1. Investigator Award in Science application

Reference number	UNS39533
Applicant name	Prof Anthony Segal
Title of application	Mechanisms and molecules involved in the pathogenesis of Crohn's disease
Total amount requested	£1,262,571.00

2. Application summary

Application title
Mechanisms and molecules involved in the pathogenesis of Crohn's disease

Proposed duration of funding (months)
60

Proposed start date	01/09/2017

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	WC1E6JF
Country	United Kingdom

Scientific area Please select from the drop-down list the Expert Review Group that you consider your research falls under
Genetics, Genomics and Population Research

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
HEFCE

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

Please specify
Medical graduate

Are you clinically active?	Yes

What is your specialty?
Gastroenterology

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

Professional recognition
Please list: (i) Prizes, honours and awards received during your career, including invited talks during the last three years; (ii) Current memberships of funding agency advisory and/or journal editorial boards.

1979 Wellcome Trust Senior Clinical Fellowship

1987 Biochemical Society Wellcome Trust Award for Research in Biochemistry Related to Medicine

1988 The European Mack-Foster Award for Clinical Investigation

1998 FRS The Royal Society, United Kingdom

1998 F.Med.Sci. (Founding fellow) Academy of Medical Sciences

1987 F.R.C.P. Royal College of Physicians of London

2002 Honorary Fellow, University College London

2008 Honorary Fellow College Physicians, The Colleges of Medicine of South Africa

Invited Talks:

2014:

February. ‘The causes of Crohn’s Disease?’. ECCO Conference, Copenhagen.

Closing lecture. This is the most important meeting on Inflammatory Bowel Disease in Europe.

April. ‘Crohn’s is an auto deficiency disease’, Human Immunology in Health and Disease, Henry Kunkel Society, New York.

May. ‘The causes of Crohn’s Disease?’, The Phagocyte Workshop, Utrecht.

June. ‘Crohn’s is an Immunodeficiency Disease’, Riken IMS-JSI International Symposium on Immunology, Tokyo, Japan.

September 2014. UCL Prize Lecture in Clinical Science 2014. ‘From bed to the bench and back: Musings by a peripatetic clinical scientist on various aspects of innate immunity and Crohn’s disease’. University College London.

https://www.ucl.ac.uk/news/slms/slms-news/slms/cl-lec2014

Peer-reviewed publications and other research outputs
List up to 20 of your most significant peer-reviewed publications or other scholarly contributions and other research outputs, e.g. patents. Please ensure that at least five of these are from the last five years. For 10 of these outputs, you may also provide a statement describing their significance (up to 50 words per output).

For original research publications indicate those arising from Trust-funded grants in **bold**, and provide the PMCID reference for each of these. Please refer to guidance notes.

*Please give citation in full, including title of paper and all authors**
*(*All authors, unless more than 10, in which case please use ‘et al’, ensuring that your position as author remains clear.)*

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 Oct;151(4):698-709. doi: 10.1053/j.gastro.2016.06.040.

PMID: 27373512

Making sense of the cause of Crohn’s – a new look at an old disease

Segal AW

F1000Research 2016, 5:2510 (doi: 10.12688/f1000research.9699.2)

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 Jan;144(1):45-55. doi: 10.1111/imm.12338.

PMID: 24943399

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 Aug 1;8(8):817-29. doi: 10.1242/dmm.020362.

PMID: 26044960

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 Aug 31;206(9):1883-97. doi: 10.1084/jem.20091233.

PMID: 19652016

Inflammatory bowel disease in CGD reproduces the clinicopathological features of Crohn's disease.

Marks DJ, Miyagi K, Rahman FZ, Novelli M, Bloom SL, Segal AW.

Am J Gastroenterol. 2009 Jan;104(1):117-24. doi: 10.1038/ajg.2008.72.

PMID: 19098859

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 Feb 25;367(9511):668-78.

PMID: 16503465

Neutrophil dysfunction in Crohn's disease.

Segal AW, Loewi G.

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

PMID: 59239

These papers describe the discovery of the underlying cause of Crohn's disease as a deficiency of the innate immune system. In (1) we showed delayed accumulation of neutrophils in skin abrasions in Crohn's (2) and (3) describe very delayed neutrophil recruitment and pro-inflammatory cytokine secretion into skin windows, the bowel, and at sites into which heat killed *E.coli* were injected in Crohn's patients. The delayed neutrophil recruitment was associated with impaired clearance of 32P-labelled bacteria injected subcutaneously. Macrophages from Crohn's disease patients demonstrate abnormally low secretion of pro-inflammatory cytokines. Transcription and mRNA stability were normal, but the cytokines were misdirected to lysosomal degradation rather than secretion. Diminished transcription of various molecules associated with vesicle trafficking could be responsible (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):e0125906. doi: 10.1371/journal.pone.0125906.

PMID: 25885273

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

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

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. doi: 10.1098/rsob.160028. Review.

PMID: 27249799

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

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

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

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

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

Novel cytochrome b system in phagocytic vacuoles of human granulocytes.

Segal AW, Jones OT.

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

PMID: 723935

These papers describe the discovery of a novel electron transport chain in neutrophil leukocytes, defects of which cause the human condition of Chronic Granulomatous Disease (CGD). I then went on to model the structure and to characterise the NADPH, FAD, haem and carbohydrate binding sites that defined the characteristics of this family of NOX flavocytochromes. More than one hundred of these systems have been identified in the animal and plant worlds where they appear to generate the electron motive force driving pH changes and ion fluxes in addition to producing superoxide and hydrogen peroxide.

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

Are you a named author on any Wellcome Trust funded original peer-reviewed research papers, published from October 2009 onwards?	Yes

Are all your Wellcome Trust funded original peer-reviewed research papers, published from October 2009 onwards, compliant with our open access policy?	Yes

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.

Grants from other funding agencies

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, PI, 5 hours Medical Research Council

Crohn's Disease in Ashkenazi Jewish patients £540,000 01/01/2012-31/12/2017, PI, 2 hours Charles Wolfson Charitable Trust.

Grants from the Trust

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

Walker, AE Teschendorff 088683/Z/09/Z £995,000.00 April 2010 - March 2015. PI, 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, ME0373, £610,195, 36 months,PI, 10 hours 1/10/07-30/9/10.

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

Determination of the mechanisms involved in the aetiology of Crohn's disease. AW Segal. £330870.67. 60 months, PI, 22/11/2004-30/11/2008.

Biology and biochemistry of neutrophils: The mechanisms by which they kill microbes and contribute to inflammatory and degenerative disease with particular emphasis on the NADPH oxidase, its mechanism of action and regulation. (067287). AW Segal with J. Godovac-Zimmermann. £1,069,810 October 2002- September 2007, PI

I had continuous funding from the Wellcome Trust by way of a Senior Clinical Fellowship and then Programme grants from 1979 until 2002.

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

The current grants from the MRC and the Charles Wolfson Charitable Trust will allow us to continue this work until the end of 2017 at which time we would hope that funding from the current application to the Trust would be forthcoming.

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 Clinical Fellow 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 Royal College of Surgeons in Dublin

Daniel Marks
PhD supervisor
MB/PhD student

Wellcome Trust.Postdoctoral Fellowship for MB/PhD Graduates UCL

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.

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 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.
With OTG Jones I discovered a novel electron transport chain in neutrophil leukocytes, defects of which cause the human condition of Chronic Granulomatous Disease (CGD). I then went on to model the structure and to characterise the NADPH, FAD, haem and carbohydrate binding sites that defined the characteristics of this family of NOX flavocytochromes. More than one hundred NOXs have been identified in the animal and plant worlds where they generate the electron motive force driving pH changes and ion fluxes in addition to producing superoxide and hydrogen peroxide. I went on to identify five of the six additional components of this NOX2 system required for its regulation and activity.
It had been believed that the pH in the phagocytic vacuole of neutrophils was the same as that in macrophage vacuoles, at about pH 5.0. We showed that the oxidase alkalinises the vacuole and that this is important for bacterial killing. This elevation in pH to about 9.0 and an accompanying flux of potassium optimise the activity of the granule enzymes, such as elastase and cathepsin G, which kill ingested microbes.
Alkalinisation of the vacuole is also important for antigen presentation in dendritic cells.
I have made several other original observations including diagnostic cell labelling, liposomal targeting, lipoprotein labelling, and 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

Crohns disease
In 2008 we described the failure to recruit neutrophils to, and the delayed clearance of *E.coli*, from, the subcutaneous tissues in Crohns patients. We also demonstrated the impaired secretion of pro-inflammatory cytokines from macrophages of these patients. To identify the molecular basis of this

abnormality we conducted transcriptomic profiling on macrophages from 120 Crohns patients and 80 healthy controls. We identified a number of under-expressed molecules shared by subgroups of patients and took two forward for further investigation. A Disintegrin And Metalloproteinase Domain-Like Protein Decysin-1 (Adamdec 1) and Optineurin, a vesicle trafficking linker, developed 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 obtained 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 NOD2, CSF2RB and LRRK2 in the pathogenesis of the disease in these subjects.There are also several other strong causal candidates. The results of this study form the basis of the current application.
Ulcerative colitis
We have conducted a transcriptome analysis on the visibly normal mucosa from patients with ulcerative colitis. We discovered the under expression of BRINP3, which could be causal, in 35% of patients (PMID:25171508).

W e have have conducted an exome sequencing and mucosal gene expression project on Icelandic patients because of their restricted gene pool and excellent patient record system. We have identified several unique strong candidate genes.
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.

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 Werner Muller, Manchester Immunology Group

Werner is expert in the construction of mutant mouse models. He has made the *DUOX2* knock-in mouse and is half way to constructing the *CSF2RB* knock-in mouse. His research interests are in cellular cytokine networks, cell adhesion and Immunogenetics. He has considerable experience of mouse immunology and is a perfect partner to determine whether immunological changesdevelop in our mutant mice.

Professor Dario Alessi, MRC Protein Phosphorylation and Ubiquitylation Unit Dundee

Dario's laboratory focuses on determining the roles that protein phosphorylation and ubiquitylation pathway components play in biology by analysing molecules that emerge from the genetic analysis of human disease. They are currently focusing on dissecting signalling pathways associated with Parkinson's Disease and LRRK2 is of primary interest. As a consequence they have an array of assays and knock-out and knock-in mice that will be invaluable for our analysis of the LRRK2

variants associated with Crohn's disease. Our findings relating to binding partners and to signalling pathways will be of interest to his group. We have already started doing some joint experiments on neutrophil signal transduction.

Professor Mathias Chamaillard, Centre for Infection and Immunity, Institut Pasteur de Lille

They have two main avenues of research (i) biochemical, anatomical and immunological features of commensals which overcome or preserve (either individually or as a whole organ) epithelial barrier function, and (ii) specific cellular and molecular features through which NOD2 and NLRP6 shape a protective assembly of commensal lineages against intestinal inflammation and tumorigenesis.

They will characterise the microbiome in our patients and mutant mice. We will also collaborate on the signal transduction relating to NOD2 and NLRP2.

Dr Alexander Schmidt, Biozentrum Basel

Alexander is very experienced in proteomic analysis with particular reference to phosphorylation and biotinylation and will collaborate with us on those aspects of our research.

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?	Yes

Please describe how this application differs from the original
This application is similar to a previous application to build on the genetic study that we had performed on two very large Ashkenazi Jewish (AJ) families with Crohn's disease. In that application we proposed to validate strong candidate variants by reproducing the variants in mice, testing the animals in bowel inflammation models and then investigating their biology. In the interim: **We have published two papers on the genetics of our AJ CD in Gastroenterology**, the premier gastroenterology journal, as well as several other papers on candidate molecules we identified by other means. **We have produced the important mutant DUOX2 mice we proposed and the CSF2RB knock-in is underway.** The feedback I received after interview was largely strongly positive. However, it was stated "concerns were raised that the immunological context of the planned programme of work

was not reflective of the latest developments in the field. It also commented that the choice of infection models was poorly explained". **I have published a major review explaining how the work from my laboratory has been responsible for many of the latest developments in our understanding of the defective innate immunity that results in CD, and the inadequacies of many current animal models.**

7. Research summary

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

It is increasingly accepted that there is an underlying predisposition to Crohn's disease (CD) that involves innate immunity. We used a genetic approach to identify causal molecules. We concentrated on Ashkenazi Jews (AJs) because they have a fourfold incidence of CD, and may have large families.We identified two such families with ~800 and ~250 individuals with 54 and 26 cases of CD respectively. We have also studied large numbers of sporadic cases of CD in AJs. We have investigated genetic factors in these subjects, including exome sequencing of their DNA. We have identified several genetic variants that are likely to be causally related to CD. A frameshift mutation in *CSF2RB* has been validated, as have variants in *LRRK2,* and variants in *DUOX2 and NLRP2* are strong candidates.
Key goals are:
1. The further analysis of the large AJ CD families to identify asymptomatic carriers and phenocopies, the macrophage phenotype and their microbiome.
2. To validate candidate variants in *CSF2RB*, *DUOX2* and *LRRK2*, (+/- *NOD2*$^{-/-}$) in Knock-in and Knock-out mice, in the predisposition to bowel inflammation and immune function.
3. To study these molecules, and *NLRP2*, in cell lines to determine their binding partners and signalling networks.

Lay summary
Please provide a summary of your proposed research, including key goals, for a non-expert audience

The primary objective of this research is to identify the molecular lesions that cause Crohn's disease. We will then characterise their biochemistry and cell biology and determine how their disordered function produces the observed pathology.
The incidence of Crohn's disease in Ashkenazi Jews (AJ) is 3-4 times that in Caucasians and some subsets have very large families, both of which are very advantageous for the identification of causal genetic mutations. We have identified two very large AJ families, with ~200 and ~800 family members in which there is a very high incidence of Crohn's disease (10% and 5% respectively). We have also studied hundreds of AJ Crohn's disease subjects with, and without, a family history. We have sequenced their DNA and have found mutations that could be causing the Crohn's. We will determine whether these mutations predispose to bowel inflammation in mutant mice and to disordered innate immune function. We will determine how these molecules normally work and how they become defective in Crohn's disease. Understanding these mechanisms might help us to develop tests to aid diagnosis and to design novel approaches to the treatment of Crohn's disease.

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 before completing this section.

1. Research questions

The overall purpose of this investigation is to identify, and to characterise the products of, genes that predispose to Crohn's disease (CD) when mutated. We will:

a. Verify genetic variants identified as strong candidates to be causal in the aetiopathogenesis of CD in the Ashkenazi Jewish (AJ) population.

b. The determination of the physiological function of these abnormal AJ CD gene products and the mechanism(s) of their pathological effect(s)?

c. The investigation of the interaction of these AJ CD genetic variants with other CD associated proteins like NOD2.

2. Why are these research questions important?

CD is a chronic debilitating condition that has a seriously detrimental effect upon the physical, social, sexual and professional lives of patients[12]. The incidence in developed countries has shown a dramatic increase in recent years such that it now affects approximately one in five hundred of the population[3] and has a major societal impact. The annual cost to the NHS is approximately 1 billion pounds[4].

We have discovered that CD is an immunodeficiency disease in which the common pathogenesis is the failure to remove antigenic material penetrating the bowel wall. The accumulation of neutrophils, which normally kill and digest bacteria and remove organic material[5]is compromised because of the inadequate secretion of pro-inflammatory cytokines by macrophages[67].

CD has been the target for several large scale GWAS investigations. Although statistically highly significant, the effect size of the many associated genetic variants is small. Together these GWAS findings have been estimated to contribute only 11% of the heritability of CD[8]. The majority of these variants are in genes associated with inflammation and overlap significantly with other inflammatory diseases[9].

Large family studies can be particularly powerful in revealing the genetic cause of disease[10].Accordingly we exome sequenced large Ashkenazi Jewish (AJ) families[11] with CD and have identified two validated, and several putative, genes associated with the development of the disease. We have subsequently sequenced approximately 600 familial CD cases, and 500 sporadic cases, the latter of which have been undertaken as part of a collaboration with the Broad Institute In which about 2500 AJ CD cases and controls have been exome sequenced. The Broad study is a very useful resource of accurate AJ allele frequencies and variant affect sizes.

This investigation is aimed at understanding the biological function of the identified genes and defining their precise involvement in the evolution of CD. In addition to my clinical experience of CD, my laboratory has the important experience in animal models of bowel disease, and in biochemistry and cell biology,that will allow us to explore the functional consequences of the identified genetic aberrations.

3. How will this research advance your field?

A clear understanding of the causes of CD will help to promote a more lucid appreciation of the nature of the disease in the minds of scientists, patients, clinicians and pharmaceutical companies. The development of molecular techniques for diagnosis will be useful for disease management, antenuptial and pre-conceptual planning. Genetic defects may be amenable to targeted therapy in the future.

The generation of accurate animal models will be useful for the development of novel therapeutic agents.

In addition, advances in our understanding of factors regulating the innate immune response will have important implications for understanding the pathogenesis of other common infectious and inflammatory diseases such as tuberculosis, leprosy, sarcoidosis and inflammatory rheumatic conditions[12].

4. What approach will you take?

The proposed study will employ a multifaceted approach encompassing genetics, biochemical, cellular biological, animal and human studies to further our understanding of molecules involved in the pathogenesis of CD.

We initially chose to investigate AJs as they are enriched for mutations associated with rare Mendelian[13], and common complex diseases, and have an approximately 4-fold increased prevalence of CD[14].

We employed a family based approach because the study of multiply affected families has the ability to identify rare, more highly penetrant variants. We identified two very large families, one with >800 and > 200 members, with 54 and 26 cases of CD respectively[11].

Through the study of these families and sporadic AJ CD cases, and through our Broad collaboration, we identified a number of candidate genes for which there is encouraging genetic evidence of their involvement in the pathogenesis of CD. These are CSF2RB, DUOX2, LRRK2 and NLRP2.

1. **CSF2RB**

We identified a frameshift truncating mutation in CSF2RB that was significantly enriched in affected individuals in the larger family and was replicated in a study of sporadic AJ CD[15]. CSF2RB is the common or shared β subunit of the receptors for GM-CSF, IL-3, and IL-5[16], and plays a pivotal role in the inflammatory and immune responses.

2. **DUOX2**

In the smaller family we identified a damaging missense mutation in DUOX2 (P303R) that impaired the function of the protein and showed a possible epistatic interaction with **NOD2**.

DUOX2 generates H_2O_2 at the mucosal surface which acts as substrate for the generation of microbicidal hypothiocyanite[17]. It might also attract neutrophils to inflammatory sites[18]. The expression of DUOX2 is induced in the bowel epithelium by the microbiota[19]. Knockdown of the DUOX2 homologue in invertebrates and mice resulted in an impaired tolerance to enteric bacteria[20]. A physical and functional interaction between DUOX2 and NOD2 has been demonstrated in epithelial and HEK23 cells[21]. In collaboration with Phil Crosier (New Zealand) we showed in zebrafish larvae that knocking down DUOX and NOD2 increased their susceptibility to *Salmonella* infection and the dual knock-down was even more susceptible. Whereas the wild-type and single knock-downs survived, the double knock-downs were lethal (Figure 1e in additional information).

3**. NLRP2**

We have found a damaging mutation in NLRP2 (E522G) in our sporadic cases of CD with a p value of $5x10^{-7}$. The same mutation had an odds ratio of 46 and p value of $<10^{-5}$ in the Inflammatory Bowel Disease Exomes Browser (https://ibd.broadinstitute.org/).

NLRP2 is a member of the NALP family of cytoplasmic proteins that form the inflammasome which activates pro-inflammatory caspases in response to activation of Toll-like receptors (TLRs) during the cell's response to microbial infection[22]. Unlike other NLRPs, very little is known about NLRP2.

4. **LRRK2**

The Broad consortium study identified statistically significant variants in LRRK2 in the patients (Rivas MA http://biorxiv.org/content/early/2016/09/25/077180).

LRRK2 is found in immune cells such as in lamina propria macrophages, B-lymphocytes, dendritic cells, and neutrophils, and levels are markedly increased in the bowel in CD[23]. It is involved in vesicle trafficking and autophagy[2425] and could be important for the elimination of intracellular *Salmonella*[26] and *Legionella*[27]. LRRK2 enhances Nod1/2-mediated inflammatory cytokine production by promoting Rip2 phosphorylation[28]. Mutations in LRRK2 cause familial and sporadic Parkinson´s disease (PD) and a clear association exists between CD and PD[2930]. LRRK2 $^{-/-}$ mice are more susceptible to DSS colitis[31].

Research Plans

Further investigation of the large families

To strengthen the genotype/phenotype correlation, we will first attempt to identify endophenotypes and phenocopies in the two families, the inclusion or exclusion of which would strengthen the genetic analysis. Faecal calprotectin and bowel permeability[33] are elevated in bowel inflammation and will be measured in as many affected and unaffected family members as possible.

In CD, cytokine secretion by monocyte derived macrophages in response to heat killed *E. coli* and a panel of TLR ligands[7] is generally impaired, as are the emigration of neutrophils into skin windows[6] and the clearance of bacteria from the subcutaneous tissues[7]. These parameters have not been examined in members of these families. Where possible we will perform these investigations on these subjects[67], to determine the *in vivo* inflammatory response. In addition to providing clues as to the pathogenesis of the disease in these families, if these investigations are found to be abnormal in affected individuals, they could be very helpful in identifying endophenotypes, which may permit the identification of further high penetrance genetic variants.

We will measure H_2O_2 generation[34] by mucosa obtained from rectal biopsies from affected and unaffected family members with and without the DUOX2 mutation. Western blotting and immunohistochemistry will be used to determine tissue levels and cellular and subcellular distribution of the DUOX2 protein (good antibodies are available). DUOX2 is thought to sterilise the overlying mucus layer and we will determine whether the DUOX2 mutant individuals have an increased number of organisms in the mucus and/or alteration in their gut microflora (dysbiosis) [3536].

We will attempt to generate intestinal mucosa organoids[37] from intestinal biopsies from these patients, which will simplify investigations of DUOX2 function.

Several studies have described an association between CD-associated genetic variants and dysbiosis of the gastrointestinal tract in CD[3839]. We will characterise the microbiota in affected and unaffected individuals in both families. It could explain the difference in the prevalence in the subfamilies, and show whether or not the mutation in DUOX2 has an influence on the microbiota. Bacterial DNA samples will be isolated and the microbiota determined using 16S ribosomal metagenomic sequencing[40] (in collaboration with Professor Mathias Chamaillard, University of Lille).

Determination of the effects of observed variants on mouse susceptibility to gastrointestinal inflammation and the examination of their biological effects in cultured cells.

Mutant mice

We have the mutant mice we require for these investigations. The natural mutations in CSF2RB and in DUOX2 in families A and B have been knocked in to mice by our collaborator, Professor Werner Muller, University of Manchester. The CSF2RB gene is duplicated in the mouse, making genotyping difficult and raising the possibility of redundancy or compensatory effects. We have knocked out the duplicated pseudogene and knocked in the truncating mutation associated with AJ CD in to the homologous region

in the mouse (Figure 2 in additional information) . LoxP sites have been inserted into this construct so that a complete KO can also be generated. These mice are viable and fertile. Professor Dario Alessi (MRC PPU unit, Dundee) has mutant mice with activating and inactivating LRRK2 mutations and Professor Chamaillard has the NLRP2 $^{-/-}$ mouse. Studies will also be performed in animals on a NOD2 $^{-/-}$ [41]background (JAX laboratories).

i. Susceptibility to gastrointestinal inflammation and assays of DUOX2 activity

We have experience of several different mouse models to investigate gastrointestinal inflammation. Colonic inflammation will be induced through the exposure of mice to 2% DSS for seven days, oral gavage of *Citrobacter rodentium* or *Salmonella typhimurium*[42,43]. Bowel inflammation and systemic alterations will be determined through the use of multi-parameter FACS analysis of the leukocyte population within the gastrointestinal tract, changes in tissue and serum cytokines, histological scoring of the tissue, bacterial translocation to the spleen and lymph nodes, and changes in body weight, bowel length, stool consistency and mortality.

In the DUOX2 mutant mice we will directly measure rates and extent of H_2O_2 generation[34] at different regions of the bowel, both by explants, by inverted sacs, and by perfused, vascularised, intestinal loops *in situ*[44]. We will be able to determine how these change with bowel inflammation and what effects DUOX2 has in the attraction of neutrophils[18].
We will characterise the microbiota in the mucus layer[35],[36] and faeces[45], of the mutant and wild type littermate controls.

ii. Immunological studies

In all our mouse models, the composition of the immune system will be analysed by flow cytometry and histology. We will determine the differential cellular composition of the bone marrow, blood and primary and secondary lymphoid organs. Immunological studies into the adaptive immune system in these mice will be conducted by Professor Werner Muller. Functional immunological investigations will be conducted if and when indicated.

Cell based systems

Cell lines derived from our mutant mice

We will initially investigate the effects of mutating or deleting CSF2RB, DUOX2, NLRP2 and LRRK2 in the whole animals and in cells derived from them. Subsequently we will work on cultured cells to reduce the numbers of mice experimented upon and to facilitate molecular manipulation.

We will generate immortalised monocyte derived macrophage cell lines from the mutant mice, and wild type controls, using the methodology developed by Wang et al[46]. In parallel work, we have already obtained C57Bl/6 Hoxb8-SCF progenitor cell lines and have differentiated them into neutrophils with G-CSF for 4 days and have shown them to phagocytose bacteria, generate superoxide and generate the rise in vacuolar pH seen in bone marrow neutrophils. We have successfully knocked out a gene important in neutrophil function with the predicted effect. These progenitor cells can also be differentiated into macrophages with G-CSF plus GM-CSF for 10 days. The macrophages are fully responsive to TLR stimulation, secrete cytokines, phagocytose bacteria and are capable of processing and presenting antigen. In contrast to bone marrow derived neutrophils and macrophages the immortalized cell line can be easily genetically manipulated and grown in high numbers, providing an ideal *in vitro* cell assay system to study cellular functions.

We will also attempt to measure DUOX2 biology and function in intestinal mucosal organoids[37] generated from the mutant mice.

Transfection of THP-1 cells

We will use a lentivirus-based CRISPR/Cas9 system[47] to create stable mutations in macrophage like cell lines. The THP-1 human leukaemia monocytic cell line, has been extensively used to study monocyte/macrophage function[48]. We have already made NOD2 mutations in THP-1 cells, a global knock-out, a frameshift (3020insC) associated with CD, two activating mutations (R334W, R334Q) that produce Blau syndrome[49] and we are currently knocking-out RIPK2.
It is highly plausible that CD associated molecules do not work in isolation and the combination of numerous proteins cooperate and interact resulting in increased susceptibility to disease. Double and triple knockdowns will be constructed where indicated.

Cytokine expression and secretion

The cell lines derived from our mutant mice and the transfected THP-1 cells will be stimulated with MDP, LPS and other TLR ligands, and live and heat-killed *E.coli*, and the induction of pro-inflammatory genes quantified by qPCR. Secreted cytokines will be measured using Meso Scale Discovery multiplex cytokine assays.
Intracellular location and trafficking of cytokines will be visualized and characterised through the use of GFP-cytokine constructs and confocal microscopy. Intracellular trafficking compartments will be identified using antibodies against well-recognized markers (eg EEA1, GM130).

Signalling pathways

Cell signalling molecules are generally activated and induced to interact by modifications, the most common being **phosphorylation and ubiquitination**, which can be detected by mass spectrometry. Initial studies will be performed to determine the phosphorylation of macrophage proteins after stimulation with the ligands described above. Initially we will determine time courses and overall patterns with Western blots using anti-phosphotyrosine, threonine and serine antibodies. Once the parameters have been defined our primary experimental technique will be quantitative mass spectrophotometry (in collaboration with Dr Alexander Schmidt, Biozentrum Basel). We will use SILAC[50] to quantitatively differentiate the compared cell lines and will attempt to measure the proteome-wide dynamics of phosphorylation[51] and ubiquitination.
We will investigate the mutant **NLRP2** on the **activation and regulation of the inflammasome** which will be measured through the cleavage of Caspase 1 and the release of IL-1[52].The formation of the inflammasome complex will be visualize and monitored using a GFP tagged adapter protein apoptosis associated speck-like protein containing a caspase activation domain adapter termed ASC. ASC helps the formation of a multi-protein complex which contain all the inflammasome components and precedes IL-1processing[53].The ASC-specks can be observed as they reach a size of around 1 μm and can be used as an indicator of inflammasome activation[54].
LRRK2. Naturally occurring *LRRK2* mutations in the CD patients will be tested for functional effects in collaboration with Professor Dario Alessi by co-transfection of HEK293 cells with *LRRK2* and *RAB10*. LRRK2 activity causes the phosphorylation of RAB10 and of Ser935[55] of LRRK2 that can be identified by Western blotting. Functional variants will then be transfected into THP-1 cells and tested as described above.

Protein/protein interactions

The majority of the molecules which we have identified are poorly characterised with respect to their role in the immune response, protein complex composition, binding partners and regulatory domains. Another way in which binding partners can be identified is by a proximity-dependent biotin identification (BioID) technique, which allows the identification of proteins in the close vicinity of a protein of interest in living cells[56]. Biotinylation of proteins in close proximity to NOD2 will be identified by transfecting the NOD2 deficient cells with biotinolase A (BirA*) fused to wild-type, constitutively active, and inactive NOD2. Other molecules of interest, such as NRLP2 will be treated in a similar way. The stably transfected cell lines will be stimulated with MDP, LPS or live or heat-killed whole bacteria, and biotinylated proteins identified by mass spectrometry.
As an additional means of identifying binding partners we will also perform immunoprecipitations of our target proteins in cells in the resting and activated states. They will be tagged with the HaLo- tag[57] which is fluorescent, allowing subcellular localisation by confocal microscopy, and binds specific antibody.

The molecules in the signalling pathways and vesicle transport systems that we identify will be compared with the GWAS hits, variants in the Broad study (Rivas MA http://biorxiv.org/content/early/2016/09/25/077180) such as HEATR3 and TRIM 26 and 31, and other

Crohn's and colitis databases. This will allow us to develop an understanding of the normal physiological processes and mutations that compromise their function.

Investigation of potential future diagnosis and treatment

The identification of proteins involved in the macrophage activation pathways will, in all likelihood, identify molecules, mutations of which predispose to CD. As a greater number of these predisposing, or causal, variants are identified, it will become feasible to develop diagnostic microarrays that will be helpful in identifying individuals at risk of CD that may be useful in prenuptial, preconception planning and in disease diagnosis.

Bone marrow transplantation can cure CD disease[58] but suitable donors are rare and the procedure carries a high mortality. The demonstration that in the majority of cases the macrophage is defective in its response to bacterial stimuli indicates that those cases should be amenable to gene therapy because macrophages in the bowel are regenerated from blood monocytes[59] whose progenitor cells are present in bone marrow. It should be feasible to aspirate marrow, transfect the progenitor cells with the correct gene, or edit out the mutation with CRISPR technology, and re-inject the cells into the conditioned patient[60]. We will be in a position to test this in our mice.

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).

Structural modelling, and *in vitro* and *in vivo* studies of *DUOX2* function. (a) The position of P303R in the extracellular peroxidase domain, visualised using comparative modelling, shown relative to the membrane as hypothesised; all other domains have been represented using homologous crystal structures. (b) HEK293 cells transfected with varying doses of the mutant P303R *DUOX2* construct produce less hydrogen peroxide as compared with the wild type construct. (c) The surface expression of the mutant protein (upper panel) is reduced while the total protein level remains constant (lower panel). (d) Intestinal colonisation one-day post infection with *Salmonella enteric*a in control, single *DUOX2* and *NOD2* knockdowns and dual knockdown zebrafish and (e) survival two-days post infection.

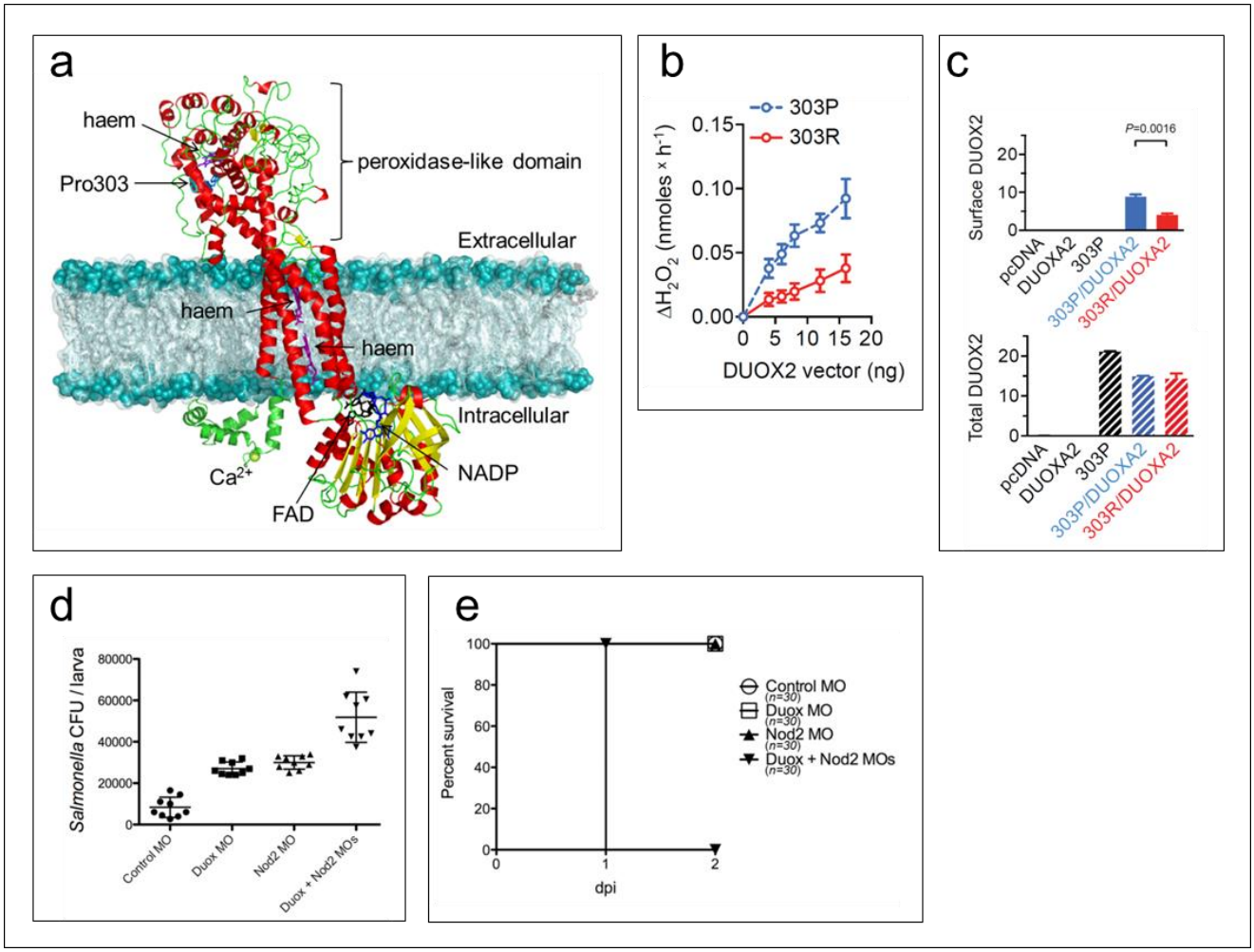
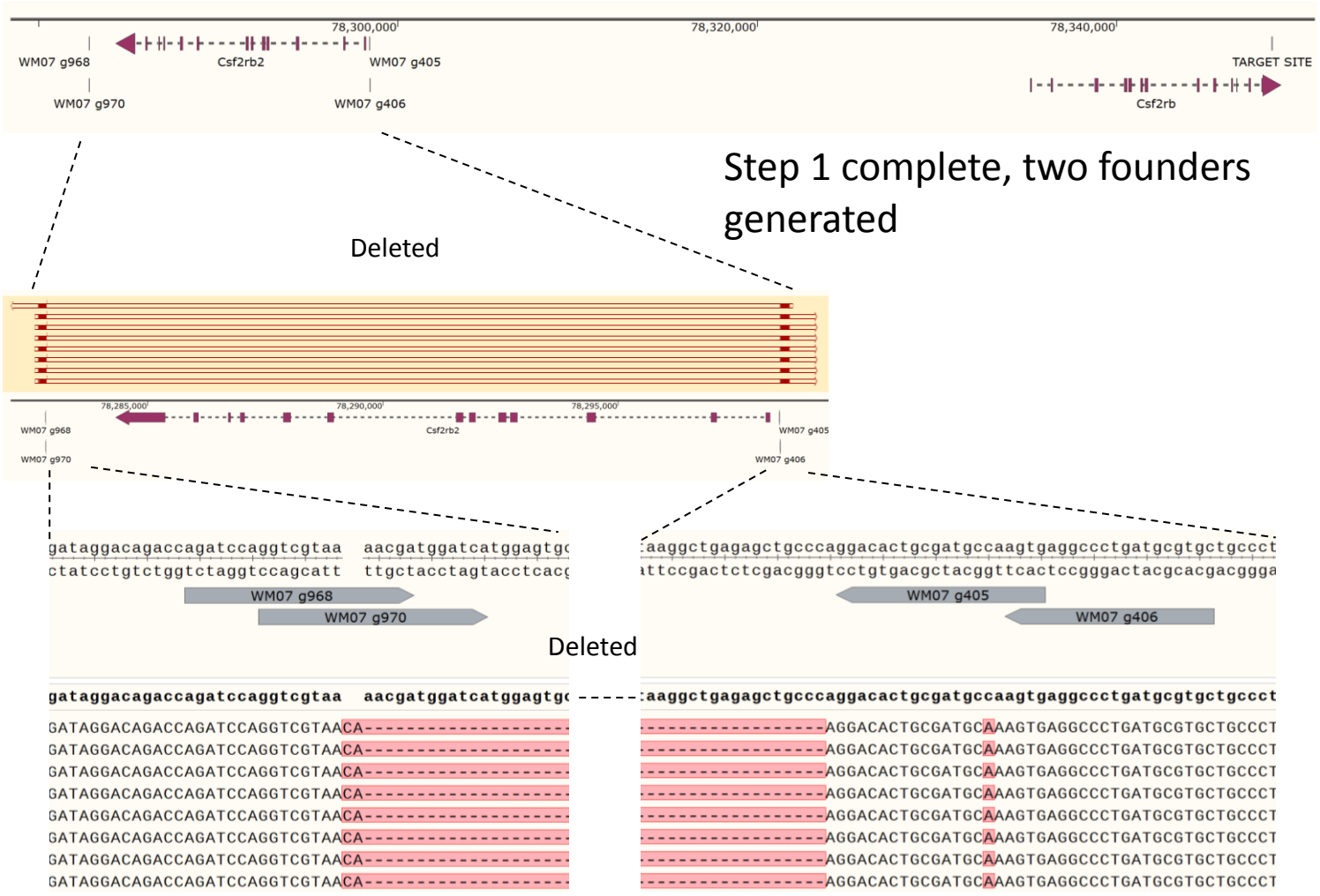


Figure 2. Scheme for the Knock-In of the *CSF2RB* mutation involving the deletion of a single guanine base, which is predicted to cause a frameshift at codon 708 of *CSF2RB*. This would result in the expression of a 729–amino acid sequence, compared with 897 residues encoded by the canonical transcript. The construction of this mutant mouse was complicated by the presence of a copy of the *CSD2RB* gene, the *CSF2RB2 gene,* which we have deleted as depicted below.



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

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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.

Future plans for my laboratory:

I run one of the most innovative and effective laboratories investigating inflammatory bowel disease. The current application is required to permit the continuity of this work until I can hand the laboratory over to my successor. Adam Levine did most of the work on these Ashkenazi Jewish families with me and is an exceptionally talented academic who plans to enter into a full time research career after completing his basic medical training. He is currently doing his Foundation yrears after which he will do his three years of Core Medical Training in an academic post, during which time he will return to do some research in the laboratory. He should then be ready to take over the direction of the laboratory.

I also accommodate Andrew Smith and Daniels Marks in my laboratory. Andrew is a Senior Lecturer at the Eastman Dental Hospital, part of UCL, whose primary interest is in mucosal immunity and inflammatory bowel disease. Daniel is a clinician of consultant status who is currently working on the aetiology of ulcerative colitis, supported by the Trust on a Postdoctoral Fellowship for MB/PhD Graduates.

These three groups form a complimentary critical mass that is making good progress in understanging the aetiology and pathogenesis of inflammatory bowel disease.

UCL is currently in talks to recruit an academic gastroenterologist to bridge the gap between basic laboratory science and clinical medicine.

We require the support afforded by this Investigator Award to maintain this critical mass and to advance the research that we have developed to a stage at which we have established some of the genetic basis of CD. The laboratory can then be handed on to a successor, or successors, to take to the next stage for the development of diagnostic and therapeutic strategies for CD and the further investigation of the aetiology and pathogenesis of ulcerative colitis.

With regard to my previous **translational activities**:

I started the use of elemental diets for the primary treatment of CD. It is now firstline treatment for children with the condition.

I discovered the molecular cause of Chronic Granulomatous Disease. It is now being treated by gene therapy at a Gene Therapy Institute at the Institute for Child Health, run by my previous PhD student, Adrian Thrasher, that I started together with Roland Levinsky.

I did the first study in man using III-Indium labelled neutrophils for the identification of foci of infection, and bowel inflammation.

11. Data management and data sharing

Will the proposed research generate data or software outputs that hold significant value as a resource for the wider research community?	No

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	3.00	2.00	2.00	1.00	1.00	1.00			
Postdoctoral research assistants	3.00	3.00	2.00	2.00	2.00	2.00	2.00	2.00		
Technical support staff	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00		
Other (please specify below)										
Total	7.00	7.00	5.00	5.00	4.00	4.00	4.00	3.00	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

13. Public engagement

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

Do you wish to apply for a provision of funds for public engagement as part of	No

your research proposal? Please refer to guidance for further information.	

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 IBD 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 IBD and the study of mucosal 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. Our work on Crohn's disease, has had over 250,000 hits on Google searches and we received regular requests for information and advice from IBD patients and family members. We have recently setup a Twitter account (@Mol_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 the funds awarded be allocated 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 (£)
PDRA	Un-named	34956	Research 7	60	100	263,340
PDRA	Un-named	34956	Research 7	60	100	263,340
Technician	Un-named	34956	Support 7	60	100	263,340

Materials and consumables Are you requesting materials and consumables?	Yes

Materials and consumables

Description	Total (£)
Antibodies	30,000
Plasticware	30,000
Tissue culture media	30,000
Cytokines and growth factors	20,000
General chemicals	20,000
Molecular biology reagents	15,000
Plasmids	1,000
Reagents and chemicals for mass spectrometry	30,000
Protein expression and purification columns, resins, reagents	20,000
Cytokine Eiisa kits	15,000

Animals Are you requesting animals?	Yes

Animals

Animal species	Total no. to be purchased	Total purchase cost	Total no. to be maintained	Average weeks' maintenance per animal	Total maintenance and procedures cost	Total (£)
Mouse CSF2RB truncating mutation	0	0	445	360	16704	16,704
Mouse LRRK2 -/-	0	0	445	36	16704	16,704
Mouse NOD2 -/-	8	2500	715	360	26388	28,888
Mouse DUOX2 KI	0	0	585	360	21096	21,096
Mouse	0	0	602	360	23040	23,040

Animal species	Total no. to be purchased	Total purchase cost	Total no. to be maintained	Average weeks' maintenance per animal	Total maintenance and procedures cost	Total (£)
CSF2RB/NOD2						
Mouse LRRK2/NOD2	0	0	602	360	23040	23,040
Mouse DUOX2/NOD2	0	0	602	360	23040	23,040

Justification for animals (number and species) requested
Please include evidence or calculations for experimental group sizes, and describe any plans to reduce bias (e.g. blinding, randomisation).

Our preliminary studies have provided us with the knowledge and experience to plan and conduct the procedures proposed in this application. Where possible all of the numbers have been generated from power calculation obtained from preliminary data.On average 750 mice are required to construct a congenic strain (numbers taken from 'Guidelines from the Institutional Animal Care and Use Committee Guidebook (2002)'). The number of experimental mice needed to complete our study has been calculated on average to be about 445-715 per strain - calculated from previous work in our laboratory.

Power calculations

These are based on our previous experience with thse models when we tested DSS and Citrobacter induced colitis in Adamdec and OPTN KO mice.

DSS [WT mean weight 97.7%, ADAMDEC1-/- mean weight 89%, SD 6.9 with Power 0.8 and $p<0.05$] = 10 mice per group.

Citrobacter [WT mean weight 100.3%, OPTN-/- mean weight 95.7%, SD 4.2 with Power 0.8 and $p<0.05$] = 14 mice per group.

If a significant result is achieved in the initial experiment then a further two experiments will be performed, allowing us to validate the findings and collect tissue and serum samples for further analysis. The *in vitro* study of colonic tissue, leukocytes, bone marrow derived macrophages and lamina propria cells will require additional mice. We have used our previous experience to calculate that on average 160 mice per strain (80 knockout / 80 WT controls) will be required for these studies. Our inflammatory models are highly dependent on equally age and sexed matched groups therefore we have increased the numbers of mice from the power calculations by 20% to account for mismatched litters. This number has been calculated from our previous experience in this area.

Equipment Are you requesting equipment or equipment maintenance?	Yes

Equipment

Type of equipment	No. of items	Cost per item	Cost of maintenance contract	Contribution from other sources	Total (£)

Type of equipment	No. of items	Cost per item	Cost of maintenance contract	Contribution from other sources	Total (£)
Upright -80'freezer Jencons-VWR	1	13610	0	0	13,610
Class II safety cabinet	1	7880	750	0	8,630
PCR Thermal cycler VWR	1	6514	0	0	6,514

Are you requesting a piece of equipment with a list price of £100,000 or more?	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 (£)
DNA sequencing	UCL		195	96 sequences	75	14,625
Tissue culture	UCL		5	hour	2400	12,000
qPCR	UCL		12	reaction	600	7,200
Confocal microscopy	UCL		25	hour	300	7,500
FACS analysis/ cell sorting	Arthritis and Rheumatism Council and UCL		83	hour	120	9,960
Proteomics Core Facility Biozentrum	University of Basel		40	per sample	400	16,000

Travel and subsistence Are you requesting travel and subsistence?	Yes

Travel and subsistence

Description	Total (£)
2 trips to Europe and one to the USA for each of the two postdocs and one of each for myself over the five years of the grant	13,000

Miscellaneous costs Are you requesting miscellaneous costs?	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.
Our -80'C freezer, Class II laminar flow cabinet and PCR machines are all very old and will not survive the duration of the grant. The access charges are for services that we currently use and the predicted costings have been extrapolated from current actual costs. The proteomics costs requested are for to cover the costs of the consumables, machine time and data analysis. It is clearly important for scientists, an in particular those in the early stages of their careers, to travel to meetings and integrate with their peers in the scientific community. I have requested two trips to Europe and one to the USA for each of the postdocs over the five years of the grant. I have requested one such trip to each continent for myself.

Summary of financial support requested	
	Total (£)
Salaries / Stipends	790,020
Materials and consumables	211,000
Animals	152,512
Associated animals costs	0
Equipment	28,754
Maintenance for existing equipment	0
Access charges	67,285
Travel and subsistence	13,000
Miscellaneous other	0
Total	**1,262,571**

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 (£)?	2420161

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.
Full Title of Study: The collection of blood, biopsies, saliva, intestinal fluid and surgical resection tissue from patients with inflammatory bowel disease and other inflammatory conditions to assess the functional effects of abnormal gene and protein expression. **Brief Title of Study:** Abnormal genes and proteins in inflammatory bowel disease **Health Research Authority,** London Surrey Borders Research Ethics Committee (REC) reference: **10/H0806/115** Study start date: 17 March 2011 End date: 30 November 2017 in the process of being extended till June 2018 In addition we have ethical approval from the UCL Research Ethics Committee for international recruitment. Study Title: Abnormal genes and protein in inflammatory bowel disease UCL Ethics Project ID Number: **6054/001**

In the course of your project, do you propose to use facilities within the National	Yes

Health Service (NHS) or to involve patients being cared for by the NHS?	

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.
Fully informed concent has been and will be obtained for all studies on patients

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)*
Proposal involves the use of animals

Do your proposals include procedures to be carried out on animals in the UK which require a Home Office licence? If yes, refer to notes.	Yes

Do your proposals involve the use of animals or animal tissue outside the UK? If yes, refer to notes.	No

If your proposals do involve the use of animals, what would be the severity of the procedures?
Moderate

Please provide details of any procedures of substantial or moderate severity
Live *Citrobacter rodentium* will be administered by gavage to mice. This results in the development of mild bowel inflammation in wild type mice over a two week period, but in genetically modified animals the same bacteria might induce a more aggressive inflammatory response which raises the severity limit to moderate. We also treat the animals with dextran sodium sulphate that induces bowel inflammation of moderate severity. All animals undergoing these procedures are monitored daily and strict Home Office license rules are enforced to maintain humane treatment.

Why is animal use necessary: are there any other possible approaches?
We are studying factors that affect the development of inflammation in the whole organism. This involves assessments of mucosal integrity, the secretion of pro-inflammatory mediators and the subsequent vascular response and recruitment of effector cells. It is impossible to reproduce such complex interrelated biological processes outside an intact

animal. We will use cultured cells and cell lines where ever appropriate and possible.

Indicate which of the following species will be used *(Primate, Cat, Dog, Equidae, Genetically Altered Animals, Other animals)*
Genetically Altered Animals

Why is the species to be used the most appropriate?
Our experimental animals will be mice. These are appropriate because the genes of interest are homologous to man in the regions of the variants of interest and because established models of bowel inflammation exist in these animals and we have extensive experience in their use.

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 issues are applicable to this application.

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 • a Major Overseas Programme • the Francis Crick Institute?	No