Imperial College London

Cell mediated immunity against RSV and influenza in a human experimental challenge model

RESEARCH PROTOCOL

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Study Management Group

Chief Investigator: Professor Peter Openshaw

Co-investigators: Professor Sebastian Johnston
Dr Christopher Chiu

Study Site

The study will take place at Imperial College Healthcare NHS Trust or Surrey Clinical Research Centre in Guildford.

Study Coordination Centre

For general queries, supply of study documentation, and collection of data, please contact:

Study Coordinator: Dr Christopher Chiu
Address: Department of Respiratory Medicine,Commonwealth Building, Hammersmith Campus, Imperial College London.

Position: Clinician Scientist & Honorary Consultant in Infectious Diseases

Tel: +44-20-8383 2301 E-mail: c.chiu@imperial.ac.uk

Clinical Queries

Clinical queries should be directed to Dr Christopher Chiu who will answer or direct the query to the appropriate person.

Sponsor

Imperial College London is the research sponsor for this study. For further information regarding the sponsorship conditions, please contact the Research Governance Manager (Ruth Nicholson) at:

Joint Research Office, Room 221,
Level 2, Medical School Building
Norfolk Place,
London W2 1PG
020759 41862
r.nicholson@imperial.ac.uk

Funder

The MRC has provided Clinician Scientist Fellowship funding for this study. This protocol describes the above study and provides information about procedures for entering participants. Every care was taken in its drafting, but corrections or amendments may be necessary. These will be circulated to investigators in the study. Problems relating to this study should be referred, in the first instance, to the Chief Investigator.

This study will adhere to the principles outlined in the NHS Research Governance Framework for Health and Social Care (3rd edition). It will be conducted in compliance with the protocol, the Data Protection Act and other regulatory requirements as appropriate including Good Clinical Practice.

STUDY SUMMARY

TITLE Cell mediated immunity against RSV and influenza in a human experimental challenge model

DESIGN Human viral challenge study in healthy volunteers

AIMS 1. To test the hypothesis that RSV and influenza lead to quantitative and functional differences in T-cell responses.

2. To identify the mechanisms underlying poor antigen-specific cell mediated immunity (CMI) to RSV by comparing the transcriptional changes in T-cells following experimental challenge with RSV and influenza.

POPULATION Healthy persons aged 18 to 55 years

ELIGIBILITY Healthy persons aged 18 to 55 years that fit the inclusion and exclusion criteria

DURATION 3 Years

GLOSSARY OF ABBREVIATIONS

AE	Adverse Event
BAL	Bronchoalveolar Lavage
CMI	Cell Mediated Immunity
CRI	Centre for Respiratory Infection
CTL	Cytotoxic T cell
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
ELF	Epithelial Lining Fluid
FEV_1	Forced Expiratory Volume in One Second
FI-RSV	Formalin-inactivated RSV vaccine
GC-MS	Gas Chromatography with Mass Spectrometry
GCP	Good Clinical Practice
HA	Haemagglutinin
ICRRU	Imperial Clinical Respiratory Research Unit
LAIV	Live Attenuated Influenza Vaccine
NA	Neuraminidase
NHLI	National Heart and Lung Institute
NP	Nucleoprotein
PBMC	Peripheral Blood Mononuclear Cell
PCR	Polymerase Chain Reaction
PEF	Peak Expiratory Flow
PIS	Participant Information Sheet
RSV	Respiratory Syncytial Virus
RV	Rhinovirus
SAE	Serious Adverse Event
SAM	Synthetic Absorptive Matrix
URT	Upper Respiratory Tract
VOC	Volatile Organic Compound
WHO	World Health Organisation

KEYWORDS

Respiratory Syncytial Virus, RSV, immune, virus, bronchiolitis, viral challenge, viral lung disease, influenza

INTRODUCTION

1.1. Background to research

Influenza and Respiratory Syncytial Virus (RSV) are the two most common causes of severe viral respiratory tract infection. Seasonal influenza has an overall incidence of 10-20% per annum with frequent complications, and the annual mortality in the USA has been estimated at up to 9.9 deaths per 100,000[1]. During the 2009 H1N1 influenza pandemic, the fatality rate has been 0.02% with many deaths occurring in young adults, and asthmatics at particularly high risk with up to 50% of asthma exacerbations thought to be due to influenza. According to WHO estimates, RSV causes around 64 million infections per annum and 160,000 deaths. It is the leading cause of severe respiratory illness in young children (associated with severe infant wheezing illness) and is also a significant problem in susceptible adults (including the elderly and those with airways disease) in whom RSV is responsible for around 22% of winter respiratory illnesses with a case fatality rate of 2-8%[2].

Vaccine development remains an urgent priority. While CMI is important in viral clearance and may enhance vaccine efficacy, its relative contribution in humans has not been fully determined. We aim to elucidate its role by comparing the human T cell responses to influenza and RSV. These differ in that re-infection with antigenically identical strains of influenza does not occur, while within 2 months of RSV challenge even subjects with high antibody levels may be re-infected with the same strain. The reasons for this are unclear but may involve immune modulation by viral products or impaired antigen presentation by RSV-infected dendritic cells leading to impaired T cell numbers function.

Immune targets in influenza and RSV The major surface antigenic determinants of influenza, haemaglutinin (HA) and neuraminidase (NA), both elicit protective humoral responses. However rapid mutation and reassortment of the segmented genome leads to immune escape. Internal viral components, such as the nucleoprotein (NP), which are relatively conserved, do stimulate cross-reactive T cell responses[3] but these do not appear to prevent infection with novel strains in the long term. The genome of RSV encodes 3 transmembrane proteins (the attachment glycoprotein G, fusion F and small hydrophobic SH proteins) and both the G and F proteins stimulate antibody production. Like HA and NA, the G protein undergoes sequence mutation but the F and SH proteins exhibit limited variability[4]. Cytotoxic T cell (CTL) responses have been demonstrated against the F and SH proteins as well as several internal proteins but not the G protein[5].

Correlates of protection against influenza and RSV infection The immune factors that determine whether an individual will develop symptomatic infection following exposure to a respiratory pathogen are poorly understood. Traditionally, serum antibody has been used as a correlate of protection against influenza and, indeed, high neutralising antibody titres do correlate somewhat with a decreased risk of influenza infection. However, it is clear that antibodies do not explain all aspects of immune protection as some individuals with no detectable neutralising antibody are still protected. With RSV, this is additionally complicated as there is no antibody level at which protection is assured. Increasing evidence indicates that protective immunity is mediated by a number of factors including early innate immune mechanisms and cell mediated immunity. Furthermore, these arms of the immune system are interlinked, with innate immunity critically influencing later adaptive responses. Thus a comprehensive understanding of both the early innate responses and cell mediate immunity will be essential for the development of effective vaccines.

Vaccines against influenza and RSV There are few treatments available for influenza and RSV infection, and those that exist cannot be used in the developing world, where a large proportion of disease burden lies, due to cost. For these reasons, effective vaccines remain of utmost importance. Influenza vaccination is currently achieved by subunit or live attenuated influenza vaccines (LAIV) which are re-formulated each year to maintain their antigenicity. Production of these vaccines each year is time-consuming and slow to respond to emerging viruses such as the current H1N1 pandemic strain.

No influenza vaccine has yet been shown to elicit broad protection against all virus strains. Meanwhile, an effective vaccine against RSV still does not exist. Studies using formalin-inactivated RSV carried out in the 1960s led to unexpected results as not only was there no protection but also 80% of vaccinees required hospitalisation with vaccine-enhanced disease[6]. It has therefore been essential to balance vaccine-induced immunopathology with immunogenicity, and few vaccine candidates have fulfilled both criteria.

The importance of cell mediated immunity There is evidence that cell mediated immunity (CMI) is important for protection against and clearance of viral infections. Vaccines that induce both humoral immunity and CMI may be more effective and protect against a wider range of virus serotypes. In animal models, CTL responses have been correlated with reductions in influenza viral load and some protection has been demonstrated using prior inoculation with a serologically distinct strain[7]. The protective role of CMI in humans has been more difficult to determine, and the contribution of reduced CMI in infective exacerbations of airways disease is unclear. T cells have been identified in humans that have cross-reactive potential and one study has directly demonstrated correlation between high CTL levels and reduced virus shedding[8], while in vaccinated individuals, levels of serum antibody correlate poorly with vaccine efficacy[9]. LAIV stimulates more vigorous CMI than subunit vaccines and there is an association between the number of interferon-γ (IFN-γ) producing peripheral blood mononuclear cells (PBMCs) and the protective efficacy of the vaccine[10]. These may explain why LAIV is more effective than subunit vaccines. In mice, depletion of CD4+ and CD8+ T cells suggests that both make significant contributions to the inhibition of RSV replication. Athymic nude mice infected with RSV exhibit persistent shedding of virus which can be terminated by adoptive transfer of T cells from previously primed animals[11]. In the clinical setting, children with defects in CMI suffer more severe RSV disease and shed the virus for several months compared with normal children who shed for only 7-21 days[12]. This is also true of bone marrow transplant recipients.

Contrasting immune responses to influenza and RSV In a mouse model of influenza infection, viral load falls as soon as CTLs appear in the lung[13] and effector memory CTLs act rapidly on re-challenge to recruit T cells to then expand and form a new effector pool. Along with the humoral response against HA and NA, this leads to protective immunity and re-infection with the same strain of influenza does not occur[14]. In contrast, infection with RSV does not confer full protective immunity. Almost all children will have seroconverted to RSV by 24 months of age, but re-infection is common throughout life. This occurs despite persistent antibodies against the relatively conserved F protein which are cross-reactive against the different serogroups of RSV[15]. In human experimental challenge studies, where adults naturally infected with RSV were later challenged 6 times with a similar laboratory strain, 73% could be re-infected and 50% re-infected twice or more[16]. Re-infection took place at a rate of 25% even in those subjects with the highest levels of IgG and IgA against F and G proteins within as little as 2 months. These differences are likely to be due to differential immune responses against influenza and RSV and may be quantitative or functional. In adult blood and lung, influenza-specific memory T cells are 2-4 times more frequent than those specific for RSV[17,18], while analysis of cytokine responses in the nasopharyngeal aspirates of infected children show higher levels of IFN-γ and decreased IL-4 and IL-5 in influenza compared with RSV[19].

Mechanisms of immune modulation by RSV The reasons for these contrasting immune responses and incomplete protection against RSV infection are unclear. *In vitro* studies have suggested many RSV proteins possess immunomodulatory activity. NS1 and NS3 proteins have been shown to inhibit response to interferons[20], while replication of RSV within dendritic cells (DCs) has also been shown to interfere with IFN-α and IL-12 responses, as well as inducing IFN-λ production that suppresses CD4+ T cells[21]. Furthermore, animal studies have shown RSV infection depleting DC precursors and leading to the inhibition of DC expansion on re-infection[22]. RSV may also interfere with the normal interaction between DCs and T cells. In mice, RSV-specific CTLs are short-lived and have impaired cytolytic ability and reduced production of IFN-γ[23]. These deficiencies can be reversed by incubation with exogenous IL-2, and are believed to be due to a defect in the TCR signalling pathway, which may be due to the effects of direct contact with cells expressing F protein on their surface and/or inhibition of formation of the immunological synapse[24].

Human challenge model In the early 1990s, Peter Openshaw's group carried out human volunteer challenge studies showing that individuals could be repeatedly infected with the same strain of RSV although the mechanism remained unclear (A.H. Cherrie, PhD thesis, University of London 1992). Although human challenge experiments with RSV were discontinued, Sebastian Johnston's group has since performed many volunteer challenge studies to investigate immune responses to rhinovirus. Their recent studies on exacerbations of asthma by rhinovirus have demonstrated the altered cytokine profiles of subjects, and their experience in this area will form the basis of this study[25].

Participants in the influenza arm of this study will be challenged with either a fully virulent influenza strain live attenuated influenza vaccine (LAIV). Challenge experiments with virulent influenza overcomes the many disadvantages associated with studying a patient group infected with naturally-acquired influenza: diagnosis is often delayed, the timing and dose of inoculating virus is extremely variable, many patients have underlying medical conditions influencing their immune responses, and they may be subject to therapeutic measures that alter those responses. Finally, it would not be possible to re-challenge these individuals in order to examine a recall response. Using a well-characterised, fully virulent GMP-certified challenge strain of influenza will allow us to closely replicate natural infection but also provide us with the control required to answer important questions about the essential immune responses required for protection against re-infection. Since, LAIV replicates and undergoes intracellular processing in a similar way to virulent influenza, it is also able to elicit similar immune responses including mucosal immunity and IFN-γ producing T cells in the blood[10]. In children it is highly effective in preventing subsequent infection with antigenically similar strains of seasonal influenza. However, its efficacy in adults diminishes with age and duration of protection is poor. By comparing the immune responses of LAIV to virulent influenza, we will be able to define the differences in phenotype and function that are responsible for the more robust immunity seen with natural infection.

This study will for the first time systematically investigate the mechanisms behind the differential responses to RSV and influenza. The data obtained will be essential for further understanding of the natural history of human antiviral responses, and will have direct bearing on future studies into the effect of these infections on the elderly and asthmatics as well as novel approaches to vaccine development.

1.2. Research Hypotheses

1. RSV specifically interferes with T cell activation, causing impairment of cell-mediated immunity and allowing recurrent infection with antigenically similar strains.

2. By contrast, repeated infection with the same strain of influenza is prevented by antibody and T cell dependent immune responses that show quantitative and/or qualitative differences to the responses elicited by RSV infection.

STUDY OBJECTIVES

1.3. Primary Objective:

- To test the hypothesis that RSV and influenza lead to quantitative and functional differences in T-cell responses.

1.4. Secondary Objective:

- To identify the mechanisms underlying poor antigen-specific CMI to RSV by comparing the transcriptional changes in T-cells following experimental challenge with RSV and virulent influenza.

1.5. Recruitment

Subjects will be recruited by advertisement in local newspapers, around College sites, and online: the Imperial Trust website, Gumtree, Student Union websites, ICRF website and on Social Media such as the Imperial CRF twitter page. Additionally, respondents to adverts for prior research projects in the department that were not subsequently enrolled, but were otherwise eligible for this study, will be contacted and invited to take part in our study (they have previously given their consent to be contacted). If interested, they will be invited for screening.

1.6. Pre-registration evaluations

1.6.1. Screening Visit

The screening visit will involve each participant attending the Imperial Clinical Respiratory Research Unit (ICRRU) at St Mary's Hospital, Paddington or Imperial Clinical Research Facility (ICRF) at Hammersmith Hospital for a brief interview and medical examination to find out if they are suitable for the study. Potential subjects will be given a participant information sheet (PIS) detailing the study and experimental procedures. When the subject has had enough time to consider their participation in this study, ask any questions they may have, and only when they have agreed to take part will they be asked to read, sign and date a consent form in the presence of the study doctor who will also sign the consent form. Consent will be obtained prior to any history-taking, examination or tests are carried out. A copy will be kept in the research file, a copy given to the patient and a copy put into their medical notes.

A medical history will then be taken and clinical examination, lung function tests, chest X-ray, ECG and blood tests performed by the study doctor. Women of childbearing potential will be asked about current contraceptive use, and be required to use effective contraception (barrier, oral contraceptive pill, depot injection, implant, or total abstinence) throughout the study. Blood tests include general screening for underlying illness, particularly full blood count, urea and electrolytes, liver function tests, coagulation C-reactive protein, lymphocyte subsets, HIV serology and baseline titres of neutralising antibodies against RSV and influenza. A urine drug screen for illicit drugs will also be performed.

These will all be done in the ICRRU at St Mary's Hospital or ICRF at Hammersmith Hospital. If the evaluation and the results of these tests show no evidence of infection or any other problems with the participants' health, and matches the inclusion / exclusion criteria then they will continue to the main part of the study.

1.7. Inclusion criteria

Healthy persons aged 18 to 55 years, able to give informed consent

1.8. Exclusion criteria

Chronic respiratory disease (asthma, COPD, rhinitis, sinusitis) in adulthood

Inhaled bronchodilator or steroid use within the last 12 months

Use of any medication or other product (prescription or over-the-counter) for symptoms of rhinitis or nasal congestion within the last 3 months

Acute upper respiratory infection (URI or sinusitis) in the past 6 weeks

Smoking in the past 6 months OR >5 pack-year lifetime history

Subjects with allergic symptoms present at baseline

Clinically relevant abnormality on chest X-ray

Any ECG abnormality

Those in close domestic contact (i.e. sharing a household with, caring for, or daily face to face contact) with children under 3 years, the elderly (>65 years), immunosuppressed persons, or those with chronic respiratory disease

Subjects with known or suspected immune deficiency

Receipt of systemic glucocorticoids (in a dose ≥ 5 mg prednisone daily or equivalent) within one month, or any other cytotoxic or immunosuppressive drug within 6 months prior to challenge

Known IgA deficiency, immotile cilia syndrome, or Kartagener's syndrome

History of frequent nose bleeds

Any significant medical condition or prescribed drug deemed by the study doctor to make the participant unsuitable for the study

Pregnant or breastfeeding women

Positive urine drug screen

Detectable baseline antibody titres against influenza challenge strains

Influenza arm only: history of hypersensitivity to eggs, egg proteins, gentamicin, gelatin or arginine, or with life-threatening reactions to previous influenza vaccinations.

All women of childbearing age will have a **pregnancy test performed prior to virus inoculation** to exclude pregnancy and be required to use contraception **throughout the study**.

1.9. Withdrawal criteria

Any subjects can withdraw from the study at any time if they wish to. Subjects can also be removed from the study if an investigator feels this is necessary or appropriate. Subjects will be closely monitored throughout by the study doctor. This is defined further in the section on 'Serious Adverse Events'.

STUDY DESIGN

Figure 1: Subject allocation

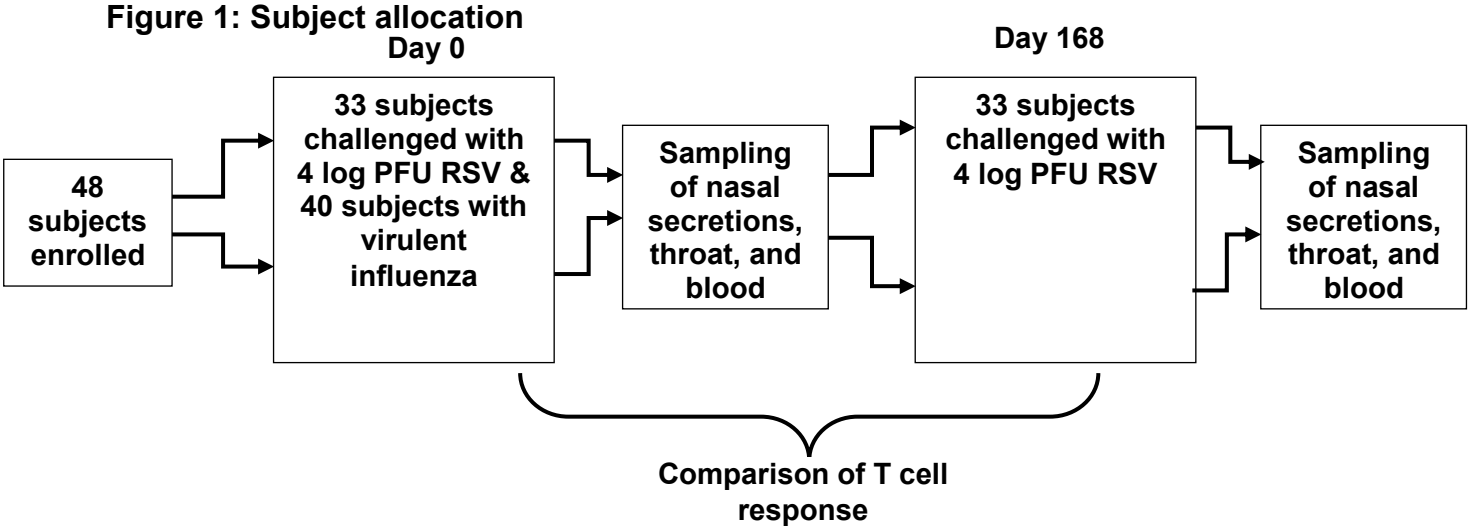

Table 1: Study procedures (Screening to day 9)

Procedures	DAY (relative to viral inoculation)											
	Screen	-14	0	1	2	3	4	5	6	7	8	9
Consent	X											
Physical Examination	X	X	X							X		
Lung Function Testing – FEV_1 , peak flow & breath tests	X	X	X	X	X	X	X	X	X	X	X	X
Screening blood tests	40											
Blood - serum			5									
Blood for HLA typing	5											
Blood - PBMCs (mls)			40									
Blood – plasma (mls)		X	6 x 2	6 x 2	6 x 2	6 x 2	6 x 2	6 x 2	6 x 2	6 x 2	6	6
Blood – RNA (mls)			7.5 x 2	7.5 x 2	7.5 x 2	7.5 x 2	7.5 x 2	7.5 x 2	7.5 x 2	7.5 x 2	7.5	7.5
Throat & stool swabs	X	X		X	X	X	X	X	X	X	X	X
Nasal lavage (daily)	X	X	X	X	X	X	X	X	X	X	X	X
Nasosorption using SAM (right nostril)	X	X		X	X	X	X	X	X	X	X	X
Nasal scrape (Rhinopro®)	X	X		X	X	X				X		
Bronchoscopy: Bronchosorption using SAM (4) Bronchial biopsy (6) Bronchial brush (4) Bronchoalveolar lavage (BAL)		X										
Virus inoculation			X									
Review symptom diaries daily	X	X	X	X	X	X	X	X	X	X	X	X
Collect symptom diaries												
Urine pregnancy test	X		X									

Table 2: Study procedures (day 10 to day 196)

Procedures	DAY (relative to viral inoculation)							
	10	**14**	**28**	**161**	**168**	**178**	**182**	**196**
Consent				X				
Physical Examination	X	X	X	X	X	X	X	X
Lung Function Testing – FEV_1, peak flow & breath tests	X	X	X	X	X	X		
Screening blood tests								
Blood - serum			5		5		5	5
Blood for HLA typing								
Blood - PBMCs (mls)	60		40	20	40	60	60	60
Blood – plasma (mls)	6	6	6					
Blood – RNA (mls)	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Throat & stool swabs	X	X	X		X	X	X	X
Nasal lavage (daily)	X	X	X	X		X		
Nasosorption using SAM (right nostril)	X	X	X		X	X	X	X
Nasal scrape (Rhinopro®)	X				X	X	X	X
Bronchoscopy: Bronchosorption using SAM (4) Bronchial biopsy (6) Bronchial brush (4) Bronchoalveolar lavage (BAL)	X		X				X	
Virus inoculation					X			
Review symptom diaries daily	X	X			X	X	X	X
Collect symptom diaries		X					X	
Urine pregnancy test			X	X				

SAM = synthetic absorptive matrix

1.10. Study visits for all subjects

RSV and Influenza Study Arm

The RSV and influenza arms of the study are divided into outpatient and confinement phases. Subjects will stay overnight for a period of 8-10 nights in total, from the day of viral challenge, to the 8-10th day after viral challenge and, for RSV only, a second subsequent 8-10-night stay after a second viral inoculation. This period of confinement has been chosen to eliminate the possibility of subjects in the study transmitting the virus to anyone not involved in the study (i.e. family, household contacts, and the wider community). Confinement is not used to enable closer monitoring or to enhance safety for study subjects, although this may be an additional benefit in some circumstances. During the confinement period, all study procedures will take place in the confinement facility (except bronchoscopy – see sections 1.10.2 and 7)(Figure 1 & Tables 1 & 2).

1.10.1. Baseline visit (, day -14)

On the first visit (baseline – around one week before infection with the virus) we will perform lung function, blood tests (serology and PBMCs), a throat swab, take washings from the participant's nose (nasal lavage), a nasal scrape, a nasal SAM and bronchoscopy. At this visit the participant will also be given a symptom diary card and asked to complete it (before any nasal procedures), detailing any respiratory symptoms they experience in their upper and lower airways. They will be asked to complete the symptom diary on a daily basis for one week following the first bronchoscopy and then daily for two weeks after infection with the virus. The first bronchoscopy will be performed on this visit

On the next visit, a week later, subject to a satisfactory assessment by the study doctor (brief interview and medical examination) and the taking of blood and nasal lavage samples, we will infect volunteers with RSV or virulent influenza. Those challenged with RSV and virulent influenza will then be confined as described (Section 1.10.2).

1.10.2. Confinement period for RSV and influenza arms (confinement, day 0 – day 8-10 and day 168 to 177-179)

On the morning of viral challenge, participants will attend the ICRRU or ICRF. A single nasal lavage sample will be taken to exclude coincident infection and blood will be taken for PBMCs. Following this, subject to a satisfactory assessment by the study doctor (brief interview and medical examination), we will infect the volunteers with RSV using a dose of 4 log PFU or with influenza at a dose of $3.5x10^6$ $TCID_{50}$. After infection, participants will be observed for a period of 30 minutes to ensure no adverse reactions have occurred. They will enter a residential research facility (confinement facility), where they will reside for the next 8-10 nights so we can monitor the development of cold symptoms and collect samples to evaluate immune responses and test the hypotheses. These will include daily blood samples, throat swabs, nasal lavage, nasal SAM, nasal scrapes, lung function tests, as well as bronchoscopy on day 7 or 10 post-infection. Participants will be seen daily by the study team. On the 8th day of residence, assuming all significant symptoms have resolved and at the discretion of the principle investigator, participants will leave the confinement facility and asked to return daily for assessment and sampling. If symptoms continue or the study doctors deems it necessary, the volunteer will be asked to remain until the 9th or 10th day of residence at which time they will be discharged, subject to a satisfactory assessment by the study doctor. If a subject is discharged before the 10th day or wishes to withdraw after virus inoculation but before the 10th day, we will strongly advise them to confine themselves in their own homes, and to strictly avoid any contact with young children, the elderly or other high-risk individuals for the remainder of the period during which viral shedding may occur. Additionally, subjects infected with influenza withdrawing before the 8th day post-inoculation will be treated with oseltamivir to prevent reduce the risk of viral shedding having left confinement.

1.10.3. Follow-up period (outpatient, day 11 – day 196)

Participants will return to the ICRRU or ICRF on days 14 and 28 post infection for assessment and sampling, including bronchoscopy on day 28. During the 6 months after challenge, subjects will be asked to report all episodes of upper respiratory tract symptoms. If an episode of natural infection occurs, the subject will be asked to re-attend for blood and upper respiratory tract samples to be collected. After 6 months, subjects will be asked to return for further samples to be taken, including bronchoscopy if subject has been included for this. A second dose of RSV administered a week later in the RSV arm only. Further samples will be collected at the peak of the secondary response 7 days post-infection. We will not do every test on every visit as can be seen from Table 2. The total study period for each subject is 210 in the RSV arm and 42 in the influenza arm.

STUDY PROCEDURES

A variety of procedures are carried out during the study period. The frequency and timing of these procedures are shown in above. For the screening, and days -14, 14, 28 and 196 visits, procedures will take place in the procedures room on ICRRU or ICRF; for days 0 to 10 and 168 to 179 subjects in the RSV and influenza arms of the study will reside in the confinement facility and procedures will take place there (with the possible exception of virus inoculation and bronchoscopy).

1.11. Virus Inoculation

GMP influenza A/California/04/2009-like (H1N1); influenza A/Belgium/4217/2015 (H3N2); or RSV Memphis 37 virus strains will be used for experimental infection of volunteers. On the day of inoculation (day 0 and day 168), RSV or influenza virus stock will be defrosted from its storage in the freezer. Subjects will be inoculated with intra-nasal drops on a single occasion with diluted inoculum at a given dose divided equally between the two nostrils. This will be done slowly with sufficient interval between each inoculation (2-3 minutes) to ensure maximum contact time between with the nasal and pharyngeal mucosa. Subjects will be asked not to swallow during the procedure to ensure maximal pharyngeal contact. The inoculation procedure will be performed using a ventilated body box, allocated isolation bay or negative pressure room in the ICRRU or ICRF. Inoculations using intranasal drops will be done using a 1mL pipette with subjects supine. Following inoculation, advice regarding hand hygiene will be given and subjects will be provided with alcohol hand gel and face-masks to reduce spread of virus in the home environment.

1.12. Swabs for microbial analysis

1.12.1. Throat swab

A sterile dry cotton-headed swab is used to obtain samples from the pharynx for bacterial 16S gene analysis. This is performed with the subject sitting. Ensure adequate lighting and use a tongue depressor if required. Remove the swab from the container carefully to ensure the tip is not contaminated, and swab the dorsal aspect of the pharynx and soft palate, avoiding the tongue. Some subjects may experience a strong gag reflex. Obtain two samples; place one used swab into a dry container and freeze at -80°C prior to analysis and another into bacteriology culture medium.

1.12.2. Stool swab

Sterile dry cotton-headed swabs will be used to obtain stool samples for bacterial 16S gene analysis. These will be collected by the subject from the toilet paper after opening their bowels.

Procedure:

- Remove the swab from the collection tube by holding it firmly by the red cap. DO NOT TOUCH THE COTTON PART WITH YOUR BARE HANDS!
- Collect a small amount of fecal material by rubbing the cotton tip of the swab on a faecal sample: a piece of used bathroom tissue is the best material possible. A small amount is enough: it should cover half of the cotton tip. Do not try to collect too much biomass.
- Replace the swab in the collection tube and close it by pushing firmly on the cap.
- Store the swab at -80°C within 48h. If it is not possible to store at -80°C, store the sample at 4°C until transfer into a cryogenic environment.

1.13. Nasal sampling procedures

All nasal procedures will be performed in the order below **prior** to the bronchoscopy to avoid contamination by the local anaesthetic gel and bronchoscope introduced into the nose.

1.13.1. Nasal scrape using Rhinopro®

Rhinopro® curettes will be used to obtain a sample of nasal epithelial cells from each nostril. This is a painless procedure and will not require local anaesthetic. The following technique is used:

The subject should be sat comfortably, ideally with their head fixed, looking forward, while their chin rests on a support (if available)

Tear bag and remove the flexible plastic Rhinopro® without contaminating the scoop end

Place a speculum in the nose to keep the cavity open and employ good lighting

Under direct visual inspection, insert the cupped probe onto the surface of the mid-inferior portion of the inferior turbinate. Note: Avoid the anterior bulb.

The Rhinopro® should be 3cm up the nose; the floor of the nostril can be used to rest on

Have the cup of the Rhinopro® at the correct angle

Gently press the cupped tip on mucosal surface and move out and in of nostril 3mm up to 3 times

Note that this area has limited sensitivity and the subject should not find this procedure painful, although a nasolacrimal reaction usually occurs

The cell harvest is epithelial cells, goblet cells and mast cells. It does not contain deeper layers of the mucosa. The sample obtained should be placed immediately into a tube containing RNA Cell Protect® (Qiagen) or Trizol and frozen at -80°C for storage prior to analysis.

1.13.2. Nasosorption

Up to four strips of SAM will be used (2 per nostril, one after the other) for 2 minutes to obtain repeated samples of neat nasal ELF. This is a painless minimally invasive procedure that will not require any local anaesthetic. Following sampling, SAM will be placed in a 1mL microfuge spin filter tube containing 250µL of elution buffer (PBS/1% bovine serum albumin/0.05% azide/0.05% Triton®). Further details are given in the SOP Human Sampling Procedures for Challenge Study.

The SAM will be transported on ice to the laboratory.

1.13.3. Nasal Lavage

Nasal lavage is performed using the following technique:

- 5mL of 0.9% saline is introduced into each nostril using a syringe attached to a nasal olive with the subject sitting with the head tilted forward
- The saline is then washed in and out of the nose approximately 10 times by alternately withdrawing and advancing the plunger of the syringe while the subject maintains a tight seal between the nasal olive and the nostril; the aim is to recover ~80% of the saline from the nose
- The fluid is then aliquotted into sterile microfuge tubes and centrifuged for analysis of cells

Lavage fluid will later be analysed to quantify the degree of RSV or influenza shedding. Multiplex PCR will be performed on the pre-inoculation lavage and post-inoculation lavage collected during the study to exclude the presence of other respiratory viruses. Supernatants will be frozen and stored at -80°C. Further details are given in the SOP Human Sampling Procedures for Challenge Study.

1.14. Bronchoscopy

Bronchoscopies will be performed in the endoscopy suite at St Mary's Hospital, or at Hammersmith hospital, in accordance with BTS guidelines. During the confinement period, private transport from the confinement facility to the endoscopy suite will be mandatory. Subjects will be instructed to wear face masks during this visit, and staff will use appropriate personal protective equipment (PPE).

- Subjects will sign a consent form prior to being screened for the study and will **sign a separate consent form for each bronchoscopy**. The procedure will be explained during the assessment stage and **subjects will be given written information specifically regarding bronchoscopies in addition to the Participant Information Sheet**

- Subjects will fast for four hours prior to the procedure

- Resuscitation equipment (for intubation, ECG monitoring and defibrillation) and necessary drugs (salbutamol, theophylline, adrenaline, hydrocortisone) will be available in the bronchoscopy room

- Premedication may be given including:

  - Nebulised salbutamol 2.5mg 10-15 minutes prior to bronchoscopy
  - Sedation – midazolam (2-10mg) or fentanyl (50-100µg) as necessary
  - Lignocaine – gel and solutions (1-4%) for topical anaesthesia. The total dose will not exceed 400mg

- Supplemental oxygen at a rate of $2Lmin^{-1}$ is given via a nasal cannula and oxygen saturations and heart rate are monitored with a pulse oximeter continuously. Intravenous access will be mandatory in all cases

- The subject will be monitored during the bronchoscopy by adequately trained clinical staff, according to Research Bronchoscopy SOP (V3).

- The following samples are collected in this order: (i) Bronchosorption using Synthetic Absorptive Matrix (SAM) (ii) Bronchial Brushings (iii) Bronchial Biopsy (iv) Bronchoalveolar lavage (BAL)

- All adverse events – pain, bleeding, hypoxia etc will be recorded and reported according to Section 8 of this protocol

- Subjects will be observed for a minimum of 2 hours and nil by mouth until safe

- Transportation will be arranged as subjects should not drive on the day of the procedure

- All subjects will have a contact telephone number on discharge

1.14.1. Bronchosorption:

'Bronchosorption™ FX·i is a non-sterile, single-use device consisting of a synthetic absorptive matrix (SAM™) strip enclosed within a catheter for bronchial sampling. Bronchosorption™ FX·i is designed to operate through a flexible video bronchoscope with a maximum working length of 815mm. The SAM™ strip is 1.0mm wide, designed to work with a minimum instrument channel diameter of 2.0mm.' Hunt Developments UK (Ltd).

- The device will be passed down the bronchoscope.
- The probe will be deployed for up to 120 seconds in segmental and larger bronchi to allow the SAM to absorb local epithelial lining fluid (ELF).
- The probe will then be resheathed and removed via the operating port of the bronchoscope.
- This will be repeated up to 4 times with a new device.
- The SAM will be transferred to polypropylene tubes for transport on ice to the laboratory and stored at -80 degrees C until analysis.

1.14.2. BronchoBronchial Biopsies:

Six bronchial biopsies will be taken from the segmental and sub-segmental bronchi of the right lower and middle lobe (RLL, ML). This is performed using *Keymed* 2mm biopsy channel cupped and fenestrated biopsy forceps [FB-19C-1 (1111065)]; four biopsies will be placed in 4% paraformaldehyde and stored in paraffin blocks and two placed immediately into RNAlater® (Qiagen) stabilisation solution and refrigerated for 24 hours prior to freezing at -80°C.

1.14.3. Bronchial Brushings:

Four bronchial brushings will be taken from the left lower lobe (LLL) sub-segmental bronchi with a standard cytology brush. The brush is washed in a tube containing RNA Cell Protect® (Qiagen) to preserve cells prior to freezing at -80°C. A new brush is used each time the brushes are washed and discarded.

1.14.4. Bronchoalveolar Lavage (BAL):

- BAL is performed by instillation of sterile physiological (0.9%) saline at room temperature into the left upper lobe (LUL) bronchus in 30ml aliquots to a total of 180-240ml
- Aiming for 80% volume recovery and aspirating after each instillation
- The BAL fluid is collected into a plastic chamber and transferred to polypropylene tubes for transport on ice to the laboratory
- BAL processing - Keep BAL collected at bronchoscopy on ice at all times

1.15. Blood sampling

Screening visit blood will be taken for full blood count, renal function, liver function tests, glucose, clotting, CRP, lymphocytes, immunoglobulins, HIV, hepatitis B and C. These will be processed in the Haematology and Chemical Pathology Laboratories of Imperial College Healthcare NHS Trust. Serology will be performed at screening and day -14 by IgG microneutralisation assay.

The total amount of blood taken at screening would amount to 40mL. Blood for peripheral blood mononuclear cells (PBMCs) will also be taken at day -14, 0, 3, 7, 14, 28, 161, 168, 175 and 196. On these occasions, 20-60mls of blood will be taken (see Table 1 & 2). In addition, during the first challenge, blood for gene expression profiling, serum and plasma will be taken from day 0 to day 10 plus day 14 and 28. Blood for gene expression profiling will be taken once or twice day. A maximum of 72 mls of blood will be taken on any single day for a total of 478.5 mls over the 28-day challenge period (see Tables 1 & 2).

1.16. Physical examination

Physical examination, including ENT, respiratory and cardiac assessment will be performed by the study doctor at screening and days -14, 0 (prior to inoculation),8, 9,10 14, 28 and 175 days post inoculation.

1.17. Lung Function Tests

Predicted Values for Lung Function Measurements: the predicted or reference values for lung function measurements are those recommended by the Report Working Party for the European Community for Coal and Steel. Also incorporated are the recommendations of the British Thoracic Society and the Association of Respiratory Technicians and Physiologists.

1.17.1. Measuring FEV_1 and FVC by Spirometry

Performing the measurement

- Posture must be consistent during a study, either standing or sitting, with no breathing limitation
- The subject should breathe in to total lung capacity. A good tight seal by the lips round the mouthpiece is essential. The subject should then exhale forcibly into the spirometer, blowing as hard as possible and continue to residual volume
- The best value of 3 attempts will be recorded

Calibration: Diagnostic spirometers will be serviced and calibrated by the relevant company for the brand. However, the calibration will be checked at least once a month using a calibrated one litre syringe.

At the screening visit and at other visits as described in the table in, measurements of FEV1 and FVC will be made as outlined above.

1.17.2. Collection and analysis of exhaled breath

Exhaled breath is believed to contain infectious virus during natural influenza infections. To collect potentially infectious particles in breath exhaled during infection, we will use an altered resuscitation mask with Teflon filter mouthpiece into which the participant will be asked to breath for 20 minutes.

1. The test will take a total of thirty minutes including twenty minutes of quiet breathing.
2. A study team member will explain what the device consists of and why the test is being performed.
3. The participant will hold the mask by the cardboard tubing and not touch any plastic surface.
4. They will gently press the mask against their face, covering the mouth and nose.
5. Then they test breathing through their nose and mouth while study staff check for leaks around the face.
6. If the participant experiences pinching of their nose, difficulty in nasal breathing or a leak is detected air can be removed with a 20 ml catheter tip syringe from the inflatable cuff on the facemask and the mask re-tested.
7. Once steps 4 and 5 have been optimised the participant will be asked to breath in through their mouth and out through their nose (normal tidal volumes and rate).
8. If their nose is blocked due to symptomatic infection mouth breathing will be performed and this will be documented in the Breath Collection Log.
9. The participant will be asked not to speak, unless it is necessary.
10. They will be asked not to remove the mask from their face, unless absolutely necessary.
11. It will be explained that if the seal around their face is broken, e.g. if they remove the mask, the test will be restarted.

1.18. Clinical symptom scores

A self-completed diary card of both upper and lower respiratory tract clinical symptoms will be made at baseline 14 days prior to inoculation (prior to nasal washing and / or bronchoscopy), on day 0, and daily for 14 days.

Additionally, the symptom diary will be completed daily for 7 days after the 1st bronchoscopy (i.e. day -14 to day -7). This is to allow the effect of bronchoscopy on symptoms to be measured and adjusted.

Individual symptom scores will be accumulated over the six-day period of maximal illness (days 2-7) after inoculation and the baseline recording (including any effect measured from the 1st bronchoscopy where appropriate) subtracted from the post inoculation recordings. Thus, for a patient who has a score of zero on day 0 prior to inoculation, the maximum cumulative score for the following six days is 144.

Upper Respiratory Tract Symptoms

A total 'upper respiratory clinical symptom score' will be derived using a four-point scale (0-3 for absent, mild, moderate and severe) for each of the following eight respiratory symptoms: sneezing, headache, malaise, fever/chills, nasal discharge, nasal obstruction, sore throat and cough according to established methods, giving a maximum clinical severity score of 24. This is an established method for studies of common cold illnesses[44]. Symptoms will be recorded at the same time of day and before any procedures such as bronchoscopy or nasal lavage are performed.

An example is shown below:

Symptom	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
Sneezing							
Headache							
Malaise							
Fever / chills							
Nasal discharge							
Nasal obstruction							
Sore throat							
Cough							
Total score							

0 = absent, 1 = mild, 2 = moderate, 3 = severe

Definition of a clinical cold

A clinical cold is diagnosed if **two or more** of the following are present:

- A cumulative clinical symptom score of 14 or greater over a 6 day period
- Nasal discharge is present on three or more days over the six-day period post viral inoculation
- A subjective impression of a cold developing. This latter criterion is used because there are a few subjects who have had a very strong subjective impression of a clinical cold but the cumulative clinical score does not reach the arbitrary cut-off level

-

System, Lower Respiratory Tract and Other Symptoms

A diary card of lower respiratory tract symptoms will be completed with a scoring system outlined below.

SYMPTOM	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Cough							
Difficulty breathing							
Hoarseness							
Chest discomfort							
Chills							
Diarrhoea							
Muscle aches							
Feeling/being sick							
Red/watery eyes							
Wheezy chest							
TOTAL SCORE							

Absent = 0 Mild = 1 Moderate = 2 Severe = 3

ADVERSE EVENTS

1.19. Definitions

Adverse Event (AE): any untoward medical occurrence in a patient or clinical study subject.

Serious Adverse Event (SAE): any untoward and unexpected medical occurrence or effect that:

- **Results in death**
- **Is life-threatening** – refers to an event in which the subject was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe
- **Requires hospitalisation**
- **Results in persistent or significant disability or incapacity**
- **Is a congenital anomaly or birth defect**

Medical judgement should be exercised in deciding whether an AE is serious in other situations. Important AEs that are not immediately life-threatening or do not result in death or hospitalisation but may jeopardise the subject or may require intervention to prevent one of the other outcomes listed in the definition above, should also be considered serious.

1.20. Expected adverse events

1.20.1. Potential adverse events related to RSV and influenza infection

We would expect subjects to experience typical symptoms of a common cold (including, but not limited to: fever, headache, malaise, rhinorrhoea, nasal congestion, sneezing, sore throat, and cough). These would not be deemed adverse events, unless in the opinion of the study doctor. However, fever greater than 38ºC for more than three consecutive days or withdrawal from the study due to intolerable symptoms in more than two subjects in any arm will lead to a suspension of the study. The safety monitoring committee will be convened to determine any systematic cause for unexpectedly severe symptoms.

Any influenza-like illness resulting in

- **Sustained elevated heart rate >120bpm AND**
- **Sustained low blood pressure SBP<100**
- **Sustained elevated respiratory rate >30/min AND**
- **Sustained low blood oxygen SaO2<94%**
- **Evidence of pneumonia on clinical examination**
- **New ECG abnormalities**

will lead to referral for assessment in Accident and Emergency. Hospitalisation of any subject will lead to immediate suspension of the trial. The safety monitoring committee will be convened to assess the clinical evidence in order to determine whether the study may proceed.

1.20.2. Potential adverse events of bronchosorption

Bronchosorption is likely to have less adverse effects than bronchoalveolar lavage, brushing and biopsy (the standard bronchoscopy tools) as it should not cause bleeding, infection or a reactive pyrexia. The only additional adverse event relating specifically to bronchosorption is dislodgement of the SAM from the forceps. If this does occur the SAM can be retrieved using standard endobronchial forceps and snares available routinely in the bronchoscopy suite. Emergency procedures SOP is in place.

1.20.3 Potential adverse effects of chest X-ray

Volunteers participating in this study will receive 1 chest Xray, which is entirely for research purposes. The estimated dose will be 0.02mSv (national Diagnostic Reference Level), which is approximately equivalent to 3 days natural background radiation and carries risk of inducing a cancer of approximately 1:1000,000 based on risk factors for a healthy adult. This is classified as a trivial risk level (ICRP 62).

1.21. Reporting procedures

All adverse events should be reported. Depending on the nature of the event the reporting procedures below should be followed. Any questions concerning adverse event reporting should be directed to the Chief Investigator in the first instance.

1.21.1. Non serious AEs

All such events, whether expected or not, should be recorded. These will be discussed by the safety monitoring committee (see Section 12)

1.21.2. Serious AEs

An SAE form should be completed and faxed to the Chief Investigator and the Sponsor within 24 hours. The safety monitoring committee (see Section 12) will also be informed and a meeting convened as soon as possible.

All SAEs should be reported to the West London REC 2 Research Ethics Committee where in the opinion of the Chief Investigator, the event was:

- 'related', i.e. resulted from the administration of any of the research procedures; and
- 'unexpected', i.e. an event that is not listed in the protocol as an expected occurrence

Reports of related and unexpected SAEs should be submitted to ethics, the sponsor and the R&D office within 15 days of the Chief Investigator becoming aware of the event, using the NRES SAE form for non-IMP studies.

Contact details for reporting SAEs
Fax: 020 7262 8913 for the attention of Professor Peter Openshaw and/or Dr Christopher Chiu
Please send SAE forms to: Respiratory Infections, Wright Fleming Institute, NHLI, Imperial College, St Mary's Campus, London, W2 1PG
Tel: 020 7594 3854 (Mon to Fri 09.00 – 17.00)

ASSESSMENT AND FOLLOW-UP

Study participants will be seen frequently during the study period, and daily for 10 days following infection. They have details to contact the study doctor and research nurses and will be offered daily telephone contact. In this way, participants will be assessed regularly by the investigating team and any adverse events detected rapidly; subjects meeting the criteria for a serious adverse event will be offered prompt treatment as appropriate.

Subjects will have completed the study when they have had final convalescence investigations, expected to be 28 days after the initial inoculation with the influenza virus and 196 days after initial inoculation with the RSV virus. The overall study will be completed when sufficient numbers of subjects have been recruited. The end of the study is defined as the last visit of the last participant.

When the study is completed they will not be routinely followed-up. Subjects will return to the care of their GP following completion of the study.

STATISTICS AND DATA ANALYSIS

Based on pilot data from de Bree et al., we calculate that a sample size of 16 in each arm will be sufficient to find a difference of 1.4 between epitope-specific T cell counts in blood and lung, with 80% power using a 2-sided unpaired t-test with 5% significance level (where the variability in each group is 1.3). T cell numbers will be compared between RSV and influenza using an unpaired t-test (if normality of the T cell distribution, or its transformation, is satisfactory as assessed by histogram with q-qplot and Shapiro-Wilks test) or a Mann-Whitney test (if normality is not satisfactory). We will perform microarray analysis on each of 6 patients prior to inoculation and at 3 post-baseline timepoints. Our main analysis will identify differentially expressed genes between influenza and RSV at each post-baseline timepoint. The sample size, calculated using the size package from bioconductor which provides pilot data of gene expression variability from the U95 package, predicts that 6 individuals in each group will give us 80% power to detect a potential fold change of 3 in up to 67% of the genes with a Bonferroni multiple testing correction with a genome-wide 5% significance level.

Quantitative assessments of symptom scores, lung function, virus load, leukocyte numbers and inflammatory markers will be compared within subjects to determine differences between baseline and during infection. Intra-subject differences will be analysed using ANOVA and 2-tailed paired Student's t-tests or Wilcoxon signed rank test as appropriate. Correlations between inflammatory cell, illness severity, viral load and leukocyte counts will be examined using Spearman's rank correlations to investigate possible causal relationships.

Data and all appropriate documentation will be stored for a minimum of 10 years after the completion of the study, including the follow-up period according to Imperial College London policy.

REGULATORY ISSUES

1.22. Ethics approval

The Chief Investigator has obtained approval from the Fulham Research Ethics Committee for this study. The study will be submitted for Site Specific Assessment (SSA) at Imperial College Healthcare NHS Trust. The Chief Investigator will require a copy of the R&D approval letter before accepting participants into the study. The study will be conducted in accordance with the recommendations for physicians involved in research on human subjects adopted by the 18th World Medical Assembly, Declaration of Helsinki 1964 and later revisions.

1.23. Consent

Consent to enter the study will be sought from each participant only after a full explanation has been given, an information leaflet offered, time allowed for consideration, and any questions participants may have answered. Signed participant consent will be obtained prior to any screening tests being carried out. The right of the participant to refuse to participate without giving reasons must be respected. After the participant has entered the study the clinician remains free to give alternative treatment to that specified in the protocol at any stage if he/she feels it is in the participant's best interest, but the reasons for doing so should be recorded. In these cases the participants remain within the study for the purposes of follow-up and data analysis. All participants are free to withdraw at any time from the protocol treatment without giving reasons and without prejudicing further treatment.

1.24. Confidentiality

The Chief Investigator and all of the research team will preserve the confidentiality of participants taking part in the study and abide by the Data Protection Act.

1.25. Indemnity

Imperial College, London as sponsor of this study holds negligent and non-negligent harm insurance policies which apply to this study. These have been arranged through the Joint Research Office.

1.26. Sponsor

Imperial College London will act as the main sponsor for this study. Delegated responsibilities will be assigned to the NHS trusts taking part in this study.

1.27. Funding

Following a successful Clinician Scientist Fellowship application the MRC is funding this study. They are acting as sole funders and this agreement is in place. The investigators will not receive any additional payment above their normal salaries. Participants in the study will have their travel costs refunded. They will also be given a donation of up to £3000 to compensate for the time and inconvenience of taking part in the study.

1.28. Audits and inspections

The study may be subject to inspection and audit by Imperial College London under their remit as sponsor and other regulatory bodies to ensure adherence to GCP and the NHS Research Governance Framework for Health and Social Care (3rd edition).

1.29. Sample storage and usage

Samples of tissue, cells and fluids will be stored at Imperial College London. Samples will be fully anonymised. These may be used for further assays or in other ethically approved studies. Samples may be shared with UK and international collaborators in studies that have been approved by local ethics committee and subject to a valid Materials Transfer Agreement.

STUDY MANAGEMENT

The day-to-day management of the study will be co-ordinated through Dr Christopher Chiu, Clinical Senior Lecturer & Honorary Consultant in Infectious Diseases, with close support from Professor Peter Openshaw. In addition, a safety monitoring committee will convene monthly during the study to discuss all adverse events, protocol deviations, and other safety issues.

PUBLICATION POLICY

Our expectation is that after analysis the data from this study will be widely distributed in the medical and scientific community. Facilitated with presentations at local, national and international meetings, we hope to publish widely in the medical literature. In addition we have an excellent media department at Imperial College and publicise research that has public interest when it is published. No identifying participant information will be published.

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