Abstract
Atherosclerosis results from the accumulation of cholesterol and other lipids in the intima, initially within macrophages that form foam cells, and subsequently free within the intestitium, leading to inflammation and scarring. While clinical factors such as hyperlipidaemia, smoking and diabetes commonly drive this process, we hypothesise that some patients, especially those without overt risk factors, may be predisposed to atherosclerosis because their macrophages have a reduced ability to digest lipids phagocytosed from lipoproteins and efferocytotic cells. Digestion within mononuclear phagocytes takes place within the phagocytic vacuole and depends upon the enzymes released into it from the cytoplasmic granules, or lysosomes, and the suitability of the conditions within this compartment for their activity. Monocytes and macrophages have more recently been separated into Classical, non-Classical and Intermediate monocytes, M1 and M2 macrophages and at least 3 subtypes of Dendritic cells based upon their surface markers.

In 20 patients with early atherosclerosis in the absence of traditional risk factors and 20 matched controls without atherosclerosis, we will:

- Separate the monocyte and macrophage subtypes and compare the profiles in cases and controls;
- Measure the pH within their phagocytic vacuoles using SNARF labelled *Candida*, a method we developed;
- Quantify efferocytosis and the digestion of radiolabelled efferocytosed cells;
- Purify the cytoplasmic granules and analyse their contents by proteomics;

Taken together these parameters will indicate whether or not aberrant mononuclear cell function may be responsible for the accumulation of atherosclerotic plaque in these patients, and as such represent a novel mechanism for predisposition to atherosclerosis.

Background to the project and pilot data
Atherogenesis occurs when monocytes are recruited to the intimal layer of the medium and large size arteries where they differentiate into macrophages and encounter a plethora of modified lipid species such as oxidized low-density lipoprotein. Macrophages employ mainly scavenger receptors to facilitate the uptake of these modified lipoproteins, leading to the formation of foam cells[1]. These lipid-rich cells secrete multiple pro-inflammatory mediators[2] that propagate the development of the necrotic core of an atherosclerotic plaque, increasing its vulnerability. Early atherogenesis involves the development of fatty streaks whereby macrophages internalise retained ApoB-containing lipoproteins, which become degraded in lysosomes, with excess free cholesterol (FC) trafficked to the endoplasmic reticulum (ER). LDL particles ingested via the LDL receptor are degraded and cholesteryl esters (CE) are hydrolysed in lysosomes to FC and fatty acids. FC is trafficked to peripheral cellular sites by a mechanism involving the proteins Niemann Pick C 1 and 2 (NPC1 & NPC2)[3]. In the ER, FC is esterified by acyl CoA:cholesterol acyltransferase (ACAT), and the resulting CE is packaged into cytoplasmic lipid droplets, which are a hallmark of foam cells[4].
Two major pathways facilitate cytoplasmic CE clearance. The first involves the hydrolysis of cytoplasmic CE by cholesterol ester hydrolase (NCEH) and the resulting free cholesterol is mobilized away from the ACAT pool[4] and made available for efflux via ATP-binding cassette transporter A1 (ABCA1), scavenger receptor class B type I (SR-BI), and aqueous diffusion. Alternatively, cytoplasmic CEs in lipid droplets are packaged into autophagosomes, which are trafficked to lysosomes, where the CE is hydrolysed by lysosomal acid lipase (LAL), generating FC for ABCA1-dependent efflux[3]. At this stage, lysosomal hydrolysis and sterol clearance is effective. However, the progression of fatty streak lesions to unstable plaques is characterized by the substantial accumulation of CEs and FC in lysosomes[5–7], indicating a failure to adequately hydrolyse and clear them. This confirms lysosome dysfunction is a key event in late-stage atherosclerotic disease. Overloading macrophages with lipids results in lysosomal leakage, leading

to activation of NLRP3 inflammasomes and the secretion of IL-1β, which has a fundamental role in establishing and driving the pathogenesis of atherosclerosis. It stimulates monocytes, macrophages, endothelial and smooth muscle cells to secrete pro-inflammatory cytokines and chemokines[8].
Another way in which material can accumulate in atheromatous plaques is through the ineffective clearance of apoptotic cells by efferocytosis. Macrophage apoptosis occurs during all stages of atherosclerosis and influences early lesion formation, plaque progression, and plaque stability[9]. Increased macrophage apoptosis reduces atheromatous lesion progression[10] whereas ineffective apoptosis has the opposite effect[11].
Apoptotic cells display "eat-me" signals on their surface that engage with a variety of receptors on the macrophage surface that leads to their engulfment in a process known as efferocytosis[12].
Once apoptotic cells have been internalized, certain autophagy-related proteins are recruited to conjugate LC3-family proteins to lipids at the phagosome membrane, a process called LC3-associated phagocytosis (LAP)[13]. LAP promotes phagosome fusion to lysosomes to drive hydrolytic degradation of apoptotic cell constituents. After apoptotic cells are degraded in phagolysosomes, macrophages have evolved elegant mechanisms to either use or efflux this cargo. For instance, cholesterol efflux is promoted from the cells[14].
Considerable recent progress has been made in classifying the lineages and subpopulations of mononuclear phagocytes 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 specialised compartment shared by the mononuclear phagocytes where processes like lipoprotein digestion and efferocytosis take place. The realisation that there are several different subtypes of these mononuclear phagocytes with considerably different functions[15,16] establishes the importance of developing our understanding of phagosome biology. 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[17–20] where the investigations largely involved electron microscopy, without much functional data[20–22].
The importance of understanding the enzymology of the phagocytic vacuole is exemplified by lysosomal acid lipase deficiency, which is a rare, autosomal recessive condition caused by mutations in the gene encoding this protein resulting in reduced or absent activity of this essential enzyme. The severity of the resulting disease depends on the nature of the underlying mutation and magnitude of its effect on enzymatic function and can vary from fatal Wolman's to cholesteryl ester storage disease[23]. The latter patients develop premature atherosclerosis and about 20% of them have normal lipid profiles. These conditions were first identified because of the gross general effects that they produce. It is possible that derangements of other enzymes, with more circumscribed activities, could lead to the accumulation of lipids in macrophages, or in the arterial wall, resulting in premature vascular disease.
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 atherosclerosis.

The measurement of vacuolar pH in blood mononuclear cells

AWS' recent work generating spatiotemporal measurements of the pH within the vacuoles of monocytes, macrophages and dendritic cells has provided surprising and important results that indicate that pH has a critical impact on the enzymology in these cells. The pH in the phagocytic vacuole of neutrophils was considered to be very acidic[24] until he demonstrated that it was in fact alkaline as a consequence of the activity of the NADPH oxidase, NOX2[25]. Subsequently he 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[26].

He 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. He found that as with neutrophils[26], 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[27]. 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). Where 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.
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[25,26]

Original hypothesis

Atherosclerotic lesions start off as fatty streaks containing macrophages stuffed with lipids to form foam cells. These apoptose and are taken up and digested by other macrophages in the process of efferocytosis. Eventually free lipids and cholesterol crystals accumulate in the extracellular interstitium of arterial walls where they induce an inflammatory response, and fibrotic reaction. The premature accumulation of lipids in fatty streaks and evolving lesions can be explained in hyperlipidaemic states. However, it is not uncommon for patients with normal or low plasma lipids, and without other important risk factors, to present with premature atherosclerosis. We propose that in this situation there may be cases in which there is a failure of the capacity of their macrophages to engulf and digest, or metabolise, exogenous lipids or apoptotic cells. The technology is now available to test this hypothesis. We can purify subsets of blood mononuclear cells and derived macrophages, purify their granules and analyse their enzyme cargoes, measure the pH in the phagocytic vacuoles and determine the uptake and digestion of apoptotic cells. These studies might provide novel insights into the atherogenic process and provide an explanation as to the cause in some patients developing premature atherosclerosis without common risk factors.

Experimental details and design of proposed investigation

1. Patient population

20 patients with early atherosclerosis, defined as <50 years of age for men and <55 for females will be identified, from among those presenting for a CT coronary angiogram, for clinical indications. Atherosclerosis will be defined as having a calcium score >90th centile for the relevant age/gender group, according to standard population normograms[28] or with diffuse, multi-vessel coronary plaques of at least 50% stenosis. Patients will be clinically stable and not yet commenced on any medical therapy.
To be eligible for the study, patients will be treatment naïve, and have (1) normal or near normal pre-treatment LDL levels of <3mmol/L (2) non-smokers; (3) non diabetics; (4) BMI will be <30kg/m2. Patients who are pregnant, <18years of age or with any inflammatory conditions or on steroids or other immunosuppressive agents will be excluded.
Control subjects will be recruited from those undergoing CT coronary angiography, confirmed as not having any atheroma (plaque free and calcium score 0) and matched for age, gender and ethnicity.
Dr Patel, consultant cardiologist, will oversee the study at the Bart's Heart Centre, within the imaging department, conducting over 5000 coronary CT angiography scans per year with a state of the art dual source 256 slice CT scanner. A research associate will help identify and recruit patients into the NIHR Bart's Bioresource project, through which samples and clinical data will be prospectively obtained for study.

2. Purification of cell populations to be studied and profiling of monocyte subsets

Cells will be purified from blood derived from these patients and from consenting healthy volunteers. Mononuclear cells (PBMCs) will be separated from whole blood by centrifugation on Ficoll-Hypaque[16,29]. 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.[30–32] 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[33].

We will also study **neutrophils** separated by density centrifugation on Lymphoprep followed by osmotic lysis of erythrocytes.

One end point will be the relative numbers of each subtype in the patients vs controls.

3. Measurement of the pH of the vacuoles after phagocytosis and efferocytosis

a. We will measure the kinetic changes in vacuolar pH in neutrophils, and monocyte and macrophage subsets using the phagocytosis of SNARF-labelled *Candida* that we developed as shown in Figure 1.

b. We have also developed a method of visualising efferocytosis of apoptotic cells by macrophages and the accompanying pH and morphological changes taking place within the vacuoles. For this we use neutrophils induced to undergo apoptosis by aging which we then label with SNARF-1. These cells are then be taken up by calcein labelled macrophages. Pilot studies on purified M1 and M2 macrophages showed the latter to be more avidly phagocytic and to have much more acidic vacuoles (Figure 2). In both of these cell types there is a progressive shrinkage of the vacuoles, which is indicative of digestion. Changes in pH over time will be quantitated and in addition to the SNARF-1, to accurately determine acidic pHs, we will also label the *Candida* and apoptotic cells with pHrodo™ iFL Red STP Ester (ThermoFisher) which gives a linear response between pHs of 5 and 8.

We will optimise the efferocytosis assay to quantitate phagocytosis of the apoptotic cells[34] 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.

4. 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[26], 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[35] and degranulation[36] 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 [37] and Classical and Non-classical monocytes[38] are very different.

Transcriptome analysis may or may not include representative levels of 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[39]. 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,[40] and there has been a proteomic characterisation of human M1 and M2 macrophages and their response to *Candida albicans*[41]. Proteomic studies have also been conducted on a variety of different dendritic cells[42]. 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.

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[43] or Percoll[44] gradients to establish the buoyant density of the granules. They will be identified by assays for lipases with the lipase Activity Assay Kit III (Sigma) which provides a simple and direct procedure for measuring lipase activity determined using a coupled enzyme reaction, which results in the generation of methylresorufin (λex = 529/λem = 600 nm) and protease activity using the universal protease substrate resorufin-labeled casein (Sigma).
We will measure phospholipase activity with fluorogenic phospholipid substrates[45]. Assays will be conducted at pHs of 5.5, 7.0 and 8.5.
Electron microscopy (EM) will be conducted on the cell preparations[36] and on the fractionated material.
Subsequently, granules will be separated on discontinuous gradients, and the interface harvested, diluted and pelleted.
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[46] . Each run compares 6 samples. Initially we will determine the variability in the relative concentrations of the different granule proteins between healthy individuals. Subsequently we will run samples from the patients against a pool of control subjects.
Relative or absolute deficiencies of proteins identified by proteomics will be confirmed by Western blotting and by enzyme assay.

5. 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.
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*.
Antibodies are available to a wide range of enzymes including to most of the lysosomal acid lipase, 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.

6. Measurement of the ability of patients cells to degrade phagocytosed organic material.

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[47]. 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 3.

We will prepare LDL by centrifugation. They will then be incubated with ^{3}H cholesterol to allow it to exchange into these carriers. The LDL with then be oxidised, causing them to aggregate, and they will be separated from the free 3H cholesterol by column chromatography or centrifugation.
The aggregated, oxidised LDL will be taken up by monocytes and macrophages[48] and their digestion and the liberation of cholesterol measured by its efflux by reversed transport, using Cyclodextrin as the acceptor[49] at the surface.
To measure the rate of degradation of cellular DNA, protein, carbohydrate and cholesterol, human HL-60 cells, will be cultured in a medium containing tritiated thymidine[50], ^{35}S-methionine, ^{14}C glucose and ^{14}H-cholesterol. The cells will be matured to neutrophils[51,52] and apoptosis will be induced by aging. 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.
We will also quantitate digestion of the lipid, protein, carbohydrate and DNA components by determining the solubilisation of radioactive tracers incorporated into the apoptotic cells. The 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[53,54].

7. Demonstration of importance of specific enzymes in digestion of efferocytotic cells

Inhibition of enzyme activities associated with the phagosomes

It is possible that we will identify deficiencies of specific enzymes is some of the patients in this population. If we do we will wish to confirm that these observations are biologically relevant to the digestion of organic matter which will be presented as described above.
We will employ general and specific inhibitors, where available, for example from Tocris Bioscience.
If an enzyme or enzymes appear to be of particular importance, in a subsequent study we will target the genes monocyte/macrophage cell lines. We will use Mono Mac 6 or THP-1 cells and target the genes by CRISPR[55]. These methods have been successfully employed in AWS' laboratory. Digestion and efferocytosis will then be measured.

Power calculations

We will subject the results of the different measurements described above to direct comparisons between the two groups using Student's t-test or paired t-test as appropriate. A p-value $< 0{\cdot}05$ will be considered significant. The causes of premature atherosclerosis are likely to be highly heterogenous, and as such might not be suitable for comparisons by t-tests and results might be better compared on Bayesian principles. A useful analysis might be an outlier analysis, which AWS has previously used to assess mRNA expression levels, and demonstrated under expression of Optineurin in macrophages from Crohn's disease patients[56]. We will use a Z-score of 2.5 to identify outliers of possible significance.

Expected value of results

Atherosclerosis is the major cause of mortality and morbidity in the modern world. There is currently inadequate knowledge of the underlying causal mechanisms. If we are able to determine the processes responsible for the handling of lipid cargoes and efferocytotic cells by normal macrophages and to identify mechanisms by which defects in these predispose to premature atherosclerosis, it will provide important information into atherogenesis in general, identify genes that can be screened as risk factors for diagnostic profiling, and define processes that might be manipulated to prevent or reverse vascular lesions.

Time Line All the methods required for this study are up and running in the Segal laboratory. Patients will be studied as and when they present to Dr Patel.

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

56. Smith, A. M. *et al.* Disruption of macrophage pro-inflammatory cytokine release in Crohn's disease is associated with reduced optineurin expression in a subset of patients. *Immunology* **144,** 45–55 (2015).

9. Contribution to be made by each co-applicant

Dr Patel will be responsible for recruiting suitable patients and matched healthy control subjects, explaining the project to them and obtaining informed consent.

Professor Segal will be responsible for laboratory based experimental work.

This will be a truly joint project. The co-investigators will meet regularly to discuss, analyse and to publish the results.

10. Relevant publications

R Patel – Publications relating to premature atherosclerosis

Kofink D, Muller SA, Patel RS et al. Routinely measured hematological parameters and prediction of recurrent vascular events in patients with clinically manifest vascular disease. PloS One. Sep 2018;13(9) 3.

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Forssen H, Patel R, et al. Evaluation of Machine Learning Methods to Predict Coronary Artery Disease Using Metabolomic Data. Studies in health technology and informatics. 2017;235:111-115.

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Rubbo B, Fitzpatrick NK, Denaxas S, et al. Use of electronic health records to ascertain, validate and phenotype acute myocardial infarction: A systematic review and recommendations. International journal of cardiology. 2015;187:705-711.

Patel RS, Li Q, Ghasemzadeh N, et al. Circulating CD34+ progenitor cells and risk of mortality in a population with coronary artery disease. Circulation research. Jan 16 2015;116(2):289-297.

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AW Segal – Publications relating to vacuolar pH, subcellular fractionation and intracellular digestion

Imaging the Neutrophil Phagosome and Cytoplasm Using a Ratiometric pH Indicator.
Foote JR, Levine AP, Behe P, Duchen MR, Segal AW.
J Vis Exp. 2017 Apr 5;(122). doi: 10.3791/55107.
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The NADPH Oxidase and Microbial Killing by Neutrophils, With a Particular Emphasis on the Proposed Antimicrobial Role of Myeloperoxidase within the Phagocytic Vacuole.
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Microbiol Spectr. 2016 Aug;4(4).
PMID: 27726789

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Reeves EP, Nagl M, Godovac-Zimmermann J, Segal AW.
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Immunity. 2000 Feb;12(2):201-10.
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Roberts PJ, Segal AW.
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PMID: 2448226

Defective degradation of bacterial DNA by phagocytes from patients with systemic and discoid lupus erythematosus.
Roberts PJ, Isenberg DA, Segal AW.
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PMID: 2443291

The subcellular localization of ubiquinone in human neutrophils.
Cross AR, Jones OT, Garcia R, Segal AW.
Biochem J. 1983 Dec 15;216(3):765-8.
PMID: 6320799

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

Iodination by stimulated human neutrophils. Studies on its stoichiometry, subcellular localization and relevance to microbial killing.
Segal AW, Garcia RC, Harper AM, Banga JP.
Biochem J. 1983 Jan 15;210(1):215-25.
PMID: 6303312

Analytical subcellular fractionation of neutrophils from patients with chronic granulomatous disease. Demonstration of the enzyme defect in four cases.
Segal AW, Peters TJ.
Q J Med. 1978 Apr;47(186):213-20.PMID: 684156

Kinetics of fusion of the cytoplasmic granules with phagocytic vacuoles in human polymorphonuclear leukocytes. Biochemical and morphological studies.
Segal AW, Dorling J, Coade S.
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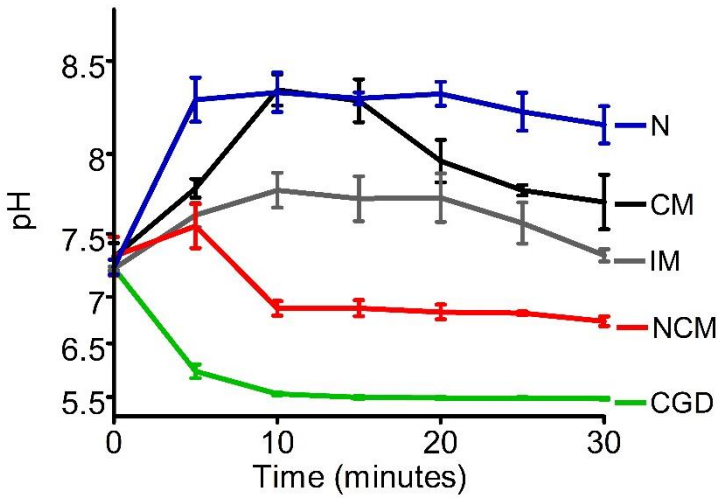
Analytical subcellular fractionation of human granulocytes with special reference to the localization of enzymes involved in microbicidal mechanisms.
Segal AW, Peters TJ.
Clin Sci Mol Med. 1977 Apr;52(4):429-42. No abstract available.PMID: 862338

11. Appendices:

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, and (**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 lacking the NADPH oxidase gave the same results as shown for neutrophils (CGD). Bar = 10 µm

A



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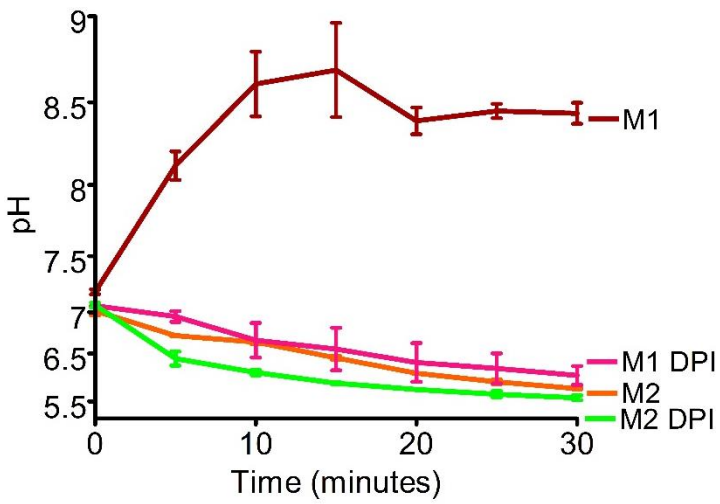

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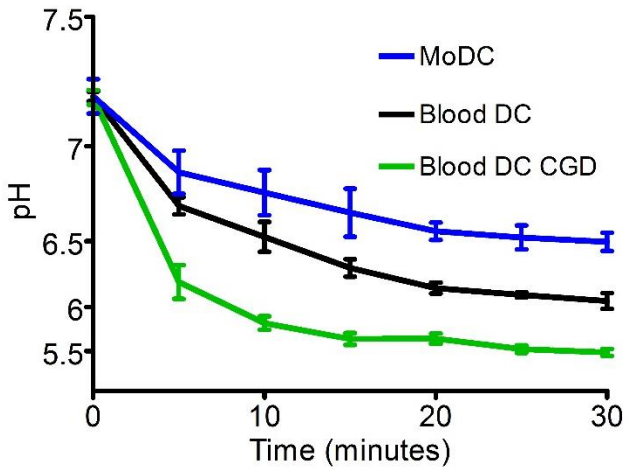


D

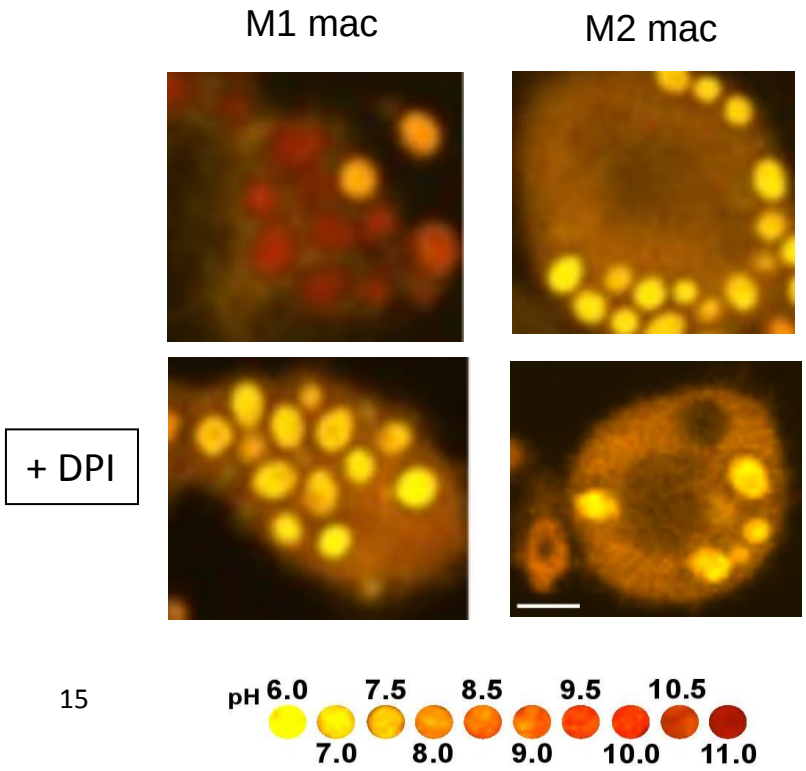
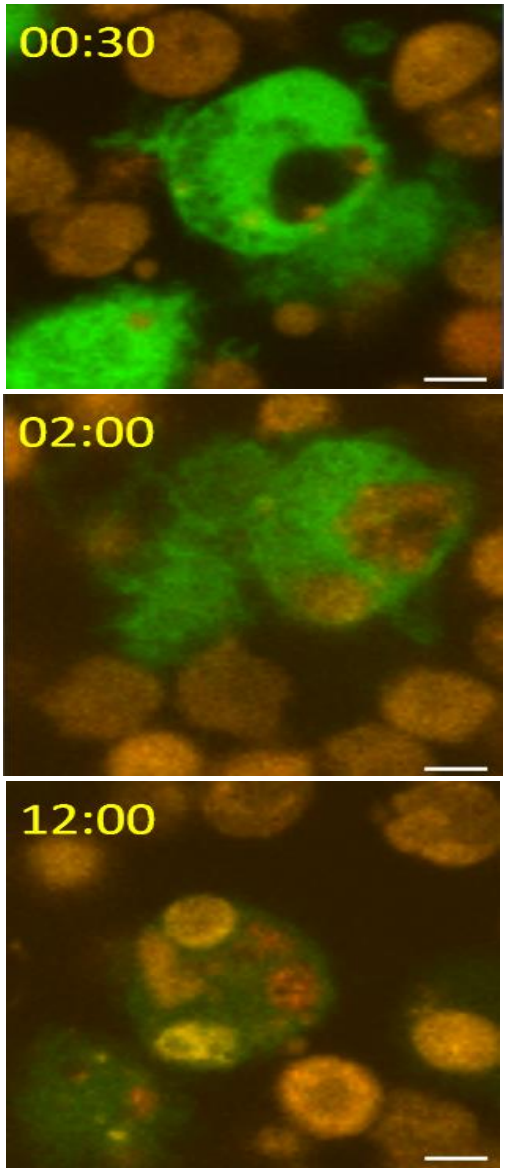


Figure 2. **Efferocytosis in M1 and M2 macrophage**

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.

M1 macrophage



M2 macrophage

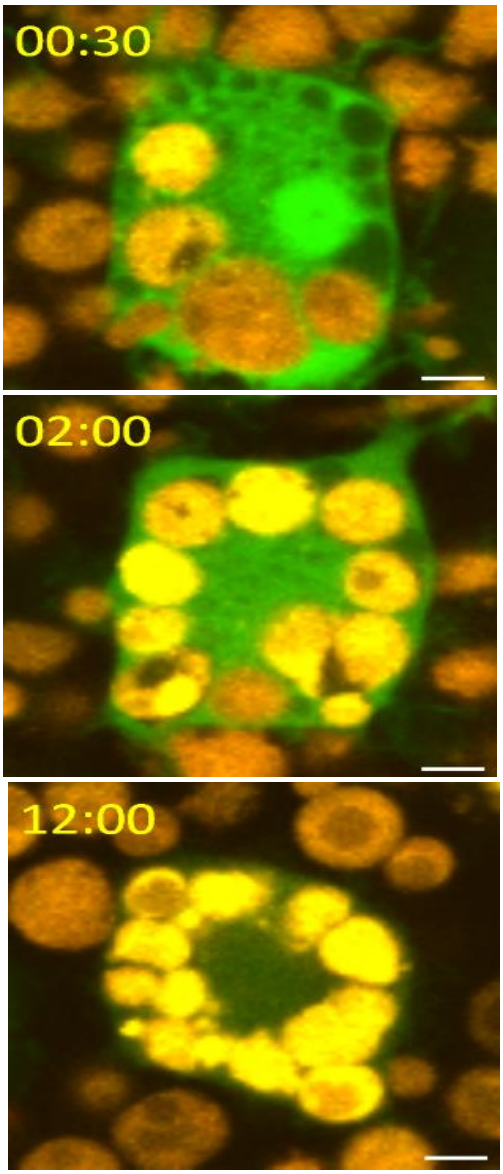


Figure 3. **Elastase activity in neutrophil and monocyte**
ThermoFisher bisamide rhodamine 110 elastase substrate (CBZ-Ala-Ala-Ala-Ala)2-R110 fluorescence demonstrating elastase activity after phagocytosis of unlabelled *Candida.*

Neutrophil

Monocyte