1. Investigator Award in Science application

Reference number	UNS66042
Applicant name	Prof Anthony Segal
Title of application	The effect of pH on phagocytic vacuole biology in monocytes, macrophages and dendritic cells
Total amount requested	£608,806.00

2. Application summary

Application title
The effect of pH on phagocytic vacuole biology in monocytes, macrophages and dendritic cells

Proposed duration of funding (months)
48

Proposed start date	01/01/2019

Name of administering organisation
University College London

Lead applicant's address at administering organisation	
Department/Division	Medicine
Organisation	UCL
Street	5 University Street
City/Town	London
Postcode/Zipcode	WC1E6JJ
Country	United Kingdom

Research funding area Please select from the drop-down list the funding area that you consider your research falls under
Immune System in Health and Disease

3. Lead applicant

Lead applicant details	
Full Name	Prof Anthony Segal

Department	
Division	Medicine
Organisation	University College London
Address Line 1	Dept of Medicine, The Rayne Institute
City/Town	LONDON
Postcode	WC1E 6JF
Country	United Kingdom
Telephone No.	4420 7679 6175
Email Address	t.segal@ucl.ac.uk

ORCID iD	
ORCID iD	0000-0001-7602-9043

Career history (current/most recent first)

From	To	Position	Organisation
10/1986	12/2024	Charles Dent Professor of Medicine	University College London
10/1979	09/1986	Wellcome Trust Senior Clinical Fellow	University College London
09/1976	10/1979	MRC Clinical Scientist	Northwick Park Institute for Medical Research
01/1969	08/1976	Various house officer, registrar and senior registrar	Northwick Park and Hammersmith hospitals
01/1968	10/1969	House Physician and Surgeon	Groote Schuur Hospital

Education/training

From	To	Qualification	Subject	Organisation
01/1984	02/1984	DSc	Science	University of London
01/1976	06/1979	Doctor of Philosophy (PhD;DPhil)	Science	University of London
01/1974	02/1974	Doctor of Medicine (MD)	Medicine	University of Cape Town
08/1971	06/1973	Master of Science (MSc)	Biochemistry	University of London
01/1971	07/1971	MRCP	Medicine	Royal College of Physicians
01/1962	01/1967	Primary Med Qual (BM;MBChB;MBBS;MD)	Medicine	University of Cape Town

Source(s) of personal salary support
Higher Education Funding Council

Clinical status Do you have a medical/veterinary degree?	Yes

Please specify
Medical graduate

Are you clinically active?	No

What is your specialty?
Immunology

Career breaks Have you had any career breaks or periods of part-time work, for example parental or long-term sick leave?	No

Do you wish to undertake this award part time?	No

Research outputs
List up to 20 of your most significant research outputs, ensuring that at least five of these are from the last five years. For 10 of these outputs, provide a statement describing their significance and your contribution (up to 50 words per output).

Research outputs may include (but are not limited to):

- Peer-reviewed publications and preprints
- Datasets, software and research materials
- Inventions, patents and commercial activity

For original research publications indicate those arising from Wellcome funded grants in **bold**, and provide the PubMed Central ID (PMCID) reference for each of these. Please refer to guidance notes.

Please give citation in full, including title of paper and all authors. Citations to preprints should state "Preprint", the repository name and the articles persistent identifier (e.g. DOI).*

*(*All authors, unless more than 10, in which case please use 'et al', ensuring that your position as author remains clear.)*

Publications relating to the neutrophil NADPH oxidase (NOX) and to mechanisms of microbial killing by these cells.

1. The LRRC8A Mediated "Swell Activated" Chloride Conductance Is Dispensable for Vacuolar Homeostasis in Neutrophils.

Behe P, Foote JR, Levine AP, Platt CD, Chou J, Benavides F, Geha RS, Segal AW.

Front Pharmacol. 2017 May 12;8:262. doi: 10.3389/fphar.2017.00262. eCollection 2017. PMID: 28553230

2. An Exploration of Charge Compensating Ion Channels across the Phagocytic Vacuole of Neutrophils.

Foote JR, Behe P, Frampton M, Levine AP, Segal AW.

Front Pharmacol. 2017 Feb 28;8:94. doi: 10.3389/fphar.2017.00094. eCollection 2017. PMID:

28293191

3. Alkalinity of neutrophil phagocytic vacuoles is modulated by HVCN1 and has consequences for myeloperoxidase activity.

Levine AP, Duchen MR, de Villiers S, Rich PR, Segal AW.

PLoS One. 2015 Apr 17;10(4):

PMID: 25885273

Showed that the pH in the phagocytic vacuoles of neutrophils is elevated to 9.0 for at least 30 minutes by the NADPH oxidase. This is optimal for the activity of the neutral proteases but inhibitory of the peroxidatic function of myeloperoxidase. The role of the Hvcn1 channel is defined. **PI**

4. NADPH oxidases as electrochemical generators to produce ion fluxes and turgor in fungi, plants and humans.

Segal AW.

Open Biol. 2016 May;6(5). pii: 160028.

PMID: 27249799

5. Reassessment of the microbicidal activity of reactive oxygen species and hypochlorous acid with reference to the phagocytic vacuole of the neutrophil granulocyte.

Reeves EP, Nagl M, Godovac-Zimmermann J, Segal AW.

J Med Microbiol. 2003 Aug;52(Pt 8):643-51.

PMID: 12867557

6. Killing activity of neutrophils is mediated through activation of proteases by K+ flux.

Reeves EP, Lu H, Jacobs HL, Messina CG, Bolsover S, Gabella G, Potma EO, Warley A, Roes J, Segal AW.

Nature. 2002 Mar 21;416(6878):291-7.

PMID: 11907569

Demonstrated that NOX2 induces a flux of potassium into the phagocytic vacuole that, together with the elevated pH is important for the activation of the granule enzymes. Knock-out mice lacking

cathepsin G and elastase are susceptible to bacterial and fungal infection, organisms that their separated neutrophils could not kill. **PI**

7. Cytochrome b-245 is a flavocytochrome containing FAD and the NADPH-binding site of the microbicidal oxidase of phagocytes.

Segal AW, West I, Wientjes F, Nugent JH, Chavan AJ, Haley B, Garcia RC, Rosen H, Scrace G.

Biochem J. 1992 Jun 15;284 (Pt 3):781-8.

PMID: 1320378

The characterisation of gp91phox as containing the NADPH and FAD binding sites of NOX2. This was very controversial at the time. **PI**

8. Activation of the NADPH oxidase involves the small GTP-binding protein p21rac1.

Abo A, Pick E, Hall A, Totty N, Teahan CG, Segal AW.

Nature. 1991 Oct 17;353(6345):668-70.

PMID: 1922386

First description of the involvement of the small GTP binding protein, Rac1, as an essential component of the NADPH oxidase, and demonstration that it was complexed to GDP dissociation inhibition factor GDI. **PI**

9. The X-linked chronic granulomatous disease gene codes for the beta-chain of cytochrome b-245.

Teahan C, Rowe P, Parker P, Totty N, Segal AW.

Nature. 1987 Jun 25-Jul 1;327(6124):720-1.

PMID: 3600769

10. Absence of both cytochrome b-245 subunits from neutrophils in X-linked chronic granulomatous disease.

Segal AW.

Nature. 1987 Mar 5-11;326(6108):88-91.

PMID: 3821877

11. Stimulated neutrophils from patients with autosomal recessive chronic granulomatous disease fail to phosphorylate a Mr-44,000 protein.

Segal AW, Heyworth PG, Cockcroft S, Barrowman MM.

Nature. 1985 Aug 8-14;316(6028):547-9.

PMID: 4033752

12. Absence of cytochrome b-245 in chronic granulomatous disease. A multicenter European evaluation of its incidence and relevance.

Segal AW, Cross AR, Garcia RC, Borregaard N, Valerius NH, Soothill JF, Jones OT.

N Engl J Med. 1983 Feb 3;308(5):245-51.

PMID: 6848934

Unequivocal demonstration of the molecular lesion, an absence of the flavocytochrome b, as the cause of X-linked CGD and the demonstration that this molecule was present, but did not function, in patients with autosomal recessive CGD. **PI**

13. The respiratory burst of phagocytic cells is associated with a rise in vacuolar pH.

Segal AW, Geisow M, Garcia R, Harper A, Miller R.

Nature. 1981 Apr 2;290(5805):406-9.

PMID: 7219526

14. Novel cytochrome b system in phagocytic vacuoles of human granulocytes.

Segal AW, Jones OT.

Nature. 1978 Nov 30;276(5687):515-7.

PMID: 723935

Discovery of molecular basis of the neutrophil NADPH oxidase. These NOXs have subsequently been found throughout the biological world, from plants to humans. **PI**

Discovery of the underlying cause of Crohn's disease

15. Genetic Complexity of Crohn's Disease in Two Large Ashkenazi Jewish Families.

Levine AP, Pontikos N, Schiff ER, Jostins L, Speed D; NIDDK Inflammatory Bowel Disease Genetics Consortium., Lovat LB, Barrett JC, Grasberger H, Plagnol V, Segal AW.

Gastroenterology. 2016; 151(4):698-709.

PMID: 27373512

Exome sequences on two of the largest families ever studied demonstrated a causal mutation for Crohn's disease as a truncating mutation in the beta chain of the GM-CSF, IL-3 and IL-5 receptors. This is one of the very few genetic mutations shown to be causal for this disease. **PI**

16. Optineurin deficiency in mice contributes to impaired cytokine secretion and neutrophil recruitment in bacteria-driven colitis.

Chew TS, O'Shea NR, Sewell GW, Oehlers SH, Mulvey CM, Crosier PS, Godovac-Zimmermann J, Bloom SL, Smith AM, Segal AW.

Dis Model Mech. 2015; 8(8):817-29.

PMID: 26044960

17. Disruption of macrophage pro-inflammatory cytokine release in Crohn's disease is associated with reduced optineurin expression in a subset of patients.

Smith AM, Sewell GW, Levine AP, Chew TS, Dunne J, O'Shea NR, Smith PJ, Harrison PJ, Macdonald CM, Bloom SL, Segal AW.

Immunology. 2015; 144(1):45-55.

PMID: 24943399

18. Disordered macrophage cytokine secretion underlies impaired acute inflammation and bacterial clearance in Crohn's disease.

Smith AM, Rahman FZ, Hayee B, Graham SJ, Marks DJ, Sewell GW, Palmer CD, Wilde J, Foxwell BM, Gloger IS, Sweeting T, Marsh M, Walker AP, Bloom SL, Segal AW.

J Exp Med. 2009; 206(9):1883-97.

PMID: 19652016

After E.coli injection, accumulation of (111)In-labelled neutrophils and clearance of (32)P-labelled

bacteria were markedly impaired in Crohn's disease. Secretion of pro-inflammatory cytokines by their macrophages was grossly impaired because an abnormal proportion of cytokines are routed to lysosomes and degraded rather than being released through the normal secretory pathway. **PI**

19. Defective acute inflammation in Crohn's disease: a clinical investigation.

Marks DJ, Harbord MW, MacAllister R, Rahman FZ, Young J, Al-Lazikani B, Lees W, Novelli M, Bloom S, Segal AW.

Lancet. 2006; 367(9511):668-78.

PMID: 16503465

The first study to show in humans that neutrophil recruitment and cytokine production after acute trauma, interleukin 8 secretion by cultured monocyte-derived macrophages after exposure to inflammatory mediators, and local inflammatory and vascular changes in response to subcutaneous injection of heat-killed E.coli were all abnormal in Crohn's. **PI**

20. Neutrophil dysfunction in Crohn's disease.

Segal AW, Loewi G.

Lancet. 1976 Jul 31;2(7979):219-21.

PMID: 59239

First description of the pathophysiology of Crohn's disease as a deficiency of the innate immune system with the failure of neutrophils to accumulate at sites of acute inflammation.

Total number of peer-reviewed publications which you have authored/co-authored. Please exclude abstracts and literature reviews.	220

Current and recent research funding (including Wellcome Trust grants)
Please list all held in the last five years and any key prior grants (list the most recent first). State the name of the awarding body, name(s) of grantholder(s), title of project, amounts awarded, your role in the project, and start and end dates of support. For all active grants, indicate the number of hours per week that are spent on each project.

Identification and characterisation of causal molecules for Crohn's disease and ulcerative colitis A Segal and AM Smith Pis £888,203 1/01/2014-31/12/2017 5 hours Medical Research Council

Crohn's Disease in Ashkenazi Jewish patients £668,000 01/01/2014-31/12/2014 2 hours Charles Wolfson Charitable Trust

Studentship to Juliet Foote from Irwin Joffe Memorial Trust Supervisor 5 hours/week - terminates October 2018..

Wellcome Trust

Studies into the molecular causes of Crohn's Disease AW Segal with A Smith, SL Bloom, A Walker,

AE Teschendorff 088683/2/09/Z £995,000.00 April 201O - March 2015. 10 hours.

Ion channels related to neutrophil NADPH The Wellcome Trust 7/2007 - 12/2012 £778,157.00 WT08195 5 hours

Defective innate immunity in inflammatory bowel disease. AW Segal with A Smith, SL Bloom, A Walker, 081731/2/06/Z, £610,195, 36 months, 10 hours 1/10/07-30/9/1O

Ion channels related to neutrophil NADPH oxidase. AW Segal, M Duchen. 081695/2/06/Z £778,157 60 months 10 hours 01/10/07-30/09/10

Please describe how the currently active grants listed above relate to this application

The current application is based upon the findings of NADPH oxidase activity resulting in vacuolar alkalinisation in monocyte, macrophage and dendritic cell subsets by my PhD student, Juliet Foote, with support from the Irwin Joffe Memorial Trust. She will be finishing her studentship at the end of October 2018.

Training record
Please name *up to five individuals* you have trained, if any. Describe in brief your contribution to their career development, and state both their position at the time you were training them (e.g. postgraduate student, postdoctoral research assistant) and their current position.

Adrian Thrasher PhD supervisor, Graduate student

Professor of Paediatric Immunology and Wellcome Trust Senior Investigator, Institute for Child Health.

Edward Odell PhD supervisor, Graduate student

Professor of Oral Pathology and Medicine, Kings College London.

Anil Mehta PhD supervisor Graduate student

Reader, Dundee School of Medicine.

Emer Reeves PhD supervisor Research assistant

Lecturer and Deputy Head of the School of Postgraduate Studies, Royal College of Surgeons in Dublin

Daniel Marks PhD supervisor MBPhD student

Director, Discovery Medicine, GSK

Career contributions
What are your most important research-related contributions to date? These may include contributions to health policy or practice, or to technology or product discovery and development.

NOXs and Chronic Granulomatous Disease

I discovered the NADPH oxidase electron transport chain in neutrophil leukocytes, and other professional phagocytes, and demonstrated that defects in this oxidase cause the human condition of Chronic Granulomatous Disease (CGD). I then went on to characterise this enzyme complex in terms of the NADPH, FAD, haem and carbohydrate binding sites and identified five of the six additional components of this NOX2 system required for its regulation and activity.

More than one hundred NOXs have been identified in the animal and plant worlds where they are required for essential processes like root and pollen tube growth, stomatal closure and the invasion of plants and insects by fungi. All these NOXs conform to the basic structural characteristics I characterised in the human leukocyte molecule.

Mechanism of microbial killing by neutrophils

It had been believed that the pH in the phagocytic vacuole of neutrophils was very acidic at about pH 5.0. We showed that the oxidase alkalinises the vacuole. This elevation in pH to about 9.0 and an accompanying influx of potassium into the vacuole optimise the activity of the granule enzymes, such as elastase and cathepsin G, which kill and digest the ingested microbes.

The demonstration of alkalinisation of the vacuole of classical monocytes and M1 macrophages indicates granules of these cells will have a complement of enzymes adapted to this environment

Crohn's disease

I was responsible for developing the understanding that the underlying pathology in Crohn's disease is a failure of innate immunity resulting in delayed or incomplete removal of intestinal contents penetrating into the wall of the bowel. We demonstrated that the impaired neutrophil accumulation at sites of acute inflammation stems from a failure of the secretion of pro-inflammatory cytokines by macrophages. This commonly results from disordered vesicle trafficking, directing the cytokines to degradation rather than secretion. We have identified a number of the molecules responsible for this anomalous behaviour and several genes that predispose to the disease.

I have also made several other original observations including diagnostic cell labelling, liposomal targeting, lipoprotein labelling, and the occurrence of bowel inflammation in rheumatoid arthritis.

Summary of research over the last five years
Please provide a summary of the research that you have carried out over the last five years

Neutrophil biology

We developed a novel technique of measuring vacuolar pH in phagocytic cells using the ratiometric fluorophore SNARF coupled to opsonised *Candida*. We used this to accurately measure the vacuolar pH of neutrophils at about 9.0 which has important implications for the function of enzymes within this compartment.and has redefined the mechanisms underlying the killing and digestion of bacteria and fungi by neutrophils.

The fluxes of protons and other ions that compensate the charge induced across the membrane by electron transport are balanced to maintain the pH at 9.0. To identify the ion channels involved we studied cells from 22 knock-out mice or human mutations. The results suggest a chloride conductance out of the vacuole and of cation/s into it. The identity of these channels remains to be established.

Crohn's disease

We described the failure to recruit neutrophils to, and the delayed clearance of *E.coli* from, the subcutaneous tissues in Crohn's patients. We also demonstrated the impaired secretion of proinflammatory
cytokines from macrophages of these patients.. This is now believed to involve defective bacterial recognition (NOD2, TLR4), autophagy activation (ATG16L1, IRGM, OPTN, NDP52) and cytokine regulation and is currently one of the major area of research in the field. We demonstrated impaired transcription of Optineurin, a vesicle trafficking linker in Crohn's and showed exaggerated inflammation in knock-down zebrafish and KO mouse models.
We have also taken a genetic approach to solving this problem. We have characterised and exome sequenced DNA from two very large Ashkenazi Jewish (AJ) families, one hundred smaller families and a thousand sporadic cases. These studies have led to the identification of mutations in CSF2RB and LRRK2 in addition to those in NOD2 in the pathogenesis of the disease in these subjects. We have also identified several additional strong causal candidates.

4. Joint applications

Is this a joint Investigator Award application?	No

5. Collaborators

Will you require any key collaborators for this proposal?	Yes

Please list any key collaborators* (name and organisation) and provide a very brief outline of their role in the proposed research.
**The collaborators named may be replaced with suitable alternatives should it be necessary or appropriate to do so.*

Professor Kathryn Lilley is a Professor in the Department of Biochemistry and the Cambridge Systems Biology Centre. She is also Director of the Cambridge Centre for Proteomics. She will undertake the proteomic analyses.

Dr Pierre Guermonprez, King's College, will collaborate on studies of antigen presentation and protein digestion.

Professor David Isenberg, Rheumatology, UCL, will provide me with blood samples from patients with SLE.

I confirm that the collaborators named above have agreed to be involved, as described, in the proposed research and are willing for their details to be included as part of this application.

Confirmed

6. Related applications

Is this or a similar application for funding currently under consideration elsewhere?	No

Is this a resubmission of an application submitted to the Trust within the last 24 months?	No

7. Research summary

Research summary
Please provide a summary of your proposed research, including key goals, for an expert audience

We developed a novel method for the measurement of pH within the phagocytic vacuole of neutrophils. We found the pH in this compartment to be alkaline, at about 9.0, optimal for neutral proteases which kill and digest microbes. We have applied this measurement of pH to monocytes, macrophages and dendritic cells and found the pH to be alkaline in Classical monocytes and M1 macrophages and acidic in Non-classical monocytes, M2 macrophages and blood and monocyte derived dendritic cells.

The pH of the phagocytic vacuoles will have a major impact on the enzymic action within that compartment and on the function of the cell as a whole. Accordingly I wish to identify the constituent proteins in the granules that are released into, and dictate the function of the vacuoles, of these different cell types, and monocyte/macrophage cell lines. The cells will be sorted, granules purified and their components identified using proteomics..

In the different cell types we will:

Study the cell biology of the enzymes.

Investigate bacterial killing.

Measure digestion, antigen presentation and efferocytosis.

Examine the consequences of targeting genes for the major enzymes on the above processes.

Measure pH changes and granule components in patients with SLE .

Lay summary
Please provide a summary of your proposed research that people who may not be familiar with the subject can understand. We may edit your summary and then use it to describe your research on our website and elsewhere.

The professional phagocytic cells in the circulation, neutrophils, monocytes, macrophages and dendritic cells, are responsible for phagocytosing, killing and digesting microbes, removing cellular debris and presenting antigens for immune responses.

We have devised a method of accurately determining the pH within the phagocytic vacuoles and have demonstrated markedly different values in different subsets of these cells.

The pH within these compartments will determine the activity of the enzymes in these compartments, which are therefore likely to differ between cell types and subtypes.

We will isolate these different cells , isolate the cytoplasmic granules containing the granules, and determine the constituent enzymes by proteomics..

The function of these enzymes on bacterial killing, on the digestion of engulfed materials and dead cells, and in immunological processes, will be measured. SLE is an autoimmune disease in which digestion might be abnormal and this possibility will be investigated in cells from patients.

8. Research vision

Please describe your research vision. You should ensure that this addresses the aims and key research questions, how this research will advance your field and the research approaches you will take (3,000 words maximum).
Please refer to guidance notes (blue question mark icon, below) before completing this section

The biology of phagocytic vacuoles of human monocytes, macrophages and dendritic cells

Aims of Research

To characterise the enzyme cargoes carried in the cytoplasmic granules of subsets of human monocytes, macrophages, and dendritic cells and to relate their activity to the specific functions of the different cell types, concentrating on microbial killing, digestion and antigen presentation.

To determine the optimal conditions for the function of these enzymes, and to examine the effects on the aforementioned immunological functions by removing them from human cells by gene editing with CRISPR.

Having characterised the pH changes, oxidase activity, and digestive functions in normal mononuclear cells, we will investigate these parameters in cells from patients with systemic lupus erythematosus (SLE), where aberrations of oxidase activity could influence autoantibody production and disease pathogenesis.

Background

Blood monocytes, macrophages and dendritic cells play a pivotal role in innate and adaptive immunity, performing essential functions including microbial killing, microbial and cellular digestion, and antigen presentation. All these processes take place in the specialised compartment that they share, the phagocytic vacuole.

Considerable recent progress has been made in classifying the lineages and subpopulations of these cell types by determining their surface marker characteristics and examining their general immunological functions. However, there has been very little advance in our understanding of the biology and biochemistry of their vacuoles. The last systematic work on their morphology and the beginnings of enzymology on these cells occurred in the laboratory of Zanvil Cohn in the 1960s-1980s [1–4]where the investigations largely involved electron microscopy, without much functional data[4–6].

The realisation that there are several different subtypes of these mononuclear phagocytes with considerably different functions[7,8] establishes the importance of developing our understanding of phagosome biology. Major recent technical developments including those in proteomics, confocal and ultra-high resolution microscopy and gene editing have provided the tools with which to explore the biochemical, cell biological and functional characteristics of these different subtypes of mononuclear blood cells, or cells derived from them. Studying phagosome biology has the potential to uncover new biological paradigms with implications for our pathophysiological understanding of a number of diseases.

I have considerable experience of the investigation of neutrophil granules, degranulation, digestion, and the overall biology of the phagocytic vacuoles of neutrophils having worked with this cell type for 45 years. My group's recent work generating spatiotemporal measurements of the pH within the vacuoles of monocytes, macrophages and dendritic cells have provided surprising and important results that indicates that pH must have an critical impact on the enzymology in these cells and is worthy of detailed characterisation.

The measurement of vacuolar pH in blood mononuclear cells

The pH in the phagocytic vacuole of neutrophils was considered to be very acidic[9] until we demonstrated that it was in fact alkaline as a consequence of the activity of the NADPH oxidase, NOX2[10]. Subsequently we developed a superior kinetic assay using the ratiometric,

pH sensitive dye, SNARF, coupled to *Candida,* to measure the pH in the phagocytic vacuoles of mouse and human neutrophils at high spatiotemporal resolution which replicated and further resolved this observation[11].

We then applied this assay to human blood mononuclear cells which include Classical (CM), Non-classical (NCM) and Intermediate monocytes (IM), M1 and M2 macrophages, and monocyte derived (MoDC) and blood (Blood DC) dendritic cells. We found that as with neutrophils[11], the phagocytic vacuoles of Classical monocytes and M1 macrophages became very alkaline, with pHs of 8.0-9.0. In M1 macrophages this alkaline state was maintained for at least 30 minutes whereas in the Classical monocytes after about 10 minutes it started to fall to around 7-7.5 (Figure 1). Vacuoles of Non-classical monocytes and M2 macrophages rapidly became acidic after a brief alkaline phase, and Intermediate monocytes maintained a relatively neutral pH. Similar results have previously been reported by others for M1 and M2 macrophages[12]. Blood and monocyte-derived dendritic cells had acidic phagosomes, with the pH falling to 5.5-6.0 without an alkaline phase.

The activity of the NADPH oxidase, NOX2, is responsible for the alkalinity observed in the vacuoles of all these cells (Figure 1). When this electron transport chain is defective, in Chronic Granulomatous Disease (CGD), or when the oxidase is inhibited by diphenylene iodonium (DPI), all the vacuoles in all the different cell types, become acidic without exhibiting an alkaline phase. Although the vacuoles in the dendritic cells remain acidic from the outset, the pH is depressed further by DPI or in CGD cells. This indicates that the pH is still modulated to an extent in dendritic cells by the oxidase, supporting the view that this is important for their ability to process and present antigen[13,14].

We propose that the different pHs in the vacuoles of these cells will have a major influence on their killing, digestive and antigen presentation activities by modulating the enzyme action of the granule proteins released into the vacuoles, as we have clearly demonstrated to be the case in neutrophils[10,11]

Research Plans

Cell populations to be studied

Monocyte subsets and blood DCs will be FACS sorted from normal human blood after separation of mononuclear cells (PBMCs) on Ficoll-Hypaque[8,15]. In the majority of cases the blood will be derived from consenting healthy volunteers but when working with rarer subsets of mononuclear cells we may need to purify the cells from buffy coat residues obtained from the NHS blood transfusion service or from packs of blood from patients venesected for iron overload or polycythaemia.

The three **monocyte subsets** will be FACS sorted based on cell-surface markers, in particular CD14 and CD16, to separate Classical ($CD14^{++}CD16^{-}$), Intermediate ($CD14^{++}CD16^{+}$), and Non-classical ($CD14+CD16^{++}$) monocytes.[16–18]To purify the **macrophages**, the PBMCs will be adhered to plastic and incubated with GM-CSF for five days followed by interferon-gamma and LPS to produce M1 macrophages. For M2 macrophages the PBMCs will be incubated with M-CSF followed by IL-4[19].

Monocyte derived DCs will be prepared from Classical monocytes isolated using the EasySep™ purification kit (STEMCELL Technologies), and cultured with GM-CSF and IL-4 for 7 days[20].

We will also study **neutrophils**, and two human cell lines: **Mono Mac 6**, that can be differentiated into mature monocytes[21], and **THP-1 cells** that can be matured into M1 and M2 macrophages[22].

1. The protein composition of the cytoplasmic granules

The biological processes that take place within the phagocytic vacuoles of these cells are determined by the nature of the proteins released into them from the cytoplasmic granules. Given the pH sensitivity of enzymic activity[11], the very different pHs we have observed within this compartment in the different cell populations are likely to require very different enzymes.

Compared with neutrophils, where granule protein composition[23] and degranulation[24] have been well characterised, very little is known about the granules of mononuclear cells.

Transcriptomes of the different cell types have been analysed; M1 and M2 macrophages [25] and Classical and Non-classical monocytes[26] are very different.

Transcriptome analysis may or may not include mRNA for the granule proteins, many of which might be preformed while the cells develop in the bone marrow, depending upon cell type.

Proteomics has also been performed on monocytes and macrophages[27]. A multicentre proteomic study has been undertaken on Classical and Non-classical monocyte populations derived from healthy donors. Macrophage proteomics has been undertaken comparing round and spindle shaped cells,[28] and there has been a proteomic characterisation of human M1 and M2 macrophages and their response to *Candida albicans*[29]. Proteomic studies have also been conducted on a variety of different dendritic cells[30]. These proteomics investigations have been performed on whole cells and the output has been dominated by the abundant cytoskeletal and housekeeping proteins. No clear picture has emerged as to the constituent proteins of the granules or their relative abundance.

Methods

Preparation of granules

Cells will be homogenised by physical methods (Dounce or two syringe homogenisers) and a post-nuclear supernatant (PNS) will be prepared.

Initially the PNS will be centrifuged into continuous sucrose[31] or Percoll[32] gradients to establish the buoyant density of the granules. They will be identified by assays of protease activity using the universal protease substrate resorufin-labeled casein (Sigma) which will be assayed at pHs of 6.5 and 8.5. Electron microscopy (EM) will be conducted on the cell preparations[24] and on the fractionated material. Subsequently, granules will be separated on discontinuous gradients, and the interface harvested, diluted and pelleted.

The granule preparations will be examined for heterogeneity by electron microscopy.

At a later stage in the studies, for example if there is evidence of enzymes being present in subpopulations of granules, they will be fractionated on continuous density gradients, or by isokinetic fractionation, then pelleted and analysed.

Proteomics

Protein components of the pooled granules will be identified by mass spectrometry (MS) in collaboration with Professor Kathryn Lilley at the Cambridge Centre for Proteomics (University of Cambridge).

The relative quantities of the proteins contained in the granules will be checked by staining one or two dimensional SDS-PAGE, and the identity of the bands/spots confirmed by MS.

Relative concentrations of the same proteins between the different cells will be established by Tandem Mass Tagging (TMT) protein quantitation[33] . This will be important because if the enzyme composition of the granules of the Mono Mac 6 and THP-1 cells are similar to that of the primary monocytes and macrophages, then manipulation of the enzymes in the cell lines can be undertaken with confidence that the alterations will accurately reflect changes that would occur in the primary cells.

Knowledge of the enzyme composition of the granules in the different cell types will provide valuable background information of general importance to the scientific community.

2. Cell biology and biochemistry of enzymes

When we know the identity of the enzymes in the granules of the different cell types we will examine their biochemistry and role in cellular function.

Antibodies are available to a wide range of enzymes including to most of the cathepsins, myeloperoxidase and lysozyme. If antibodies are unavailable to major constituent enzymes, these will be raised to peptide antigens. Antibodies will be used for immunohistochemistry to determine their distribution in the granules, to resolve co-location or otherwise with granular proteins, and to examine the kinetics of degranulation.

We will choose two of the most abundant enzymes that would not be expected to degranulate at the same times, for example a neutral protease and an acid hydrolase, and transfect Mono Mac 6 and THP-1 cells with a lentiviral vector carrying a fluorescent reporter tagged to the protein of interest. We will use these stably transfected cells lines to examine the kinetics of the enzymes' release into the phagocytic vacuole. It would also be possible to purify their phagocytic vacuoles and measure the kinetics of degranulation as we have done previously for neutrophils[24].

We will then determine the optimal conditions for the function of these enzymes. Where available we will purchase the pure proteins. Otherwise we will purify them from bulk leukocyte preparations, or express them in bacterial or mammalian cells. We will establish their pH optima and salt requirements.

The timing of the download of these enzymes from their granules into the phagocytic vacuole should reflect their properties and in particular their pH optima should relate to the pH in the vacuole at the time of degranulation, which we will be able to confirm with SNARF-Candida.

Inhibition of enzyme activities associated with the phagosomes

To determine the functional importance of the enzymes we will employ general and specific inhibitors, where available, for example from Tocris Bioscience, and by targeting their genes in Mono Mac 6 or THP-1 cells by CRISPR[34]. We have successfully targeted the gene for *NOD2* in THP-1 cells in my laboratory (Figure 4).

Investigation of inhibition of enzyme function on cellular function

We will study the following cellular functions:

i. Microbial killing

Although it is generally accepted that the predominant cell involved in killing mycobacteria is the macrophage, there is a recurrent theme in the literature that neutrophils might be more effective in this function, and that other mononuclear phagocytes might also be involved[35,36]. I have extensive experience of microbial killing assays and will compare and contrast killing by the different cell types of *M.bovis BCG-DSRed*[37]*, mCherry-Salmonella and mCherry-E. coli,* all of which we have in the laboratory.

We will measure the loss of fluorescence of the organisms as indicative of their digestion[38], and will also perform plating assays.

ii. Digestion and efferocytosis

To measure proteolytic activity, 3 μM non degradable latex beads will be crosslinked at their surface with Cy3 fluorescently-labelled ovalbumin (OVA). After phagocytosis, the phagocytes will be lysed with detergent to release the phagocytic cargos. Phagosomal proteolysis will be analysed by flow cytometry, by loss of OVA staining using anti-OVA antibodies coupled to Alexa488[39]. We will also measure enzyme activity in the vacuoles of live cells with specific fluorescent substrates. An example of elastase activity is shown in Figure 2.

We have recently devised a method of measuring vacuolar pH during efferocytosis of apoptotic neutrophils by labelling them with SNARF-1 after which they are engulfed by macrophages labelled with calcein. The engulfed cells are then digested and the vacuoles acidify and shrink (Figure 3). We will also label the apoptotic cells with pHrodo™ iFL Red STP Ester (ThermoFisher) which gives a linear response between pHs of 5-8.

We will optimise this assay to quantitate phagocytosis of the apoptotic cells[40] and subsequent digestion which will be measured by determining the changes in the volume of the vacuole, and its contents, on Z-stacked confocal images. We will also examine this process by super-resolution microscopy.

To measure the rate of degradation of DNA, protein and cholesterol, retrovirus transformed murine ER-HOXB8 cells, or human HL-60 cells, will be cultured in a medium containing tritiated thymidine[41], 35S-Methionine or 14C-Cholesterol. The cells will be matured to neutrophils[42,43] and apoptosis will be induced by oestrogen deprivation, which prevents HOXB8 nuclear translocation, or by aging HL-60 cells. These cells will be efferocytosed by the different subsets of phagocytic cells under investigation and the time course of solubilisation of the different radiolabels measured after TCA precipitation.

iii. Antigen presentation by MHCI and MHCII

These studies will be conducted in collaboration with Dr Pierre Guermonprez, Laboratory of Phagocyte Immunobiology, and King's College.

Amigorena and others[13,14] demonstrated a central role for NADPH oxidase in modulating the efficiency of antigen degradation and MHC-I cross-presentation to $CD8^+$ T cells by DCs. We have demonstrated that the vacuolar pH does fall in these cells, but less so in the presence of an active oxidase, which is consistent with their conclusions (Figure 1).

Antigen presentation will be analysed using phagocytic cargo carrying protein antigens with defined epitopes restricted by MHCI (HLA-A2 02:01, peptide Mart1/MelanA$_{26\text{-}35}$) and MHCII (HLA-DR1 04:05, peptide WT1$_{322}$).

Three types of antigenic particle will be used:

1 - Micron-sized, latex beads coated with streptavidin and biotin-coupled to long peptides necessitating intracellular protein degradation for MHCI or MHCII loading;

2 - *E. coli*-expressed GFP-fused antigenic peptides to model bacterial phagocytosis and associated antigen presentation;

3 - Conditionally immortalised, estrogen dependent ER-HOXB8 leukaemia cells stably expressing model antigens (delivered by a retroviral vector) will be used for the presentation of cell-associated antigens.

Donors of mononuclear phagocytes will be selected to express HLA-A2 02:01 and/or HLA-DR1 04:05 enabling antigen presentation assays.
These experiments will be performed immediately following sorting or *in vitro* differentiation of $CD14^+$ monocytes into M1 or M2 effector macrophages.

Bead-sorted, syngeneic, polyclonal, $CD8^+$ or $CD4^+$ T cells will be cultured with an IL2/IL15/aCD3/aCD28 cocktail and transduced for antigen-specific TCR recognition. T cell activation will be assessed by IL2 and IFN-γ secretion at day 2, and day 5 in the supernatants of co-culture experiments.
In an alternative approach, TCR-negative J76 clone will be transduced stably for the same MHCI-restricted (HLA-A2 02:01, peptide $MelanA_{26-35}$)[44] or MHCII-restricted (HLA-DR1 04:05, peptide $WT1_{322}$)[45] TCRs. In this case, a NF-AT-luciferase reporter construct activated downstream of TCR signalling will be used.

3. Investigation of patients with SLE

We have chosen to study the pathophysiological aspects of phagocytic cell function in SLE for the following reasons:

i) Several CGD patients have been described with SLE or discoid lupus erythematosus[46–49]. In addition, lupus has also been widely reported in female carriers of X-CGD[46,47,50,51].

ii) Genes encoding oxidase subunits have been associated with inflammatory and autoimmune disorders[52, 53]. Variants in NCF4 are associated with Crohn's disease[54], and NCF2 (p67phox) activates the electron transporting core of the enzyme system, gp91phox and the gene encoding NCF2 has been identified as an important risk factor for SLE[55,56]. However, the mechanism by which variation in NCF2 predisposes to SLE this is unknown.

iii) It is generally accepted that there is likely to be a failure of the clearance of autologous material in SLE[57]. DNase has been postulated to be responsible for the removal of DNA from nuclear antigens and an impairment of this process is thought to be partially responsible for the development of anti-DNA antibodies that are implicated in the pathogenesis of SLE[58]. The pH in vacuoles of monocyte/macrophage/DCs might differ from the optimum of 6.5 for DNAse 1[59]. It is of interest that hydroxychloroquine, a weak base that partitions into phagocytic vacuoles and elevates the pH[60,61], is a widely used treatment for SLE.

Blood samples from SLE patients on no treatment will be provided from the UCH lupus clinic (director Professor D Isenberg). Approximately one newly diagnosed patient on no treatment per month will be available to be studied. If vacuolar pH is abnormal in cells from these patients, we will measure their oxidase activity with the Seahorse Analyzer (Agilent) and quantitate *phox* proteins by Western blotting.

The studies described above will define the protein composition of the mononuclear phagocytes of human blood, will compare, contrast and characterise the variable functions of these different cell types, and the role that the specific granule proteins play to execute these functions.

Does your proposal involve a clinical trial?	No

Additional information
You may submit up to two A4 pages of additional information (such as graphs, figures, tables and essential unpublished data).

Figure 1. **Time course of phagosomal pH in:**
(**A**) neutrophils (N), classical (CM), intermediate (IM) and non-classical (NCM)monocytes,
(**B**) M1 and M2 macrophages with and without DPI
(**C**) blood and monocyte-derived (MoDC) dendritic cells.
Some representative images and a guide to pHs are shown in D.
All cells types from CGD patient gave the same results as shown for neutrophils (CGD).

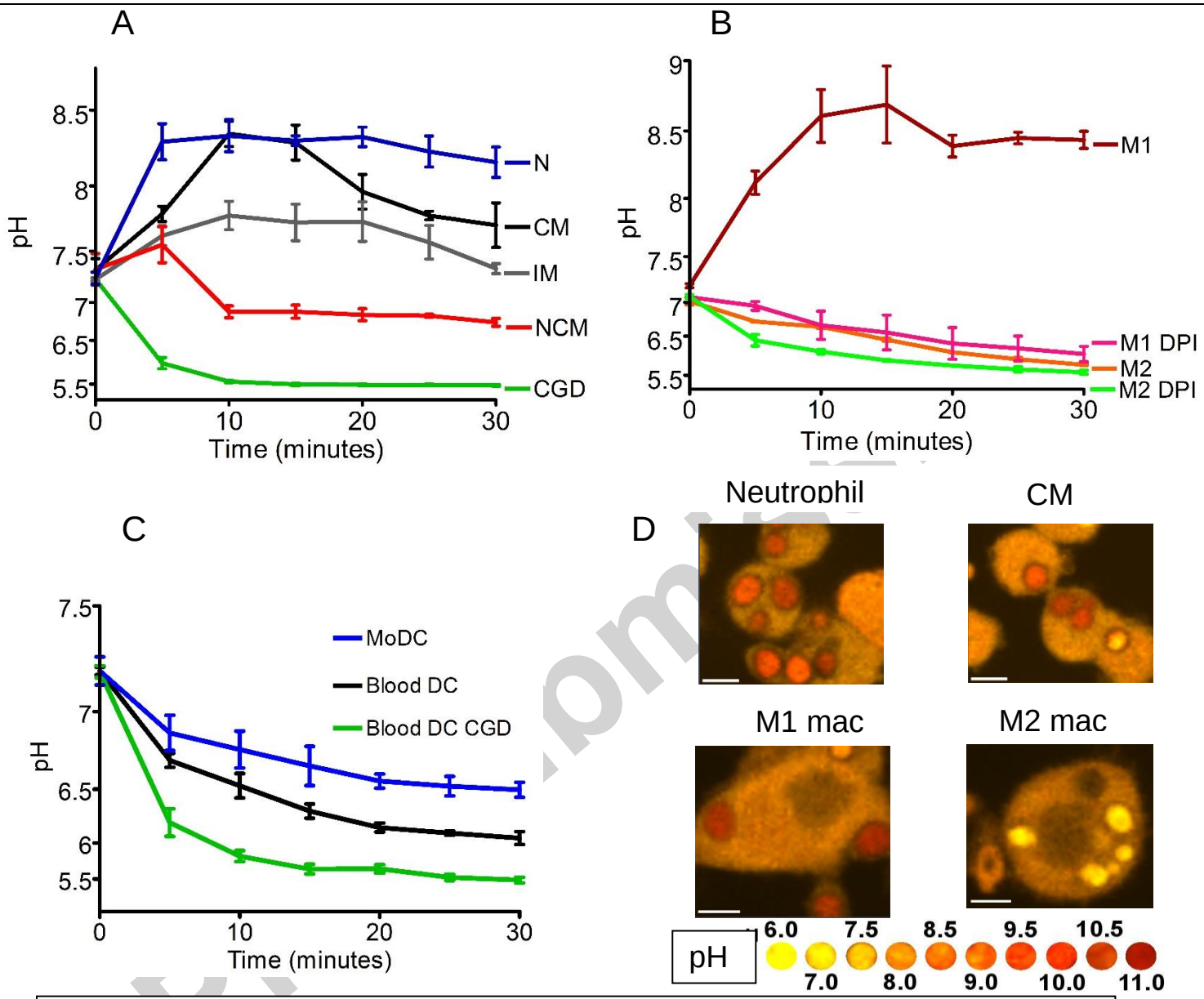


Figure 2. **Elastase activity in neutrophil and monocyte**
ThermoFisher bisamide rhodamine 110 elastase substrate (CBZ-Ala-Ala-Ala-Ala)2-R110 fluorescence demonstrating elastase activity after phagocytosis of unlabelled *Candida*.

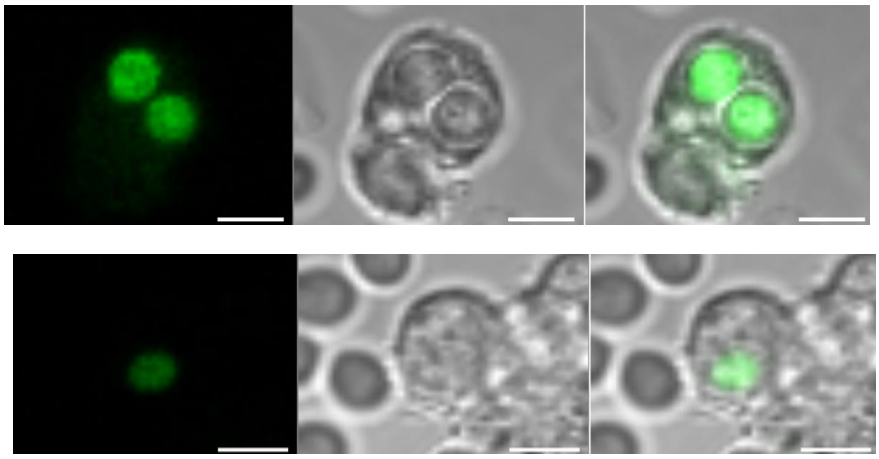


Figure 3. **Efferocytosis in M1 and M2 macrophage**
The phagocytosis of aged apoptotic neutrophils is seen. The phagocytic vacuoles and their contents then decrease in size. The neutrophils are taken up much more avidly by the M2 macrophages, in which the vacuoles are more acidic. The time after phagocytosis is shown in hours.

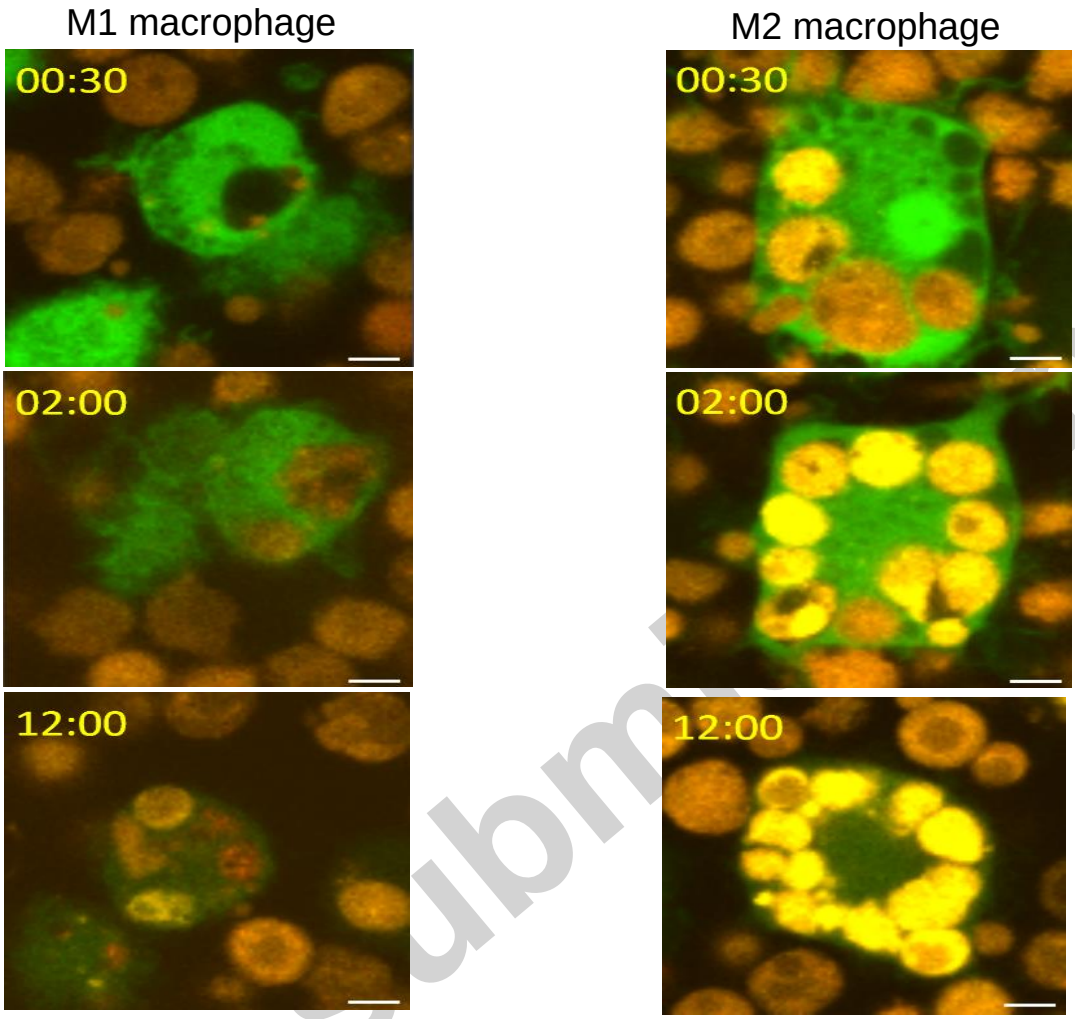
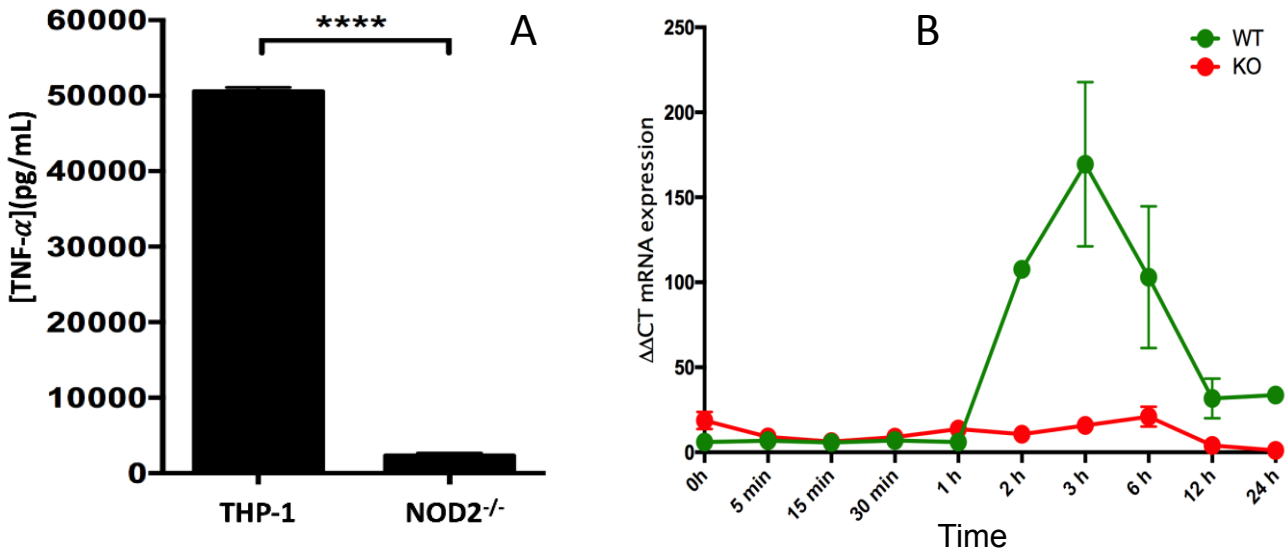


Figure 4. **CRISPR-based gene editing of NOD2 in THP-1 cells.**
A. TNF-α measured by ELISA 24 hours after MDP stimulation of THP-1 (WT) and THP-1 (NOD2-KO) cells. B. Time course of TNF-α mRNA levels measured by RT-PCR.



Key references
You should give the citation in full, including title of paper and all authors.

1. Fels, A. O. & Cohn, Z. A. The alveolar macrophage. *J. Appl. Physiol.* **60,** 353–369 (1986).

2. Werb, Z. & Gordon, S. Secretion of a specific collagenase by stimulated macrophages. *J. Exp. Med.* **142,** 346–60 (1975).

3. Nichols, B. A., Bainton, D. F. & Farquhar, M. G. Differentiation of monocytes. Origin, nature, and fate of their azurophil granules. *J. Cell Biol.* **50,** 498–515 (1971).

4. Cohn, Z. A., Fedorko, M. E. & Hirsch, J. G. The in vitro differentiation of mononuclear phagocytes. V. The formation of macrophage lysosomes. *J. Exp. Med.* **123,** 757–66 (1966).

5. Bennett, W. E. & Cohn, Z. A. The isolation and selected properties of blood monocytes. *J. Exp. Med.* **123,** 145–60 (1966).

6. van Furth, R., Hirsch, J. G. & Fedorko, M. E. Morphology and peroxidase cytochemistry of mouse promonocytes, monocytes, and macrophages. *J. Exp. Med.* **132,** 794–812 (1970).

7. Jakubzick, C. V., Randolph, G. J. & Henson, P. M. Monocyte differentiation and antigen-presenting functions. *Nat. Rev. Immunol.* **17,** 349–362 (2017).

8. Patel, A. A. *et al.* The fate and lifespan of human monocyte subsets in steady state and systemic inflammation. *J. Exp. Med.* **214,** 1913–1923 (2017).

9. Jensen, M. S. & Bainton, D. F. Temporal changes in pH within the phagocytic vacuole of the polymorphonuclear neutrophilic leukocyte. *J. Cell Biol.* **56,** 379–88 (1973).

10. Segal, A. W., Geisow, M., Garcia, R., Harper, A. & Miller, R. The respiratory burst of phagocytic cells is associated with a rise in vacuolar pH. *Nature* **290,** 406–409 (1981).

11. Levine, A. P., Duchen, M. R., de Villiers, S., Rich, P. R. & Segal, A. W. Alkalinity of Neutrophil Phagocytic Vacuoles Is Modulated by HVCN1 and Has Consequences for Myeloperoxidase Activity. *PLoS One* **10,** e0125906 (2015).

12. Canton, J., Khezri, R., Glogauer, M. & Grinstein, S. Contrasting phagosome pH regulation and maturation in human M1 and M2 macrophages. *Mol. Biol. Cell* **25,** 3330–41 (2014).

13. Savina, A. *et al.* NOX2 controls phagosomal pH to regulate antigen processing during crosspresentation by dendritic cells. *Cell* **126,** 205–218 (2006).

14. Rybicka, J. M., Balce, D. R., Chaudhuri, S., Allan, E. R. O. & Yates, R. M. Phagosomal proteolysis in dendritic cells is modulated by NADPH oxidase in a pH-independent manner. *EMBO J.* **31,** 932–944 (2012).

15. Haniffa, M. *et al.* Human tissues contain CD141hi cross-presenting dendritic cells with functional homology to mouse CD103+ nonlymphoid dendritic cells. *Immunity* **37,** 60–73 (2012).

16. Lambert, C., Preijers, F. W. M. B., Yanikkaya Demirel, G. & Sack, U. Monocytes and macrophages in flow: an ESCCA initiative on advanced analyses of monocyte lineage using flow cytometry. *Cytometry B. Clin. Cytom.* **92,** 180–188 (2017).

17. Ziegler-Heitbrock, L. *et al.* Nomenclature of monocytes and dendritic cells in blood. *Blood* **116,** e74-80 (2010).

18. Weber, C. *et al.* Role and analysis of monocyte subsets in cardiovascular disease. Joint consensus document of the European Society of Cardiology (ESC) Working Groups "Atherosclerosis & Vascular Biology" and "Thrombosis". *Thromb. Haemost.* **116,** 626–37 (2016).

19. Canton, J. Phagosome maturation in polarized macrophages. *J. Leukoc. Biol.* **96,** 729–738 (2014).

20. Ohradanova-Repic, A., Machacek, C., Fischer, M. B. & Stockinger, H. Differentiation of human monocytes and derived subsets of macrophages and dendritic cells by the HLDA10 monoclonal antibody panel. *Clin. Transl. Immunol.* **5,** e55 (2016).

21. Erl, W., Weber, C., Wardemann, C. & Weber, P. C. Adhesion properties of Mono Mac 6, a monocytic cell line with characteristics of mature human monocytes. *Atherosclerosis* **113,** 99–107 (1995).

22. Chanput, W., Mes, J. J. & Wichers, H. J. THP-1 cell line: an in vitro cell model for immune modulation approach. *Int. Immunopharmacol.* **23,** 37–45 (2014).

23. Cowland, J. B. & Borregaard, N. Granulopoiesis and granules of human neutrophils. *Immunol. Rev.* **273,** 11–28 (2016).

24. Segal, A. W., Dorling, J. & Coade, S. Kinetics of fusion of the cytoplasmic granules with phagocytic vacuoles in human polymorphonuclear leukocytes. Biochemical and morphological studies. *J.Cell Biol.* **85,** 42–59 (1980).

25. Galvan-Pena, S. & O'Neill, L. A. J. Metabolic Reprograming in Macrophage Polarization. *Front. Immunol.* **5,** 420 (2014).

26. Anbazhagan, K., Duroux-Richard, I., Jorgensen, C. & Apparailly, F. Transcriptomic Network Support Distinct Roles of Classical and Non-classicalClassical Monocytes in Human. *Int. Rev. Immunol.* **33,** 470–489 (2014).

27. Castagna, A., Polati, R., Bossi, A. M. & Girelli, D. Monocyte/macrophage proteomics: recent findings and biomedical applications. *Expert Rev. Proteomics* **9,** 201–215 (2012).

28. Eligini, S. *et al.* Human monocyte-derived macrophages are heterogenous: Proteomic profile of different phenotypes. *J. Proteomics* **124,** 112–123 (2015).

29. Reales-Calderón, J. A., Aguilera-Montilla, N., Corbí, Á. L., Molero, G. & Gil, C. Proteomic characterization of human proinflammatory M1 and anti-inflammatory M2 macrophages and their response to Candida albicans. *Proteomics* **14,** 1503–18 (2014).

30. Ferreira, G. B., Mathieu, C. & Overbergh, L. Understanding dendritic cell biology and its role in immunological disorders through proteomic profiling. *PROTEOMICS - Clin. Appl.* **4,** 190–203 (2010).

31. Segal, A. W. & Peters, T. J. Analytical subcellular fractionation of human granulocytes with special reference to the localization of enzymes involved in microbicidal mechanisms. *Clin. Sci. Mol. Med.* **52,** (1977).

32. Clemmensen, S. N., Udby, L. & Borregaard, N. in *Methods in molecular biology (Clifton, N.J.)* **1124,** 53–76 (2014).

33. O'Connell, J. D., Paulo, J. A., O'Brien, J. J. & Gygi, S. P. Proteome-Wide Evaluation of Two Common Protein Quantification Methods. *J. Proteome Res.* **17,** 1934–1942 (2018).

34. Baker, P. J. & Masters, S. L. in *Methods in molecular biology (Clifton, N.J.)* **1714,** 41–55 (2018).

35. Warren, E., Teskey, G. & Venketaraman, V. Effector Mechanisms of Neutrophils within the Innate Immune System in Response to Mycobacterium tuberculosis Infection. *J. Clin. Med.* **6,** 15 (2017).

36. Srivastava, S., Ernst, J. D. & Desvignes, L. Beyond macrophages: the diversity of mononuclear cells in tuberculosis. *Immunol. Rev.* **262,** 179–192 (2014).

37. Vázquez, C. L. *et al.* The proneurotrophin receptor sortilin is required for Mycobacterium tuberculosis control by macrophages. *Sci. Rep.* **6,** 29332 (2016).

38. Schwartz, J., Leidal, K. G., Femling, J. K., Weiss, J. P. & Nauseef, W. M. Neutrophil Bleaching of GFP-Expressing Staphylococci: Probing the Intraphagosomal Fate of Individual Bacteria. *J. Immunol.* **183,** 2632–2641 (2009).

39. Savina, A., Vargas, P., Guermonprez, P., Lennon, A.-M. & Amigorena, S. Measuring pH, ROS production, maturation, and degradation in dendritic cell phagosomes using cytofluorometry-based assays. *Methods Mol. Biol.* **595,** 383–402 (2010).

40. Marée, A. F. M., Komba, M., Finegood, D. T. & Edelstein-Keshet, L. A quantitative comparison of rates of phagocytosis and digestion of apoptotic cells by macrophages from normal (BALB/c) and diabetes-prone (NOD) mice. *J. Appl. Physiol.* **104,** 157–169 (2008).

41. Choudhury, C. & Sparks, R. Cell cycle status of stromal cells in long-term haematopoietic cultures. *Cell Prolif.* **24,** 461–8 (1991).

42. Wang, G. G. *et al.* Quantitative production of macrophages or neutrophils ex vivo using conditional Hoxb8. *Nat. Methods* **3,** 287–293 (2006).

43. Roberts, P. J., Cross, A. R., Jones, O. T. G. & Segal, A. W. Development of cytochrome b and an active oxidase system in association with maturation of a human promyelocytic (HL-60) cell line. *J. Cell Biol.* **95,** (1982).

44. Johnson, L. A. *et al.* Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. *Blood* **114,** 535–546 (2009).

45. Katsuhara, A. *et al.* Transduction of a novel HLA-DRB1*04:05-restricted, WT1-specific TCR gene into human CD4+ T cells confers killing activity against human leukemia cells. *Anticancer Res.* **35,** 1251–61 (2015).

46. Winkelstein, J. A. *et al.* Chronic granulomatous disease. Report on a national registry of 368 patients. *Medicine (Baltimore).* **79,** 155–69 (2000).

47. van den Berg, J. M. *et al.* Chronic granulomatous disease: the European experience. *PLoS One* **4,** e5234 (2009).

48. Manzi, S. *et al.* Systemic lupus erythematosus in a boy with chronic granulomatous disease: case report and review of the literature. *Arthritis Rheum.* **34,** 101–5 (1991).

49. Martin-Villa, J. M., Corell, A., Ramos-Amador, J. T., Ruiz-Contreras, J. & Arnaiz-Villena, A. Higher incidence of autoantibodies in X-linked chronic granulomatous disease carriers: random X-chromosome inactivation may be related to autoimmunity. *Autoimmunity* **31,** 261–4 (1999).

50. Cale, C. M., Morton, L. & Goldblatt, D. Cutaneous and other lupus-like symptoms in carriers

of X-linked chronic granulomatous disease: incidence and autoimmune serology. *Clin. Exp. Immunol.* **148,** 79–84 (2007).

51. De Ravin, S. S. *et al.* Chronic granulomatous disease as a risk factor for autoimmune disease. *J. Allergy Clin. Immunol.* **122,** 1097–103 (2008).

52. Singel, K. L. & Segal, B. H. NOX2-dependent regulation of inflammation. *Clin. Sci.* **130,** 479–490 (2016).

53. Olsson, L. M. *et al.* A case-control study of rheumatoid arthritis identifies an associated single nucleotide polymorphism in the NCF4 gene, supporting a role for the NADPH-oxidase complex in autoimmunity. *Arthritis Res. Ther.* **9,** R98 (2007).

54. Wu, P.-B. *et al.* Association between NCF4 rs4821544T/C polymorphism and inflammatory bowel disease risk in Caucasian: a meta-analysis. *Inflamm. Res.* **64,** 825–831 (2015).

55. Jacob, C. O. *et al.* Lupus-associated causal mutation in neutrophil cytosolic factor 2 (NCF2) brings unique insights to the structure and function of NADPH oxidase. *Proc. Natl. Acad. Sci.* **109,** E59–E67 (2012).

56. Alarcón-Riquelme, M. E. *et al.* Genome-Wide Association Study in an Amerindian Ancestry Population Reveals Novel Systemic Lupus Erythematosus Risk Loci and the Role of European Admixture. *Arthritis Rheumatol.* **68,** 932–943 (2016).

57. Tsokos, G. C., Lo, M. S., Reis, P. C. & Sullivan, K. E. New insights into the immunopathogenesis of systemic lupus erythematosus. *doi.org* **12,** 716–730 (2016).

58. Martínez Valle, F., Balada, E., Ordi-Ros, J. & Vilardell-Tarres, M. DNase 1 and systemic lupus erythematosus. *Autoimmun. Rev.* **7,** 359–63 (2008).

59. Fujihara, J., Yasuda, T., Ueki, M., Iida, R. & Takeshita, H. Comparative biochemical properties of vertebrate deoxyribonuclease I. *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.* **163,** 263–73 (2012).

60. Klempner, M. S. & Styrt, B. Alkalinizing the intralysosomal pH inhibits degranulation of human neutrophils. *J. Clin. Invest.* **72,** 1793–800 (1983).

61. Henriet, S. S. V *et al.* Chloroquine modulates the fungal immune response in phagocytic cells from patients with chronic granulomatous disease. *J. Infect. Dis.* **207,** 1932–9 (2013).

9. Institutional support

Upload your institutional statement of commitment here. This must be a statement of support on behalf of your host organisation, signed by the most senior authority in the relevant Faculty, School or Division. Please refer to guidance notes.

10. Supporting information

Is there anything you would like to add in support of your application for an Investigator Award? You might wish to highlight, for example:

- any special circumstances relating to your research career;
- evidence of your commitment to public engagement;
- your translational activities;
- key members of your team who will contribute to your research programme.

I believe that I have a body of experience that makes me uniquely qualified to undertake the work to define the enzymology of the vacuoles of these cells.

The professional phagocytic mononuclear leukocytes play a central role in innate and adaptive immunity. Some of the most essential functions of these cells relate to processes that take place in the phagocytic vacuoles, yet we know surprisingly little about the identity and functions of the proteins that are released into these compartments from the cytoplasmic granules.

Most of the work on these cells has been on subset analysis and lineage definition with little on their enzymology and the differential function of these proteins. Our discovery of different pHs in the vacuoles of the different monocyte and macrophage subsets and the acidic environment in dendritic cell vacuoles strongly indicates the existence of granule enzymes that will have different pH optima and function differently in the different cells.

The pH in the vacuoles is differentially and temporally regulated by the **NADPH oxidase, NOX2**. I discovered and characterised this system and demonstrated how it elevates vacuolar pH, activates granule neutral proteases and causes digestion of bacteria.

Nature. 1978; 276(5687):515-7; Nature. 1981; 290(5805):406-9; Nature. 1985 ;316(6028):547-9; Nature. 1987;326(6108):88-91; Nature. 1987;327(6124):720-1; Nature. 1991;353(6345):668-70; Nature. 2002;416(6878):291-7; Biochem J. 1992;284 (Pt 3):781-8; Biochem J. 1993 ;296 (Pt 3):557-61.

I have experience of **subcellular fractionation**:

Biochem J. 1984;219(1):233-42; Biochem J. 1979;182(1):181-8; Q J Med. 1978;47(186):213-20; Clin Sci Mol Med. 1977;52(4):429-42; Clin Sci Mol Med. 1976;51(4):421-5.

I also have experience of measurements of **degranulation kinetics** of neutrophil granules:

J Cell Biol. 1980 ;85(1):42-59.

I have extensive experience of **protein purification**:

Biochem J. 1984;219(2):519-27; Biochem J. 1985;227(3):783-8.

And some experience of **proteomic analysis**:

Proteomics. 2009;9(7):2037-49. Biochem J. 2004;377:469-77; J Med Microbiol. 2003 ;52(Pt 8):643-51.

I have knowledge of **electron and confocal microscopy**:

J Cell Biol. 1980;85(1):42-59; Nature. 1981; 290(5805):406-9

My collaborator, Dr Pierre Guermonprez, is knowledgeable on **vacuolar digestion and antigen processing.**

Professor David Isenberg is the leading authority on SLE in the UK, and has access to suitable numbers of untreated patients with this disease.

We are well suited to undertake this important research.

11. Outputs management and sharing

Will the proposed research generate outputs of data, software, materials or intellectual property that hold significant value as a resource for the wider research community?	Yes

Which approach do you intend to use to maximise the impact of your significant research outputs to improve health and benefit the wider research community?
Make research outputs available for access and re-use

Please provide an outputs management plan. Ensure this describes any significant data, software, materials or intellectual property outputs, their management, and resources required (refer to guidance).
Proteomics data will be deposited in a publicly available repository such as PRIDE or ProteomeXchange https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4409848/ The results of these studies will be published and presented and discussed at scientific meetings. Disease related results will be communicated to patients through the appropriate patient support groups.

12. Research group size

Please provide details of the number of people in your research group reporting to you: during the previous two years, currently, and projected over the proposed duration of your award, including any research staff supported by recurrent or core funding.

Research group size data should be calculated on a full-time equivalent (FTE) basis.

	Year -2	Year -1	Current	Year 1	Year 2	Year 3	Year 4	Year 5	Year 6	Year 7
PhD students / research assistants (inc. shared)	3.00	1.00	2.00	2.00	2.00	2.00				
Postdoctoral research assistants	3.00	3.00	3.00	0.00	0.00	0.00				

Technical support staff	1.00	1.00	1.00	1.00	1.00	1.00				
Other (please specify below)										
Total	7.00	5.00	6.00	3.00	3.00	3.00	0.00	0.00	0.00	0.00

Please specify the categories of any staff included under 'Other'. If this does not apply, please enter N/A.
N/A

If desired, provide additional clarification of research group numbers
I share my laboratory with Dr Andrew Smith, Senior lecturer, Eastman Dental Hospital, UCL. He was a postdoctoral scientist in my group before he obtained this permanent position at UCL. He has four PhD students and a postdoctoral scientist in his group, and we are integrated, although we work on related, but separate, projects, so there is a critical mass and stimulating environment within the laboratory.

13. Public engagement

Do you have plans for engaging with the non-academic public about your work?	Yes

Please provide a brief outline of your public engagement plans.
The results of our research will be published on our university websites. We are closely associated with CGD charities and regularly attend meetings and discuss our research with patients, family members and healthy volunteers. We encourage summer school students to work in our laboratory where they get real experience of science in action. UCL run numerous events for the general public and at least once a year we will endeavour to setup a stall which deals with immunity in order to maximise our public exposure. We will work closely with UCL public engagement unit (http://www.ucl.ac.uk/public-engagement) who are dedicated experts in this field. This unit is one of six in the United Kingdom to be funded by the Beacons for Public Engagement programme set up by HEFCE, Research Councils UK and the Wellcome Trust. The Public Engagement Unit supports UCL staff and students involved in activities directed at the local communities, schools and interested parties outside of the University. We have a track record in raising public awareness, including two major pieces of previous work that were of interest to the public and both were widely publicised in broad sheet newspapers and over the radio. We have setup a Twitter account (@MoI_Med_UCL) which we use as a platform to publicise our groups current research.

Please note that we provide support for Wellcome Trust funded researchers to engage with the non-academic public. Do you wish to receive information about training, funding and other public engagement opportunities?	No

14. Location of activity

Will the funded activity take place at more than one location?	No

Will you require funds to be awarded directly to more than one location?	No

15. Costs requested and justification

Please select the currency in which you wish to apply.
GBP - Pound Sterling

Is the selected currency your local currency?	Yes

Salaries Are you requesting salaries? Please refer to guidance notes and definition of terms for further details	Yes

Salaries

Staff category	Name (if known)	Basic starting salary (p.a.)	Salary grade / scale	Period on project (months)	% time	Total (£)
Research technician	Francesca Semplici	39992	Research 7	48	100	237,046
Postdoctoral scientist	To be appointed	41212	Research 8	48	100	247,020

Materials and consumables Are you requesting materials and consumables?	Yes

Materials and consumables

Description	Total (£)
Antibodies	10,000
Cell lines	3,000
Cell separation media	6,000
Chemicals	8,000
Growth factors and cytokines	12,000
Molecular Biology reagents	5,000
Plasticware	12,000
Radio Isotopes	5,000
Tissue Culture media	8,000

Description	Total (£)
Western blot membrane, X-ray film, developers	10,000
T-cell isolation kits	3,500
Magnetic beads and columns for sorting monocytes and DCs	1,500

Animals Are you requesting animals?	No

Equipment Are you requesting equipment or equipment maintenance?	No

Synchrotron radiation sources Will the proposed research require access to a synchrotron source?	No

Access charges Are you requesting access charges?	Yes

Access charges

Details of equipment/ facility	Original source of funding	Wellcome Trust grant number, if applicable	Standard access charge per unit	Specify unit	No. of units to be used for this project	Total (£)
Flow cytometer analysis	Arthritis and Rheumatism Council		18	hourly	400	7,200
Confocal microscopy	Wellcome Trust	: 048858	25	hourly	600	15,000
Elecron microscopy	HEFCE		27	hour	20	540

Travel and subsistence Are you requesting travel and subsistence?	Yes

Travel and subsistence

Description	Total (£)
Travel to and from Cambridge	1,000
Overseas conference travel	8,000

Miscellaneous costs Are you requesting miscellaneous costs?	Yes

Miscellaneous costs

Type	Description	Total (£)
Other	Proteomics costs	9,000

Are you requesting research management costs under the miscellaneous costs heading? (for applicants from low- and middle-income countries only)	No

Justification for resources requested
Please provide a complete justification for all the resources requested, ensuring that you present this information according to the cost headings requested above.

Staff

Postdoctoral Scientist Grade 8. The focus of the grant is an in depth investigation of the cell biology and biochemistry of the vacuoles of a major group of phagocytic cells. It will require an able scientist with experience of handling and manipulating cells, proteins and bacteria. Therefore I will need an individual with expertise in cell biology, immunology, molecular biology, protein biochemistry and microscopy. The individual will be expected to work independently to conduct the requisite experiments. In areas where they are lacking knowledge or experience, I, or one of our collaborators, will provide guidance and support. In order to achieve all of these aims we require an experienced postdoctoral scientist.

I require a technician to help me run my laboratory. I have a very competent person in Francesca Semplici, who is my laboratory manager. She will also help with the more routine tasks like cell culture, bacterial killing assays and cell sorting. I do not have support for her after the end of 2018.

I spend a considerable amount of my time in and out of the laboratory, and will be constantly involved in the design and conduct of this work and have allocated 12.5% of my time to it.

Travel

We will need travel money to undertake specific research associated with proteomics as part of our collaborations. I have also requested £1000 a year for travel within the UK. The bulk of this will be used to travel backwards and forwards to Cambridge to transport samples and to discuss results..

We will present our results at national and international scientific conferences (including annual meetings held by British Society of Immunology). We have requested money to travel to and attend a selection of these conferences through the duration of the grant. Requested £8,000 over the four years to allow for travel to annual meetings in Europe and to two international meetings.

Consumables

I have requested £21,000 per year for the four years which reflects the actual expenditure by a postdoctoral scientist and technician in my laboratory.

We will require general laboratory consumables such as plastic ware, culture media, microscopy reagents, electrophoresis materials, PVDF and photographic film. Growth factors and cytokines will be required for cell polarization. We will require a range of primary and secondary antibodies for western blotting, immunocytochemistry and FACS analysis and cell sorting. We will need radioisotopes for digestion studies.

A variety of resins and columns will be needed for the purification of proteins.

Specific inhibitors to test the actions of granule enzymes in intact cells.

We will perform our proteomics work with the Cambridge Centre for Proteomics. Each run costs £300 including data analysis. I estimate that we will require 30 runs at a cost of £9000.

Access charges within UCL

We will require a substantial amount of FACS analysis and cell sorting and I have costed 400 hours

at £18/hour. Much of our work will be done with high-resolution confocal imaging performed on a Zeiss LSM 800 Confocal Laser Scanning Microscope (access charge of £25.33/hour) and we will require 600 hours over the duration of the project. 50 hours of Super high resolution microscopy to image efferocytosis is included in that figure.

We will require transmission electron microscopy to determine changes to microbes within the phagocytic vacuoles and we will need 20 hours at £27/hour for which I have requested £540.

Summary of financial support requested	
	Total (£)
Salaries / Stipends	484,066
Materials and consumables	84,000
Animals	0
Associated animals costs	0
Equipment	0
Maintenance for existing equipment	0
Access charges	22,740
Travel and subsistence	9,000
Miscellaneous other	9,000
Total	**608,806**

16. Full economic costing

Is your organisation based in the UK?	Yes

Is your organisation calculating the full economic cost of this proposal?	Yes

What is the total full economic cost (£)?	1085224

17. Research involving human participants, human biological material and identifiable data

Does your project involve human participants, human biological material, or identifiable/potentially identifiable data?	Yes

Please confirm that you have read the Trust's guidance on the feedback of health-related findings in research and that you are in the process of considering your approach to this.
Confirmed

Please state by whom and when the ethics of the project has been, or will be, reviewed and specify any other regulatory approvals that have been obtained, or will be sought.

We reserve the right to see relevant approval documents at any point during the lifetime of the grant, in accordance with our policy position on research involving human participants.
Abnormal genes and proteins in inflammatory bowel disease **REC: 10/H0806/115** Ethical and research governance approval is provided by The National Research Ethics Service London Surrey Borders Committee (10/H0906/115) IRAS:28159

In the course of your project, do you propose to use facilities within the National Health Service (NHS) or to involve patients being cared for by the NHS?	Yes

Is a formal sponsor required for the project, for example under the Medicines for Human Use (Clinical Trials) Regulations or the Research Governance Framework for Health and Social Care and equivalent guidance?	No

If any potentially commercially exploitable results may be based upon tissues or samples derived from human participants, please confirm that there has been appropriate informed consent for such use.
N/A

18. Proposals involving animals

Please indicate which of the following apply: *(Proposal involves the use of animals, Proposal involves the use of animal tissue, Neither of the above)*
Neither of the above

19. Risks of research misuse

Please confirm that you have considered whether your proposed research could generate outcomes that could be misused for harmful purposes.
Confirmed

Have you identified any tangible risks of this type?	No

20. Freedom to operate/conflicts of interest

Describe any freedom to operate issues or potential conflicts of interest that have been identified or that might arise and how these will be or have been addressed.

In particular, please consider the following:

- Do any of the individuals involved in the project hold any consultancies or equities in, or directorships of, companies or other organisations that might have an interest in the results of the proposed research?

• Will the proposed research use technology, materials or other inventions that are subject to any patents or other form of intellectual property protection? • Will any element of the research be subject to agreements with commercial, academic or other organisations, including arrangements with collaborators named in the grant application, that might lead to intellectual property issues or restrictions?
None of these apply

21. Wellcome Trust supported facilities

Will the project be based in one of the following Wellcome Trust supported facilities: • the Wellcome Trust Sanger Institute • a Wellcome Trust Centre • an Africa and Asia Programme • the Francis Crick Institute?	No