 15(2).

54. Tutuncu EE, Ozturk B, Gurbuz Y, et al. Clinical characteristics of 74 pandemic H1N1 influenza patients from Turkey. Risk factors for fatality. *Saudi Med J* 2010; 31(9): 993-8.

55. Blakemore S, Gopalan P. Pandemic influenza A (H1N1) 2009: A case series from intensive care units in port Shepstone, South Africa. *Southern African Journal of Anaesthesia and Analgesia* 2010; 16(3): 17-22.

56. Chacko J, Gagan B, Ashok E, Radha M, Hemanth HV. Critically ill patients with 2009 H1N1 infection in an Indian ICU. *Indian Journal of Critical Care Medicine* 2010; 14(2): 77.

57. Chien Y-S, Su C-P, Tsai H-T, et al. Predictors and outcomes of respiratory failure among hospitalized pneumonia patients with 2009 H1N1 influenza in Taiwan. *Journal of Infection* 2010; 60(2): 168-74.

58. Yang P, Deng Y, Pang X, et al. Severe, critical and fatal cases of 2009 H1N1 influenza in China. *Journal of Infection* 2010; 61(4): 277-83.

59. Vasoo S, Singh K, Trenholme GM. Predicting Need for Hospitalization of Patients with Pandemic (H1N1) 2009, Chicago, Illinois, USA. *Emerging Infectious Diseases* 2010; 16(10): 1594-7.

60. Poggensee G, Gilsdorf A, Buda S, et al. The first wave of pandemic influenza (H1N1) 2009 in Germany: from initiation to acceleration. *BMC Infect Dis* 2010; 10: 155.
61. van't Klooster T, Wielders CCHL, T D, L I, A M, van den Wijngaard C. Surveillance of hospitalisations for 2009 pandemic influenza A(H1N1) in the Netherlands, 5 June-31 December 2009. *Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin* 2010; 15(2): 14.
62. Estenssoro E, Rios FG, Apezteguia C, et al. Pandemic 2009 influenza A in Argentina: a study of 337 patients on mechanical ventilation. *American journal of respiratory and critical care medicine* 2010; 182(1): 41-8.
63. Parcevaux M, Boisson V, Lemant J, et al. [Outbreak of influenza A(H1N1)/2009: description of cases and crisis management in a ICU in Reunion Island]. *Annales francaises d'anesthesie et de reanimation* 2010; 29(12): 902-8.
64. Nguyen-Van-Tam JS, Openshaw PJ, Hashim A, et al. Risk factors for hospitalisation and poor outcome with pandemic A/H1N1 influenza: United Kingdom first wave (May-September 2009). *Thorax* 2010; 65(7): 645-51.
65. Fezeu L, Julia C, Henegar a, et al. Obesity is associated with higher risk of intensive care unit admission and death in influenza A (H1N1) patients: a systematic review and meta-analysis. *Obesity reviews : an official journal of the International Association for the Study of Obesity* 2011; 12: 653-9.
66. Van Kerkhove MD, Vandemaele KaH, Shinde V, et al. Risk factors for severe outcomes following 2009 influenza A (H1N1) infection: a global pooled analysis. *PLoS medicine* 2011; 8: e1001053.
67. Louie JK, Acosta M, Samuel MC, et al. A novel risk factor for a novel virus: obesity and 2009 pandemic influenza A (H1N1). *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2011; 52: 301-12.

68. Yokota RT, Skalinski LM, Igansi CN, et al. Risk factors for death from pandemic (H1N1) 2009, southern Brazil. *Emerg Infect Dis* 2011; 17(8): 1467-71.
69. Diaz E, Rodriguez A, Martin-Loeches I, et al. Impact of obesity in patients infected with 2009 influenza A(H1N1). *Chest* 2011; 139(2): 382-6.
70. Hung IF, To KK, Lee CK, et al. Convalescent Plasma Treatment Reduced Mortality in Patients With Severe Pandemic Influenza A (H1N1) 2009 Virus Infection. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2011; 52(4): 447-56.
71. Riquelme R, Jimenez P, Videla AJ, et al. Predicting mortality in hospitalized patients with 2009 H1N1 influenza pneumonia. *Int J Tuberc Lung Dis* 2011; 15(4): 542-6.
72. Yu H, Feng Z, Uyeki TM, et al. Risk factors for severe illness with 2009 pandemic influenza A (H1N1) virus infection in China. *Clin Infect Dis* 2011; 52(4): 457-65.
73. Zolotusca L, Jorgensen P, Popovici O, et al. Risk factors associated with fatal influenza, Romania, October 2009 - May 2011. *Influenza and other respiratory viruses* 2014; 8: 8-12.
74. Kok J, Blyth CC, Foo H, et al. Viral pneumonitis is increased in obese patients during the first wave of pandemic A(H1N1) 2009 virus. *PloS one* 2013; 8: e55631.
75. Ren Y-y, Yin Y-y, Li W-q, et al. Risk factors associated with severe manifestations of 2009 pandemic influenza A (H1N1) infection in China: a case-control study. *Virology journal* 2013; 10: 149.
76. Cocoros NM, Lash TL, Demaria A, Klompas M. Obesity as a risk factor for severe influenza-like illness. *Influenza and other respiratory viruses* 2014; 8: 25-32.

77. Kreijtz JHCM, Fouchier RaM, Rimmelzwaan GF. Immune responses to influenza virus infection. *Virus research* 2011; 162: 19-30.
78. García-Sastre A. Induction and evasion of type I interferon responses by influenza viruses. *Virus research* 2011; 162: 12-8.
79. Iwasaki A, Pillai PS. Innate immunity to influenza virus infection. *Nature reviews Immunology* 2014; 14: 315-28.
80. McNab F, Mayer-Barber K, Sher A, Wack A, O'Garra A. Type I interferons in infectious disease. *Nature reviews Immunology* 2015; 15(2): 87-103.
81. Travar M, Petkovic M, Verhaz A. Type I, II, and III Interferons: Regulating Immunity to Mycobacterium tuberculosis Infection. *Archivum immunologiae et therapiae experimentalis* 2015.
82. Donnelly RP, Kotenko SV. Interferon-Lambda: A New Addition to an Old Family. *Journal of Interferon & Cytokine Research* 2010; 30(8): 555-64.
83. Short JAL. Review Viral evasion of interferon stimulated genes. 2009; 2: 212-24.
84. Schoggins JWJ, Wilson SJS, Panis MM, et al. A diverse range of gene products are effectors of the type I interferon antiviral response. *Nature* 2011; 472(7344): 481-5.
85. Sadler AJ, Williams BRG. Interferon-inducible antiviral effectors. *Nature reviews Immunology* 2008; 8: 559-68.
86. Seo J-Y, Yaneva R, Cresswell P. Viperin: a multifunctional, interferon-inducible protein that regulates virus replication. *Cell host & microbe* 2011; 10: 534-9.

87. Wang X, Hinson ER, Cresswell P. The interferon-inducible protein viperin inhibits influenza virus release by perturbing lipid rafts. *Cell host & microbe* 2007; 2(2): 96-105.
88. Desai TM, Marin M, Chin CR, Savidis G, Brass AL, Melikyan GB. IFITM3 restricts influenza A virus entry by blocking the formation of fusion pores following virus-endosome hemifusion. *PLoS pathogens* 2014; 10: e1004048.
89. Hale B, Albrecht RA, García-Sastre A. Innate immune evasion strategies of influenza viruses. 2010.
90. Pauli E-K, Schmolke M, Wolff T, et al. Influenza A virus inhibits type I IFN signaling via NF-kappaB-dependent induction of SOCS-3 expression. *PLoS pathogens* 2008; 4: e1000196.
91. Pothlichet J, Chignard M, Si-Tahar M. Cutting Edge: Innate Immune Response Triggered by Influenza A Virus Is Negatively Regulated by SOCS1 and SOCS3 through a RIG-I/IFNAR1-Dependent Pathway. *The Journal of Immunology* 2008; 180: 2034-8.
92. Almond M, McAuley D. Influenza-related Pneumonia. *Clinical ...* 2012; 12: 67-70.
93. Yoshimura A, Naka T, Kubo M. SOCS proteins, cytokine signalling and immune regulation. *Nature reviews Immunology* 2007; 7: 454-65.
94. Jia D, Rahbar R, Chan RWY, et al. Influenza virus non-structural protein 1 (NS1) disrupts interferon signaling. *PloS one* 2010; 5(11): e13927.
95. Ramírez-Martínez G, Cruz-Lagunas A, Jiménez-Alvarez L, et al. Seasonal and pandemic influenza H1N1 viruses induce differential expression of SOCS-1 and RIG-I genes and cytokine/chemokine production in macrophages. *Cytokine* 2013; 62: 151-9.

96. Wei H, Wang S, Chen Q, et al. Suppression of Interferon Lambda Signaling by SOCS-1 Results in Their Excessive Production during Influenza Virus Infection. *PLoS pathogens* 2014; 10: e1003845.
97. Huang Y, Zaas AK, Rao A, et al. Temporal Dynamics of Host Molecular Responses Differentiate Symptomatic and Asymptomatic Influenza A Infection. *PLoS Genetics* 2011; 7(8): e1002234.
98. Kedzierski L, Linossi EM, Kolesnik TB, et al. Suppressor of cytokine signaling 4 (SOCS4) protects against severe cytokine storm and enhances viral clearance during influenza infection. *PLoS pathogens* 2014; 10: e1004134.
99. Nelli RK, Dunham SP, Kuchipudi SV, et al. Mammalian innate resistance to highly pathogenic avian influenza H5N1 virus infection is mediated through reduced proinflammation and infectious virus release. *Journal of virology* 2012; 86(17): 9201-10.
100. Smith AG, Sheridan PA, Harp JB, Beck MA. Diet-induced obese mice have increased mortality and altered immune responses when infected with influenza virus. *The Journal of nutrition* 2007; 137(5): 1236-43.
101. Milner JJ, Beck Ma. The impact of obesity on the immune response to infection. *The Proceedings of the Nutrition Society* 2012; 71: 298-306.
102. Smith AG, Sheridan PA, Tseng RJ, Sheridan JF, Beck MA. Selective impairment in dendritic cell function and altered antigen-specific CD8⁺ T-cell responses in diet-induced obese mice infected with influenza virus. *Immunology* 2009; 126(2): 268-79.
103. Karlsson EA, Sheridan PA, Beck MA. Diet-induced obesity impairs the T cell memory response to influenza virus infection. *Journal of immunology (Baltimore, Md : 1950)* 2010; 184(6): 3127-33.

104. Karlsson EA, Sheridan PA, Beck MA. Diet-induced obesity in mice reduces the maintenance of influenza-specific CD8⁺ memory T cells. *The Journal of nutrition* 2010; 140(9): 1691-7.
105. Easterbrook JD, Dunfee RL, Schwartzman LM, et al. Obese mice have increased morbidity and mortality compared to non-obese mice during infection with the 2009 pandemic H1N1 influenza virus. *Influenza and other respiratory viruses* 2011; 5(6): 418-25.
106. O'Brien KB, Vogel P, Duan S, et al. Impaired wound healing predisposes obese mice to severe influenza virus infection. *The Journal of infectious diseases* 2012; 205(2): 252-61.
107. Milner JJ, Wang J, Sheridan Pa, Ebbels T, Beck Ma, Saric J. ¹H NMR-Based Profiling Reveals Differential Immune-Metabolic Networks during Influenza Virus Infection in Obese Mice. *PloS one* 2014; 9: e97238.
108. Zhang AJX, To KKW, Li C, et al. Leptin Mediates the Pathogenesis of Severe 2009 Pandemic Influenza A(H1N1) Infection Associated With Cytokine Dysregulation in Mice With Diet-Induced Obesity. *The Journal of infectious diseases* 2013; 207(8): 1270-80.
109. Radigan KA, Morales-Nebreda L, Soberanes S, et al. Impaired clearance of influenza A virus in obese, leptin receptor deficient mice is independent of leptin signaling in the lung epithelium and macrophages. *PloS one* 2014; 9(9): e108138.
110. Stienstra R, Duval C, Muller M, Kersten S. PPARs, Obesity, and Inflammation. *PPAR research* 2007; 2007: 95974.
111. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor- α : direct role in obesity-linked insulin resistance. *Science* 1993; 259(5091): 87-91.

112. Trayhurn P, Wood IS. Signalling role of adipose tissue: adipokines and inflammation in obesity. *Biochemical Society transactions* 2005; 33(Pt 5): 1078-81.
113. Florez H, Castillo-Florez S, Mendez A, et al. C-reactive protein is elevated in obese patients with the metabolic syndrome. *Diabetes research and clinical practice* 2006; 71(1): 92-100.
114. Malli F, Papaioannou AI, Gourgoulisanis KI, Daniil Z. The role of leptin in the respiratory system: an overview. *Respiratory research* 2010; 11: 152.
115. Dixon AE, Pratley RE, Forgione PM, et al. Effects of obesity and bariatric surgery on airway hyperresponsiveness, asthma control, and inflammation. *The Journal of allergy and clinical immunology* 2011; 128: 508-15.e1-2.
116. Mackey-Lawrence NM, Petri Wa. Leptin and mucosal immunity. *Mucosal immunology* 2012; 5: 472-9.
117. Mancuso P. Obesity and lung inflammation. *Journal of applied physiology (Bethesda, Md : 1985)* 2010; 108(3): 722-8.
118. Physiological significance of resistin and resistin-like molecules in the inflammatory process and insulin resistance. 2012: 1-.
119. Steppan CM, Wang J, Whiteman EL, Birnbaum MJ, Lazar MA. Activation of SOCS-3 by Resistin. *Molecular and Cellular Biology* 2005; 25(4): 1569-75.
120. Moschen AR, Kaser A, Enrich B, et al. Visfatin, an adipocytokine with proinflammatory and immunomodulating properties. *Journal of immunology (Baltimore, Md : 1950)* 2007; 178: 1748-58.
121. Hong SB, Huang Y, Moreno-Vinasco L, et al. Essential role of pre-B-cell colony enhancing factor in ventilator-induced lung injury. *American journal of respiratory and critical care medicine* 2008; 178(6): 605-17.

122. White RT, Damm D, Hancock N, et al. Human adiponin is identical to complement factor D and is expressed at high levels in adipose tissue. *The Journal of biological chemistry* 1992; 267(13): 9210-3.
123. Gabrielsson BG, Johansson JM, Lonn M, et al. High expression of complement components in omental adipose tissue in obese men. *Obes Res* 2003; 11(6): 699-708.
124. Moilanen S, Lehtimäki L, Nieminen R, et al. Adipokine adiponin is associated with the degree of lung fibrosis in asbestos-exposed workers. *Respiratory medicine* 2012; 106(10): 1435-40.
125. Sauni R, Oksa P, Lehtimäki L, et al. Increased alveolar nitric oxide and systemic inflammation markers in silica-exposed workers. *Occupational and environmental medicine* 2012; 69(4): 256-60.
126. Ali Assad N, Sood A. Leptin, adiponectin and pulmonary diseases. *Biochimie* 2012; 94: 2180-9.
127. Miller M, Cho JY, Pham A, Ramsdell J, Broide DH. Adiponectin and functional adiponectin receptor 1 are expressed by airway epithelial cells in chronic obstructive pulmonary disease. *Journal of immunology (Baltimore, Md : 1950)* 2009; 182(1): 684-91.
128. Holguin F, Rojas M, Brown LA, Fitzpatrick AM. Airway and plasma leptin and adiponectin in lean and obese asthmatics and controls. *The Journal of asthma : official journal of the Association for the Care of Asthma* 2011; 48(3): 217-23.
129. Folco EJ, Rocha VZ, Lopez-Ilasaca M, Libby P. Adiponectin Inhibits Pro-inflammatory Signaling in Human Macrophages Independent of Interleukin-10. *Journal of Biological Chemistry* 2009; 284(38): 25569-75.

130. Wulster-Radcliffe MC, Ajuwon KM, Wang J, Christian JA, Spurlock ME. Adiponectin differentially regulates cytokines in porcine macrophages. *Biochem Biophys Res Commun* 2004; 316(3): 924-9.
131. Ahmad D, Morgan WK. Obesity and lung function. *Thorax* 2001; 56(9): 740-1.
132. Lessard A, Alméras N, Turcotte H, Tremblay A, Després J-P, Boulet L-P. Adiposity and pulmonary function: relationship with body fat distribution and systemic inflammation. *Clinical and investigative medicine Médecine clinique et expérimentale* 2011; 34(2): E64-70.
133. Sideleva O, Suratt BT, Black KE, et al. Obesity and asthma: An inflammatory disease of adipose tissue not the airway. *American journal of respiratory and critical care medicine* 2012; 186: 598-605.
134. Sood A, Qualls C, Arynchyn A, et al. Obesity-asthma association: is it explained by systemic oxidant stress? *Chest* 2009; 136(4): 1055-62.
135. Mackey-Lawrence NM, Petri WAJ. Leptin and mucosal immunity. *Mucosal immunology* 2012; 5(5): 472-9.
136. La Cava A, Matarese G. The weight of leptin in immunity. *Nature Reviews: Immunology* 2004; 4(5): 371-9.
137. Tsuchiya T, Shimizu H, Horie T, Mori M. Expression of leptin receptor in lung: leptin as a growth factor. *European journal of pharmacology* 1999; 365: 273-9.
138. Bruno A, Pace E, Chanez P, et al. Leptin and leptin receptor expression in asthma. *The Journal of allergy and clinical immunology* 2009; 124: 230-7, 7.e1-4.

139. Vernooy JHJ, Drummen NEa, van Suylen RJ, et al. Enhanced pulmonary leptin expression in patients with severe COPD and asymptomatic smokers. *Thorax* 2009; 64: 26-32.
140. Lord GM, Matarese G, Howard JK, Baker RJ, Bloom SR, Lechler RI. Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. *Nature* 1998; 394(6696): 897-901.
141. Lugogo NL, Hollingsworth JW, Howell DL, et al. Alveolar macrophages from overweight/obese subjects with asthma demonstrate a proinflammatory phenotype. *American journal of respiratory and critical care medicine* 2012; 186: 404-11.
142. Al-Hassi HO, Bernardo D, Murugananthan aU, et al. A mechanistic role for leptin in human dendritic cell migration: differences between ileum and colon in health and Crohn's disease. *Mucosal immunology* 2013; 6: 751-61.
143. Mattioli B, Straface E, Quaranta MG, Giordani L, Viora M. Leptin Promotes Differentiation and Survival of Human Dendritic Cells and Licenses Them for Th1 Priming. *The Journal of Immunology* 2005; 174: 6820-8.
144. Tsiotra PC, Pappa V, Raptis SA, Tsigos C. Expression of the long and short leptin receptor isoforms in peripheral blood mononuclear cells: implications for leptin's actions. *Metabolism* 2000; 49(12): 1537-41.
145. Bjørbaek C, Elmquist JK, Frantz JD, Shoelson SE, Flier JS. Identification of SOCS-3 as a potential mediator of central leptin resistance. *Molecular cell* 1998; 1: 619-25.
146. Nave H, Mueller G, Siegmund B, et al. Resistance of Janus kinase-2 dependent leptin signaling in natural killer (NK) cells: a novel mechanism of NK cell dysfunction in diet-induced obesity. *Endocrinology* 2008; 149(7): 3370-8.

147. Papathanassoglou E, El-Haschimi K, Li XC, Matarese G, Strom T, Mantzoros C. Leptin receptor expression and signaling in lymphocytes: kinetics during lymphocyte activation, role in lymphocyte survival, and response to high fat diet in mice. *Journal of immunology* 2006; 176(12): 7745-52.
148. Münzberg H, Myers MG. Molecular and anatomical determinants of central leptin resistance. *Nature neuroscience* 2005; 8(5): 566-70.
149. Maffei M, Halaas J, Ravussin E, et al. Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nature medicine* 1995; 1(11): 1155-61.
150. Considine RV, Sinha MK, Heiman ML, et al. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *The New England journal of medicine* 1996; 334: 292-5.
151. Friedman JM, Halaas JL. Leptin and the regulation of body weight in mammals. *Nature* 1998; 395: 763-70.
152. Bjorbaek C, El-Haschimi K, Frantz JD, Flier JS. The Role of SOCS-3 in Leptin Signaling and Leptin Resistance. *Journal of Biological Chemistry* 1999; 274: 30059-65.
153. Gainsford T, Willson TA, Metcalf D, et al. Leptin can induce proliferation, differentiation, and functional activation of hemopoietic cells. *Proceedings of the National Academy of Sciences of the United States of America* 1996; 93(25): 14564-8.
154. Santos-Alvarez J, Goberna R, Sánchez-Margalet V. Human leptin stimulates proliferation and activation of human circulating monocytes. *Cellular Immunology* 1999; 194(1): 6-11.

155. Gabay C, Dreyer M, Pellegrinelli N, Chicheportiche R, Meier CA. Leptin directly induces the secretion of interleukin 1 receptor antagonist in human monocytes. *The Journal of clinical endocrinology and metabolism* 2001; 86(2): 783-91.
156. Martín-Romero C. Human Leptin Activates PI3K and MAPK Pathways in Human Peripheral Blood Mononuclear Cells: Possible Role of Sam68. *Cellular Immunology* 2001; 212(2): 83-91.
157. Sanchez-Margalet V, Martin-Romero C. Human Leptin Signaling in Human Peripheral Blood Mononuclear Cells: Activation of the JAK-STAT Pathway. *Cellular Immunology* 2001; 211(1): 30-6.
158. Dreyer MG, Juge-Aubry CE, Gabay C, et al. Leptin activates the promoter of the interleukin-1 receptor antagonist through p42/44 mitogen-activated protein kinase and a composite nuclear factor kappa B/PU.1 binding site. *Biochemical Journal* 2003; 370(Pt 2): 591-9.
159. Mancuso P. Leptin augments alveolar macrophage leukotriene synthesis by increasing phospholipase activity and enhancing group IVC iPLA2 (cPLA2) protein expression. *American journal of physiology Lung cellular and molecular physiology* 2004; 287(3): L497-L502.
160. Shen Y, Wang Q, Zhao Q, Zhou J. Leptin promotes the immune escape of lung cancer by inducing proinflammatory cytokines and resistance to apoptosis. 2009: 295-9.
161. Kiguchi N, Maeda T, Kobayashi Y, Fukazawa Y, Kishioka S. Leptin enhances CC-chemokine ligand expression in cultured murine macrophage. *Biochemical and Biophysical Research Communications* 2009; 384(3): 311-5.
162. Taideman J, Pérez-Novo CA, Rottiers I, et al. Human mast cells express leptin and leptin receptors. *Histochemistry and Cell Biology* 2009; 131(6): 703-11.

163. Gomez R, Scotece M, Conde J, Gomez-Reino JJ, LAGO F, GUALILLO O. Adiponectin and leptin increase IL-8 production in human chondrocytes. *Annals of the Rheumatic Diseases* 2011; 70(11): 2052-4.
164. Bruno A, Alessi M, Soresi S, et al. Increased leptin/leptin receptor pathway affects systemic and airway inflammation in COPD former smokers. *Journal of inflammation research* 2011; 4: 51-9.
165. Handy JA, Fu PP, Kumar P, et al. Adiponectin inhibits leptin signalling via multiple mechanisms to exert protective effects against hepatic fibrosis. *Biochemical Journal* 2011; 440(3): 385-95.
166. Teran-Cabanillas E, Montalvo-Corral M, Caire-Juvera G, Moya-Camarena SY, Hernandez J. Decreased interferon-alpha and interferon-beta production in obesity and expression of suppressor of cytokine signaling. *Nutrition* 2013; 29(1): 207-12.
167. Terán-Cabanillas E, Montalvo-Corral M, Silva-Campa E, Caire-Juvera G, Moya-Camarena SY, Hernández J. Production of interferon α and β , pro-inflammatory cytokines and the expression of suppressor of cytokine signaling (SOCS) in obese subjects infected with influenza A/H1N1. *Clinical nutrition (Edinburgh, Scotland)* 2013; 1-5.
168. Contoli M, Message SD, Laza-Stanca V, et al. Role of deficient type III interferon-lambda production in asthma exacerbations. *Nature medicine* 2006; 12: 1023-6.
169. Wark PA, Johnston SL, Bucchieri F, et al. Asthmatic bronchial epithelial cells have a deficient innate immune response to infection with rhinovirus. *The Journal of experimental medicine* 2005; 201(6): 937-47.

170. Sykes A, Edwards MR, Macintyre J, et al. Rhinovirus 16-induced IFN- α and IFN- β are deficient in bronchoalveolar lavage cells in asthmatic patients. *The Journal of allergy and clinical immunology* 2012; 129: 1506-14.e6.
171. Mallia P, Message SD, Gielen V, et al. Experimental rhinovirus infection as a human model of chronic obstructive pulmonary disease exacerbation. *American journal of respiratory and critical care medicine* 2011; 183(6): 734-42.
172. Forbes RL, Wark PA, Murphy VE, Gibson PG. Pregnant women have attenuated innate interferon responses to 2009 pandemic influenza A virus subtype H1N1. *The Journal of infectious diseases* 2012; 206(5): 646-53.
173. Parker D, Cohen TS, Alhede M, Harfenist BS, Martin FJ, Prince A. Induction of type I interferon signaling by *Pseudomonas aeruginosa* is diminished in cystic fibrosis epithelial cells. *American journal of respiratory cell and molecular biology* 2012; 46: 6-13.
174. Sajjan U, Ganesan S, Comstock AT, et al. Elastase- and LPS-exposed mice display altered responses to rhinovirus infection. *American journal of physiology Lung cellular and molecular physiology* 2009; 297(5): L931-44.
175. Schneider D, Ganesan S, Comstock AT, et al. Increased cytokine response of rhinovirus-infected airway epithelial cells in chronic obstructive pulmonary disease. *American journal of respiratory and critical care medicine* 2010; 182(3): 332-40.
176. Huttunen R, Syrjänen J. Obesity and the risk and outcome of infection. *International journal of obesity (2005)* 2012.
177. Chairat K, Jittamala P, Hanpithakpong W, et al. Population pharmacokinetics of oseltamivir and oseltamivir carboxylate in obese and Non-obese volunteers. *Br J Clin Pharmacol* 2016.

178. Ashrafian H, le Roux CW, Rowland SP, et al. Metabolic surgery and obstructive sleep apnoea: the protective effects of bariatric procedures. *Thorax* 2012; 67(5): 442-9.
179. Yamauchi M, Tamaki S, Tomoda K, et al. Evidence for activation of nuclear factor kappaB in obstructive sleep apnea. *Sleep & breathing = Schlaf & Atmung* 2006; 10(4): 189-93.
180. Akinnusi M, Jaoude P, Kufel T, El-Solh AA. Toll-like receptor activity in patients with obstructive sleep apnea. *Sleep & breathing = Schlaf & Atmung* 2013; 17(3): 1009-16.
181. Sharma S, Malur A, Marshall I, et al. Alveolar macrophage activation in obese patients with obstructive sleep apnea. *Surgery* 2012; 151(1): 107-12.
182. Baraldo S, Contoli M, Bazzan E, et al. Deficient antiviral immune responses in childhood: distinct roles of atopy and asthma. *The Journal of allergy and clinical immunology* 2012; 130(6): 1307-14.
183. Forbes RL, Gibson PG, Murphy VE, Wark PA. Impaired type I and III interferon response to rhinovirus infection during pregnancy and asthma. *Thorax* 2012; 67(3): 209-14.
184. Consoli A, Devangelio E. Thiazolidinediones and inflammation. *Lupus* 2005; 14(9): 794-7.
185. Terblanche M, Almog Y, Rosenson RS, Smith TS, Hackam DG. Statins and sepsis: multiple modifications at multiple levels. *The Lancet Infectious diseases* 2007; 7(5): 358-68.
186. Standardized lung function testing. Report working party. *Bulletin europeen de physiopathologie respiratoire* 1983; 19 Suppl 5: 1-95.

187. Nelson DW, Blair KS, Martin MJ. Analysis of obesity-related outcomes and bariatric failure rates with the duodenal switch vs gastric bypass for morbid obesity. *Archives of surgery* 2012; 147(9): 847-54.
188. O'Brien PE, MacDonald L, Anderson M, Brennan L, Brown WA. Long-term outcomes after bariatric surgery: fifteen-year follow-up of adjustable gastric banding and a systematic review of the bariatric surgical literature. *Annals of surgery* 2013; 257(1): 87-94.
189. Hutter MM, Schirmer BD, Jones DB, et al. First report from the American College of Surgeons Bariatric Surgery Center Network: laparoscopic sleeve gastrectomy has morbidity and effectiveness positioned between the band and the bypass. *Annals of surgery* 2011; 254(3): 410-20; discussion 20-2.
190. Du Rand IA, Blaikley J, Booton R, et al. British Thoracic Society guideline for diagnostic flexible bronchoscopy in adults: accredited by NICE. *Thorax* 2013; 68 Suppl 1: i1-i44.
191. Jackson DJ, Makrinioti H, Rana BM, et al. IL-33-dependent type 2 inflammation during rhinovirus-induced asthma exacerbations in vivo. *American journal of respiratory and critical care medicine* 2014; 190(12): 1373-82.
192. BioRad. BioPlex Pro Cytokine, chemokine, and growth factor assays. In: BioRad, editor. BioRad Luminex Manual; 2015.
193. WHO WHO. Obesity - Situation and Trends. 2015. http://www.who.int/gho/ncd/risk_factors/obesity_text/en/.
194. Pearce N, Pekkanen J, Beasley R. How much asthma is really attributable to atopy? *Thorax* 1999; 54(3): 268-72.

195. Fisman DN, Savage R, Gubbay J, et al. Older age and a reduced likelihood of 2009 H1N1 virus infection. *The New England journal of medicine* 2009; 361(20): 2000-1.
196. Nguyen AM, Noymer A. Influenza mortality in the United States, 2009 pandemic: burden, timing and age distribution. *PloS one* 2013; 8(5): e64198.
197. Klein SL, Passaretti C, Anker M, Olukoya P, Pekosz A. The impact of sex, gender and pregnancy on 2009 H1N1 disease. *Biology of sex differences* 2010; 1(1): 5.
198. Zhao H, Harris RJ, Ellis J, Pebody RG. Ethnicity, deprivation and mortality due to 2009 pandemic influenza A(H1N1) in England during the 2009/2010 pandemic and the first post-pandemic season. *Epidemiology and infection* 2015: 1-9.
199. Khaitov MR, Laza-Stanca V, Edwards MR, et al. Respiratory virus induction of alpha-, beta- and lambda-interferons in bronchial epithelial cells and peripheral blood mononuclear cells. *Allergy* 2009; 64(3): 375-86.
200. Edwards MR, Regamey N, Vareille M, et al. Impaired innate interferon induction in severe therapy resistant atopic asthmatic children. *Mucosal immunology* 2013; 6(4): 797-806.
201. Wiwanitkit V. Human bird flu infection: is there any relationship to hypertension? *Hypertension* 2006; 47(6): e28.
202. Vernooij JHJ, Ubags NDJ, Brusselle GG, et al. Leptin as regulator of pulmonary immune responses: involvement in respiratory diseases. *Pulmonary pharmacology & therapeutics* 2013; 26: 464-72.
203. Yu WC, Chan RW, Wang J, et al. Viral replication and innate host responses in primary human alveolar epithelial cells and alveolar macrophages infected with influenza H5N1 and H1N1 viruses. *Journal of virology* 2011; 85(14): 6844-55.

204. Zucchini N, Bessou G, Robbins SH, et al. Individual plasmacytoid dendritic cells are major contributors to the production of multiple innate cytokines in an organ-specific manner during viral infection. *International immunology* 2008; 20(1): 45-56.
205. Lambrecht BN. Alveolar macrophage in the driver's seat. *Immunity* 2006; 24(4): 366-8.
206. Shieh WJ, Blau DM, Denison AM, et al. 2009 pandemic influenza A (H1N1): pathology and pathogenesis of 100 fatal cases in the United States. *The American journal of pathology* 2010; 177(1): 166-75.
207. Vareille M, Kieninger E, Edwards MR, Regamey N. The airway epithelium: soldier in the fight against respiratory viruses. *Clinical microbiology reviews* 2011; 24(1): 210-29.
208. Mordstein M, Neugebauer E, Ditt V, et al. Lambda interferon renders epithelial cells of the respiratory and gastrointestinal tracts resistant to viral infections. *Journal of virology* 2010; 84(11): 5670-7.
209. Hsu AC, Barr I, Hansbro PM, Wark PA. Human influenza is more effective than avian influenza at antiviral suppression in airway cells. *American journal of respiratory cell and molecular biology* 2011; 44(6): 906-13.
210. Hsu AC, Parsons K, Barr I, et al. Critical role of constitutive type I interferon response in bronchial epithelial cell to influenza infection. *PloS one* 2012; 7(3): e32947.
211. Chan MC, Chan RW, Yu WC, et al. Tropism and innate host responses of the 2009 pandemic H1N1 influenza virus in ex vivo and in vitro cultures of human conjunctiva and respiratory tract. *The American journal of pathology* 2010; 176(4): 1828-40.

212. Chan RW, Yuen KM, Yu WC, et al. Influenza H5N1 and H1N1 virus replication and innate immune responses in bronchial epithelial cells are influenced by the state of differentiation. *PloS one* 2010; 5(1): e8713.
213. Dicker D, Salook MA, Marcoviciu D, Djaldetti M, Bessler H. Role of peripheral blood mononuclear cells in the predisposition of obese individuals to inflammation and infection. *Obesity facts* 2013; 6(2): 146-51.
214. Yen CL, Chao WC, Wu CH, et al. Phosphorylation of glycogen synthase kinase-3beta in metabolically abnormal obesity affects immune stimulation-induced cytokine production. *International journal of obesity* 2015; 39(2): 270-8.
215. Ahmad R, Al-Mass A, Atizado V, et al. Elevated expression of the toll like receptors 2 and 4 in obese individuals: its significance for obesity-induced inflammation. *Journal of inflammation* 2012; 9(1): 48.
216. Paich HA, Sheridan PA, Handy J, et al. Overweight and obese adult humans have a defective cellular immune response to pandemic H1N1 influenza A virus. *Obesity (Silver Spring, Md)* 2013; 21(11): 2377-86.
217. Hao X, Kim TS, Braciale TJ. Differential response of respiratory dendritic cell subsets to influenza virus infection. *Journal of virology* 2008; 82(10): 4908-19.
218. Gardner EM, Beli E, Clinthorne JF, Duriancik DM. Energy intake and response to infection with influenza. *Annual review of nutrition* 2011; 31: 353-67.
219. O'Shea D, Corrigan M, Dunne MR, et al. Changes in human dendritic cell number and function in severe obesity may contribute to increased susceptibility to viral infection. *International journal of obesity* 2013; 37(11): 1510-3.
220. Schneider C, Nobs SP, Heer AK, et al. Alveolar macrophages are essential for protection from respiratory failure and associated morbidity following influenza virus infection. *PLoS pathogens* 2014; 10(4): e1004053.

221. Wang J, Oberley-Deegan R, Wang S, et al. Differentiated human alveolar type II cells secrete antiviral IL-29 (IFN-lambda 1) in response to influenza A infection. *Journal of immunology* 2009; 182(3): 1296-304.
222. Goritzka M, Makris S, Kausar F, et al. Alveolar macrophage-derived type I interferons orchestrate innate immunity to RSV through recruitment of antiviral monocytes. *The Journal of experimental medicine* 2015; 212(5): 699-714.
223. Kotani N, Hashimoto H, Sessler DI, et al. Intraoperative modulation of alveolar macrophage function during isoflurane and propofol anesthesia. *Anesthesiology* 1998; 89(5): 1125-32.
224. Steurer M, Schlapfer M, Steurer M, et al. The volatile anaesthetic sevoflurane attenuates lipopolysaccharide-induced injury in alveolar macrophages. *Clinical and experimental immunology* 2009; 155(2): 224-30.
225. Djukanovic R, Harrison T, Johnston SL, et al. The effect of inhaled IFN-beta on worsening of asthma symptoms caused by viral infections. A randomized trial. *American journal of respiratory and critical care medicine* 2014; 190(2): 145-54.
226. Steppan CM, wang J, Whiteman EL, Birnbaum MJ, Lazar MA. Activation of SOCS-3 by Resistin. *Journal of molecular and cellular biology* 2005; 25(4): 1-7.
227. Jackson DJ, Sykes A, Mallia P, Johnston SL. Asthma exacerbations: origin, effect, and prevention. *The Journal of allergy and clinical immunology* 2011; 128: 1165-74.
228. Dong Q, Wright JR. Expression of C-reactive protein by alveolar macrophages. *Journal of immunology (Baltimore, Md : 1950)* 1996; 156: 4815-20.
229. Vos R, Vanaudenaerde BM, De Vleeschauwer SI, et al. C-Reactive Protein in Bronchoalveolar Lavage Fluid is Associated With Markers of Airway Inflammation After Lung Transplantation. *Transplantation Proceedings* 2009; 41: 3409-13.

230. Rugsarash W, Tungtrongchitr R, Petmitr S, et al. The genetic association between alpha-2-macroglobulin (A2M) gene deletion polymorphism and low serum A2M concentration in overweight/obese Thais. *Nutritional neuroscience* 2006; 9(1-2): 93-8.
231. Loffreda S, Yang SQ, Lin HZ, et al. Leptin regulates proinflammatory immune responses. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 1998; 12(1): 57-65.
232. Galve-de Rochemonteix B, Wiktorowicz K, Kushner I, Dayer JM. C-reactive protein increases production of IL-1 alpha, IL-1 beta, and TNF-alpha, and expression of mRNA by human alveolar macrophages. *J Leukoc Biol* 1993; 53(4): 439-45.
233. Gielen V, Sykes A, Zhu J, et al. Increased nuclear suppressor of cytokine signaling 1 in asthmatic bronchial epithelium suppresses rhinovirus induction of innate interferons. *Journal of Allergy and Clinical Immunology* 2015; 136: 177-88.e11.
234. Le Goffic R, Pothlichet J, Vitour D, et al. Cutting edge: Influenza A virus activates TLR3-dependent inflammatory and RIG-1-dependent antiviral responses in human lung epithelial cells. *Journal of immunology* 2007; 178: 3368-72.
235. Vuolteenaho K, Koskinen a, Moilanen T, Moilanen E. Leptin levels are increased and its negative regulators, SOCS-3 and sOb-R are decreased in obese patients with osteoarthritis: a link between obesity and osteoarthritis. *Annals of the Rheumatic Diseases* 2012; 71: 1912-3.
236. Bourdonnay E, Zas ona Z, Penke LRK, et al. Transcellular delivery of vesicular SOCS proteins from macrophages to epithelial cells blunts inflammatory signaling. *Journal of Experimental Medicine* 2015; 212: 729-42.
237. Kim HJ, Kim C-H, Ryu J-H, et al. Reactive oxygen species induce antiviral innate immune response through IFN- λ regulation in human nasal epithelial cells. *American journal of respiratory cell and molecular biology* 2013; 49: 855-65.

238. Dalpke A, Heeg K, Bartz H, Baetz A. Regulation of innate immunity by suppressor of cytokine signaling (SOCS) proteins. *Immunobiology* 2008; 213: 225-35.
239. Myers MG, Leibel RL, Seeley RJ, Schwartz MW. Obesity and leptin resistance: Distinguishing cause from effect. *Trends in Endocrinology and Metabolism* 2010; 21: 643-51.
240. Milner JJ, Karlsson EA, Beck MA. Leptin treatment alters the response of respiratory epithelial cells to influenza infection. 2010: FASEB J.; 2010.
241. Feng H, Liu Q, Zhang N, et al. Leptin promotes metastasis by inducing an epithelial-mesenchymal transition in A549 lung cancer cells. *Oncology research* 2013; 21(3): 165-71.
242. Woo H-J, Yoo WJ, Bae CH, et al. Leptin up-regulates MUC5B expression in human airway epithelial cells via mitogen-activated protein kinase pathway. *Experimental lung research* 2010; 36: 262-9.
243. Suzukawa M. Leptin Enhances ICAM-1 Expression, Induces Migration and Cytokine Synthesis, and Prolongs Survival of Human Airway Epithelial Cells. *Pharmacoeconomics* 2015; 28 Suppl 1: i.
244. Li S, Wang L, Berman M, Kong YY, Dorf ME. Mapping a dynamic innate immunity protein interaction network regulating type I interferon production. *Immunity* 2011.
245. Hussell T, Cavanagh MM. The innate immune rheostat: influence on lung inflammatory disease and secondary bacterial pneumonia. *Biochemical Society transactions* 2009; 37(Pt 4): 811-3.
246. Memoli MJ, Czajkowski L, Reed S, et al. Validation of the wild-type influenza A human challenge model H1N1pdMIST: an A(H1N1)pdm09 dose-finding investigational new drug study. *Clin Infect Dis* 2015; 60(5): 693-702.

