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Immune complex-induced anti-inflammatory neutrophil functions

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VERSUS ARTHRITIS

Declaration

The research detailed within this thesis has been carried out during my PhD studentship under the supervision of Dr. Sonja Vermeren and Prof. Ian Dransfield from October 2017 to November 2021. I declare that this thesis was composed by myself, that the work contained herein is my own except where explicitly stated otherwise in the text, and this work has not been submitted for any other degree or professional qualification except as specified. Where published work has been consulted and referenced, the source has been clearly cited.

Utsa Karmakar

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I wish you all the best.

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Abstract

Neutrophils are short lived innate immune cells that perform a range of functions, including chemotaxis, phagocytosis, degranulation, generation of reactive oxygen species (ROS) and production of cytokines which equip them well to defending the host from bacterial or fungal infections. At the end of their short lives neutrophils undergo apoptosis, and are subsequently cleared by professional phagocytes in a specialised process referred to as efferocytosis which helps to promote the resolution of inflammation. Inappropriate recruitment and activation of neutrophils can result in important host damage.

Antibodies, in the shape of opsonins of particles or as immune complexes (ICs) bind to, and cross-link Fc receptors on the neutrophil surface, acting as powerful stimuli. In the context of autoimmunity, e.g. rheumatoid arthritis (RA) and other chronic inflammatory events, (auto) antibodies can trigger neutrophilic inflammation. However, observations by our group and others have suggested that neutrophils can promote repair as well as driving inflammation.

I addressed the hypothesis that insoluble immune complexes (iICs) trigger anti-inflammatory neutrophil functions. I investigated (i) the mechanism of iIC-internalisation, (ii) whether iIC internalisation and induction of neutrophil apoptosis are linked and (iii) whether these functions are dysregulated in neutrophils derived from RA patients.

By performing a range of functional experiments I showed that insoluble ICs (iICs), which had previously already been shown to induce neutrophil apoptosis, are internalised by receptor-mediated macropinocytosis and subsequently digested by neutrophils. My experiments identified that this internalisation mechanism coincided

with, but was separately controlled to the induction of neutrophil apoptosis, with neither event dependent upon the other, suggestive of a mechanism that is distinct from phagocytosis-induced cell death (PICD). However, as with PICD, iIC-induced neutrophil apoptosis was dependent upon ROS production. While both iIC-induced macropinocytosis and apoptosis were both chiefly mediated by FcγRII, simultaneous iICs induced ROS production was dependent upon additional receptors, FcγRI and αMβ2. My results suggest therefore that iICs promote both pro-inflammatory (ROS) and anti-inflammatory neutrophil functions (their own clearance; neutrophil apoptosis). I moreover demonstrated that induction of neutrophil apoptosis via iICs promotes improved neutrophil efferocytosis by macrophages, suggestive of a third anti-inflammatory function induced by iICs. I finally compared the induction of some pro- and anti-inflammatory neutrophil functions induced in healthy donor- and RA patient-derived neutrophils, observing increased ROS production, but no differences in terms of iIC internalisation and iIC-mediated induction of apoptosis of RA neutrophils. Taken together, my results suggest that iICs mediate previously underappreciated anti-inflammatory neutrophil functions in all neutrophils, even those obtained from RA patients.

Lay Abstract

Human blood comprises red blood cells, white blood cells, and platelets. Among the different types of white blood cells, neutrophils are most abundant. They are the first immune cells to fight against an attack. In a sudden war against germs, neutrophils defend our bodies by eating the bugs and producing toxic chemicals that kill them. In the end, exhausted by killing germs, the neutrophils commit suicide. They do this by a special cell death process called ‘apoptosis’. Like a green traffic light, apoptotic neutrophils signal for other, bigger immune cells, the macrophages, to come to clean up the battlefield by cleaning up their corpses. Clearing away the apoptotic neutrophils from the injured tissue brings the body back to its normal safe status. In certain uncontrolled situations such as in chronic inflammatory disease, neutrophils remain on high alert for a long time, struggle to eat germs, live longer than they should and end up causing damage to the body’s own tissues. Promoting neutrophil apoptosis in such situations, especially in autoimmune diseases such as rheumatoid arthritis, is key to controlling excessive inflammation.

The presence of so-called auto-antibodies, which do not recognise bugs, but the body’s own tissues, is a hallmark of autoimmune diseases, in which the body is under attack by its own immune system. Autoantibodies frequently aggregate. Antibody aggregates, so-called ‘immune complexes’, can circulate in bodily fluids, e.g. the blood or the fluid inside the arthritic joint, either in a soluble or an insoluble form. Alternatively immune complexes may get deposited on tissue surfaces, e.g. the inner lining of the arthritic joint. All of these immune complexes can powerfully stimulate neutrophils and are recognised as important drivers of inflammation. In this work, I demonstrate unexpected anti-inflammatory functions of insoluble immune complexes (iICs). I used freshly prepared neutrophils derived from the blood of healthy donors to test this.

First, I demonstrated that iIC-stimulation caused neutrophil apoptosis. Second, I showed that neutrophils drink the iICs together with the fluid surrounding them. By doing this, they get rid of them. Third, the apoptotic neutrophils that had cleared away iICs were subsequently eaten by macrophages, preventing runaway inflammation. I concluded that iICs cause anti-inflammatory responses in healthy neutrophils in these three different ways.

I also performed similar experiments with neutrophils derived from rheumatoid arthritis patients. This work unfortunately suffered from COVID-19 related lack of access to patient samples so that I was only able to analyse a small number of blood samples from patients. My preliminary results indicated that patient derived neutrophils were more inflammatory than those from healthy donors. Despite this, iICs seemed to induce some protective responses even in the patient-derived neutrophils. In particular, patient-derived neutrophils were as able to drink iICs as those from healthy donors.

The insights gained from my work will help with the design of improved future therapies for treating those suffering from autoimmune diseases.

List of publications

1. Karmakar U, Chu JY, Sundaram K, Astier AL, Garside H, Hansen CG *et al.* Immune complex-induced apoptosis and concurrent immune complex clearance are anti-inflammatory neutrophil functions. *Cell Death & Disease* 2021; **12**: 296.

2. Karmakar U, Vermeren S. Crosstalk between B cells and neutrophils in rheumatoid arthritis. *Immunology* 2021; **164**: 689–700.

3. Vermeren S, Karmakar U, Rossi AG. Immune complex-induced neutrophil functions: A focus on cell death. *European Journal of Clinical Investigation* 2018; **48**: e12948.

Please see the appendix for copies of publications.

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List of abbreviations

ACPA	Anti-citrullinated protein antibodies
ACR	The American College of Rheumatology criteria
AF	Alexa fluor
ANA	Anti-nuclear antibodies
ANCA	Anti-neutrophil cytoplasmic antibody
ANOVA	Analysis of variance
Anti-CarP	Anti carbamylated peptide antibodies
Apaf1	Apoptotic protease activating factor-1
APLA	Anti-phospholipid antibody
APRIL	A proliferation-inducing ligand
Atg5	autophagy gene 5
ATP	Adenosine triphosphate
AUC	Area under the curve
BAFF	B cell-activating factor
BAI	Brain-specific angiogenesis inhibitor
Bax	Bcl-associated X protein
Bcl-2	B-cell lymphoma 2
BSA	Bovine serum albumin
$CaCl_2$	Calcium Chloride
CAD	caspase-activated DNAse
CAIA	anti-collagen antibody induced arthritis model
CCL	C-C motif ligand, chemokine
CD	Cluster differentiation
CDK	Cyclin dependent kinases
CGD	Chronic granulomatous disease
CLIC	Clathrin & dynamin-Independent Carriers
CO_2	Carbon dioxide
CR	Complement receptor
CSF	Colony stimulating factor
CTLA	Cytotoxic T-lymphocyte-associated protein
CXCL	CXC motif chemokine ligand
CXCR	CXC motif chemokine receptor
Cyt-B	Cytochalasin B
Cyt-C	Cytochrome C
DFP	Diisopropylfluorophosphate
DMARD	Disease modifying anti-rheumatic drugs
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPI	Diphenyleneiodonium chloride
dsDNA	Double stranded DNA
EDTA	Ethylenediaminetetraacetic acid
EEA1	early endosomal antigen 1
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
ECL	Enhanced chemiluminescence

EIPA	5-(N-Ethyl-N-isopropyl)-amiloride	Ig	Immunoglobulin
Erk	Extracellular regulated kinase	ICs	Immune complexes
EULAR	The European League Against Rheumatism	iICs	Insoluble immune complexes
FADD	Fas-associated death domain protein	IFN	Interferon
FcRn	Neonatal Fc receptor	IL	Interleukin
FcγR	Fc gamma receptor	ILD	Interstitial lung disease
FITC	Fluorescein isothiocyanate	IMDM	Iscove's Modified Dulbecco's Medium
fMLF	N-formyl-methionyl-leucyl-phenylalanine	ITAM	Immunoreceptor tyrosine-based activation motif
FR	FR180204 (Erk inhibitor)	ITIM	Immunoreceptor tyrosine-based inhibition motif
FSC	Forward scatter	IV	Intravenous
G2A	G-protein-coupled receptor 132	Kd	The dissociation constant
GEEC	GPI-Enriched Endocytic Compartments	LAMP	Lysosomal-associated membrane proteins
GM-CSF	Granulocyte-macrophage colony-stimulating factor	Lat-B	Latrunculin-B
GPI	Glycosylphosphatidylinositol	LB	Latex beads
GTP	Guanine triphosphate	LFA	Lymphocyte function-associated antigen
H_2O_2	Hydrogen peroxide	LPC	Lysophosphytidlycholine
HBSS	Hank's Balanced Salt Solution	LPS	Lipopolysaccharide
HCL	Hydrochloric acid	LTB4	Leukotriene B4
HD	Healthy donor	Mac-1	Macrophage-1 antigen
HOCL	Hypochlorous acid	macs	Macrophages
HSA	Human serum albumin	MAPK	Mitogen-activated protein kinase
HRP	Horseradish peroxidase	Mcl-1	Myeloid cell leukaemia 1
ICAM	Intercellular adhesion molecules	MDM	Monocyte derived macrophages

MerTK	Mer protooncogene, tyrosine kinase	PBS	Phosphate buffer saline
MES	2-(N-morpholino) ethanesulfonic acid	PBST	PBS with Tween 20
MFGE8	Milk fat globule EGF 8	PDVF	Polyvinylidene difluoride
$MgCl_2$	Magnesium Chloride	PF	PF3758309 (Pan-Pak inhibitor)
MHC	Major histocompatibility complex	PFA	Paraformaldehyde
MMP	Matrix metalloproteinase	PGE_2	Prostaglandin E_2
MPO	Myeloperoxidase	PI	Propidium iodide
Na_2VO_4	Sodium orthovanadate	PI(3,4,5)P3/PIP3	Phosphatidylinositol-(3,4,5)-trisphosphate
NaCl	Sodium Chloride	PI(4,5)P2	Phosphatidylinositol-(4,5)-bisphosphate
NADPH	nicotinamide adenine dinucleotide phosphate	PI3K	Phosphoinositide 3-kinase
NaF	Sodium fluoride	PICD	Phagocytosis induced cell death
nAPC	immunogenic antigen presenting cells	PK	Protein kinases
NE	Neutrophil elastase	PMNs	Polymorphonuclear leukocytes
NETs	Neutrophil extracellular traps	PR3	Proteinase 3
NF-kB	Nuclear factor kappa light chain enhancer of activated B cells	PtdSer	phosphatidylserine
NK	Natural killer	PVDF	Polyvinylidene fluoride
$O_2^{\bullet-}$	Superoxide anion	RA	Rheumatoid arthritis
OCL-	Hypochlorite ion	RF	Rheumatoid factor
$OH^{\bullet}$	Hydroxyl ion	RIPK	Receptor interacting serine/threonine kinase
PAF	Platelet-activating factor	RLU	Relative light unit
Pak	p21-activated kinase	RPM	Revolutions per minute
PAMP	Pathogen associated molecular pattern	RNP	Ribonucleoproteins
PBMCs	Peripheral blood derived monocytes	ROS	Reactive oxygen species

Rosco	R-Roscovitine
RT	Room temperature
sIC	Soluble immune complexes
SIRPα	Signal regulatory protein α
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	Succinimidyl ester
SEM	Standard errors of the mean
SF	Synovial fluid
SHIP	SH2-containing inositol 5-phosphatase
SLE	Systemic lupus erythematosus
SMAC	Second mitochondria-derived activator of caspases
SOD	Superoxide dismutase
SSC	Side scatter
SQ	Subcutaneous
TEMED	Tetramethylethylenediamine
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Tx 100	Triton X-100
VEGF	Vascular endothelial growth factor
XIAP	x-linked inhibitor of apoptosis protein

Others

h	Hour/s
min	Minutes
sec	Second
mM	Millimolar
mm	Millimetre
µM	Micromolar
µm	Micrometre
nM	Nanomolar
nm	Nanometre
U/L	Unit per litre
U/ml	Unit per millilitre
V	Volt

1. Introduction

1.1. Neutrophil Biology

Human blood comprises red blood cells, white blood cells, and platelets. Polymorphonuclear leukocytes (PMNs) are the most abundant circulating white blood cells in humans. Neutrophils are a subset of PMNs and contain an arsenal of toxic substances that kill and degrade microbes. They represent up to 70% of all peripheral blood leukocytes in humans and act a first line of defence against bacterial and fungal infections constituting an essential part of innate immune system.[1–3]

Neutrophils are terminally differentiated, short-lived immune cells. It is estimated that 10^9 neutrophils/kg of body weight are turned over every day in the absence of inflammation.[3] Circulating neutrophils survive for 24-48 hours however, data suggest that they can survive up to 5 days under *in vivo* conditions.[4] Neutrophils are continuously produced in the bone marrow and released into in the peripheral vasculature. At the end of their life, circulating neutrophils that are not recruited to any site of inflammation, travel to liver, spleen or bone marrow where they undergo constitutive apoptosis and are cleared by resident macrophages.[5,6]

Unstimulated neutrophils circulate through the blood stream and migrate to sites of infection or injury. Upon an encounter with microbes as well as in response to activating agents e.g. damage-associated molecular pattern molecules (DAMPs) and pathogen-associated molecular pattern molecules (PAMPs) in sterile injury,[7] neutrophils become activated and exhibit a range of effector functions. Their primary function is to phagocytose and kill pathogens and degrade cell debris and any material that is foreign to the host.[8] The functional capacity of neutrophils depends on several

main processes which are summarised in **Figure 1.1** and discussed in the **section 1.2**.

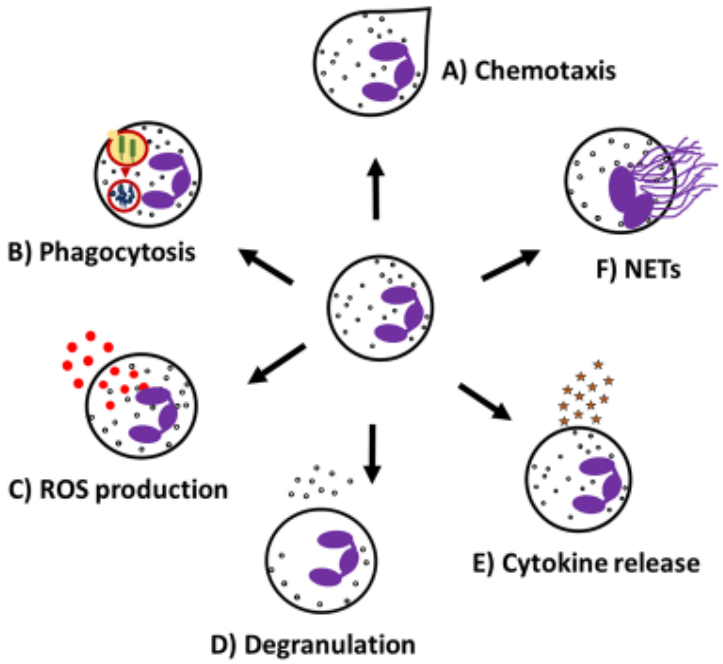


Figure 1.1. Neutrophil effector functions.

Neutrophils use a range of defence mechanisms in infection or sterile inflammation. Neutrophils travel to sites of injury by chemotaxis (**A**). Upon an encounter with microbes, they phagocytose the offending agent (**B**), generate toxic/microbiocidal reactive oxygen species (ROS, **C**) and degranulate to release preformed enzymes and proteins from intracellular granules (**D**) into a phagosome or to the outside of the cell. They release pro- and anti-inflammatory cytokines (**E**) and can also produce neutrophil extracellular traps (NETs, **F**).

1.1.1. Neutrophils in inflammation

Because neutrophils can potentially cause tissue damage, their activation and recruitment to relevant inflammatory sites are subjected to tight regulation. Neutrophil activation occurs in two stages. First, they are “primed” by either lipopolysaccharide (LPS), or cytokines/chemokines such as tumour necrosis factor (TNF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) followed by acquisition of their full functional potential. Priming is believed to be one mechanism of controlling the neutrophil response. Although an unprimed (or quiescent) neutrophil is capable of all functions associated with activation, the elicited response will be amplified if the cell has been primed as shown in **Figure 1.2.** In contrast to full activation, priming is a reversible process.[9,10]

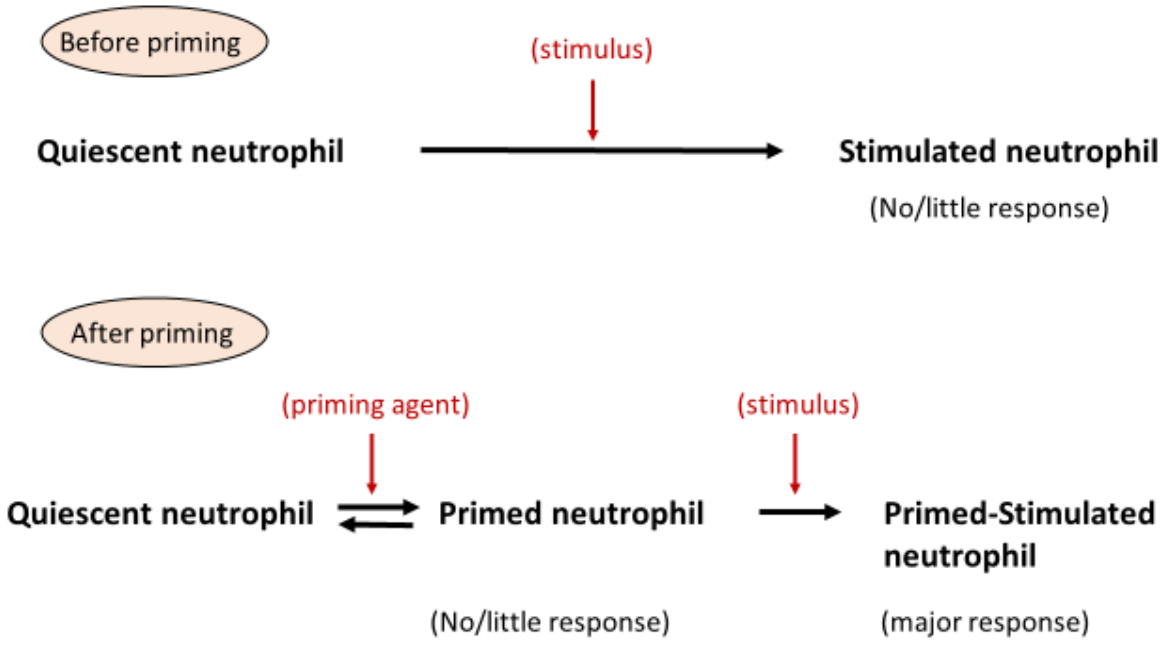


Figure 1.2. Neutrophil priming.

Quiescent neutrophils show no or little response with a stimulus. Addition of a priming agent primes the neutrophil and escalate the response of the same stimulus.

Neutrophils respond to multiple signals by producing cytokines and other inflammatory factors such as histamine and leukotrienes that influence and regulate inflammation by modulating other immune cells. These inflammatory mediators change the surface of the endothelium, promoting neutrophils marginating towards the endothelial surface. Neutrophils are then mobilised to sites of infection or inflammation through a process known as the leukocyte adhesion cascade, whereby they form increasingly strong interactions with the endothelium causing rolling, tethering, adhesion, crawling and finally, transmigration (**Figure 1.3**).[11–15]

Endothelial cells of blood vessels near the injured site get activated and express adhesion receptors such as E- and P-selectins. These receptors bind to glycoprotein ligands on neutrophils, promoting rolling on the endothelium. To ensure neutrophil adherence to the endothelium, surface expression of β2 integrins (especially αMβ2) is upregulated on the neutrophils. Chemokine-induced conformational changes in integrins is accompanied by an increased expression of complementary intercellular adhesion molecule-1 (ICAM-1) on the surface of activated endothelial cells. Neutrophil priming/activation also results in L-selectin shedding which is a further prerequisite for transendothelial migration.[16]

Subsequent extravasation of neutrophils leads to migration of the neutrophils to the site of inflammation by chemotaxis, which is dependent on gradients of chemoattractants such as Interleukin-8 (IL8), N-formyl-methionyl-leucyl-phenylalanine (fMLF), Leukotriene B4 (LTB4). In addition to being driven by nearby tissue-resident macrophages, the neutrophil recruitment is also intensified by the active neutrophils themselves which amplify gradients by producing LTB4, causing swarming behaviour.[17] Under certain circumstances, this multistep process can end with reverse migration, when neutrophils return to the vasculature.[18]

However, it is thought that the most important clearance mechanism of tissue-resident, inflammatory neutrophils is that they undergo apoptosis and are cleared through phagocytosis by resident macrophages and dendritic cells (see details in **section 1.3**). Senescent neutrophils in the blood upregulate expression of CXC motif chemokine receptor type 4 (CXCR4), which allows them to return to the bone marrow for final clearance.[19]

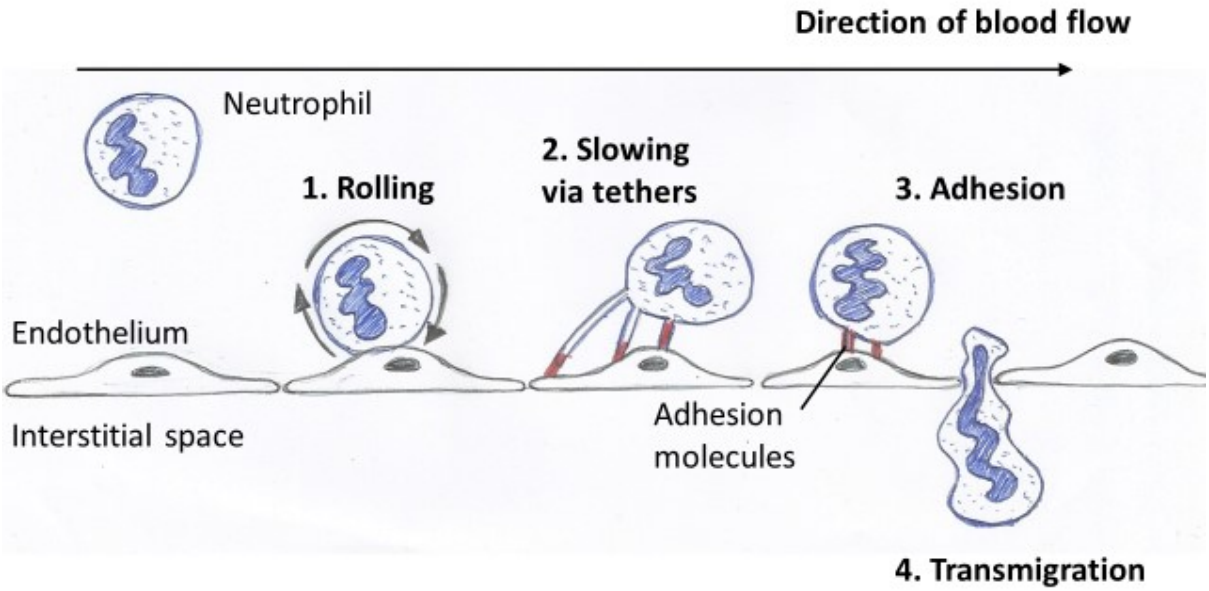


Figure 1.3. Leukocyte adhesion cascade.

A diagram illustrating the steps of the neutrophil recruitment cascade that involves a sequential events, capture, rolling (1), slow adhesion via tethers (2), followed by firm adhesion (3) via the adhesion molecules, crawling and finally, transmigration (4) through the endothelial membrane.

1.1.2. Neutrophils in the resolution of inflammation

As mentioned above, neutrophils are best known for their pro-inflammatory functions. However, a key histological feature in the resolution of acute inflammation is the disappearance of neutrophils from the local inflamed sites. Indeed, neutrophils are important in orchestrating the resolution of inflammation. Aborted neutrophil recruitment is an important step that is required to reinstate tissue homeostasis. This involves neutrophil-mediated disruption of chemokine gradients, e.g. by proteolysis of chemokines and generation of pro-resolution mediators. The best understood mechanism consists of neutrophil apoptosis and clearance of their apoptotic corpses by macrophages.[20,21]

Interestingly, neutrophils contribute to the resolution of inflammation by eliminating the microorganisms.[22–24] Wound healing in neutrophil depletion models has been shown to be delayed.[25] Blocking the leukocyte adhesion cascade in certain autoimmune diseases, such as ulcerative colitis, leads to an exacerbation of disease,[26] illustrating the pivotal role of neutrophils in the attenuation of inflammatory disease.

1.2. Neutrophil functions

1.2.1. Phagocytosis

As a professional phagocyte, the neutrophil has a unique capacity to recognise, internalise and destroy a (bacterial or fungal) pathogen or foreign particles. The exocytosis of secretory vesicles and specific granules to the neutrophil surface results in the upregulation of complement receptors (e.g. CR1 and CR3) and Fcγ receptors. These receptors bind to the complement components C3b, C3bi and the Fc region of immunoglobulin G (IgG), respectively, that decorate (opsonise) foreign particles and promote their phagocytosis. At the inflammatory site, the neutrophil recognises opsonised particles and this triggers the extrusion of pseudopodia and engulfment of the target into a phagocytic vacuole. Moreover, like macrophages, neutrophils can also phagocytose non-opsonised particles.[27,28]

Phagocytosis is a tightly regulated, actin-dependent process. Actin remodelling results in the formation of a phagocytic cup around the pathogen or opsonised particle and, fusion of the plasma membrane around this cup results in effective engulfment. The enclosed compartment containing the target is typically surrounded by a double membrane that is derived from the plasma membrane and called phagosome. The phagosome subsequently matures, acquiring the cellular machinery required for killing and disposal of the internalised microorganisms.[29]

In most professional phagocytes, phagosomes fuse with lysosomes to form phagolysosomes.[30] However in neutrophils, azurophil granules serve lysosomal functions.[31] Macrophage lysosomes are characterised by their extreme acidity (pH < 5) and elevated concentration of proteases. Prior to phagolysosome formation, early endosome and late endosomes are formed which are characterised by their associated molecules. Early endosomes are less acidic (pH 6.0-6.5) and their markers

are transferrin receptors, early endosomal antigen 1 (EEA1), and the small GTPase Rab5. Late endosomes are more acidic (pH 5.5-6.0) and their markers are the GTPases Rab7 and Rab9, as well as lysosomal-associated membrane proteins (LAMPs).[32–35] The pH change is necessary for phagosome maturation and is thought to provide a mileu for the optimal activity of proteases involved in pathogen killing.

Although macrophages undertake active endocytosis, mature neutrophils are remarkably passive. Neutrophils do not possess conventional early and late endosomes or lysosomes. Instead, they contain a variety of specialised vesicles and granules,[33] that help with the degradation of internalised microorganism. The pH change in the late phagosome in neutrophils is not very dramatic. In fact, a transient initial alkalinisation has been described, which subsides after a period of ~30 minutes. Whether the pH then remains near neutral or becomes slightly acidic remains unclear.[36,37]

Besides phagocytosis, there are other modes of internalisation by neutrophils such as pinocytosis and endocytosis that are discussed in **chapter 4**.

1.2.2. Degranulation & production of reactive oxygen species

The neutrophil phagosome acquires antimicrobial agents through fusion with secretory vesicles and granules as shown in **Figure 1.4**. Neutrophils contain four categories of granules or secretary vesicles: i) primary (azurophil) granules characterised by the presence of myeloperoxidase (MPO) and membrane bound CD63; ii) secondary (specific) granules characterised by the presence of lactoferrin and membrane bound CD66b; iii) tertiary granules which contain gelatinase; and iv) secretory vesicles which contain albumin and superoxide dismutase (SOD).[38–40]

Changes in cytosolic levels of calcium is required for granule secretion and calcium signals can trigger the fusion of granules with the phagosome in neutrophils.[41] Calcium thresholds are different in individual secretory compartments, with secretory vesicles having the lowest and azurophil granules the highest threshold.[42] Along with calcium signals, protein kinase signalling was also shown to be important in degranulation and phagocytosis.

Antimicrobial killing by neutrophils relies on the *de novo* generation of toxic compounds and delivery of pre-synthesised destructive enzymes, potent proteases such as serine proteases (neutrophil elastase, cathepsin G, proteinase 3) and cysteine proteases (cathepsin C) as well as the toolkit to generate cytotoxic reactive oxygen species from neutrophilic granules to phagosomes. While these agents contribute to a beneficial antimicrobial response inside phagosomes, their extracellular release can cause tissue damage.[31]

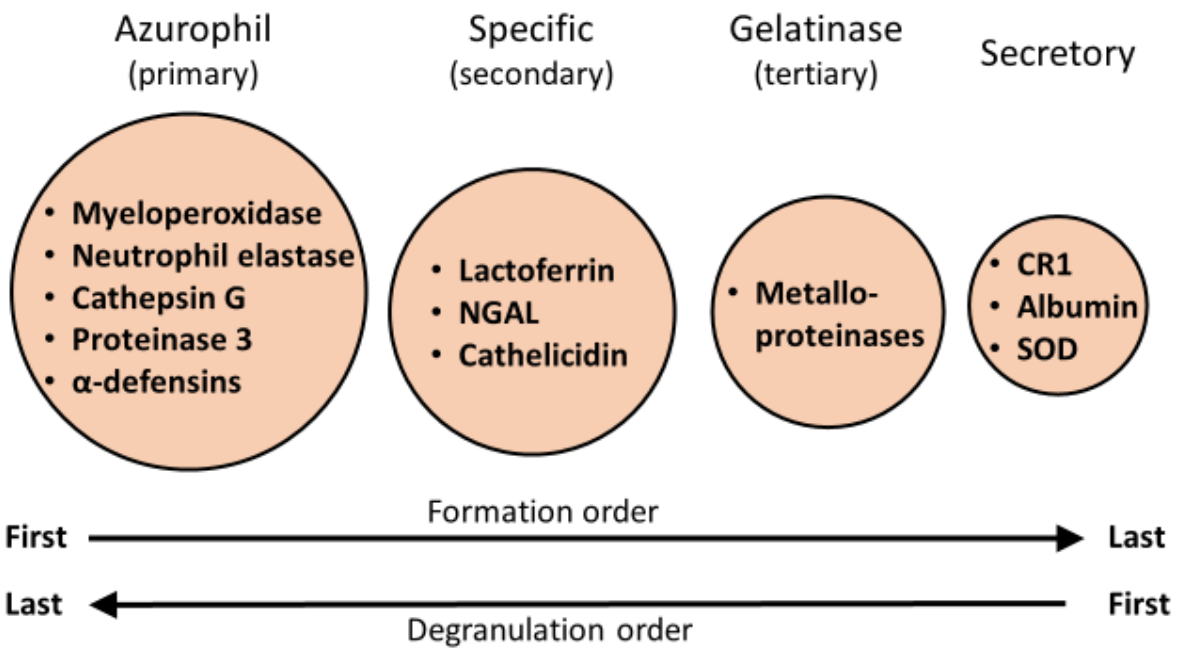


Figure 1.4. Neutrophil granules.

A diagram showing different granules found in neutrophils. The decreasing size of the circle represents the size difference in the granules. Azurophil granules are the largest, first to form and last to degranulate. On the other hand, secretory vesicles are the smallest, last to form and first to degranulate.

NGAL, Neutrophil gelatinase-associated lipocalin; CR1, complement receptor 1; SOD, superoxide dismutase.

Stimulation of a range of neutrophil receptors promotes the generation of reactive oxygen species (ROS) by a process known as the respiratory burst. In stimulated neutrophils, ROS production are initiated almost exclusively by the nicotinamide adenine dinucleotide phosphate (NADPH) that belongs to the family of NADPH oxidase (NOX) proteins. The core NADPH oxidase enzyme consists of several subunits, flavocytochrome b_{558} consisting of $gp91^{phox}$ and $p22^{phox}$ as well as $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, $gp91^{phox}$ and is subjected to complex regulatory processes. The NADPH oxidase is inactive in resting cells and its components are distributed between the cytosol and membranes. Upon activation, the cytosolic components of the NADPH oxidase translocate to the plasma membrane in tightly regulated fashion, assembling the entire oxidase.[43,44] The NADPH oxidase transfers electrons from the cytoplasmic NADPH, reducing oxygen in the phagosome or at the plasma membrane, producing the superoxide anion ($O_2^{\bullet -}$), which functions as precursor for the moderately toxic hydrogen peroxide (H_2O_2) by dismutation in the phagosome. This reaction may be catalysed by SOD, which can be delivered to the phagosome from secretory vesicles,[40] or it may occur by spontaneous dismutation. Delivery of MPO from azurophil granules into the phagosome causes oxidation of chloride and generation of the highly cytotoxic hypochlorous acid (HOCl) from hydrogen peroxide (see also **Figure 3.9** in **chapter 3**).

ROS are considered as partially reduced metabolites of oxygen that possess strong oxidising capabilities. Together with powerful proteases and antimicrobial peptides that are also released into the phagosome during degranulation, ROS are important for microbial killing.

1.2.3. Production of inflammatory mediators

At sites of inflammation, neutrophils release some pro- and anti-inflammatory cytokines, such as IL-23, IL-12, TNFα and IL10, respectively, and chemokines (especially IL-8/CXCL8). Although neutrophils are relatively poor at producing cytokines when compared to major cytokine producers such as T cells, monocytes and macrophages, this nonetheless contributes significantly to influencing the inflammatory and immune responses, and also modulates antiviral defence, haematopoiesis, angiogenesis, and fibrogenesis.[45–47]

Activated neutrophils can moreover synthesise and release an important array of additional mediators such as platelet-activating factor (PAF),[48] prostaglandin E_2 (PGE_2)[49] and LTB4.[17] The release of these pro- and anti-inflammatory mediators either singly or in combination, from large numbers of activated neutrophils at an inflammatory site fine tunes the inflammatory responses.

1.3. Neutrophil death

Neutrophils die by several different mechanisms (shown in **Figure 1.5**). Apoptosis is the main form of physiological neutrophil death and the other common mechanism is necrosis. NETosis is another form of neutrophil death which is induced by NET production in response to certain stimuli. Other types of neutrophil death exist (e.g. necroptosis and pyroptosis), but they are not discussed here.

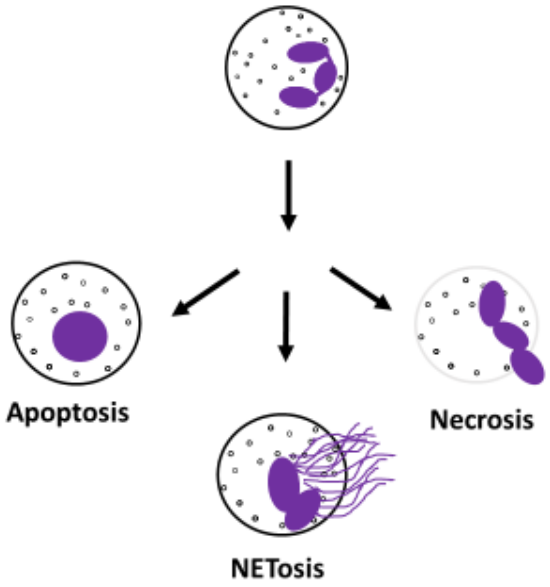


Figure 1.5. Different forms of neutrophil death.

Neutrophil death can occur via different mechanisms. The most common and physiological type of cell death is apoptosis (also known as constitutive apoptosis). Apoptotic neutrophils are characterised by condensation of nuclear material with an intact membrane. Alternative forms of cell death are found mainly in pathological conditions. These include NETosis which follows onto the generation of neutrophil extracellular trap (NET) which release protein-decorated strands of DNA as well as, necrosis which is characterised by loss of plasma membrane integrity and causes the cellular contents to spilled to the outside of the cell.

1.3.1. Apoptosis

Apoptosis is a highly organised form of cellular suicide, or programmed cell death; it is critical to control body homeostasis and to regulate tissue development. Apoptosis happens during embryonic development to promote embryogenesis and it also maintains cell populations in organs and tissues. Neutrophils undergo constitutive apoptosis after approximately 24 hours in the circulation, although the exact timing is a subject of debate.[4,8,50,51]

Cellular apoptosis is characterised by the activation of specialised regulatory pathways, which typically involve specialised proteases called caspases, as well as several morphological changes including phosphatidylserine (PtdSer) externalisation, cell shrinkage, membrane blebbing, chromatin condensation and, DNA fragmentation. The apoptotic process is terminated by phagocytosis of apoptotic corpses (efferocytosis) by tissue resident macrophages (see **section 1.5**).

1.3.1.1. Mechanism of apoptosis

Compared to most cells, neutrophils have fewer mitochondria, with a comparatively low involvement in ATP synthesis. Instead, neutrophil mitochondria have a key role in apoptosis, with a central role for cytochrome C in promoting the activation of apoptosis.

An important feature of apoptotic pathways is the activation of specialised cysteine proteases called caspases. Caspases are expressed as inactive proenzymes and are themselves activated by caspase-mediated cleavage. A cascade of events starts with the activation of initiator caspases (caspases 2, 8, 9, and 10) in a cytochrome-C dependent fashion. This progresses to the activation of effector or executioner caspases (caspases 3, 6, and 7) by the initiator caspases. Executioner caspases proteolytically cleave cellular proteins, both structural and enzymes that are localised in the cytosol, nucleus and plasma membrane. Indirectly, by activating specialised nucleases, they also induce characteristic DNA fragmentation (laddering) of apoptosis, altogether culminating in apoptotic cell death.[52–54]

Several regulatory pathways have been identified that regulate neutrophil apoptosis, the best understood of which are perhaps the intrinsic pathway, which regulates constitutive apoptosis, and the extrinsic pathway, which is induced by ligation of death receptors (TNF and FAS receptors). The caspase involvement differs between these pathways[55] (**Figure 1.6**), however these pathways have in common a key role of Bcl-2 family members, with individual family members having pro- and anti-apoptotic functions. In the neutrophil, the prominent pro-apoptotic Bcl-2 protein is Bax, which can associate with, and permeabilise the outer mitochondrial membrane to induce the release of cytochrome C. The major anti-apoptotic member is Mcl-1, and blocks Bax.[56–58] With Mcl-1 having a shorter half-life than Bax, its degradation essentially precedes the onset of apoptosis.

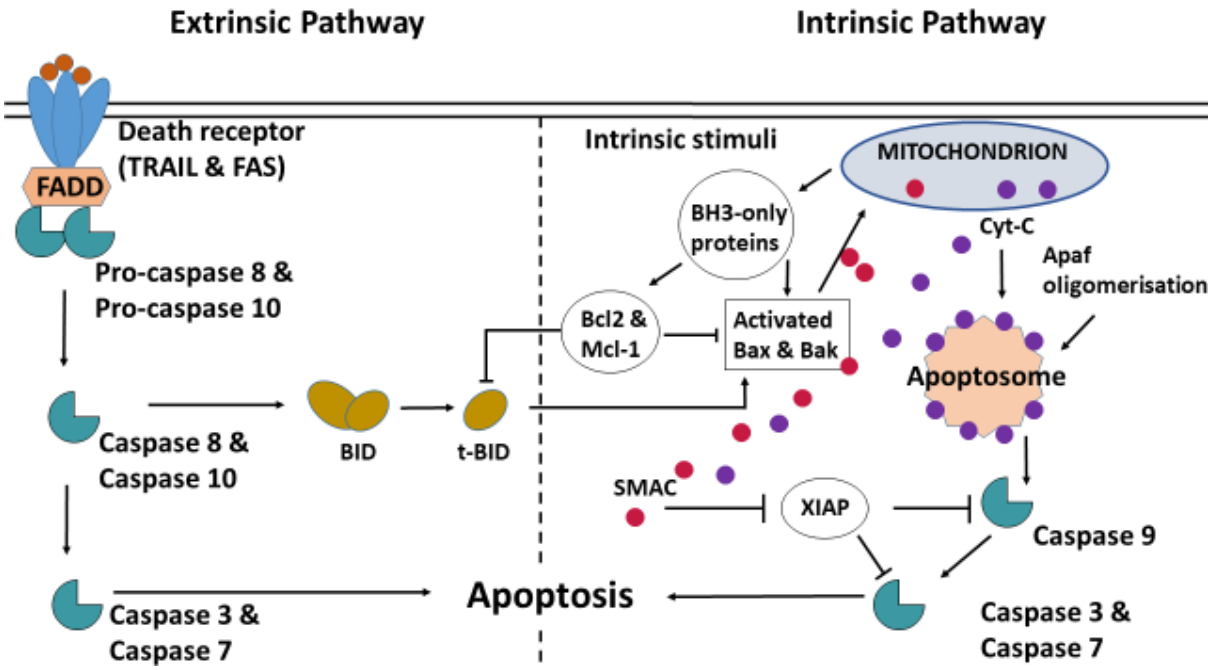


Figure 1.6. Extrinsic and intrinsic pathways of apoptosis.

Two signalling pathways of apoptosis are shown. Extrinsic apoptosis is initiated by the ligation of death receptors by death ligands such as the Fas ligand. This allows binding of FADD and the downstream activation of caspase-8 & -10. Intrinsic stimuli cause cellular stress, followed by the release of cyt C from mitochondria and, ultimately facilitating caspase 9-dependent assembly of the Apaf-1-containing apoptosome. Release of the SMAC deactivates the proteins hindering apoptosis XIAPs, thus allowing apoptosis to continue. The extrinsic and intrinsic signalling pathway meet and align upon activation of executioner caspases-3 and -7, which then continue to cleave downstream effectors to mediate apoptosis.

FADD, Fas-associated protein with death domain; cyt-C, cytochrome C; Apaf-1, Apoptotic protease activating factor-1; SMAC, second mitochondria-derived activator of caspases; XIAP, X-linked inhibitor of apoptosis protein.

1.3.1.2. Interplay between ROS, proteases and apoptosis.

Being highly toxic at elevated concentrations, ROS not only kill microbial intruders but, they also cause significant oxidative stress to the neutrophil. Moreover, if released to the outside of the neutrophil, they can promote host cell damage. Indeed, the concept of prolonged ROS production is thought to be central to the progression of inflammatory disease. In contrast, at low concentrations, ROS contribute to neutrophil survival (**Figure 1.7**). The in between concentration is referred to as 'physiological concentration when ROS play an important role in regulating cell growth, the adhesion of cells toward other cells, differentiation, senescence, and apoptosis.[59,60]

ROS and the associated oxidative damage they can trigger moreover play an important role in the interplay between cell death signalling pathways. The majority of molecular pathways causing neutrophil apoptosis depend on ROS generation.[61] However, excessive ROS production may also result in other forms of neutrophil cell death due to ROS-induced damage of essential lipids, proteins and DNA. Cells have developed several ROS scavengers such as catalase, glutathione peroxidase and glutathione reductase to prevent necrosis.[59]

Moreover, proteases within neutrophil granules can contribute to the apoptotic cascade. Azurophil granules contain three structurally similar serine proteases with microbiocidal activity, proteinase-3, cathepsin G and elastase. Cysteine proteases including cathepsins were shown to be released during apoptosis and promote caspase-3 activation.[62] Additionally, the cytosolic non-caspase cysteine protease calpain can mediate neutrophil apoptosis.[63] Calpains have been shown to cleave Bax and autophagy gene 5 (Atg5), and to deactivate X-linked inhibitor of apoptosis protein (XIAP), thus causing neutrophil apoptosis.[64–66]

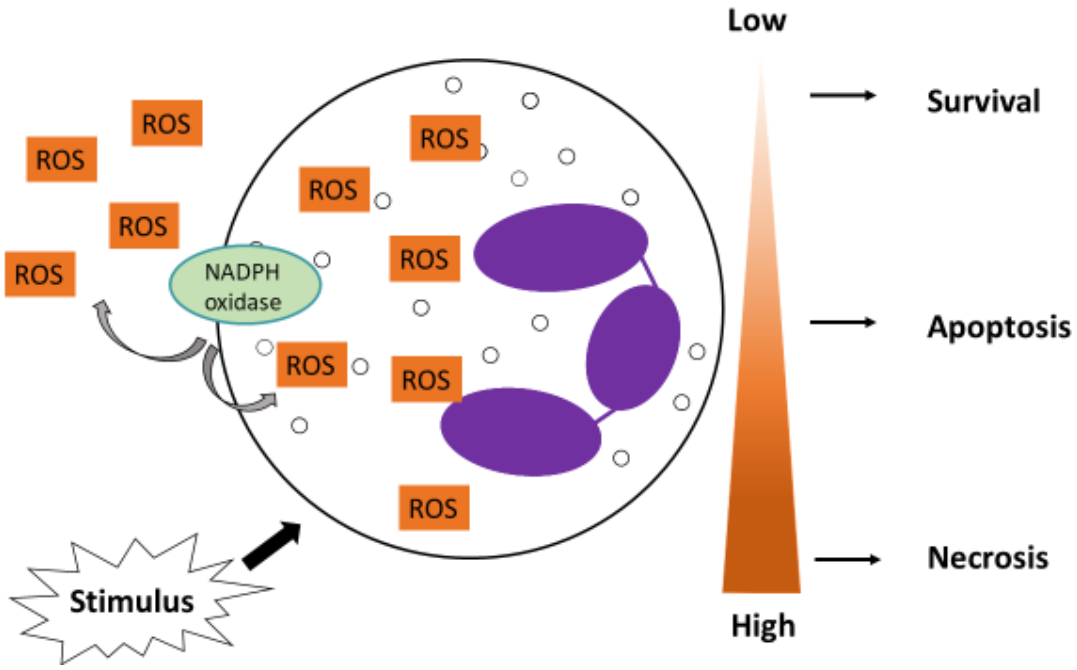


Figure 1.7. ROS mediated neutrophil cell death.

Upon exposure to various stimuli, neutrophils generate NADPH oxidase-mediated ROS. Depending on the level and localisation of ROS, neutrophils may die by apoptosis or necrosis, but may also show increased survival.

ROS, reactive oxygen species; NADPH, nicotinamide adenine dinucleotide phosphate.

1.3.1.3. Phagocytosis induced cell death

Neutrophil apoptosis not only contributes to the regulation of neutrophil cell numbers, but also guaranties the safe disposal of engulfed bacteria.[67] A specialised form of apoptosis, phagocytosis induced cell death (PICD), is a benign pathway by which neutrophils undergo apoptosis after phagocytosing and ingesting a pathogen. During this process, neutrophils also generate an immense amount of ROS, which is thought to be the driving force for the induction of apoptosis. PICD moreover drives anti-inflammatory cytokine production through clearance of the apoptotic cell by resident or infiltrating macrophages. Together these two processes limit subsequent damage to surrounding healthy tissues and promote the resolution of inflammation.[32,68–70]

Interestingly, the mechanism of PICD is not identical to constitutive apoptosis. The apoptosis mechanism in PICD was termed as ‘apoptosis differentiation programme’. The regulation of genes and transcription factors are different in PICD. This term is most commonly associated with internalisation and ingestion of bacteria followed by cell apoptosis that triggers potent antimicrobial activity. The initial stages of PICD are accompanied by changes in the expression of genes encoding apoptosis mediators, such as Bax and Bcl2,[71] as well as important regulators of detoxification and redox pathways, such as those governing glutathione-, thioredoxin-, and heme metabolism.[72] The mechanism of PICD is regulated by ROS, particularly involves NADPH oxidase-stimulated Lyn and SHIP molecules that promotes release of toxic granules into the phagocytic vacuoles. These ROS substances further promote cleavage of caspase 3 and caspase 8 to induce apoptosis. Neutrophils are the key cells in the mechanism of PICD since they are able to produce immense amount of ROS unlike the macrophages and hence, have more bactericidal properties. Moreover,

the bacteria-derived products such as *Pseudomonous aeruginosa* exotoxin pyocyanin also modulate PICD.[67]

Interestingly, reduced or absent PICD in neutrophils derived from chronic granulomatous diseases (CGD) patients is associated with delayed resolution of inflammation, which could contribute to the granuloma formation in CGD patients. Leukocytes from CGD patients cannot produce ROS due to a defect in the assembly of the NADPH-oxidase[73] establishing that PICD is a ROS dependent phenomenon.

PICD is therefore an anti-inflammatory neutrophil function that is ROS dependent and characterised by neutrophil apoptosis following pathogen internalisation. The mechanism of PICD is further investigated in the context of insoluble immune complexes in **chapter 3**.

1.3.1.4. Regulation of apoptosis as a therapeutic strategy

Prolonged survival of neutrophils can be detrimental for the immune system (as shown in **Figure 1.8**). Dysfunctional clearance of apoptotic cells has been linked to the pathogenesis of inflammatory and autoimmune diseases, in particular systemic lupus erythematosus (SLE).[74]

Therapeutic manipulation of neutrophil viability could be used as a tool to control resolution of inflammation. Some drugs are already in the clinical trials or have been approved for use in the clinic[75,76] while others are only limited to animal studies or still being conceptualised as a potential therapy. Different strategies to induce neutrophil apoptosis include neutralising XIAP[77] and inhibiting cyclic dependent kinases by Roscovitine.[78] Targeting the apoptotic pathway to promote resolution has also been investigated at the level of a mitogen-activated protein kinase (MAPK) signalling and by targeting proteins of the Bcl-2 family such as inhibiting Mcl-1 activity.[79,80]

Interestingly, in CGD patients, ROS was shown to be inversely related to inflammation. Absence of ROS was associated with enhanced inflammation according to a study conducted in a large cohort of 368 CGD patients.[81] Therefore, increasing ROS production has been considered as a therapeutic strategy in inflammatory bowel diseases and rheumatoid arthritis (RA). Sulfasalazine is one such drug that induces NADPH oxidase activity, thus promoting neutrophil apoptosis. Interestingly, sulfasalazine selectively induces neutrophil apoptosis but not in other leukocytes.[82] There is still much left to discover about the mechanisms regulating neutrophil life and death.

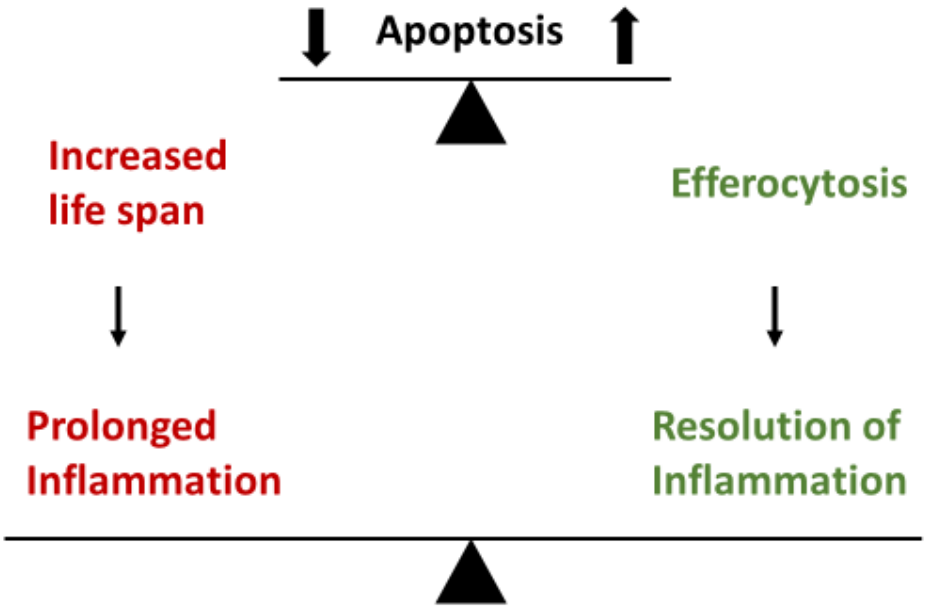


Figure 1.8. Regulation of apoptosis.

A fine balance of apoptosis is required to maintain tissue homeostasis since reduction in apoptosis may lead to prolonged survival of cells causing prolonged inflammation whereas increasing apoptosis might facilitate efferocytosis and thus, promoting resolution of inflammation.

1.3.2. NETosis

NETosis is a fast cell death programme in which neutrophils undergo self-disruption, extruding fibrillary networks [neutrophil extracellular traps (NETs)] composed of decondensed DNA with citrullinated histones, neutrophil elastase, myeloperoxidase and antimicrobial granule peptides. A key step in NET formation is citrullination, a post-translational modification of Arginine residues. Citrullination is mediated by peptidyl arginine deminiases (PADs), in particular PAD4, expression of which is restricted to leukocytes. Neutrophil elastase and MPO from the ROS pathway have also been shown to be involved in NET production.

NETosis can be triggered by microorganisms and endogenous stimuli, such as bacteria, fungi, viruses, crystals, DAMPs and immune complexes.[83,84] Ligation of a number of receptors by these stimuli activates NETosis through various downstream effector proteins. NETs are highly toxic and can mediate extracellular killing; they may entrap and kill bacteria, fungi, and protozoan pathogens. NETosis is preferentially deployed by neutrophils upon an encounter with a large microorganism such as *Candida albicans* when phagocytosis is impossible.[85]

However, in pathological conditions, NETs are associated with tissue damage in chronic inflammatory diseases including autoimmune diseases such as in RA and SLE.[86] NETs have been shown to be cleared by DNAse 1 *in vitro* however, the dynamics of NET clearance by endogenous enzymes remain poorly understood.[86,87]

Following the notion, neutrophil death can be divided into two groups, either pro- or anti-inflammatory. Selected pro- and anti-inflammatory effector functions of neutrophils are summarised in **Figure 1.9**.

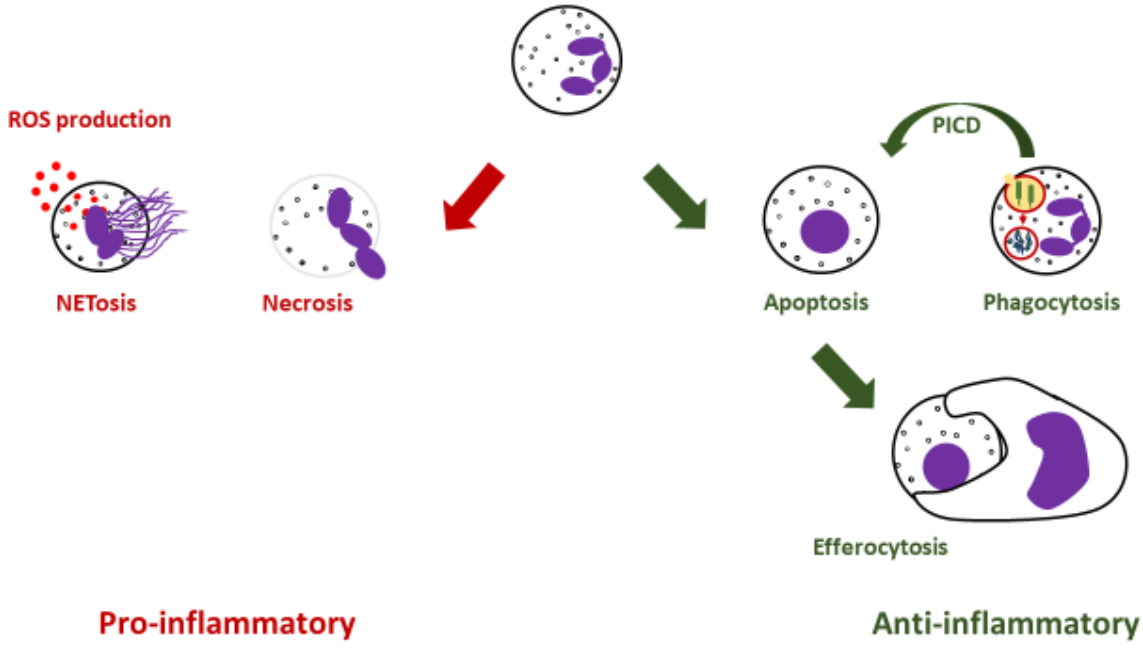


Figure 1.9. Pro- and anti-inflammatory responses of neutrophils.

Pro-inflammatory responses of neutrophils are indicated by red arrows and anti-inflammatory responses by green arrows. Abundant extracellular ROS as well as cellular death either by NETosis or necrosis are considered pro-inflammatory in nature. On the other hand, apoptosis, either constitutive or PICD-induced, promotes efferocytosis and therefore the elimination of apoptotic cells by other phagocytes such as macrophages. This is considered anti-inflammatory.

ROS, reactive oxygen species; NET, neutrophil extracellular trap; PICD, phagocytosis-induced cell death.

1.3.3. Other forms of cell death

In addition to apoptosis, increasing evidence has led to a better understanding of other forms of neutrophil cell death such as necrosis, necroptosis, pyroptosis, ferroptosis and autophagic cell death.[84] Among these different types, necrosis is the most common and acts as a trigger for inflammation. Necrosis is characterised by loss of plasma membrane integrity and release of cytosolic components to the outer environment causing cellular toxicity. Different stimuli such as pathogens, irradiation, heat, ischaemia or cytokines cause necrosis. Moreover, if apoptotic neutrophils are not properly cleared, they exhibit danger signal and progress to secondary necrosis. Initially, necrosis was suggested to be a passive mode of cell death and hence no specific mechanism was associated with it. However, it is now clear that necrotic cell death has multiple subtypes, for example necroptosis which is mediated by mixed lineage kinase domain-like, receptor interacting serine/threonine kinase 1 and 3 (RIPK1 and RIPK3),[88] whereas pyroptosis, is a caspase-1-mediated cell death.[89] Other forms of non-apoptotic cell death include ferroptosis which is dependent on iron ions[90] and autophagy which is dependent on several Atg proteins.[64] Although these new subtypes of cell death share some common mechanisms, they may be differently regulated. However, there is not enough evidence on the physiological activity of these new subtypes of cell death and on the differential response of other immune cells to these types of cell death.

1.4. Signalling in neutrophils

1.4.1. PI3K signalling in neutrophils

Phosphoinositide 3-kinase (PI3K) is a group of plasma membrane-associated lipid kinases that catalyses the phosphorylation of one or more inositol phospholipids in the 3-position of the inositol ring.[91] Both the substrates and products of these reactions are phospholipids. There are three different classes of PI3K, among which class I PI3Ks, also known as receptor-activated PI3Ks, are best understood. Class I PI3K is responsible for converting phosphatidylinositol-4,5-bisphosphate (PI4,5P2) to the lipid second messenger phosphatidylinositol-3,4,5-trisphosphate (PI3,4,5P3), in short PIP3.[92,93] Signalling downstream of PI3K occurs through effectors that are capable of binding to specific PI3K products via specialised binding domains. Class I PI3K regulates many cellular processes; in the neutrophil these include chemotaxis, ROS production, phagocytosis and apoptosis. PI3K activity is mediated through a large number of signalling pathways, including extracellular regulated kinases (Erk) and p38MAPK.[94,95] Activation of PI3K and MAPK signalling induces transcriptional activity of NF-kB, which was shown to be critically involved in enhancing neutrophil survival.[96] Another example of a pro-survival function of PI3K is GM-CSF-stimulation of neutrophils, which results in PI3K-dependent Mcl-1 protein expression.[97]

Four class I PI3K isoforms exist, α- δ, all of which are expressed by the neutrophil. PI3Kβ and δ are activated by ligation of integrins and FcγRs. PI3Kβ and δ were required for a maximal ROS production and degranulation response of neutrophils spreading on immobilised immune complexes,[98] Insoluble immune complex-induced neutrophil apoptosis was found to be dependent upon both PI3K and Erk in a non-canonical signalling pathway, PI3Kβ/δ-Cdc42-Pak-Mek-Erk, contrasting with their typically anti-apoptotic function.[99]

1.4.2. Fc Receptors in neutrophils

Fc receptors, a family of membrane proteins expressed by neutrophils, are capable of interacting with the constant region of immunoglobulins. Fc receptors are important mediators of immune responses, regulating a range of neutrophil functions such as phagocytosis, degranulation, production and secretion of chemokines, as well as, in certain situations antigen presentation.[100,101] Fc receptors are named according to the immunoglobulin heavy chain isotype they bind to, i.e. IgG binds to Fcγ receptors. Functionally, human FcγRs can be divided in two groups; the activating and inhibitory receptors (**Table 1.1**). The signal-transducing chain of activating FcγRs are associated with an immunoreceptor tyrosine-based activating motif (ITAM) in their cytoplasmic tail. In contrast, the inhibitory FcγR carries an inhibitory (ITIM) sequence in the cytoplasmic tail.[102]

Human neutrophils constitutively express the low affinity FcγRIIA (CD32A) and FcγRIIIB (CD16B) in large numbers. FcγRIIA and FcγRIIIB both promote IgG mediated neutrophil accumulation in tissues, while only FcγRIIA induces tissue injury.[103–105] In contrast, the high affinity FcγRI (CD64), which is constitutively expressed by monocytes/macrophages and dendritic cells (DCs) is restricted to activated neutrophils. Interestingly, a recent reports suggested that IgG-containing immune complexes induce neutrophil FcγRI expression in a FcγRII-dependent fashion, providing a mechanism for this.[106] Both FcγRI and FcγRIIA have been shown to promote cytotoxic functions. The human inhibitory FcγRIIB (CD32B) is highly expressed on circulating B cells and basophils, but only observed on 4% of circulating neutrophils.[107] Interestingly, FcγRIIB expression was observed on synovial fluid derived neutrophils in rheumatoid arthritis.[108] Similarly, FcγRIIC is rarely expressed in neutrophils.[109] Finally, the neonatal Fc receptor (FcRn) is best known for its function

in transporting IgG from mother to fetus, and its expression on the embryonic part of the placenta, but also some epithelial and endothelial cells. Unlike the other FcγRs, FcRn only binds IgG in the context of an acidic pH. Interestingly, FcRn was shown to be localised to neutrophil azurophil granules and secretory vesicles, and to translocate to phagosomes, to aid phagocytosis of poorly IgG-opsonised bacteria.[110]

Table 1.1. Human IgG receptors in neutrophils.

	FcγRI	FcγRIIA	FcγRIIB	FcγRIIC	FcγRIIIB	FcRn
Affinity	High	Low	Low	Low	Low	High
Function	Activation	Activation	Inhibition	Activation	?	Recycling, transport
IgG Subclass binding	1 3 4	1 2 3 4	1 3 4	1 3 4	1 3 4	1 2 3 4

1.4.3. Crosstalk between Fcγ receptors & integrin receptors in neutrophils

Integrins are a family of transmembrane receptors that is a heterodimer formed by an alpha (α) and a beta (β) chain. The name integrin was given to these receptors because they bridge the extracellular matrix with the intracellular cytoskeleton. Integrin signalling provides very important regulatory signals that control cell growth, differentiation, and survival. Regulation of the integrin ligand binding affinity is regulated by 'inside-out signalling', where the integrin responds to signals generated from inside the cell via formation of adhesion complexes.[111] This is distinct to 'outside-in signalling', where the cell responds to ligand binding by integrins. Neutrophils express a range of integrins, including the very abundant lymphocyte function-associated antigen (LFA-1, also known as αLβ2; CD11a/CD18) and αMβ2 (Mac-1; CD11b/CD18; CR3), which not only functions as an integrin but also as complement receptor-3.[112–116]

Interestingly, cross-talk between β2 integrins, specifically αMβ2, and FcγRs has been documented by a number of research groups. In human neutrophils αMβ2 contributed to sustained FcγR-mediated neutrophil adhesion to immobilised ICs, which interestingly promoted PI3K- and p21-activated kinase (Pak1)-dependent inside-out activation of αMβ2[117] (**Figure 1.10**). Both αMβ2 and FcγRIII were required to enable FcγRII-dependent ROS production.[118] Similarly, in mouse neutrophils binding of neutrophils to immobilised anti-integrin antibodies was dependent on co-stimulation of FcγRIII.[119] αMβ2 was also required for neutrophils to bind to immobilised intravascular immune complexes under flow conditions,[120] and αMβ2 deficiency contributed to milder disease in a model of immune complex-induced glomerulonephritis although initial neutrophil recruitment was unaffected.[121] These examples suggest a co-stimulatory functions of αMβ2 and FcγRs, however, the opposite has also been shown.

In a mouse model for lupus, αMβ2 conferred protection from FcγRIIA-dependent disease development.[122] A potential explanation could be αMβ2 binding to FcγRIIA which lowers FcγRIIA (but not FcγRIIIB) affinity to immune complexes in a glycosylation-dependent fashion in gene edited cell lines.[123] FcγRIIA and FcγRIIIB were also shown to interact with integrin αMβ2 on the membrane of human neutrophils.[124]

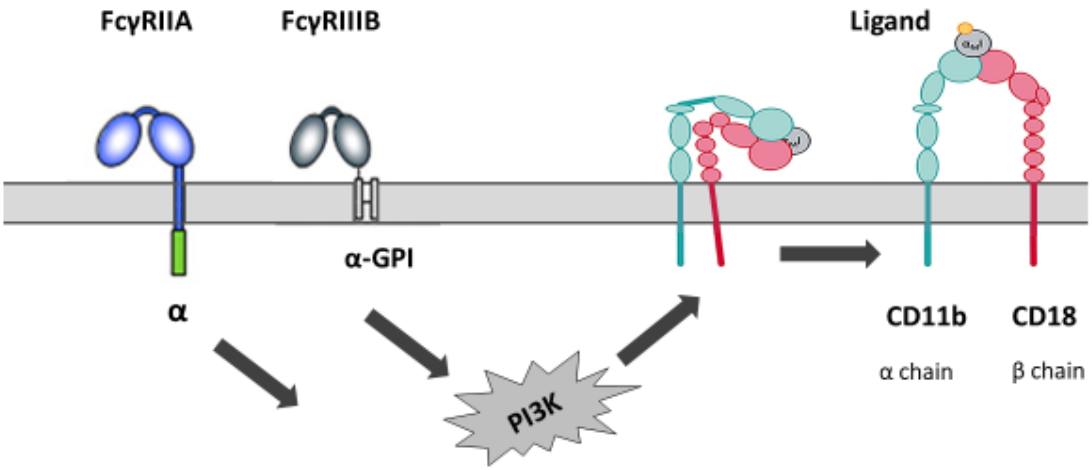


Figure 1.10. Crosstalk between Fcγ receptors & integrin receptors in the neutrophil.

"Inside-out signalling" activates αMβ2 secondary to stimulation of Fc receptors. On the neutrophil, stimulation of either FcγRIIA or FcγRIIIB leads to αMβ2 activation through a pathway that requires the activity of PI3K.

1.5. Efferocytosis and the resolution of inflammation

Although about one million cells undergo apoptosis per second in the human body, apoptotic cells are rarely accumulated in body tissues under normal physiological condition. The efficient system for prompt removal of these apoptotic cells by macrophages is called efferocytosis. It prevents secondary necrosis and the release of cellular debris, thus avoiding the escalation of inflammation. Any disturbance in efferocytosis can promote diseases including autoimmune diseases, atherosclerosis and cancer. Efferocytosis is divided into four steps: i) recruitment, ii) recognition, iii) tethering and signalling, and iv) engulfment. Each of these steps is tightly regulated to ensure that clearance of apoptotic cells occurs correctly and efficiently.[125–127]

A prerequisite for efferocytosis is the induction of apoptosis in the target cell. Apoptotic cells release 'find me' signals that include chemokines (CX3CL1) and lipid lysophosphytidlycholine (LPC). Macrophages detect these signals and are recruited to the apoptotic neutrophils as a result (first step). Upon close contact, macrophages then recognise the 'eat me' signals on the surface of apoptotic cells using specific cell surface receptors.[128] The best characterised 'eat me' signal is the exposure of phosphatidylserine (PtdSer) by the apoptotic cell (second step). Unstimulated macrophage receptors interact with, and efferocytose apoptotic cells displaying PtdSer indirectly via integrin αvβ3 (the vitronectin receptor) which recognises bridging molecules such as thrombospondin with CD36.[129] The TAM family receptor MerTK is an important efferocytosis receptor of anti-inflammatory macrophages,[130] using bridging molecules Gas6 and Protein S that are secreted by macrophages to bind PtdSer.[130,131] Interestingly, apoptotic neutrophils can also express 'don't eat me' signals on their surfaces, which include CD47, CD31, proteinase 3 and plasminogen

activator inhibitor-1 (PAI-1), and which delay their removal by macrophages.[132,133] Macrophage interaction and apoptotic cells is simplified in the **Figure 1.11**.

As a result of these interactions, a Rac1-mediated signalling pathway is activated that results in internalisation of the apoptotic cell (third step). This concludes with degradation of the apoptotic neutrophil (and any germs within it) by the lysosomal route within the phagocytes (fourth step). Phagocytes form a large vacuole containing all the lipid, proteins and nucleic acids derived from the apoptotic cells. Post engulfment anti-inflammatory cytokines are released that include PAF, IL-10 and transforming growth factor beta (TGFβ).[134]

Insufficient efferocytosis can lead to chronic autoimmune disease caused by the accumulation of unengulfed apoptotic cells. This stimulates the generation of anti-DNA and anti-RNP autoantibodies, which can induce systemic autoimmunity. Genetic studies in mice have shown that defects in the phagocytic receptor MerTK, MFGE8, and Gas6 also promote autoimmunity.[135–137]

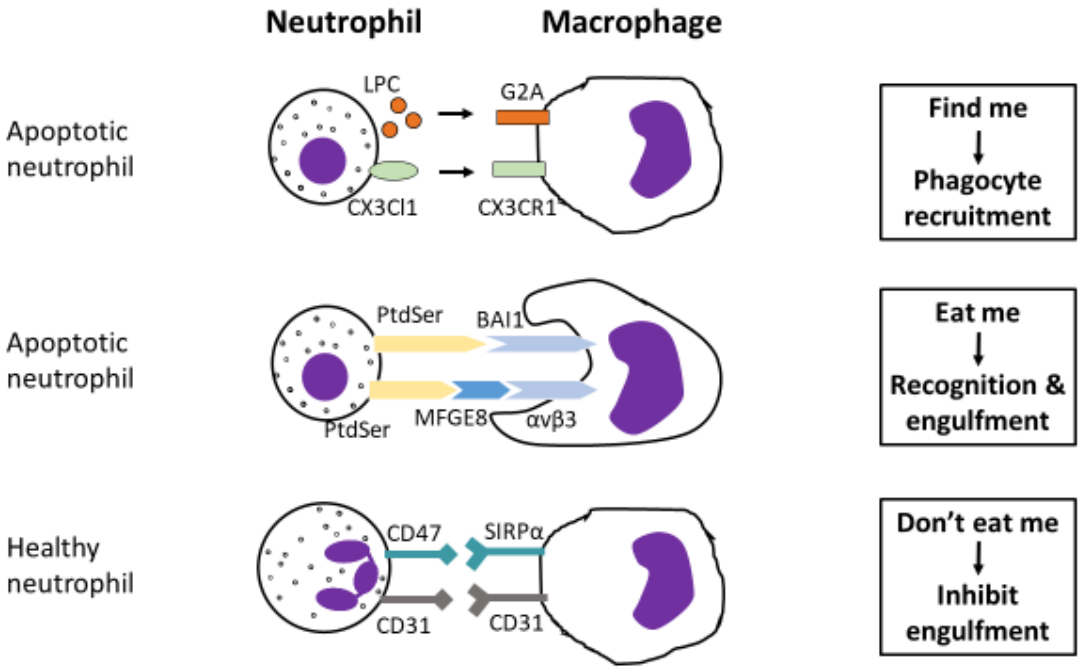


Figure 1.11. Interactions of efferocytosis signal molecules.

Apoptotic cells release "Find me" signal, such as LPC and CX3CL1, recruiting phagocytes to sites of cell death. Phagocytes such as macrophages sense these signals via cognate receptors (G2A and CX3CR1, respectively). Apoptotic cells therefore expose a variety of "Eat me" signals on their surfaces and interact with receptors on the phagocyte membrane. The most common "Eat me" signal, PtdSer, can interact with a variety of receptors on the phagocyte surface, such as directly binding to BAI1 or indirectly to αvβ3 by bridging molecules such as MFGE8. Healthy cells protect themselves from phagocytosis by expressing "Don't eat me" signals, such as CD47 and CD31, which binds to receptors (SIRPα and CD31, respectively) on phagocyte and inhibits efferocytosis (Modified from Lili Wang et al, Front Endocrinol, 2021).[137]

LPC, Lysophosphatidylcholine; G2A, G=protein-coupled receptor; CX3CL1, CX3C chemokine ligand 1; CX3CLR, CX3C chemokine receptor 1; PtdSer, Phosphatidylserine; BAI1, Brain-specific angiogenesis inhibitor 1; MFGE8, Milk fat globule EGF 8; SIRPα, Signal regulatory protein α.

1.6. Immune complexes

Whenever there is an antibody response following an encounter with a foreign particle including pathogen, the antibody meets an antigen and an immune complex is formed. Whilst the formation of immune complexes is an integral component of the immune defence mechanism, excessive immune complex formation can be detrimental to the host in that it can interfere with normal physiological processes. Although initial or a small proportion of immune complex formation could be defined as a residual product of an acute inflammatory response, they certainly play a major contributing role through impacting on chronic inflammatory processes and innate immune responses.[138-140]

When antibodies bind to antigen, a variety of immune complexes (ICs) may form, that range from an IC consisting of 1 antigen molecule and 1 antibody molecule to large IC consisting of many molecules of each reactant. The lattice of ICs thus reflects the number of antigen and number of antibody molecules in each complex. The lattice of an IC in turn influences its biological properties. The presence of ICs generated from autoantibodies is a hallmark of autoimmune diseases such as RA and SLE. They generally cause neutrophil mediated inflammation.[138–140]

In the past several years, studies have suggested that FcγRs play primary roles in diseases initiated by antibodies. IgG binding to FcγR is dependent on the size of the IC and its glycosylation status.[141] In addition to the strict requirement for FcγRs, complement is required for IC-induced inflammation and subsequent end organ damage.[142] However, other ICs such as IgM-ICs and IgA-ICs have also been shown to play important roles in autoimmunity.[143]

The tendency of ICs to form a lattice and precipitate depends upon many factors such as affinity of antibody for antigen, valence of the antigen, and the ratio of antibody to antigen.[144] At the point of equivalence (as shown in the **Figure 1.12.A**) the maximal amount of antigen- antibody precipitate is formed; neither free antigen nor free antibody are detectable in the supernatant. Addition of excess antigen beyond the point of equivalence leads to the formation of soluble immune complexes. ICs can be present in different forms, soluble, insoluble and also immobilised when deposited on the tissue surfaces such as on arthritic joints. Soluble and insoluble ICs are found in the circulation and also in other biological fluid of patients such as synovial fluid as well as in secretion. In homeostatic conditions, these ICs are removed from the circulation by the mononuclear phagocytes from liver and spleen through engagement of Fcγ receptors and complement receptors.[145-148]

Evidence in humans and animal models suggests that circulating ICs first localise within the vasculature and then translocate into extravascular tissue. Indeed, ICs are cleared less efficiently[145–147] and these ICs were shown to promote vascular leakage in the joint tissue in RA.[148] ICs size and the antibody class it is made up of both affect the extent and location of IC deposition. Moreover, the complement component C1q affects the lattice structure of ICs and facilitates deposition.[149–152]

ICs play an important role in the pathogenesis and progression of RA and SLE. Different ICs are formed in RA and SLE based on the antibodies mentioned is **Table 1.2**. In SLE, deposition of immune complexes in several organs, mainly kidney, skin, and joints, causes inflammation and tissue damage, producing a broad spectrum of clinical manifestations whereas in RA, the ICs deposit on the joint surfaces.[142,153]

Both soluble and insoluble ICs activate neutrophils but in different ways. Insoluble ICs (iICs) activate unprimed neutrophils and slowly produces moderate amount of ROS whereas soluble ICs (sICs) are unable to activate unprimed neutrophils but produce rapid and extensive amount of ROS when primed with GM-CSF.[154]

However, the ICs not necessarily cause only tissue damage but they also promote immunomodulatory functions. Different pro- and anti-inflammatory mechanisms of ICs are summarised in **Figure 1.12.B**).

Table 1.2. Most common autoantibodies produced in autoimmune diseases.

ACPA, Anti-citrullinated protein antibodies; Anti-CarP, Anti carbamylated peptide antibodies; ANA, Anti-nuclear antibodies; anti-dsDNA, anti-double stranded DNA antibodies; RNP, Ribonucleoproteins; APLA, Anti-phospholipid antibody; ANCA, Anti-neutrophil cytoplasmic antibody; SLE, systemic lupus erythematosus

Auto-antibodies	Disease	Type
Rheumatoid Factor	Rheumatoid Arthritis	IgM
ACPA	Rheumatoid Arthritis	IgG
Anti-CarP	Rheumatoid Arthritis & SLE	IgG & IgM
ANA	SLE	IgG & IgA
Anti-dsDNA	SLE	IgG, IgM & IgA
Anti-RNP	SLE	IgG & IgM
APLA	SLE- complication	IgG
ANCA	ANCA associated vasculitis	IgG, IgM & IgA
Anti-histone	Drug induced SLE	IgG & IgM
Anti-neutrophil Ab	Autoimmune neutropenia	IgG & IgM

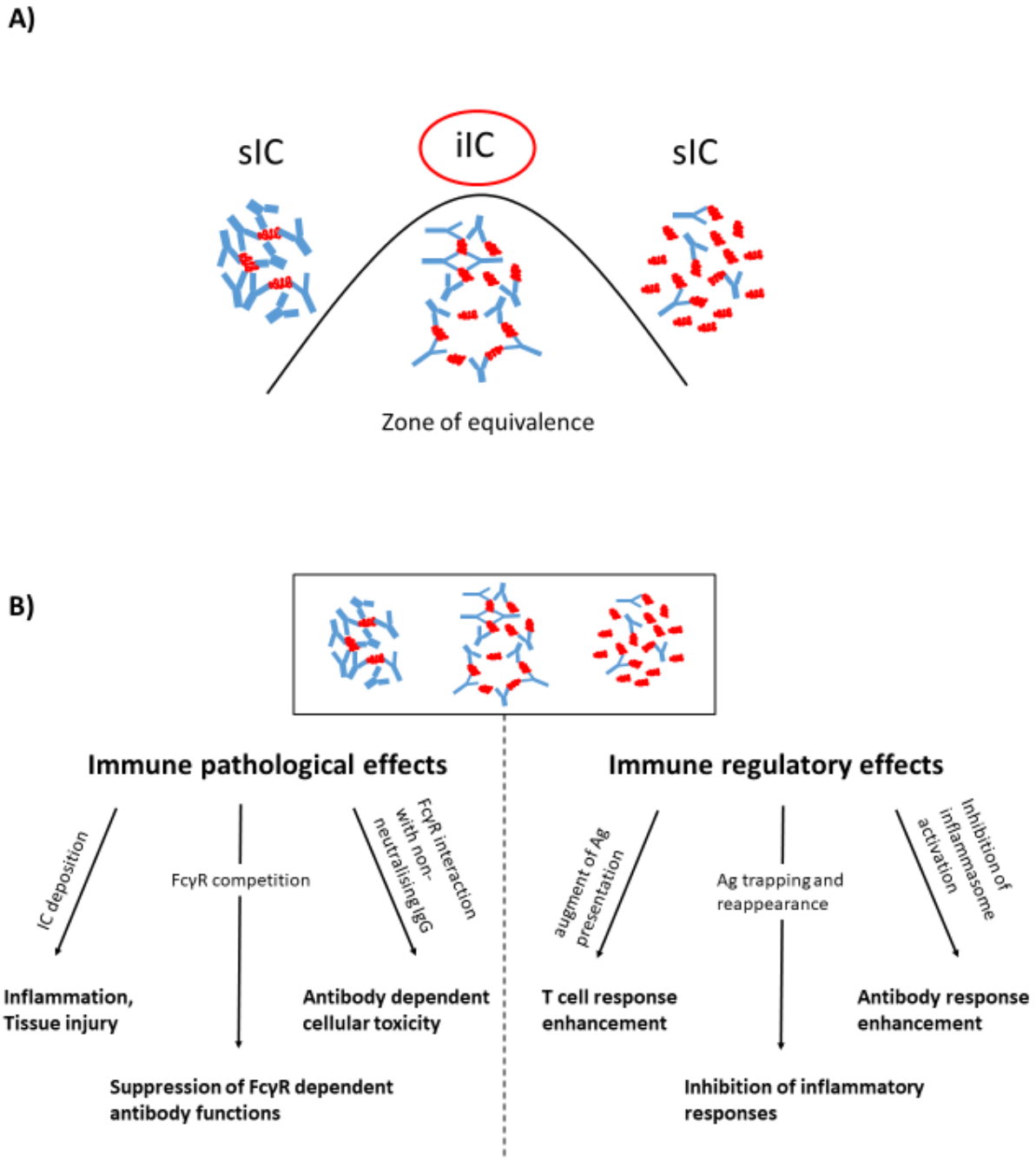


Figure 1.12. Types of immune complexes and their major functions.

A shows different forms of ICs, sIC (soluble immune complexes) and iIC (insoluble immune complexes). **B** shows iIC-induced pro- and anti-inflammatory functions and their mechanisms.

1.7. Autoimmune diseases

1.7.1. Rheumatoid arthritis

Rheumatoid arthritis is a chronic, inflammatory joint disease of autoimmune nature. Approximately 1% of the world population is suffering from RA and the symptoms include classical joint pain and stiffness. Although the exact cause of RA is still unknown, several risk factors such as genetics, female sex, environmental factors, smoking, Vitamin D deficiency, obesity and changes in the microbiota have been identified.[155] RA is characterised by autoantibodies to IgG, in particular, rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPAs) and/or anti carbamylated protein antibodies (anti-CarP), where both citrullination and carbamylation are post-translational modification of proteins (**Figure 1.13**). The presence of RF and ACPA defines the seropositive status of RA, with approximately 70% of all disease cases being seropositive. The two RA subsets, seropositive and seronegative, have different disease courses with the seropositive form typically causing more bone and joint destruction and they respond better to immunotherapy.[156]

Cardio vascular diseases are one of the major complications of autoimmune disease. Rheumatoid factor (IgM anti-Fc IgG antibody) has been reported to represent an independent risk for the development of heart disease in patients with RA. Neutrophils were found in the atheromatous plaque on atherectomy specimens from patients with myocardial infarction, suggesting that they may contribute to plaque destabilisation. Atheromatous lesions contain markers of neutrophil degranulation such as MPO and neutrophil proteases, indicating the presence of active neutrophils.[157] Widespread systemic inflammation such as pulmonary fibrosis, Sjögren's syndrome, and vasculitis are the other common complications.[158–160] Felty’s syndrome is another rare severe

complication of RA which is characterised by severe, long-standing, seropositive RA, neutropenia, and splenomegaly.[161]

The treatment of rheumatoid arthritis is based on the American College of Rheumatology criteria (ACR) or The European League Against Rheumatism (EULAR) criteria and the prognosis is observed by recording the disease activity score (DAS28). Although RA cannot be cured, with the advent of improved therapeutics in recent years remission has become an achievable goal.[162,163] Treatment strategies are summarised in **Table 1.3** which includes common biologic and non-biologic agents except for corticosteroids.

The pathogenesis of RA involves several stages that are displayed in **Figure 1.14.A**. Both genetic (such as HLA-DRB1, PTPN22) and non-genetic risk factors alter the milieu of the joint and its surrounding area, in the pre-rheumatic stage of the disease[164,165]. This phase is followed by the pre-clinical phase, when autoantibodies can be detected in bodily fluids long before the onset of any clinical symptoms. Evidence suggests that immune response in RA initiates at the mucosal sites (**Figure 1.13**). Immune cell infiltration into the joint happens in the 'early RA' phase which is followed by 'established RA' where joint pathology and deformity are obvious.[163]

RA is a complicated autoimmune disease with detrimental contributions made by many different cell types, including leukocytes (**Figure 1.14.B**). Neutrophils are abundant in joint lesions of RA patients. Despite this, they are easily overlooked due to their short lifespan, and the efficiency with which apoptotic neutrophils are cleared from tissue. In RA as in other autoimmune diseases they still are an under-investigated immune cell type.[166,167]

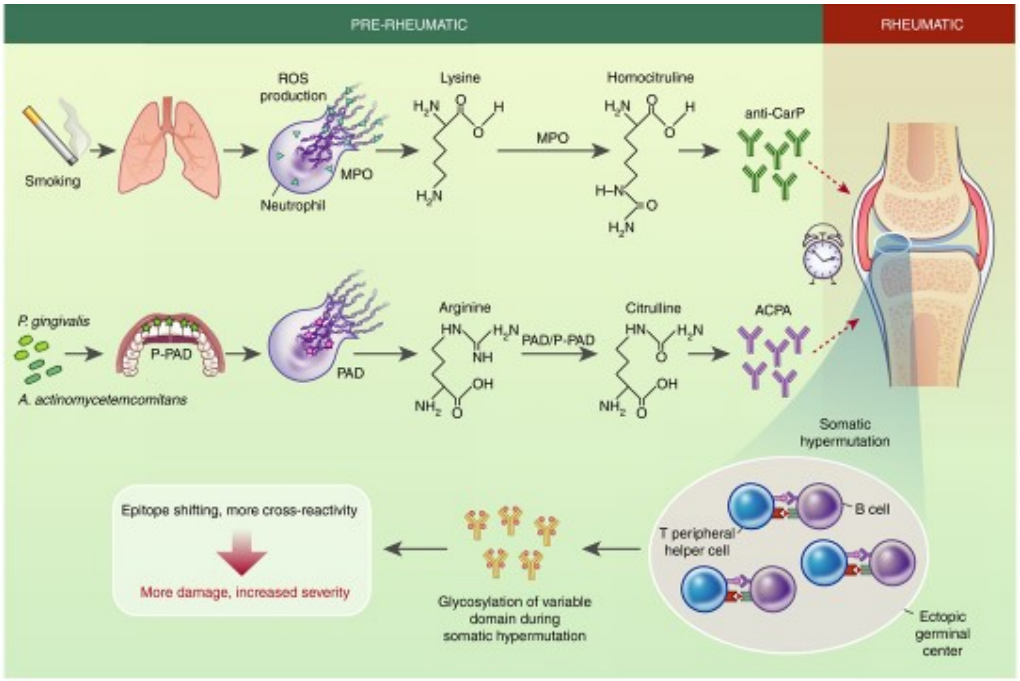


Figure 1.13. Risk factors and post-translational protein/amino acid modifications involved in the pathogenesis of RA.

The immune response in RA initiates at the mucosal sites and development of auto-antibodies such as anti Car-P and ACPA precedes the onset of clinical symptoms by up to a decade. Citrullination and homocitrullination of arginine and lysine respectively lead to generation of these autoantibodies (Pre-rheumatic). In the clinical (rheumatic) phase, autoantibody maturation occurs which involves somatic hypermutation and glycosylation of the variable domain of the antibodies, further leading to tissue damage. Figure taken from Karmakar et al., Immunology, 2021.[176]

RA, rheumatoid arthritis; ACPA, anti-citrullinated peptide antibody; Anti-CarP, Anti-carbamylated peptide antibodies.

However, at least in the context of mouse models neutrophils are critical for the generation of inflammation in RA and also other autoimmune diseases.[168,169] Specifically, neutrophils i) migrate to inflamed joints following gradients of chemoattractants, ii) release cytotoxic mediators (summarised in **Table 1.4**), iii) delay apoptosis. Circulating, and in particular synovial fluid neutrophils of RA patients are activated, more pro-inflammatory and characterised by altered transcriptional signature, suggesting they are also drivers of inflammation in the human disease.[170–175]

In addition, neutrophils are also likely to be important drivers of RA pathogenesis, and enter into a vicious circle with B cells[176] (**Figure 1.15**). ICs stimulate neutrophil effector functions, including NET generation. NETs represent an important source of citrullinated proteins that promoting ACPA formation.[177–179] ACPA predate onset of clinical disease, but their lack of affinity maturation suggests they are constantly being generated from new antigen, suggestive of a fresh source of citrullinated epitope. Given that autoantibodies form ICs, freshly produced NETs are a likely source of these epitopes.[180–184] ICs moreover stimulate MHC expression by neutrophils, turning them into antigen presenting cells[100] that are able to activate with B cells.

Table 1.3. Treatment strategies of RA.

Drugs include biologic and non-biologic (especially DMARD) agents which are prescribed alone or in combination with steroid. DMARD, disease modifying anti-rheumatic drugs; IL, interleukin; TNF, tumor necrosis factor; CTLA, cytotoxic T-lymphocyte-associated protein; SQ, subcutaneous; IV, intravenous.

Names	Type	Dose
Methotrexate	Conventional synthetic DMARD	25 mg every week
Sulfasalazaine	Conventional synthetic DMARD	3 g daily
Leflunomide	Conventional synthetic DMARD	20 mg daily
Hydroxychloroquine	Conventional synthetic DMARD	400 mg daily
Azathioprine	Conventional synthetic DMARD	50 mg once or twice daily
Tofacitinib	Targeted synthetic DMARDs (Janus kinase inhibitor)	5 mg twice daily
Baricitinib	Targeted synthetic DMARDs (Janus kinase inhibitor)	2 mg
Rituximab	Biologic agents (anti-CD20)	1000 mg every 6 months IV
Toclizumab	Biologic agents (anti-IL-6 receptor)	162 mg every 1-2 weeks SQ or 8 mg/kg every 4 weeks IV
Sarilumab	Biologic agents (anti-IL-6 receptor)	200 mg every 2 weeks SQ
Abatacept	Biologic agents (anti-CTLA-4)	125 mg every week SQ
Adalimumab	Biologic agents (TNFα specific antibodies)	40 mg every 2 weeks SQ
Certolizumab pegol	Biologic agents (TNFα specific antibodies)	200 mg every 2 weeks SQ
Etanercept	Biologic agents (TNF receptor-Fc fusion protein)	50 mg every week SQ
Golimumab	Biologic agents (TNFα specific antibodies)	50 mg every month SQ
Infliximab	Biologic agents (TNFα specific antibodies)	3-10 mg/kg every 4-8 weeks IV
Anakinra	Biologic agents (recombinant IL-1RA)	100 mg SQ daily
AMG714	Targeting IL-15	Phase 2 clinical trials
Antibodies specific for p40 & p19	Targeting IL-12 & IL-23	Pre-clinical proof of concept
Antibodies against GM-CSF	Targeting the cytokine itself or its receptor	Pre-clinical proof of concept
Other anti-cytokines	Targeting IL-17 and IL-18	Phase 1 clinical trials

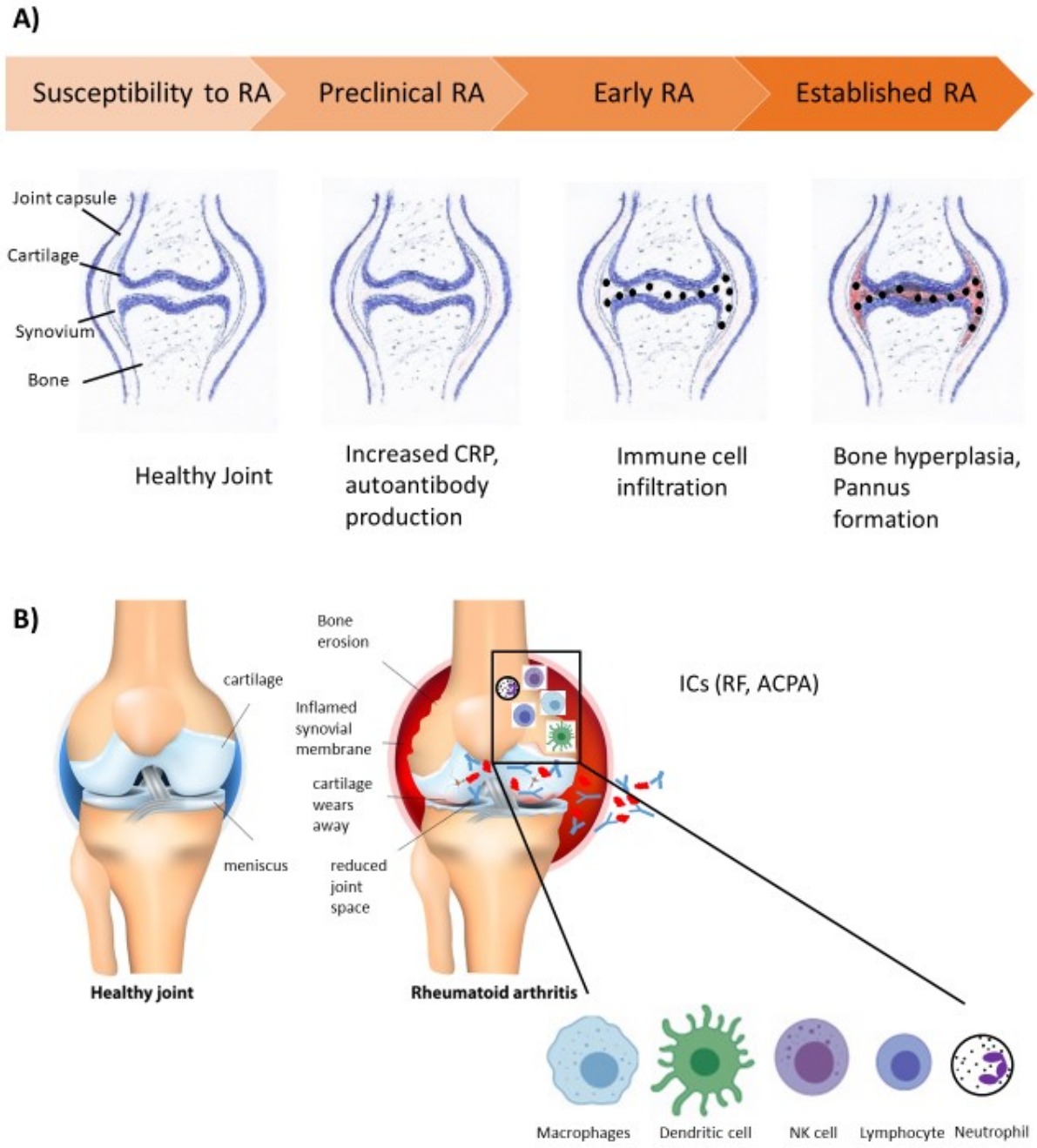


Figure 1.14. Pathogenesis of RA.

A, Different pathological and clinical stages of RA. **B** shows a healthy joint with intake cartilage and meniscus (left) and an arthritic joint characterised by bone erosion, inflamed synovial membrane, loss of cartilage and, reduced joint space (right). The characteristic immunological feature of an arthritic joint is infiltration of immune cells and presence of autoantibodies that form immune complexes.

ICs, immune complexes; RF, rheumatoid factor; ACPA, anti-citrullinated peptide antibody

Table 1.4. Pro-inflammatory cytokines and toxic mediators produced by neutrophils and their selective roles in the pathogenesis of RA.

TNF, tumor necrosis factor; IL, Interleukin; MMP, matrix metalloproteinase; BAFF, B cell-activating factor; APRIL, a proliferation-inducing ligand

Cytokines	Selective functions	Toxic mediators	Selective functions
TNFα	Monocyte activation, PMN priming, oxidative burst	**MMP8 & MMP9**	Recruitment and activation of additional neutrophils
IL-1α & IL-1β	Synovial fibroblast cytokine and chemokine production	**Proteinase 3**	Activates cytokine receptors & promotes inflammation
IL-6	B cell proliferation and antibody production	**Neutrophil elastase**	Contributes to the destruction of joints
IL-8	Chemotaxis and priming	**Cathepsin G**	Activates cytokine receptors & promotes inflammation
IL-12	T-cell and NK-cell cytotoxicity; B-cell activation	**Lactoferrin**	Inhibits chondrocyte apoptosis
IL-15	B-cell differentiation and isotype switching	**BAFF**	B cell proliferation, T cell co-stimulation
IL-18	T cell differentiation, cytotoxicity	**APRIL**	B cell proliferation
IL-17A & IL-17F secreted by Th-17 cells	↑leukocyte cytokine production, serve as neutrophil chemoattractants	**Myeloperoxidase**	Inhibits resolution of inflammation & cartilage repair

1.7.2. Systemic lupus erythematosus

Systemic Lupus Erythematosus (SLE) is an autoimmune disease characterised by autoantibody production against DNA, histone and ribonucleoproteins (RNP), immune complex deposition, and complement activation. SLE is a multi-organ disorder and among the various manifestations of SLE haematological abnormalities are common; they include haemolytic anaemia, thrombocytopenia, lymphopenia and neutropenia. The acute or continuous low-grade chronic inflammation of lupus predisposes patients to infections and cardiovascular diseases. Another dangerous complication is antiphospholipid syndrome which carried an increased risk of blood clots.[185] Neutrophils appear to be inappropriately activated in SLE patients. Apoptosis and NETosis are deregulated, and clearance of apoptotic bodies is diminished, as is the capability to degrade NETs, together providing an overload of self-antigens that under normal circumstances would not be available to be targeted by the immune system. Apoptotic cells are likely to be the most likely source of material that promotes autoantibody production. The most common autoantibodies found in SLE are anti-nuclear antibodies (ANA). SLE is also characterised by high levels of circulating ICs, which are deposited in the kidney and cause damage, ultimately resulting in lupus nephritis.[186–188]

The most common autoantibodies produced in RA and SLE are summarised in **Table 1.2.** Autoimmune diseases with RA and SLE that are associated with autoantibodies, IC production and deposition include vasculitis, glomerulonephritis and autoimmune hepatitis.[189–191]

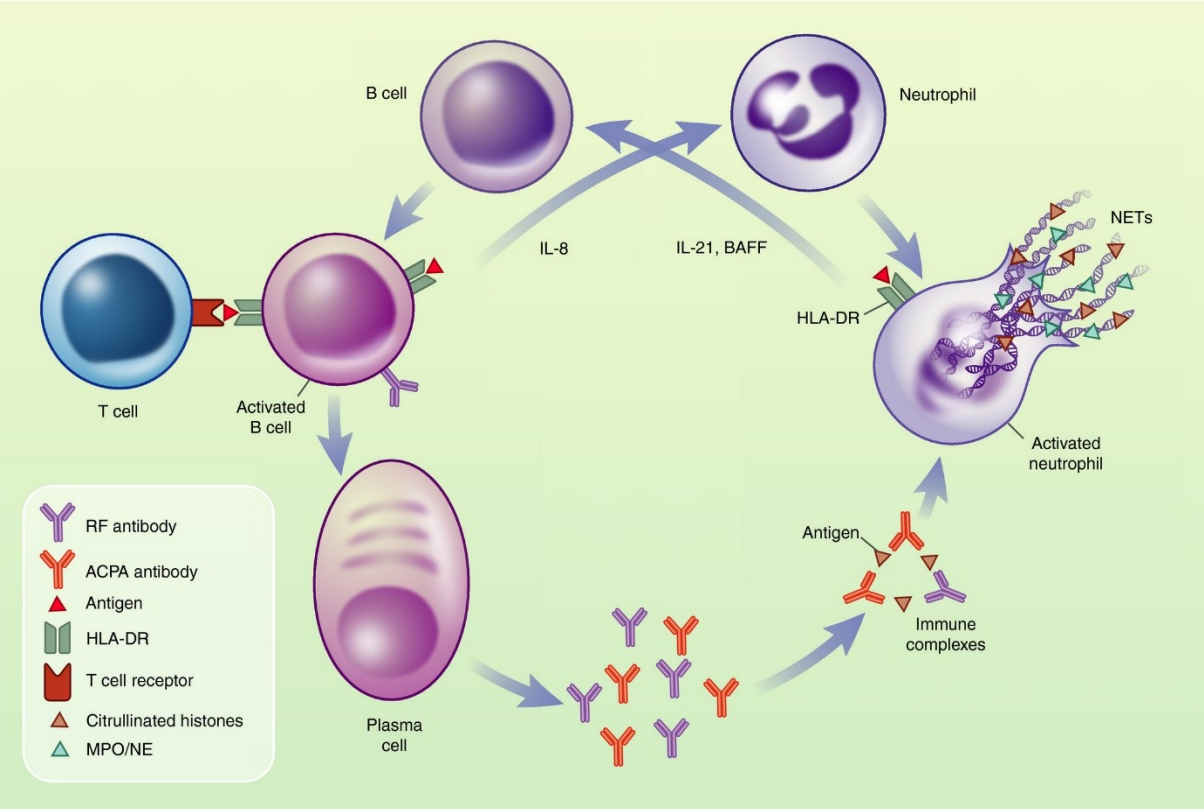


Figure 1.15. Crosstalk between B cells and neutrophils in autoimmune diseases.

Activated B cells with the help of T cells produce antibodies and release cytokine, IL-8, which recruits neutrophils to the inflamed tissue. Autoantibodies such as RF and ACPAs are produced by plasma cells and later form immune complexes. These immune complexes activate neutrophils to elicit further neutrophil responses which include BAFF production that again activates B cells forming a vicious cycle. Moreover, NETs generated from activated neutrophil act as a source of antigen for immune complex formation. In the interest of clarity, RF and ICs are simplified in this diagram. Figure taken from Karmakar et al., Immunology, 2021.[176]

HLA-DR, human leukocyte antigen–DR isotype; IL, interleukin; BAFF, B cell activating factor; RF, rheumatoid factor, ACPA, anti-citrullinated peptide antibody; MPO, myeloperoxidase; NE, neutrophil elastase.

1.8. Hypothesis and Aims

PICD is an-inflammatory mechanism by which neutrophils internalise pathogens, show an 'anti-inflammatory signature' and eventually undergo apoptosis to be eliminated by the macrophages. Previously in our lab, insoluble ICs have been shown to induce apoptosis via a novel PI3K dependent pathway (PI3K-Cdc42-Pak-Mek-Erk pathway) and a pilot experiment suggested that these insoluble ICs might be internalised by neutrophils. Considering ICs can have both pro- and anti-inflammatory effects, it is therefore of great interest to explore the mechanism of insoluble IC-induced neutrophil effector functions. To my knowledge, no study has investigated the mechanism of internalisation of insoluble immune complexes in neutrophils.

The main hypothesis I aimed to test was that **insoluble immune complexes induce neutrophil apoptosis and thereby have an anti-inflammatory function, and this is dysregulated in rheumatoid arthritis**.

Specifically, the work presented in this thesis investigates the following key aims

i) To establish whether insoluble IC-induced neutrophil apoptosis is a form of PICD

ii) To elucidate the mechanism of internalisation of insoluble immune complexes by neutrophils.

iii) To investigate whether macrophages are able to efferocytose the apoptotic neutrophils that were induced by insoluble ICs.

iv) To discover whether insoluble IC-induced neutrophil responses are dysregulated in rheumatoid arthritis.

2. Materials and Methods

2.1. Reagents

All reagents were purchased from Sigma Aldrich unless otherwise stated. All primary and secondary antibodies used in this project are summarised in **Table 2.1**. Cell media and supplements were purchased from Gibco unless specified otherwise. All cells used in the project were derived from peripheral human donor blood.

2.2. Use of human donor blood

Being a registered phlebotomist and a medical doctor, I performed most of the phlebotomy procedures involved in this project. In some cases, cells prepared by other researchers were used. I drew blood from the healthy donors using single-use needlestick method[192] with a 19 or 21 gauge butterfly needle (Nipro Safetouch PSV). Donors' consent was obtained, and donor identity was anonymised in keeping with the blood ethics protocol (approval 08/S1103/38 and, AMREC 15-HV-013, 15-HV-069 and 21-EMREC-041), for the use of healthy human donor blood was granted by the Accredited Medical Regional Ethics Committee.

Table 2.1. Working concentrations and targets of Primary antibodies used in this project.

Primary antibodies (non-fluorescent)	Clone	Working concentration	Manufacturer (Cat. No.)
Anti- CD16/FcγRIIIb	3G8	5 µg/ml	gifted by Prof. Ian Dransfield, purified by Dr. Julia Chu
Anti- CD32/FcγRIIa	IV3	0.5 µg/ml	gifted by Prof. Ian Dransfield, purified by Dr. Julia Chu
Anti- CD32/FcγRIIa	AT10	5 µg/ml	gifted by Prof. Martin Glennie
Anti- CD64/FcγRI	10.1	10 µg/ml	Biolegend (305002)
Anti- Mac1	ICRF44	12.8 µg/ml	gifted by Prof. Nancy Hogg
Anti- CD63	H5C6	1:300	Biolegend (353013)
Anti- Gelsolin	CTPC-Gelsolin 2	1:500	Biolegend (866501)
Human Trustain FcX (Fc blocking antibodies)		1:100	Biolegend (422302)
Rabbit polyclonal IgG			Sigma (I8140)
Anti-HSA (polyclonal IgG)			Sigma (A0433)

2.3. Neutrophil isolation from peripheral blood

Neutrophils were isolated from peripheral blood of healthy donors as well as rheumatoid arthritis patients. The procedure was performed in a cell culture hood with continuous laminar flow.

2.3.1. Neutrophil isolation from peripheral blood of Healthy donors

Based on the standard blood preparation protocol used in the laboratory, neutrophils were isolated using dextran sedimentation and Percoll gradient separation.[193,194]

Briefly, 40 ml blood was drawn into a 50 ml Falcon tube (BD Sciences) already containing 4 ml of 3.8% sodium citrate (0.345%, final concentration) to prevent coagulation, followed by careful and gentle mixing. Citrated peripheral venous blood was centrifuged at 350 g for 20 minutes at room temperature, with acceleration “1” and without brake (Hettich Zentrifugen, Universal 320R Centrifuge). The Platelet rich plasma layer was collected into 10 ml glass tubes and autologous serum was generated by adding $CaCl_2$ to 20 mM final concentration for 1 hour at 37°C. The platelet plug was removed from the serum, and the serum was retained for use in subsequent assays. Erythrocytes were separated from leukocytes by sedimentation with Dextran T500 (Sigma, final concentration 0.6%) and topped up to 50 ml with saline (0.9% NaCl, Baxter). The tube was kept upright to allow sedimentation of erythrocytes for maximum 30 minutes at room temperature. The leukocyte-rich, upper layer was then collected into a new 50 ml Falcon tube and washed with NaCl (0.9%, Baxter), followed by 6 minutes centrifugation at 350 g at room temperature.

Leukocytes were further fractionated using a discontinuous Percoll (GE Healthcare) gradient 49.5% /64.8% /72% that was subjected to centrifugation at 720 g for 20 minutes at room temperature, with acceleration “1” and without brake.

Polymorphonuclear cells (PMNs) were collected at the interface between 64.8% and 49.5%, and peripheral blood mononuclear cells (PBMCs) from 64.8% /72% interface. Cells were then washed twice more with 50 ml PBS (-$CaCl_2$, -$MgCl_2$, Gibco) by centrifugation at 230 g for 6 minutes at room temperature. Cells isolated from the middle cell layer contained at least 98% neutrophils according to Kwik-Diff stained cytospin preparations. Prior to functional analysis, cells were suspended in PBS^{++} (**Table 2.2**), or cultured in Iscove's Modified Dulbecco's Medium (IMDM, Gibco).

2.3.2. Neutrophil isolation from peripheral blood and synovial fluid of rheumatoid arthritis patients

The blood from RA patients was drawn by the research nurses at either an interstitial lung disease or a rheumatology clinic and transported (duration 5-30 min) to the laboratory. Samples from the first four patients mentioned in the **Table 6.1** were obtained in 2019 before the COVID-19 pandemic and the last five patients were recruited after two years in 2021, after when COVID-19 related restrictions were eased. Blood samples from the first set of patients were collected in falcon tubes with sodium citrate exactly the same way as healthy donor's blood collection. Blood samples from the second set of patients were collected in heparinised tubes as per clinic requirement. Age- and sex-matched healthy donors were recruited as a control when a patient sample was available.

Synovial fluid from an active RA patient was collected at a RA clinic. The sample was centrifuged at 300 g for 5 minutes at room temperature. The supernatant was collected and used as a supplement for culturing neutrophils for assessment of apoptosis. The cell pellet was resuspended in PBS^{++}.

2.3.3. Cytocentrifuge preparations and morphological analysis

For morphological analysis, 0.2-0.5 x10^6 cells were centrifuged onto glass slides by centrifugation at 300 g for 3 minutes in a cytocentrifuge (Shandon, SKU: 8358-30-1005). After air-drying the cells were fixed with methanol (Thermo Fisher Scientific, M/4000/17) and stained with Kwik-Diff™ by dipping the cytospin slides first in Kwik-Diff Reagent 2, eosin (Thermo Fisher Scientific, Epredia™, 9990706, catalogue no 10643649) and then Kwik-Diff Reagent 3, methylene blue (Thermo Fisher Scientific, Epredia™ 9990707, catalogue no 10549408) each for 1 minute at room temperature. Excess stain was removed by extensive washing with water. Assessment of purity and viability was performed on a wide field microscope and images were taken on the EVOS FL Auto 2 (Invitrogen) for assessment of apoptosis.

Table 2.2. Recipes of the buffers used in this project.

Buffer	Ingredients
PBS^{++}	Dulbecco's PBS (+$CaCl_2$, +$MgCl_2$), supplemented with 1 g/l glucose and 4 mM sodium bicarbonate
Annexin buffer	$HBSS^{++}$+ 2.5 mM $CaCl_2$
2x resolving buffer	90.86 g/L Tris-HCl; pH 8.8, 0.2% SDS, 42% H_2O
2x stacking buffer	30.28 g/L Tris-HCl; pH 6.8, 0.2% SDS, 42% H_2O
Sample buffer	45.53% glycerol, 10% β-mercaptoethanol, 2% SDS, 4% 1 M Tris (pH 6.8), 0.01% bromophenol blue
Transfer buffer	2.9 g/L Tris, 14.5 g/L glycine and 10% methanol
Lysis Buffer	Hepes (pH 7.4 @4 °C), Na_2VO_4, Tx 100, β- glycerophosphate, NaF, NaCl, EDTA, EGTA

2.4. Treatment with inhibitors and FcγR blocking antibodies

Neutrophils were incubated with inhibitors for 10 minutes at 37 °C prior to stimulation with iICs/beads unless otherwise specified. Incubations with FcγR blocking antibodies were for 30 minutes on ice to prevent internalisation of the receptors. The concentration of the inhibitors were chosen after optimisation or as previously described in Chu et al.[99] Inhibitors used in this project are detailed in **Table 2.3**. The cytotoxicity of inhibitors was tested by assessing apoptosis (as detailed in **section 2.10.1**) (**Figure 2.1**).

Diisopropylfluorophosphate (DFP) treatment of neutrophils was performed in a fume hood in the presence of a buddy, with double gloved hands and protective sleeves. The DFP stock was kept on ice at all times. Neutrophils in a maximal volume of 1.5 ml in PBS were incubated with DFP (7 mM) for 10 minutes at room temperature prior to being washed twice with PBS. Following DFP treatment, DFP-containing waste was inactivated by treatment in 2% aqueous sodium hydroxide for 1 day prior to disposal.

Table 2.3. Working concentrations and targets of inhibitors used in this project.

Inhibitors	Target	Working concentration	Manufacturer	Catalogue No
LY294002	Pan-PI3K	10 µM	Synkinase	SYN-1108-m001
Wortmannin	Pan-PI3K	100 nM	Sigma	W1628
FR180204	Erk	10 µM	Selleckchem	S7524
Cytochalasin B	Actin polymerisation	10 µM	PanReac applichem	A7657
Latrunculin B	Actin polymerisation	10 µM	Calbiochem	CALB428020-1
QVD-OPH	Pan-caspase	10 µM	R&D systems	OPH001-01M
Z-VAD-FMK	Pan-caspase	100 µM	Bachem	4027403
Roscovitine	CDK2/7	20 µM	Cayman	10009569
DFP*	Serine proteases	7 mM	Sigma	D0879
E64	Cysteine proteases	2.5 µM	Sigma	E3132
Chloroquine	Endocytosis	100 µM	Sigma	C6628
EIPA**	Pinocytosis	50 µM	Sigma	A3085
DPI	NADPH oxidase	10 µM	Cayman	81050
DETC	Superoxide dismutase	10 µM	Sigma	D93503
MPO inhibitor 1	Myeloperoxidase	0.5 µM	Sigma	475944
Catalase (ROS scavenger)	Hydrogen peroxide	25 µg/ml	Sigma	C1345

* pre-treatment for 10 min at room temperature, ** pre-treatment for 30 min at 37 °C

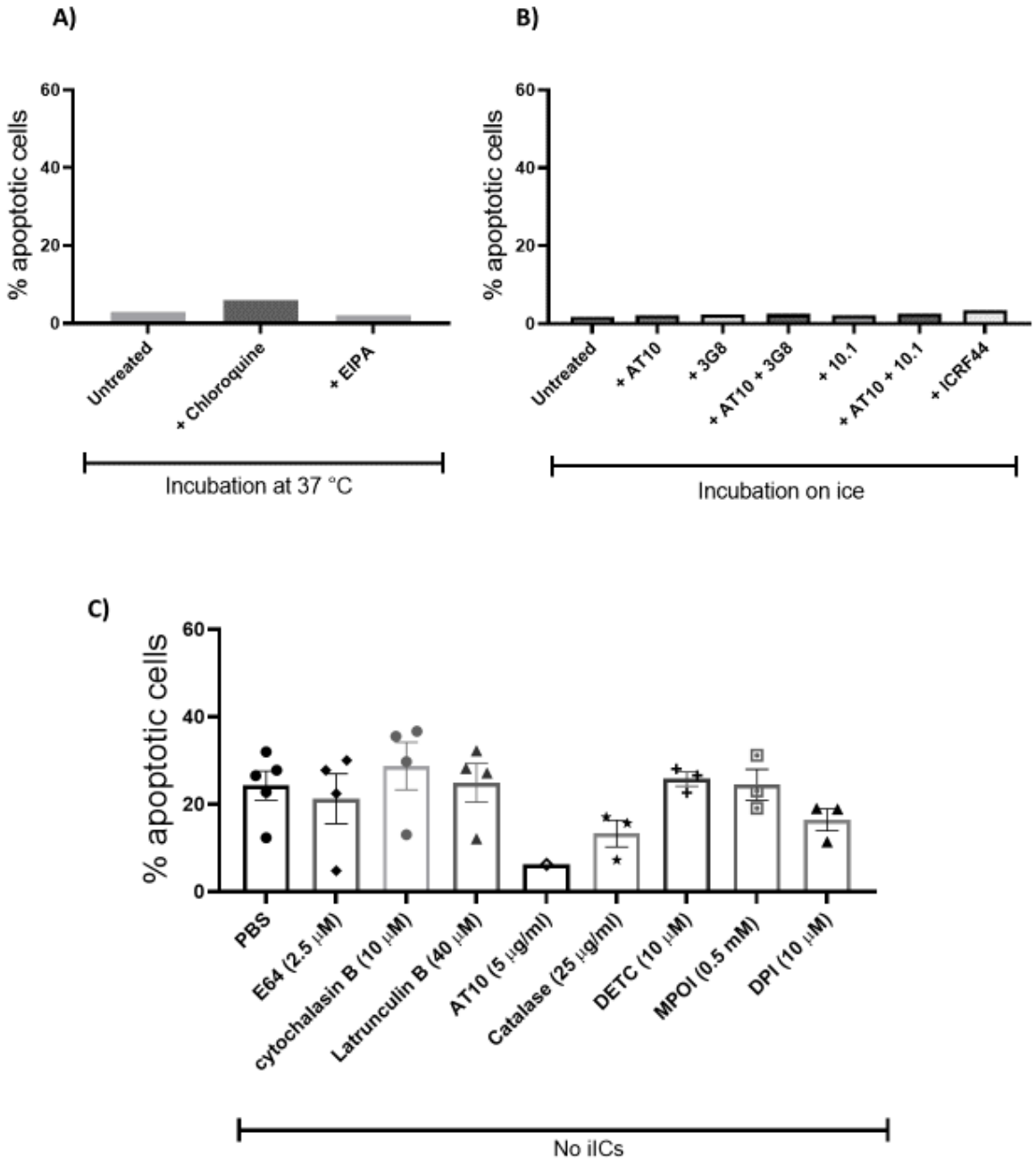


Figure 2.1. Inhibitors used are not cytotoxic within the timeframes used in this study.

Neutrophils were treated with inhibitors as indicated and in the absence of any further stimulus and apoptosis was measured by Annexin V-PI staining using flow cytometry. The concentrations of the inhibitors are detailed in **Table 2.3** unless labelled otherwise on the X-axis in the graph. **A** and **B,** cells were incubated for 30 minutes in PBS at 37 °C or on ice, respectively. No statistical test was applied since data were obtained from a single experiment. **C,** cells were incubated for 6 hours in IMDM with 10% autologous serum. Data are displayed with columns representing the mean value obtained and each symbol representing the value obtained in the individual experiments. The groups were compared using ordinary one way ANOVA with Dunnett's post hoc test. No statistical difference was observed between any two groups.

2.5. Preparation of insoluble HSA-anti HSA immune complexes

Insoluble immune complexes (iICs) were prepared in batches using human serum albumin (HSA) and rabbit anti-human serum albumin antibody (see **Table 2.1**) following a protocol provided by Professor Steve Edwards at University of Liverpool[154]. The zone of equivalence between antigen and antibody was established for each individual batch of antibody, as described. In brief, HSA and rabbit anti-HSA were made up in PBS to a final concentration of 5 mg/ml, and loaded in different ratios into wells of a 96 well plate. The plate was incubated for 1 hour at 37°C with gentle shaking. Absorbance was then measured at 450 nm to detect the zone of equivalence which has highest optical density. The remaining antibody was mixed with antigen according to that ratio and incubated for 1 hour at 37 °C. iICs were washed three times with PBS followed by centrifugation (Heraeus biofuge pico, 75003235) at 4000 g for 5 minutes at room temperature to deplete any soluble complexes. iICs were stored at 4 °C in PBS.

2.6. Opsonisation of latex beads with rabbit IgG

Three different sizes of latex beads (diameter; 3 µm, 0.8 µm and 0.3 µm) were used in this project. Latex beads were opsonised with polyclonal rabbit IgG (**Table 2.1**).

5 mg latex microspheres were washed three times in 5 ml MES buffer [25mM (2-(N-morpholino) ethanesulfonic acid) in PBS, pH 6]. After the final centrifugation step (3000 g for 20 min), beads were carefully resuspended in 5 ml MES buffer. 5 ml of 1 mg/ml polyclonal rabbit IgG in MES buffer was added to 5 ml latex bead suspension, and the mixture was incubated overnight at room temperature with gentle mixing. On the next day, the opsonised latex beads were pelleted. The protein content of the supernatant was determined using a BCA Protein Determination Kit (Pierce). The amount of protein coated on the particles was determined by subtracting the residual

protein concentration from the original one. Beads were washed three times with 10 ml PBS to eliminate any unbound IgG prior to storing in the fridge.

2.7. Detection of reactive oxygen species

A real-time, luminol-based chemiluminescence assay was performed in white polystyrene 96 well plates (Nunc). The plates were blocked with 1% non-fat milk power in PBS for at least 1 hour at room temperature and then washed three time with PBS prior to being used. $0.5x10^6$ Neutrophils per well were pre-warmed with 150 µM luminol and 18.75 U/ml horseradish peroxidase (HRP) in PBS^{++} prior to stimulation with 4 µg/ml iICs or 25 beads per cell. Luminol was used to detect internal reactive oxygen species (ROS), whereas luminol and HRP together were used to detect total ROS. Luminescence production was detected in real-time for 45 minutes with minimum read interval (~ 30 sec) using a Synergy H1 plate reader (BioTek Instruments). Data output was in relative light units (RLU). To quantitatively analyse the data, the area under curve (AUC) for each condition was calculated and compared.

2.8. Assessment of internalisation and immunostaining

2.8.1. Cell treatment

Freshly isolated neutrophils at 1-2x10^6 /ml were incubated with cell mask deep red (Life Technologies) for 10 minutes at room temperature in the dark. Cells were then treated with inhibitor/vehicle for 10 minutes at 37 °C prior to being incubated with iICs (2 µg/ml) or opsonised latex beads (5 beads per cell) for 30 minutes at 37 °C. In some experiments, cells were incubated with differentially pre-stained iICs and/or IgG-opsonised beads. As a negative control, cells were kept on ice throughout to prevent internalisation. Cells were then allowed to adhere to glass slides (conventional or electrostatic) for an hour on ice. In some experiments, 0.5-1x10^6 /ml cells used for a cytocentrifuge preparation. Neutrophils were fixed for 10 minutes on ice with 2% paraformaldehyde.

2.8.2. Immunostaining for microscopy

All fluorescent antibodies and dyes used in this study are detailed in **Table 2.4**. Immunostaining was performed following the general protocol mentioned in **Figure 2.2**, unless specified otherwise. Cellular markers (e.g. CD63) were identified using indirect immunofluorescence labelling with secondary anti-mouse antibodies (Life Technologies) for 30 minutes at room temperature with 1% BSA. Following extensive washing, slides were mounted with ProLong Gold anti-fade mount media (Life Technologies, P36934) and analysed microscopically. The percentage of internalisation was determined by manually counting the cells that contained internalised particles.

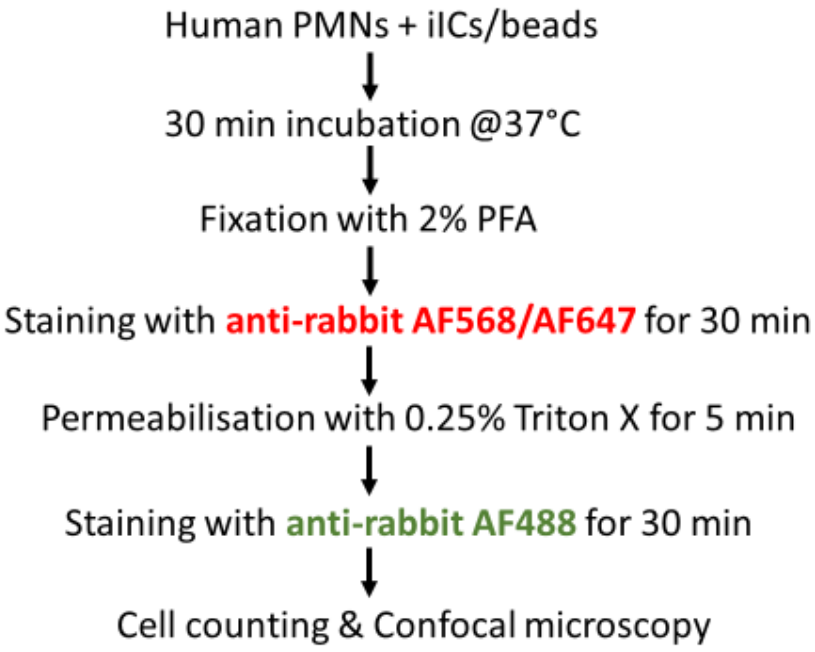


Figure 2.2. Immunostaining protocol to assess internalisation of iICs or IgG-beads.

2.8.3. Immunostaining for flow cytometry

Internalisation of iICs was also detected by flow cytometry. For these assays, Cell were stimulated with pre-stained iICs for 30 minutes at 37 °C in the dark, followed by addition of a secondary antibody that was coupled to a different fluorochrome. Cells were then fixed with BD FACS lysing solution (Fisher Scientific, Product no. 10141013), resuspended in PBS and analysed by flow cytometry (Attune NxT Flow Cytometer, Thermofisher Sceintific, product no A24858). The gating strategy employed together with appropriate controls used are shown in **Figure 2.3**.

2.8.4. Immunostaining for time-lapse imaging

Freshly isolated cells were resuspended in PBS^{++} in an imaging dish with a polymer coverslip bottom for high-end microscopy (Ibidi, product no 81156). Cells were left undisturbed for 15-20 minutes to allow them to settle down to the bottom of the dish. Pre-stained iICs were then added to the cells immediately prior to imaging in the spinning disk microscope (Andor) equipped in a heated, humidified and CO_2 controlled chamber. Images were captured every 30 sec for 45 minutes. In a standalone experiment, pHrodo dextran (see **Table 2.4**) was first added to the cells to determine the pH change in the medium. Cells were incubated with pHrodo dextran for 15 minutes at 37 °C in the dark prior to iIC-stimulation.

2.8.5. Assessment of pinocytosis

To test for pinocytosis, neutrophils were pre-incubated for 5 minutes at room temperature with buffer containing lucifer yellow (1:1000) or FITC conjugated dextran (2 mg/ml) prior to assessment of internalisation in the presence of the labelled buffer. The percentage of pinocytosis was then calculated using methods described in **section 2.8.2 and 2.8.3**.

Table 2.4. Working concentrations and targets of fluorescent antibodies/dyes used in this project.

Fluorescent antibodies/dyes	Working concentration	Manufacturer
Alexa fluor 488 goat anti-rabbit IgG (H+L)	1:400	Invitrogen (A11008)
Alexa fluor 488 goat anti-mouse IgG (H+L)	1:400	Invitrogen (A11029)
Alexa fluor 568 goat anti-rabbit IgG (H+L)	1:400	Invitrogen (A11011)
Alexa fluor 647 goat anti-rabbit IgG (H+L)	1:400	Invitrogen (406414)
Alexa fluor 488 goat anti-human IgG (H+L)	1:400	ThermoFisher Scientific (A-11013)
Alexa fluor 568 goat anti-human IgG (H+L)	1:400	ThermoFisher Scientific (A-21090)
Anti-human CD64 (conjugated with Brilliant Violet 421)	1:200	Biolegend (305020)
Anti-human Cleaved caspase 3, clone D3E9 (conjugated with Alexa fluor 488)	1:50	Cell Signalling Technology (9603)
Lucifer yellow	1:100	Merck Life Sciences (L0144)
FITC dextran	2 mg/ml	Merck Life Sciences (FD10S)
Cell mask deep red	1:1000	Life Technologies (C10046)
pHrodo red SE	1:10000	Life Technologies (36600)
pHrodo dextran	20 µg/ml	Life Technologies (P35368)
Annexin V	1:1000	Roche (11828681001)
Propidium Iodide	0.1 µg/ml	Merck Life Sciences (P4170)
Hoechst	1:10000	Abcam (33342)

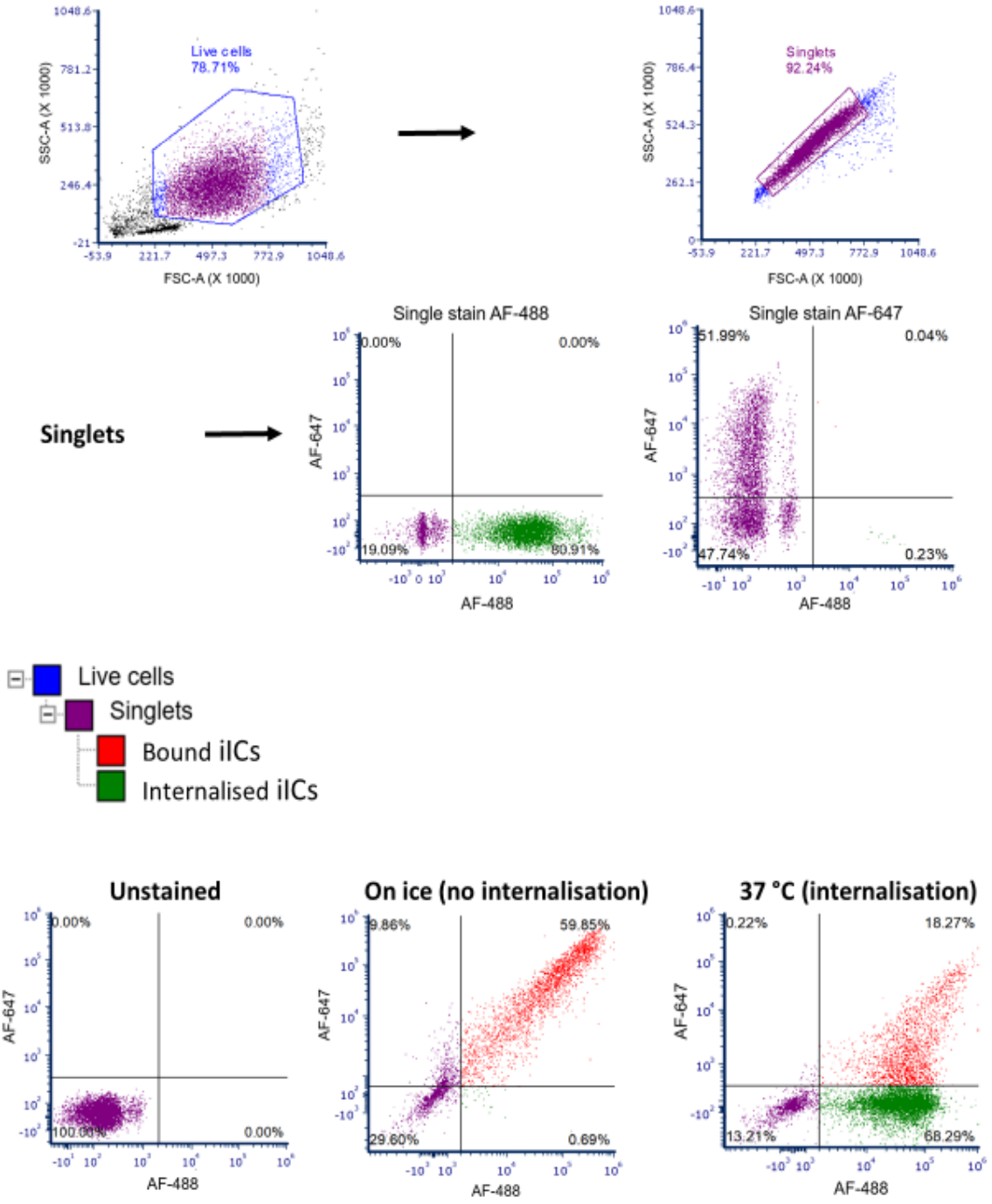


Figure 2.3. Gating strategy for estimation of internalisation.

Neutrophils were stimulated with pre-stained iICs (AF488) for 30 minutes at 37°C followed by adding another label (AF647) to detect internalised and bound iICs. A control sample was kept on ice throughout to prevent internalisation. Other control samples were prepared with single label or in the absence of any secondary dye to determine the position of quadrant gate. AF488- AF647- cells with no internalised particles; AF488+ AF647- cells with internalised iICs; and AF488+ AF647+ cells with bound iICs. The analysis was done using FCS express.

2.9. Microscopy and Image analysis

Images were analysed by epifluorescence for primary screening using an EVOS FL Auto 2 microscope and z stacks were obtained by using confocal microscopes. Files were mainly saved as either .lsm or .tiff format. ImageJ and Leica software (detailed in **Table 2.5**) were mainly used to analyse the images obtained.

Table 2.5. Microscopes and the analysis software used in this project.

Microscope	Type	Objectives used	Manufacturer	Analysis Software
EVOS FL Auto 2	Fluorescence	40-100x	Thermo Scientific™ Invitrogen™	ImageJ/Fiji
Zeiss LSM780	Confocal	63x	Carl Zeiss NTS Ltd.	Zeiss and ImageJ
Leica SP8	Confocal	63x	Leica microsystems	LAS X and ImageJ
Andor Spinning disk	Spinning disk confocal	60x	Oxford Instruments	Imaris Bitplane & ImageJ
Opera Phenix Plus	Spinning disk confocal	40x	Perkin Elmer	Harmony

2.10. Assessment of apoptosis

2.10.1. Cell culture and induction of apoptosis

Freshly isolated neutrophils at $5x10^6$ /ml were cultured in IMDM supplemented with 100 U/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco) and 10% autologous serum for up to 12-24 hours at 37 °C, 5% CO_2 to induce apoptosis. Neutrophils were pre-treated with inhibitors (**Table 2.3**) or vehicle, and then stimulated with iICs (10 µg/ml), zymosan (3 particles per cell) latex beads (5-25 beads per cell) or vehicle.

2.10.2. Annexin V-PI flow assay

To quantify apoptosis, cells were incubated in 2 ml Eppendorf tubes as described in **2.10.1**. Every three hours, a 50 µl aliquot of cells was stained with FITC-conjugated Annexin V in 250 µl Annexin buffer (**Table 2.2**) for 30 minutes on ice in the dark followed by adding Propidium iodide (**Table 2.4**). Analysis by flow cytometry was performed immediately [5 LSR Fortessa (BD LSRFortessa™ Cell analyzer) or Attune NxT Flow Cytometer]. 10,000 events from each condition were recorded to quantify the proportions of apoptotic and necrotic events, with Annexin V-PI- cells scored as viable, Annexin V+PI- as apoptotic and Annexin V+PI+ as secondary necrotic (**Figure 2.4**). Data were analysed quantitatively with FCS Express 7 (De Novo Software, version 7.10.0007). The gating strategy is shown in **Figure 2.4**.

2.10.3. Detection of cleaved Caspase-3

Activated caspase-3 was measured using an AF488-coupled antibody specific for cleaved human caspase-3 according to manufacturer's instructions. The gating strategy employed is shown in the Results chapter (**Figure 3.5.A)**.

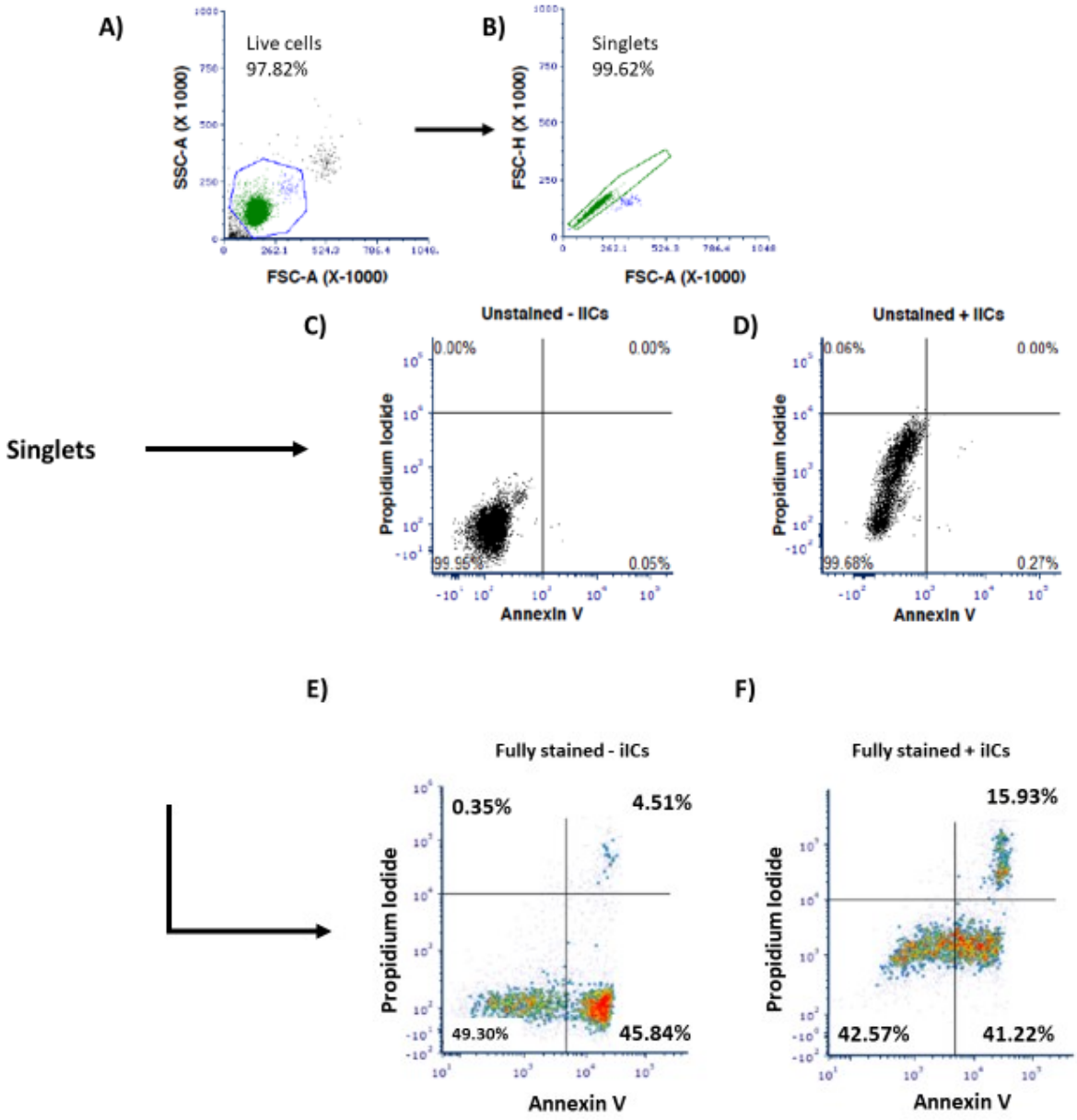


Figure 2.4. Gating strategy for analysis of neutrophil apoptosis.

Freshly isolated neutrophils were cultured in IMDM with 10% autologous serum for either 0 hour or 18 hours with or without iICs (10 µg/ml). Neutrophils (**A**) were gated based on their forward and scatter properties followed by gating the singlets (**B**). A control sample was prepared without the flow antibodies to determine gates for the quadrant plots (**C** and **D**). The unstained cells were processed for flow cytometry immediately after stimulation. Note that iIC-stimulated cells are autofluorescent in the PI channel, influencing the choice of gate used for these experiments. **(E** and **F)**, example of neutrophils that were subjected to Annexin V and PI staining after 18 hours of culture without or with iIC stimulation respectively, so that viable, apoptotic and secondarily necrotic neutrophils are present. Data were analysed using FCS express. Figure adapted from Karmakar et al.,Cell death and disease, 2021.[326]

2.11. Western Blot

2.11.1. Protein gel preparation for SDS-PAGE and transfer onto PDVF membrane

Protein gels were cast between glass plates at 1.0 mm depth, with a resolving gel (typically 12% acrylamide) overlaid by a 3.75% stacking gel. The resolving gel was made by mixing 37.5:1 acrylamide/bisacrylamide (Sigma), 2x resolving buffer (**Table 2.2**) and H_2O in appropriate ratios. Polymerisation was induced by adding TEMED (0.1%) and ammonium persulphate (0.5%, Sigma). A flush finish was ensured by overlaying the polymerising resolving gel with water-saturated butanol. Once the resolving gel had fully polymerised, it was overlaid with the stacking gel (made with 2x stacking buffer, acrylamide and H_2O, see **Table 2.2**). Wells for loading samples were formed by inserting a 1.0 mm thick Teflon gel comb into the stacking gel prior to polymerisation.

Cells were then lysed for 5 minutes with ice-cold lysis buffer using lysis buffer with added anti-proteases (recipe shown in **Table 2.2**). Protein samples as well as molecular weight standards that had been boiled in sample buffer (**Table 2.2**) were added onto the protein gel. SDS-PAGE was performed by gel electrophoresis (equipment from Biorad). Voltage was applied until the dye front reached the bottom of the gel. Proteins were wet transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore) in ice-cold transfer buffer (**Table 2.2**) under cooling for 1 hour at 100 V in a transfer tank (Biorad).

2.11.2. Visualisation of Western Blots

Membranes were blocked with 5% fat-free milk in PBS supplemented with 0.1% Tween-20 (PBST) for 1 hour at room temperature with gentle shaking, followed by incubation with IgG labelled with HRP-conjugated anti-rabbit antibody (1:3000, Santa

Cruz Biotechnology) for 30 minutes at room temperature. Membranes were washed three times with PBST, followed by incubation with an enhanced chemiluminescence (ECL) substrate (Bløk-CH buffer, Millipore) for 5 minutes at room temperature. Chemiluminescence was detected using X-ray films that were developed in an automated developer (Kodak). The exposure time varied from 5 sec to 1 minute.

2.12. Efferocytosis assay

Uptake of pHrodo labelled apoptotic neutrophils by macrophages was quantified using Flow cytometry as described.[195] Neutrophils and the macrophages were derived from different donors.

2.12.1. Preparation of apoptotic neutrophils

Neutrophils (see **2.10.1**) were stimulated with vehicle (for constitutive apoptosis), iICs (10 µg/ml) or Roscovitine (20 µM) and cultured for 21 hours to induce apoptosis. Apoptotic neutrophils were then labelled with pHrodo^{TM} Red SE (**see Table 2.4**) for 30 minutes at room temperature in the dark. Sometimes, neutrophil nuclei were also labelled with Hoechst (see details in **Table 2.4**). Neutrophils were then washed with PBS and resuspended at 1.2x10^6 /ml in IMDM supplemented with 10% autologous serum.

2.12.2. Preparation of monocyte-derived macrophages

Macrophages were derived from PBMCs by culturing in well plates for 7-8 days in IMDM supplemented with 10% autologous serum and in some cases with 250 nM Dexamethasone, with feeding on Day 4. Prior to being stimulated with apoptotic neutrophils, macrophages were labelled with anti-CD64 (See **Table 2.4**) for 30 minutes on ice and with a cell permeable nuclear stain, Hoechst (Abcam 33342) for 15 minutes at room temperature. Cells were treated with Fc block (**Table 2.1**) to avoid non-specific binding prior to labelling with the specific antibody.

2.12.3. Macrophage-apoptotic neutrophil co-culture & detection of efferocytosis

Adherent macrophages were overlaid with pHrodo-labelled human neutrophils at a macrophage : neutrophil ratio of 1:3, centrifuged at 300 g for 5 minutes and incubated

for 45 minutes in a humidified incubator at 37°C with 5% CO_2. Non-ingested apoptotic neutrophils were removed by carefully aspirating medium and washing twice with PBS. Macrophages were then detached from the culture plate with 0.05% trypsin-EDTA for 15 minutes at 37 °C and incubated on ice.

Fluorescent macrophages (i.e. those that had ingested apoptotic cells) were determined by flow cytometry based on the pHrodo SE signal (as shown in **Figure 2.5**). For imaging, macrophages were cultured in 8-well chamber slides (Ibidi), fixed with 4% PFA for 10 minutes and mounted with DABCO for analysis by confocal microscopy.

For live imaging, 8-well chambers containing the macrophages in 250 µl of media first placed under the microscope (Opera Phenix plus spinning disk microscope, see details in **Table 2.5**) with appropriate settings applied. Fluorescently labelled apoptotic neutrophils were then added on top of the macrophages. Images were captured every 30 sec for 2 hours.

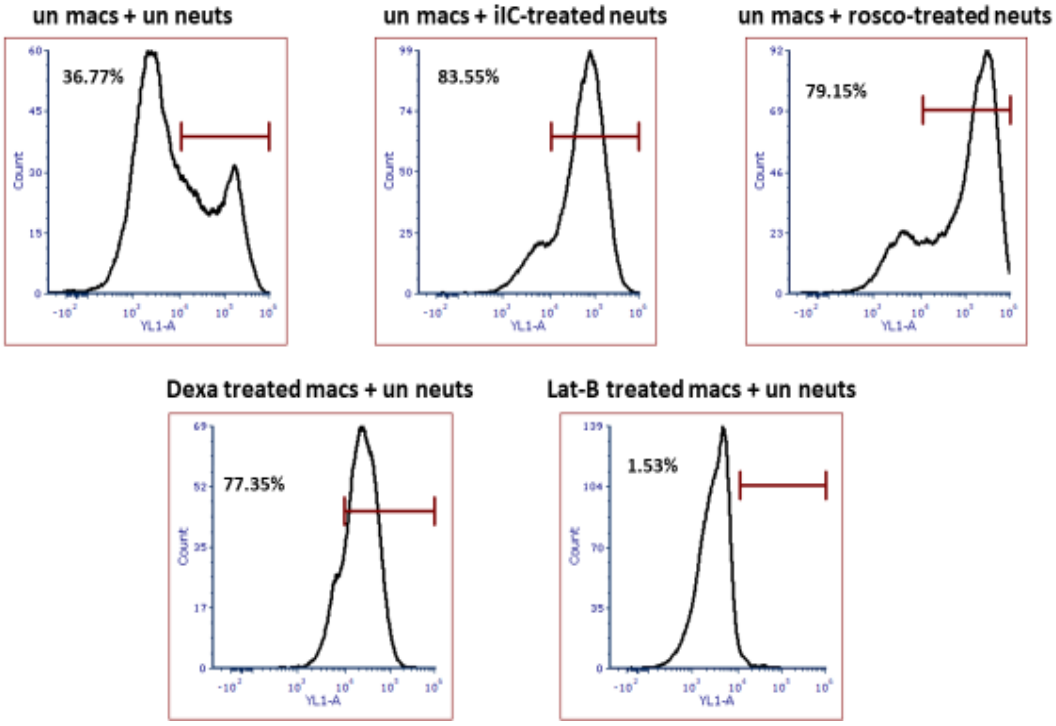


Figure 2.5. Gating strategy used to determine efferocytosis.

Monocyte-derived macrophages (MDMs) were co-cultured with pHrodo red SE-stained apoptotic neutrophils that had undergone constitutive apoptosis or that in which apoptosis had been induced by stimulation with ilCs (10 µg/ml) or Roscovitine (20 µM). Histograms are presented. A marker was employed at the junction of two distinct peaks of pHrodo SE signal to distinguish pHrodo SE-positive and negative cells. pHrodo-positive peaks in macrophages denote the percentage of efferocytosis. The data were analysed using FCS express. Rosco = Roscovitine, Dexa = Dexamethasone, Lat-B = latrunculin B. and 'un' = unstimulated/untreated.

2.13. Cytokine analysis of serum derived from RA patients

Serum was collected from the citrated whole blood of RA patients as described in **section 2.3.1** and prepared for cytokine analysis as per manufacturer's instructions (Bio-Techne Ltd, R&D systems, ARY022B).

2.14. Statistical analysis

All statistical analysis was performed on raw data using GraphPad Prism (version Prism 9). Power calculations were not performed in this project. For kinetic experiments, the area under the curve was calculated and used for statistical analysis. Where data met the assumptions for parametric tests, statistical analysis was performed by two tailed student's T tests or by one-way ANOVA. Post hoc analysis for multiple comparisons was carried out between groups of interest using Dunnett's or Bonferroni's test. Otherwise, the non-parametric Kruskal Wallis test with Dunn's post hoc test was performed. For multiple variables, two way ANOVA with Bonferroni post hoc test was applied. '*' denotes significant p-value ≤ 0.05. '**' p = ≤ 0.01, and '***' p = ≤ 0.001.

3. Results: iIC-induced neutrophil apoptosis is distinct from phagocytosis-induced cell death

3.1. Introduction

Neutrophils are recruited to sites of infection and rapidly phagocytose invading microbes. Following phagocytosis, neutrophils destroy pathogens by releasing highly microbiocidal NADPH oxidase-derived ROS substances and powerful proteases during degranulation. Uptake of pathogens induces phagocytosis-induced cell death (PICD) which is dependent upon ROS production and is important for the resolution of inflammation. In PICD, most ROS release occurs internally into the phagosome.[60]

Under physiological conditions, neutrophils mostly remain quiescent. Neutrophil activation is a multi-step process which starts with their recruitment and partial activation (priming) as they migrate to the site of inflammation/injury. The cells subsequently become fully activated in response to a range of internal and external pro-inflammatory stimuli, one of which is insoluble immune complexes (iICs).[196]

Priming plays a critical role in neutrophil-mediated tissue injury both *in vitro* and *in vivo*[10] and produces a state of enhanced responsiveness. Neutrophil priming by agents such as TNF-α, GM-CSF and LPS leads to a dramatic increase in the cell response (e.g. in terms of superoxide anion generation, degranulation and release of lipid mediators such as LTB4 and arachidonic acid). Historically, the term 'priming' was assigned to describe increased ROS production by the neutrophils, nevertheless priming can alter other neutrophil functions such as phagocytosis[197,198] and delaying apoptosis,[199] hence lengthening the survival of these cells at the inflamed site. Soluble immune complexes requires priming of neutrophils to generate ROS while insoluble immune complexes can activate unprimed neutrophils.[154]

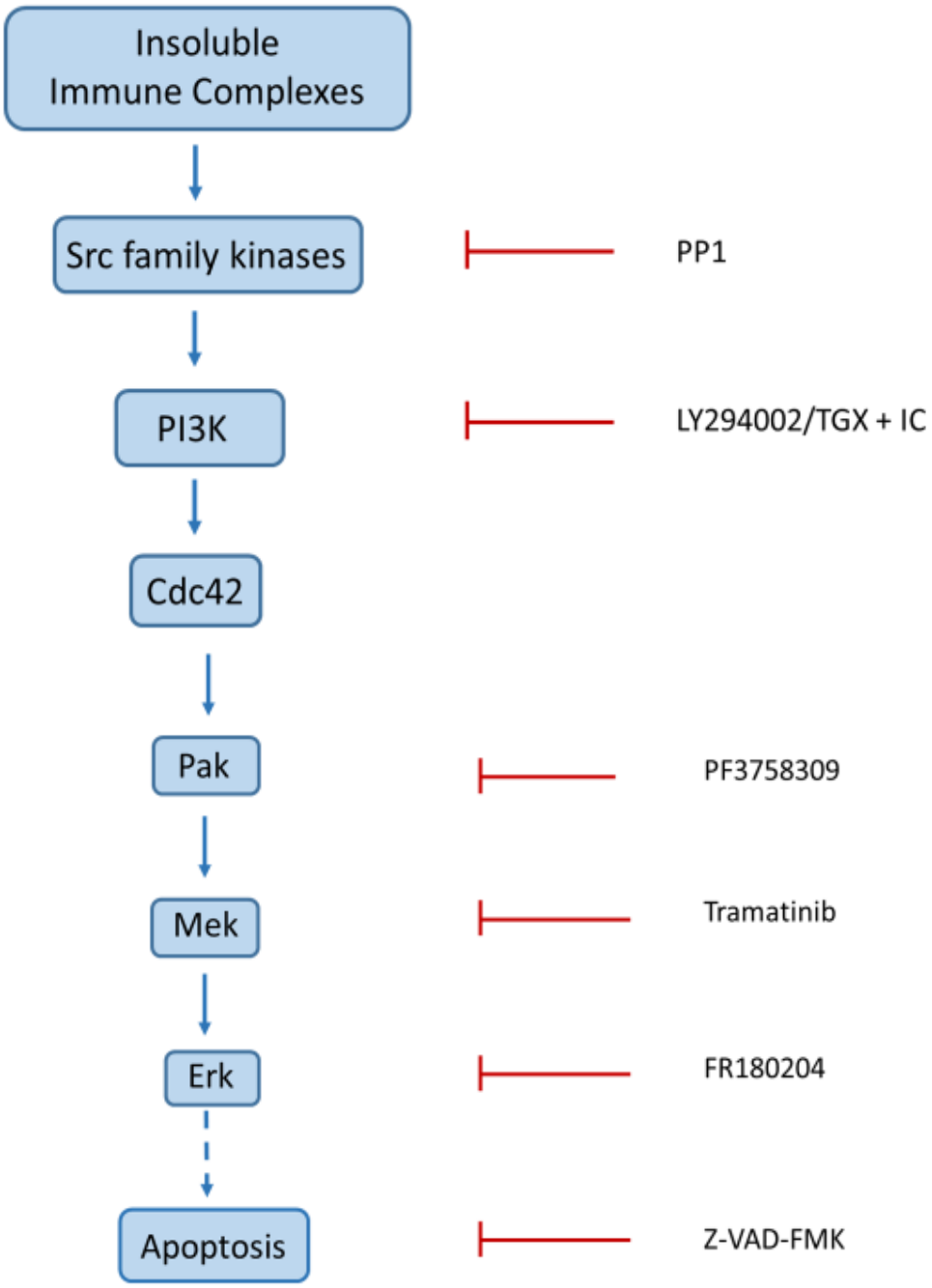


Figure 3.1. Apoptosis pathway induced by insoluble immune complexes.

iICs induce neutrophil apoptosis via a non-canonical pathway. Inhibitor used to target the regulatory molecules involved in the apoptosis pathway. Figure adapted from Chu et al., Cell Reports, 2016.[99]

iICs were previously shown to induce neutrophil apoptosis[200,201] and previously in the lab, a non-conventional signalling pathway (PI3K-Cdc42-Pak-Mek-Erk, **Figure 3.1**) was discovered which drives iIC-induced apoptosis.[99]

In her experiments, Julia Chu observed that iIC-stimulation changes the forward scatter properties of neutrophils (**Figure 3.2**). This was inhibited entirely by a Src family kinases inhibitor and partially by PI3K inhibitors. This alteration in forward scatter was noticeable as early as after 30 min after iIC-stimulation (data not shown). Since forward scatter changes are related to changes in cell morphology, particularly cell volume,[202] the observed change in neutrophils in **Figure 3.2** raised the possibility that iICs might be internalised. A pilot experiment to test whether the altered forward scatter might be due to neutrophils internalising iICs suggested that this was indeed the case (**Figure 3.3**). Similar to the flow data, inhibiting Src family kinases and PI3K, but not by Pak, Mek or Erk (the cascade controlling iIC-induced neutrophil apoptosis), interfered with iIC internalisation. This suggested that iIC internalisation and iIC-induced apoptosis might be regulated by separate signalling pathways.

I therefore set out to test whether iIC-induced neutrophil apoptosis might be a form of PICD. Opsonised latex beads and zymosan were chosen as a comparative tool since they are widely used to analyse neutrophil phagocytosis.[203,204] This chapter compares the induction of apoptosis of neutrophils in response to stimulation with iICs, serum-opsonised zymosan and IgG-opsonised latex beads.

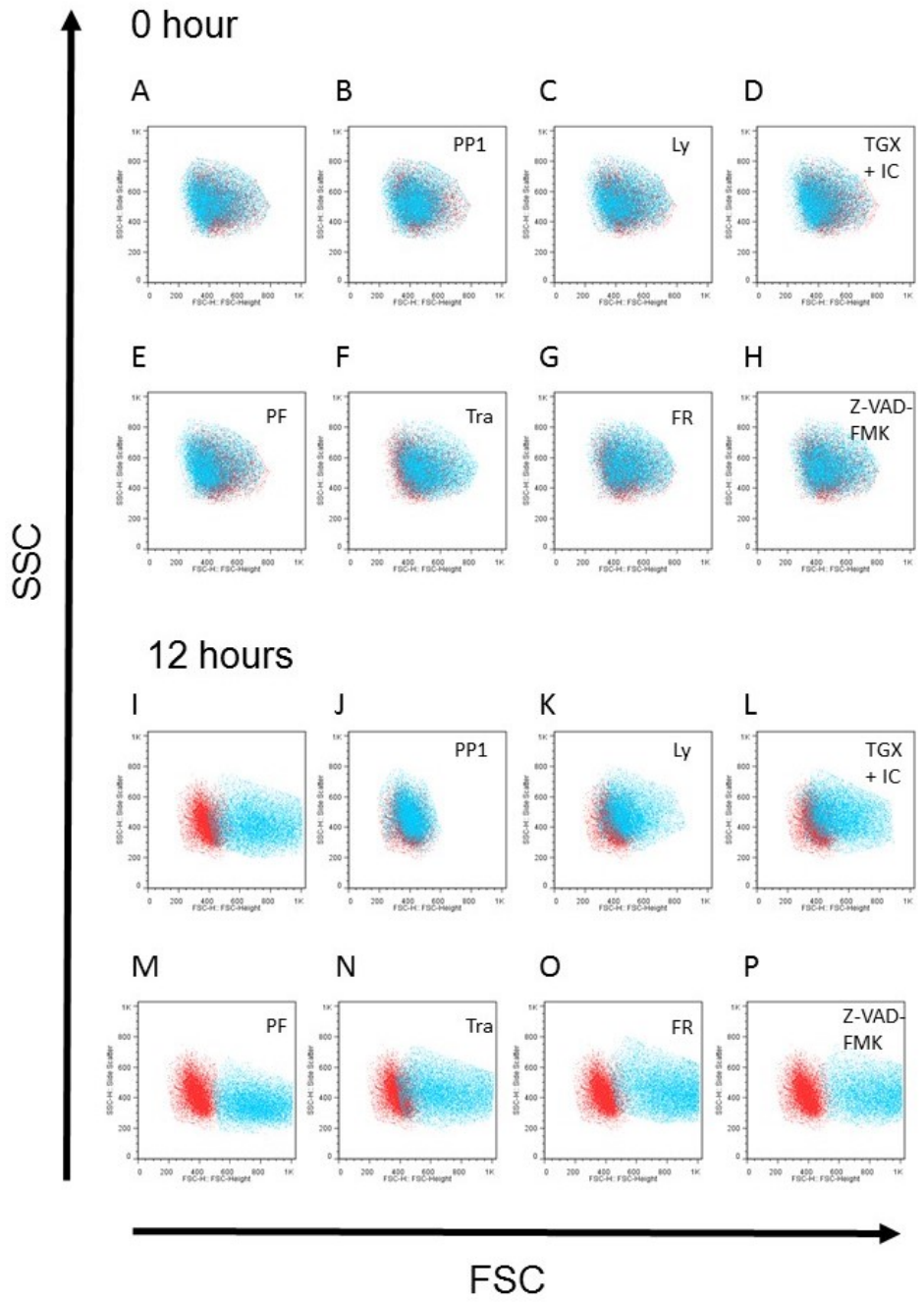


Figure 3.2: iIC-stimulation changes the forward scatter properties of neutrophils.

Neutrophil forward and side scatter was analysed by flow cytometry (5 LSR Fortessa). For ease of viewing, FSC and SSC plots of cells that had been vehicle treated (red) were copied into iIC-treated cells (blue). Cells were pre-treated with inhibitors as indicated in the upper right corner of each plot with PP1 (a Src kinase inhibitor, 10 μM), Ly (LY294002, a pan-PI3K inhibitor ,10 μM), TGX (TGX221, a PI3Kβ inhibitor 40 nM), IC (IC87114, a PI3Kδ inhibitor, 1 μM), PF (PF3758309, a pan-Pak inhibitor 5 μM), Tra (Tramatinib, a Mek inhibitor, 1 μM), FR (FR180204, an Erk inhibitor, 10 μM) and, z-VAD-FMK (a pan-caspase inhibitor, 100 μM) for 10 min at 37 °C prior to stimulation with iICs (blue) or vehicle (PBS; red) Plots shown are representative of at least 3 independent experiments. **A-H**) FSC and SSC plots of cells incubated with or without iICs in the presence or absence of inhibitors for 0 hour. **I-P**) FSC and SSC plots of cells incubated with or without iICs in the presence or absence of inhibitors for 12 hours. Experiment performed by Dr. Julia Chu.

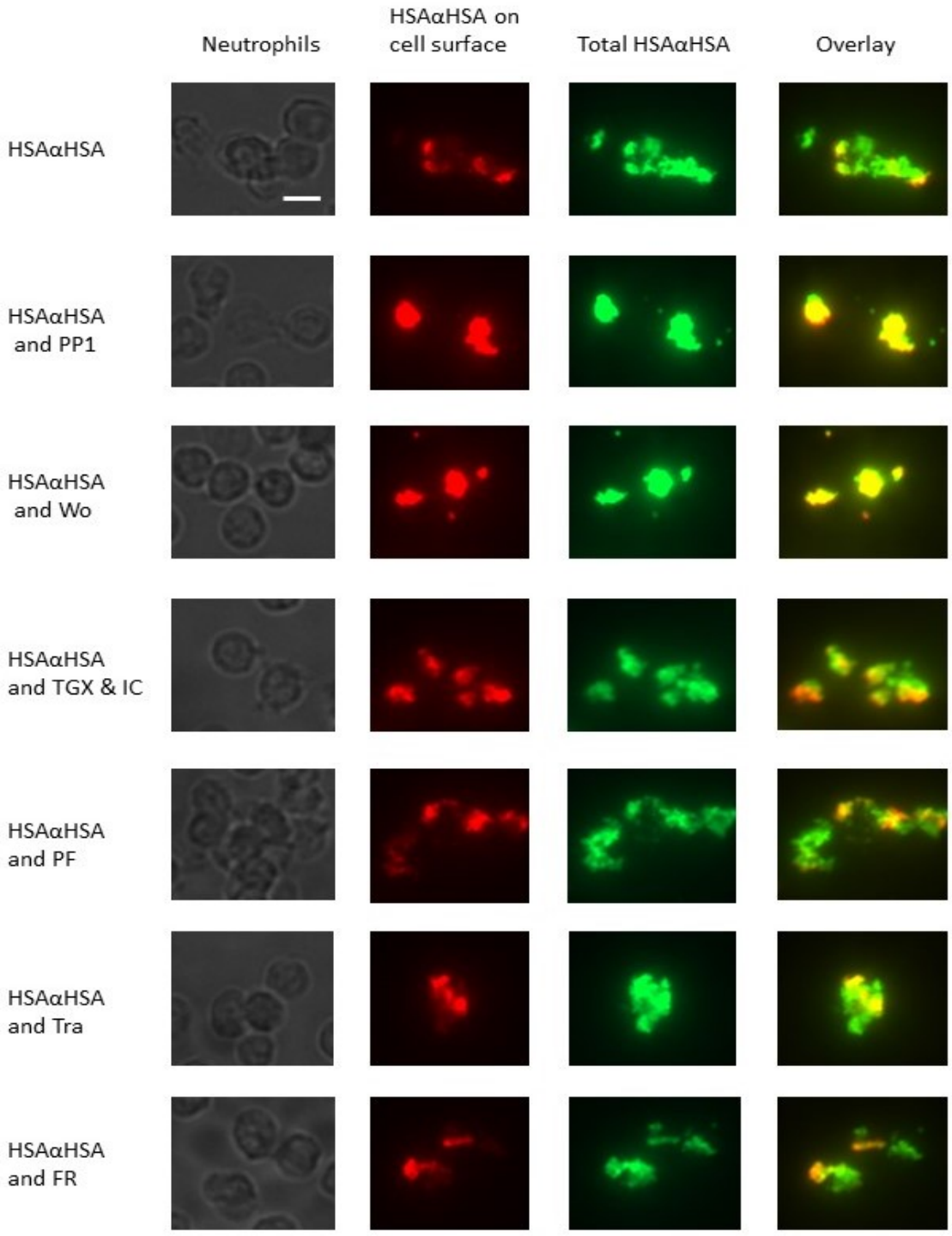


Figure 3.3: Neutrophils internalise insoluble immune complexes.

Cells were pre-treated with inhibitors as indicated for 10 min at 37 °C prior to stimulation with iICs or vehicle for 30 min at 37 °C. Cells were then adhered to glass slides and iICs were labelled with two fluorescent secondary antibodies to distinguish internalised and bound iICs (detailed method is in **chapter 2**, **section 2.8**). Experiment performed by Dr. Julia Chu.

3.1.1. Hypothesis

The work described in this chapter examines the hypothesis that iIC-induced neutrophil apoptosis is distinct from PICD.

3.1.2. Aims

The aforementioned hypothesis was examined by addressing three aims

a) To compare the apoptosis induced by iICs and opsonised particles

b) To investigate the regulators of iIC-induced apoptosis with a focus on cell surface receptors

c) To elucidate the role of ROS production in iIC-induced neutrophil apoptosis

3.2. Results

3.2.1. iICs induce neutrophil apoptosis

Initially I repeated experiments that had previously been performed by Julia Chu, and confirmed that iICs also induced neutrophil apoptosis in my hands. Apoptosis was assessed by analysis of Annexin V/propidium iodide binding using flow cytometry. **Figure 3.4** depicts that iICs induced neutrophil apoptosis after 7 hours following neutrophil stimulation that is inhibited by pan-caspase inhibitors QVD and z-VAD.

I confirmed the results obtained in the flow cytometry based analysis by staining cytocentrifuge preparations of cells from all the conditions (bottom panel of **Figure 3.4**).

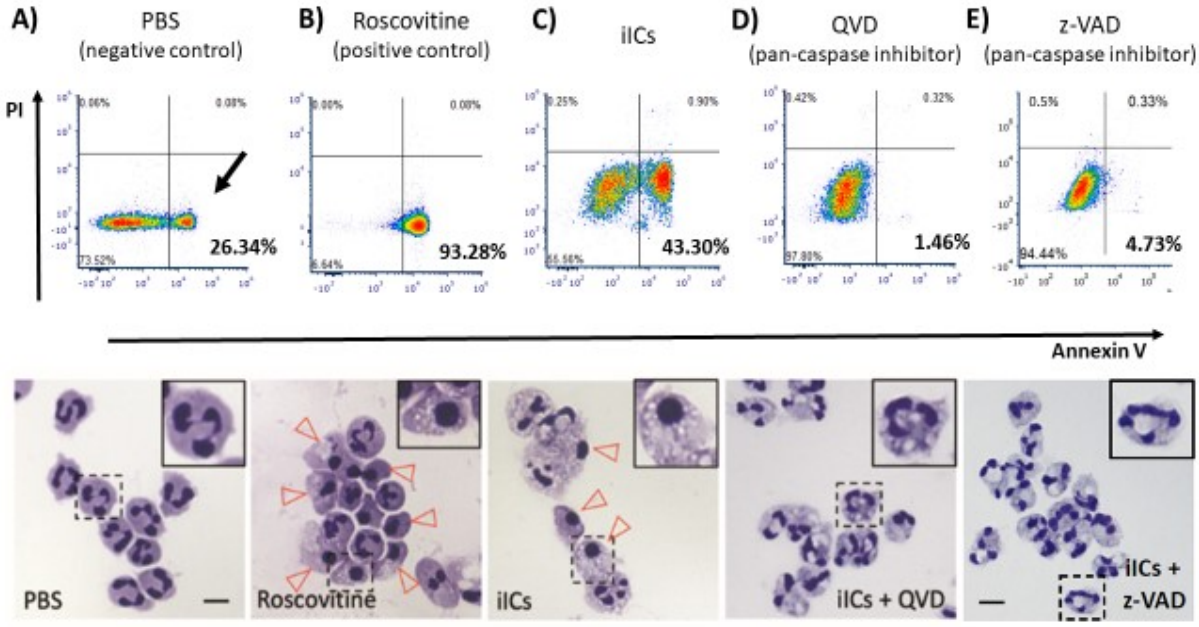


Figure 3.4: Characterisation of iIC-induced neutrophil apoptosis.

Freshly isolated neutrophils were incubated with **A)** PBS, **B)** Roscovitine (20 µM), **C)** iICs (10 µg/ml) **D)** QVD; (QVD-OPH, 10 µM), **E)** z-VAD (Z-VAD-FMK, 100 µM) in IMDM with 10% autologous serum in a humidified, CO_2 controlled incubator at 37 °C. Top panel, after 7 hours in culture cells were labelled with annexin V and PI and analysed on a flow cytometer as detailed in **chapter 2**. AnnV-positive and PI-negative cells were deemed apoptotic (population in the right bottom quadrant, here indicated by a black arrow). Bottom panel, cytocentrifuge preparations of neutrophils were stained with Kwik-Diff. Apoptotic neutrophils with characteristically condensed nuclei are indicated using red arrowheads; for ease of viewing boxed individual cells are shown enlarged in the inserted panels. Scale bars, 10 µM. A representative experiment is shown. Figure adapted from Karmakar et al.,Cell death and disease, 2021.[326]

3.2.2. iIC-induced apoptosis is associated with increased cleavage of caspase targets

As laid out in **chapter 1**, apoptosis is a cell death programme that is dependent upon a cascade of caspase activation and substrate cleavage by activated caspases. Caspase-3 is known as an executioner caspase in apoptosis because of its role in activating enzymes such as caspase-activated DNAse (CAD) and therefore, coordinating the destruction of cellular structures (DNA fragmentation or degradation of cytoskeletal proteins).[54] I performed experiments to quantify cleaved caspase-3 in iIC-stimulated neutrophils by flow cytometry, which showed that significant caspase-3 activation was induced upon stimulating neutrophils with iICs (**Figure 3.5.A** and **Figure 3.5.B**). A significant reduction in cleaved caspase-3 positive cells was observed with QVD and z-VAD, similar to that observed with untreated cells at 7h (**Figure 3.5.B**).

The caspase-3 substrate Gelsolin is an actin binding protein and key regulator of actin filament assembly and disassembly. Caspase 3-induced gelsolin fragmentation contributed to actin cytoskeletal collapse followed by apoptosis.[205] **Figure 3.5.C** shows the cleaved products of gelsolin alongside the full-length protein. iIC treatment induced the production of a notable amount of cleaved gelsolin in a caspase-dependent fashion. Taken together these data show that iIC-induced apoptosis requires caspase-3.

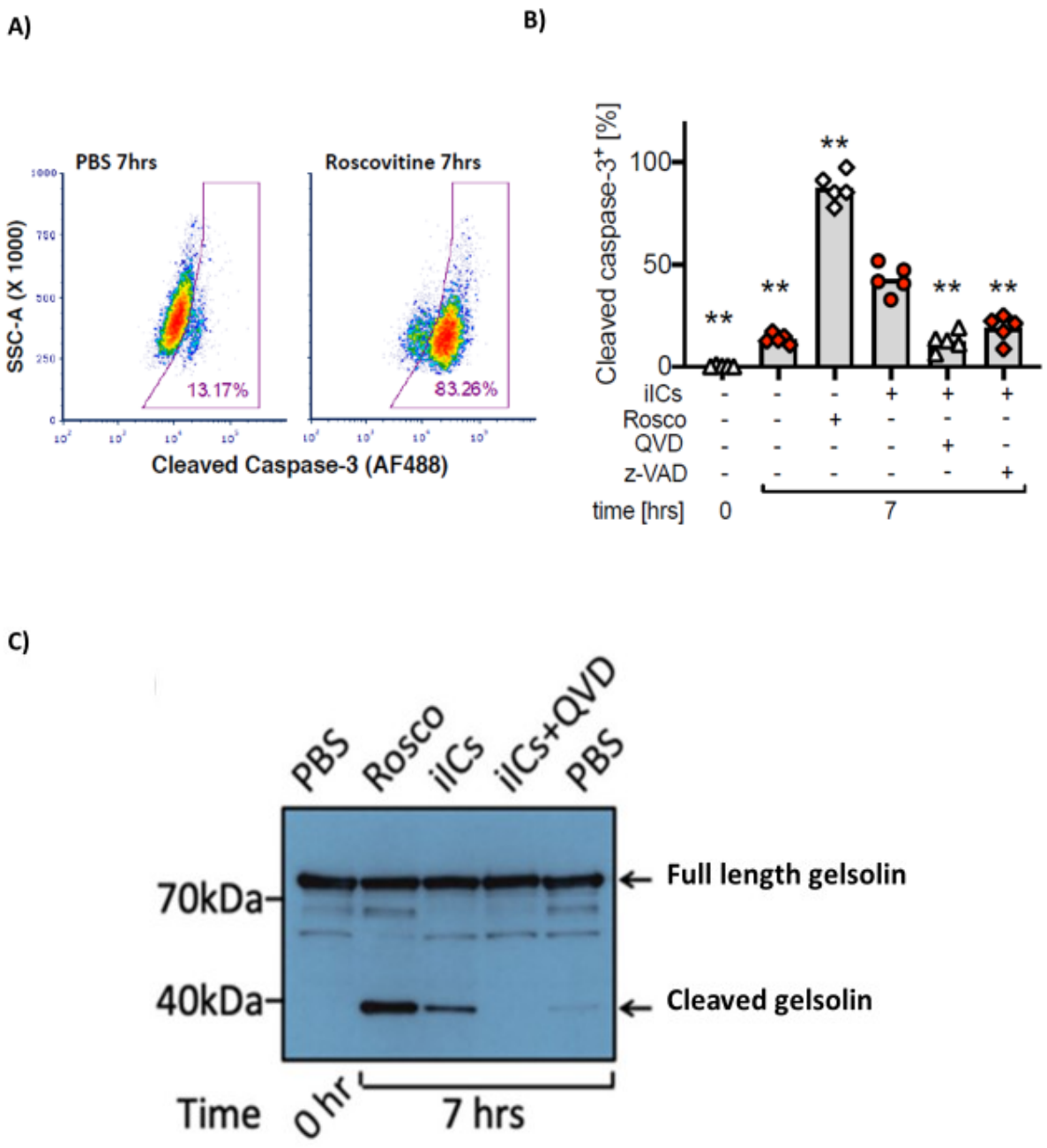


Figure 3.5: Detection of cleaved caspase 3 by flow cytometry and western blot.

Freshly isolated neutrophils were stimulated with iICs or vehicle (PBS) for 7 hours in IMDM with 10% autologous serum with or without prior incubation with inhibitors Roscovitine (Rosco), QVD-OPH (QVD) or Z-VAD-FMK (z-VAD). **A** shows the gating strategy used to identify the cleaved caspase-3-positive apoptotic population using flow cytometry. Each symbol represents the value obtained in one experiment. **, $p<0.01$; analysis was by one-way ANOVA with Bonferroni post hoc test; all comparisons were to the iIC-stimulated condition. The graph in **B** shows the cumulative data obtained in five separately conducted experiments. **C**, cell lysates were prepared and processed for Western blotting to detect full-length gelsolin and its cleavage products. My supervisor helped with this Western blot. A representative example is shown. Figure adapted from Karmakar et al.,Cell death and disease, 2021.[326]

3.2.3. iIC-induced apoptosis is distinct from PICD.

Next, I compared the apoptosis induced by iICs and IgG-opsonised latex beads (for ease of reading these are simply called “IgG-beads” hereafter). As shown in **Figure 3.6**, iICs induced neutrophil apoptosis, but IgG-beads (‘LB 0.8 µm’) did not. IgG-beads failed to induce apoptosis when used at a range of 5-25 beads per cell and also with different sizes of beads (0.8 µm, 3 µm and 0.3 µm; data not shown). There are conflicting reports relating to the induction of apoptosis by IgG-beads.[71,206] and therefore the effects of IgG-beads on apoptosis were not pursued further.

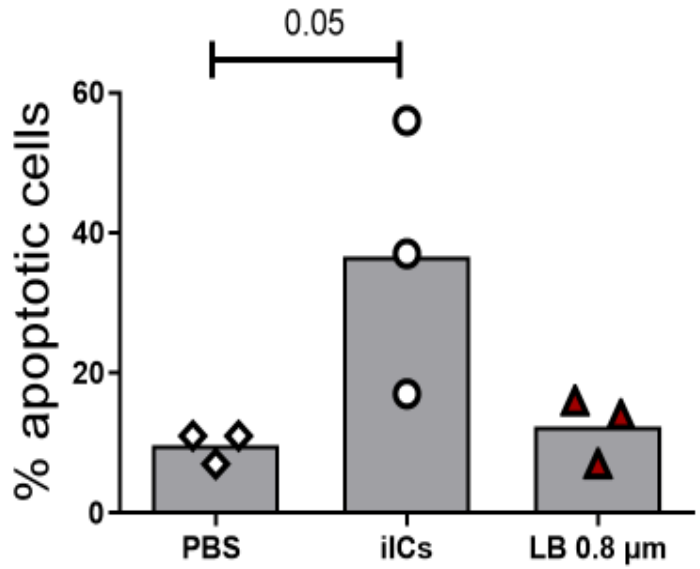


Figure 3.6: IgG-beads (0.8 µm) do not induce apoptosis.

Cytospin data of percentage of apoptotic cells are shown in the bar graph. Neutrophils were cultured with 10% autologous serum for 0h, 3h, 6h, 9h & 12h after iICs or bead stimulation (5 beads per cell are shown here). Cells were treated with vehicle only as a negative control. The induction of apoptosis by iICs was detected as early as 6h. Each symbol is representative of a separately conducted experiment. Data were analysed by One way ANOVA with Bonferroni post hoc test p=0.05.

Since IgG-beads failed to induce neutrophil apoptosis, serum-opsonised zymosan[207] (for ease of reading called zymosan hereafter) were used instead. The induction of neutrophil apoptosis in response to stimulation with iICs and zymosan were compared in collaboration with Dr. Julia Chu. Autologous serum opsonisation led to human IgG coating on the zymosan as observed by immunofluorescent analysis (not shown). To identify whether the two stimuli use the same pathway, inhibitors directed against two signalling intermediates of the pathway regulating iIC-induced neutrophil apoptosis were employed, LY294002 and FR180204. Both inhibitors significantly reduced the percentage of iIC-induced apoptosis (**Figure 3.7.A**) while there was no effect on zymosan-induced apoptosis (**Figure 3.7.B**).

PICD is dependent upon internalisation of particles, requiring dynamic actin reorganisation.[208] I next used two inhibitors of actin polymerisation with distinct mechanisms of action. Cytochalasin inhibits both the rate of actin polymerisation and the interaction of actin filaments in solution, whereas latrunculin binds to G-actin monomers; thus preventing polymerisation. Latrunculin is more potent than cytochalasin.[209] Both latrunculin B and cytochalasin B significantly reduced zymosan-induced apoptosis while iIC-induced apoptosis was unaffected (**Figure 3.7.C** and **Figure 3.7.D**).

These results suggested that iIC-induced apoptosis is not a form of PICD. In contrast, induction of both types of apoptosis was significantly reduced when ROS production was inhibited using the NADPH oxidase inhibitor DPI, suggesting that both processes were ROS dependent.

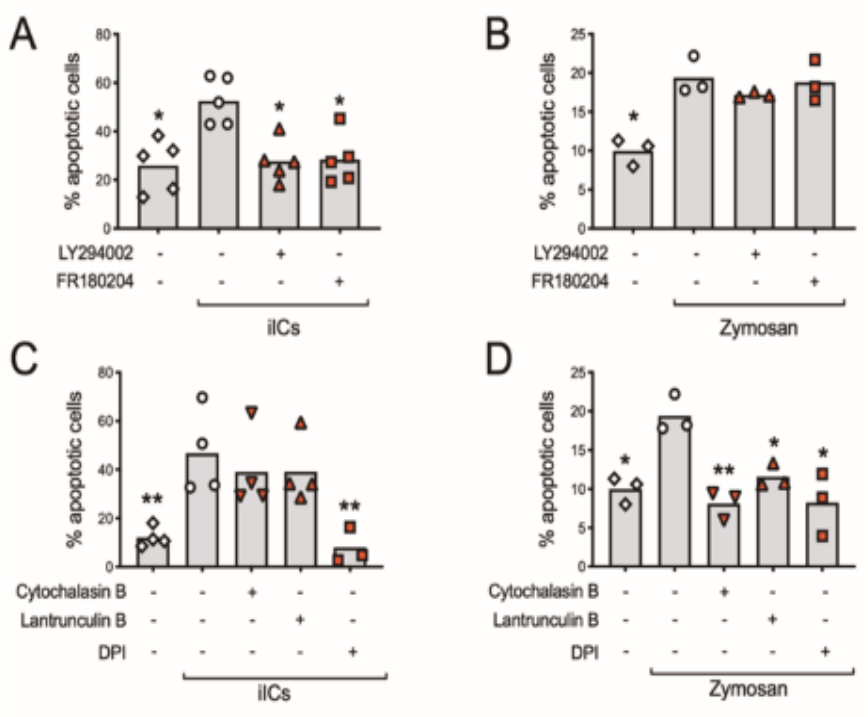


Figure 3.7: iIC-induced apoptosis is distinct from phagocytosis-induced cell death (PICD).

Freshly isolated neutrophils were pre-incubated at 37 °C for 10 min with or without inhibitors as indicated. Cells were then stimulated with opsonised zymosan (3 particles per neutrophil) or iICs (10 µg/ml) or vehicle for 12 hours in IMDM with 10% autologous serum in a humidified CO_2 incubator at 37 °C. Panels **A** and **B** show the percentage of apoptosis induced by iICs and serum opsonised zymosan respectively and also, the effects of LY294002 and FR180204. Panels **C** and **D** show the effects of Cytochalasin B, Latrunculin B and DPI (diphenyleneiodonium) on iIC-induced and zymosan-induced apoptosis respectively. The results combine three to five separately conducted experiments. Each symbol represents the result obtained in one experiment. Data were analysed by Kruskal Wallis test with Dunn's post hoc test. All comparisons were to the iIC-stimulated condition. *, $p<0.05$; **, $p<0.01$. This experiment was performed in a collaboration with Dr. Julia Chu. Figure adapted from Karmakar et al.,Cell death and disease, 2021.[326]

3.2.4. iIC-induced apoptosis is FcγRII dependent.

I also investigated the receptor involvement of iIC-induced apoptosis in a series of experiments performed together with Dr. Julia Chu. Neutrophils were pre-treated with FcγR blocking monoclonal antibodies either alone or in combination. IV.3 and AT10 are directed against FcγRII while 3G8 binds to FcγRIII. Apoptosis was then assessed with neutrophils that had or had not been stimulated with iICs. Blocking FcγRII significantly reduced the induction of apoptosis (**Figure 3.8**), while blocking FcγRIII did not, indicating that iIC-induced apoptosis is FcγRII-dependent. No further reduction was observed with cells in which both receptors had been blocked, suggesting that FcγRIII is not involved in regulating iIC-induced apoptosis.

Interestingly, these experiments showed that AT10 was a more efficient blocking antibody than IV.3, with AT10 completely abolishing iIC-induced apoptosis while IV.3 resulted only in a partial inhibition. AT10 has been shown to have a higher affinity for the FcγRII receptor with a K_D value of 2 nM while that of IV.3 is 5 nM,[210,211] providing a possible reason for this observation. Another possible explanation could be their distinct epitope recognition. I therefore used AT10 to block FcγRII in further experiments when analysing Fcγ receptor involvement (see **section 3.2.8**).

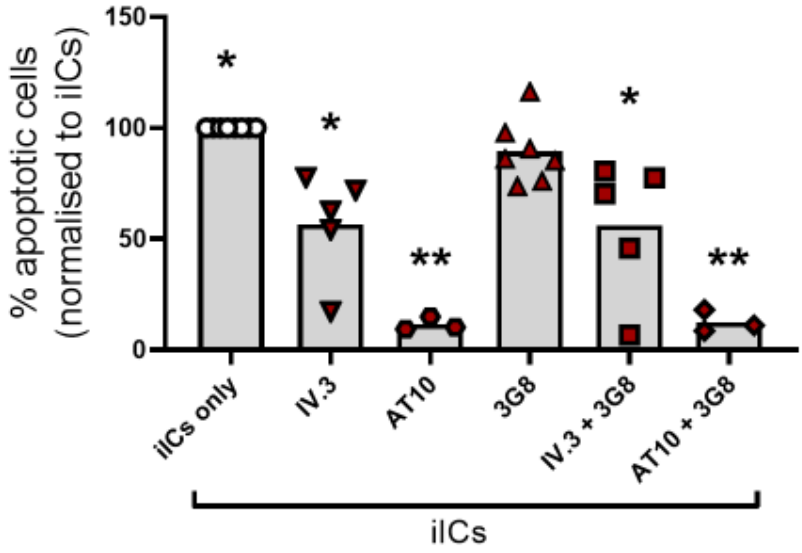


Figure 3.8: iIC-induced apoptosis is FcγRII mediated.

Freshly isolated neutrophils were pre-incubated at 4 °C for 30 min with FcγR blocking antibodies (as indicated). Cells were then stimulated with iICs (10 µg/ml) and cultured in IMDM with 10% autologous serum in a humidified CO_2 incubator at 37 °C. The results presented are from five to eight separate experiments with each symbol representing the result of one experiment. Raw results were analysed by Kruskal Wallis test with Dunn's post hoc test. The percentage of apoptosis with each treatment was compared to the 'iICs only' condition. *, $p < 0.05$, **, $p < 0.01$. Figure adapted from Karmakar et al.,Cell death and disease, 2021.[326]

3.2.5. iIC-induced apoptosis is dependent on NADPH oxidase, catalase and myeloperoxidase.

Since both PICD and iIC-induced apoptosis were NADPH oxidase-dependent (**Figure 3.7.C** and **Figure 3.7.D**), I investigated the ROS pathway in more detail to determine which substrates are required for iIC-induced neutrophil apoptosis.

Several enzymatic reactions are involved in ROS generation and lead to the production of toxic free radicals. These toxic free radicals damage the cells and have been proposed to lead to cell death, including by apoptosis.[61,212] **Figure 3.9** depicts a simplified ROS regulation pathway. Oxygen is reduced to superoxide by NADPH oxidase in the initial step of ROS generation. Then, superoxide dismutase (SOD) catalyses the production of hydrogen peroxide (H_2O_2) which serves as the substrate for myeloperoxidase (MPO) in the generation of the highly toxic hypochlorite ion (OCL^-) in a third reaction. Catalase is an enzyme which breaks down H_2O_2 into water and oxygen, thereby preventing the production of toxic hypochlorite and hydroxyl radicals.[213]

To assess the involvement of these enzymes, I performed apoptosis experiments in which neutrophils were treated with four inhibitors, DPI (NADPH oxidase inhibitor), DETC (SOD inhibitor), 475944 (myeloperoxidase inhibitor) and commercial catalase to facilitate H_2O_2 breakdown prior to stimulation with iICs to induce apoptosis.

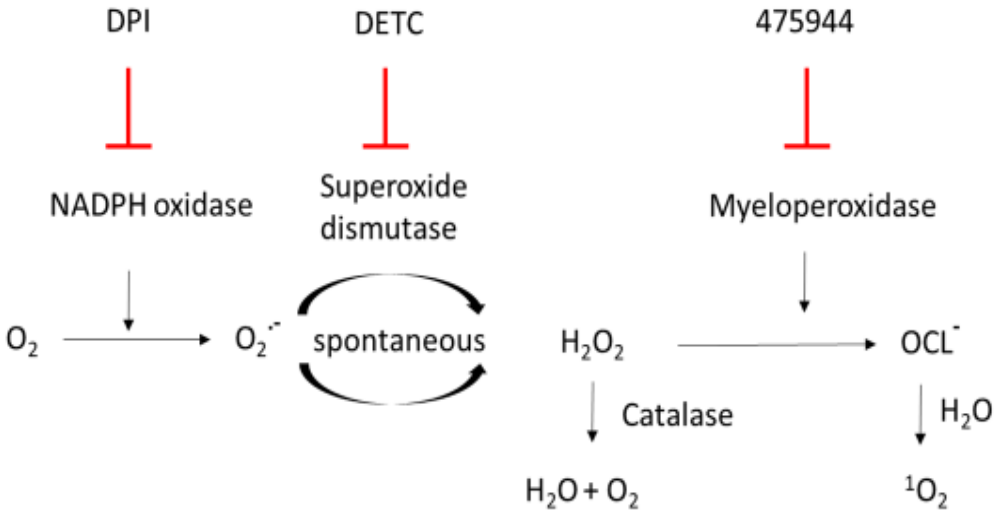


Figure 3.9: A simplified pathway of ROS regulation.

Cellular stress causes generation of ROS which eventually lead to cell damage and death including apoptosis. Several toxic free radicals are produced along the pathway that initiates the damage. NADPH oxidase is involved in the first step of ROS generation where O_2 is reduced to superoxide. Superoxide dismutase helps in H_2O_2 production followed by breaking down to hypochlorite ion by myeloperoxidase. Catalase is a key enzyme which uses hydrogen peroxide as its substrate. This enzyme is responsible for neutralisation through decomposition of hydrogen peroxide, thereby maintaining an optimum level of the molecule in the cell which is also essential for cellular signalling processes. DPI, DETC and 475944 inhibit the effects of NADPH oxidase, superoxide dismutase and myeloperoxidase respectively.

Inhibition of the NADPH oxidase (the first step of the ROS pathway) blocked ROS generation completely (**Figure 3.10.A** and **Figure 3.10.B)**. Next, I tested the effects of other ROS inhibitors on neutrophil apoptosis, revealing that DPI, catalase and MPO inhibitor all reduced apoptosis significantly, with DPI being the most prominent one (**Figure 3.10.C**).

Catalase is a non cell permeable scavenger; it can therefore only act on external ROS. The fact that catalase significantly reduced the percentage of apoptosis induced by iICs, shows that iICs induce external ROS and, more importantly, that iIC-induced apoptosis requires external ROS. Moreover, DETC which inhibits SOD did not affect the iIC-induced apoptosis suggesting the conversion of superoxide to H_2O_2 is not dependent on SOD, but rather occurs via spontaneous dismutation.[214]

In addition, I sought to investigate the role of mitochondrial ROS production in the context of iIC-induced apoptosis using two mitochondrial ROS inhibitors, Rotenone and FCCP. Although Rotenone significantly reduced the percentage of apoptosis, FCCP did not have any effect on iIC-induced apoptosis (not shown). Both compounds have a range of off-target effects, and are not thoroughly characterised, making it difficult to draw any conclusions from these results. Mitochondrial ROS were therefore not further explored.

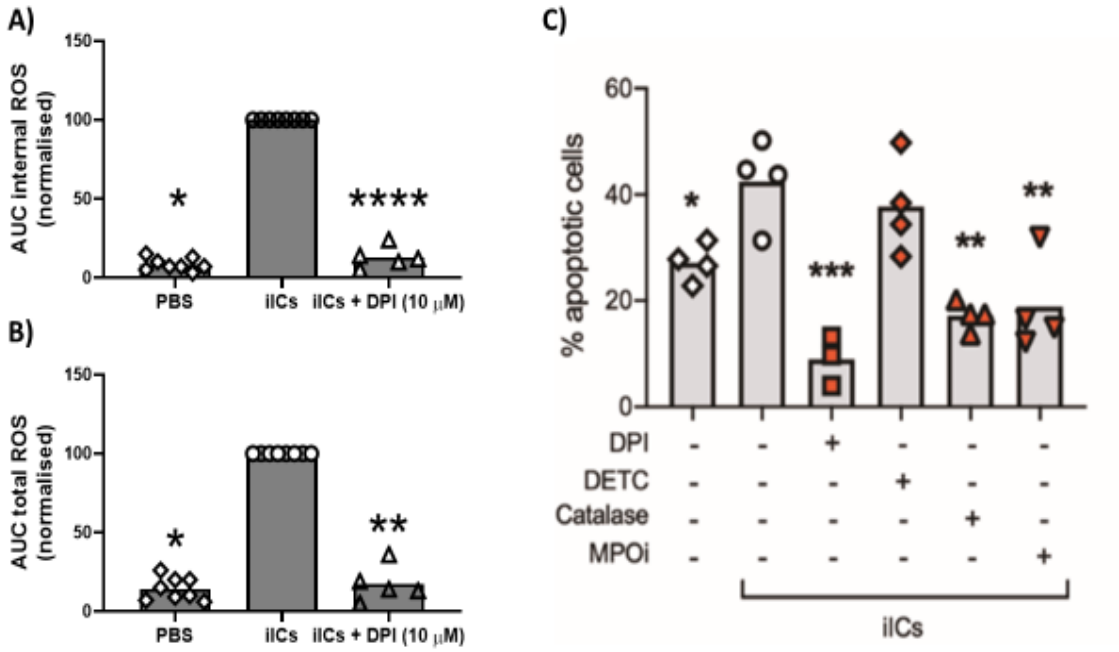


Figure 3.10: Effects of different ROS inhibitors on iIC-induced apoptosis.

Normalised internal (**A**) and total (**B**) ROS data with vehicle, iICs and iICs+ DPI treatment. Isolated neutrophils were pre-treated with the different ROS inhibitors, DPI (10 μM), DETC (10 μM), MPOi (500 μM) and catalase (25 μg/ml) for 10-30 min at 37 °C prior to iIC (10 μg/ml) treatment with 10% autologous serum for 9h (**C**). The percentages of apoptosis were compared with iIC-induced apoptosis. The results presented are from four to eight separate experiments with each symbol representing the result of one experiment. Raw results were analysed by One way ANOVA with Bonferroni post hoc test. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$. Figure **C** taken from Karmakar et al.,Cell death and disease, 2021.[326]

3.2.6. Opsonised bead-induced ROS and iIC-induced ROS are regulated differently.

The above results suggest that iICs generate both internal and external ROS, while phagocytosis is thought to result in internal ROS. I therefore next compared iIC-induced ROS with bead-induced ROS.

Since, PI3K has been shown to play an important role in ROS production downstream of protein tyrosine kinase signalling,[92,98] an inhibitor against pan-PI3K was used to assess ROS generation. Both internal and total iIC-induced ROS and bead-induced ROS were inhibited by LY294002 in an equal fashion (**Figure 3.11.A** and **Figure 3.11.B**) indicating that both types of ROS require PI3K as expected.

In contrast, differences between the stimuli were noticed with latrunculin–B treated samples (**Figure 3.11.C** and **Figure 3.11.D**). Both internal and total bead-induced ROS were abrogated by latrunculin-B (**Figure 3.11.B** and **Figure 3.11.D**), suggesting that IgG-beads induced internal ROS with only negligible external ROS being produced. In contrast, latrunculin B treatment caused a shift from internal to external ROS with iIC-stimulated neutrophils (**Figure 3.11.C**). This difference in behaviour of the two stimulation conditions suggests that different regulatory mechanisms are at play, although both events are triggered in an antibody-dependent fashion, presumably via FcγRs. To better understand how iICs regulate ROS, I next investigated iIC-induced internal and external ROS, as well as the receptor involvement in more depth.

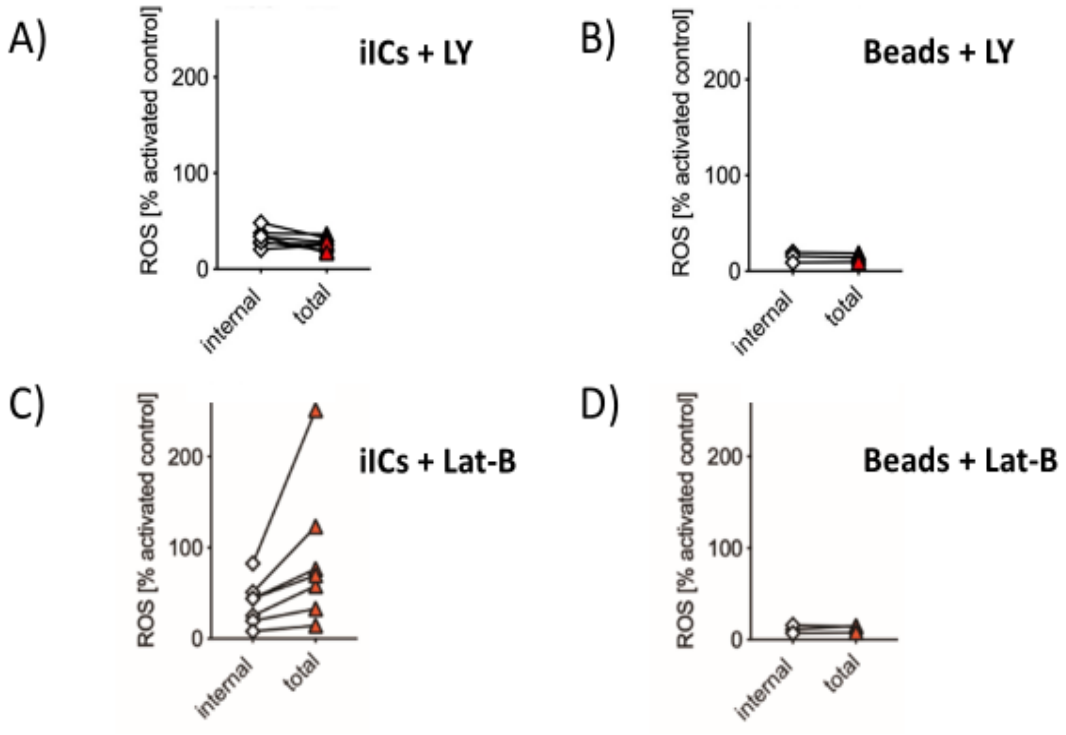


Figure 3.11: IgG bead-induced ROS and iIC-induced ROS are regulated via different pathways.

Freshly isolated neutrophils were pre-incubated with the inhibitors LY (LY294002, pan-PI3K inhibitor, 10 µM, **A** and **B**) and Lat-B (Latrunculin B, actin polymerisation inhibitor, 10 µM, **C** and **D**) at 37 °C for 10 min prior to mixing with luminol or luminol with HRP to determine internal and total ROS as detailed in **chapter 2**. Cells were then stimulated with iICs (4 µg/ml) or IgG-beads (0.8 µm, 25 beads per cell). Luminescence was measured in a plate reader. The results presented are from three to seven separately conducted experiments. Each symbol represents the result obtained in one experiment. This pairwise data analysis was performed by Mann Whitney test of the raw data. Figure adapted from Karmakar et al.,Cell death and disease, 2021.[326]

3.2.7. iICs generate both internal and external ROS.

The assay I used to analyse ROS production relies on the observation that luminol exhibits chemiluminescence when it is being oxidised. Since luminol is cell permeable, it will detect internal ROS production when H_2O_2 activates MPO inside the cell. To detect external H_2O_2 as well (total ROS), the assay depends on the addition of HRP. Taking advantage of the fact that the chemiluminescence reaction triggered by ROS production can be observed in real-time, I compared internal and total ROS production of aliquots of the same neutrophils, in response to stimulation with iICs or IgG-beads. Both iICs and IgG-beads triggered a lengthy peak of internal ROS (**Figure 3.12.A**). In the parallel analysis of total ROS, a similar looking curve with a single peak was produced in response to stimulation with IgG-beads (note that the values obtained in the presence of HRP are lower due to the brown colour of this reagent). In contrast, iIC stimulation resulted in two peaks of total ROS (**Figure 3.12.B**) suggesting that both external and internal ROS are generated following iIC stimulation. I therefore concluded that iIC-induced ROS and bead-induced ROS are different.

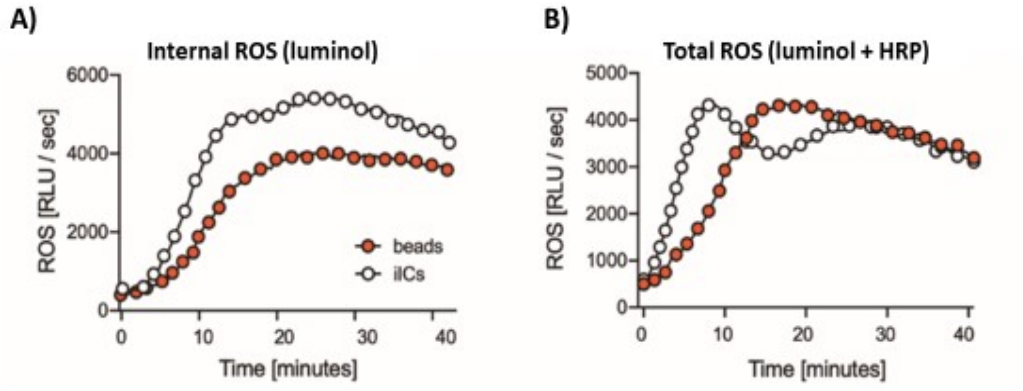


Figure 3.12: iICs generate both internal and external ROS.

Freshly isolated neutrophils were mixed with luminol (**A**, internal ROS) and luminol with HRP (**B**, total ROS) and then treated with iICs (4 µg/ml) or IgG-beads (0.8 µm, 25 beads per cell). The luminescence was measured at the plate reader every 30 sec up to 45 min. A representative graph of at least five separately conducted experiments. Figure adapted from Karmakar et al.,Cell death and disease, 2021.[326]

3.2.8. iIC-induced ROS depends on FcγR and also on Integrin αMβ2.

I next performed experiments to identify the receptors involved in iIC-induced ROS generation. Due to extensive crosstalk between the Fcγ receptors and integrins (as discussed in **chapter 1**), I designed experiments to test all the receptors that might be involved.

Three FcγR blocking antibodies and one αMβ2 blocking antibody were used either separately or in combination to evaluate their effects on internal and total ROS. As expected, blocking FcγRII receptor led to a significant reduction of both internal and total ROS. However, to our surprise both FcγRI and αMβ2 were identified as equally involved according to the extent to which they blocked iIC-induced ROS production (**Figure 3.13.A**). Comparing the generation of internal and total ROS in parallel in real-time again allowed me to gain a deeper understanding. Interestingly, blocking FcγRI or αMβ2 did not have any effect on the initial peak of external ROS, while blocking FcγRII abrogated external ROS production (**Figure 3.13.B**). Blocking FcγRIII did not interfere with ROS production, identifying that this receptor has no major involvement here (**Figure 3.13.C** and **Figure 3.13.D**). I concluded that the external ROS is entirely dependent upon FcγRII, while internal ROS relied on FcγRI, FcγRII and αMβ2.

The blocking antibodies were next used in different combinations to further characterise the receptor dependency of iIC-induced ROS generation (**Figure 3.13.E** and **Figure 3.13.F**). When used in any combination, all four blocking antibodies significantly reduced both iIC-induced internal and total ROS, however, none of the conditions resulted in complete inhibition of ROS generation, suggesting that further factors might be involved in this complex regulatory mechanism.

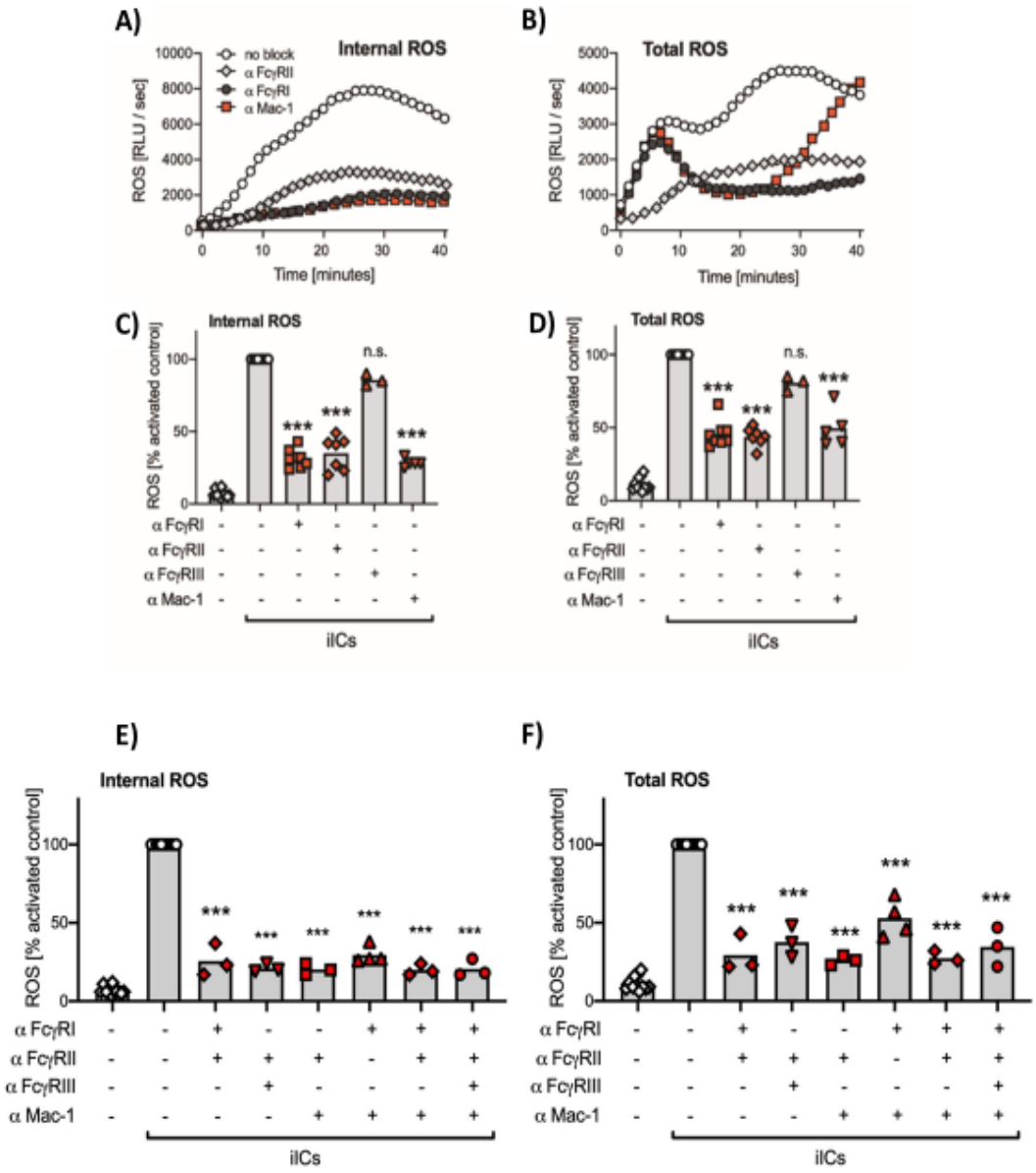


Figure 3.13: Involvement of FcγR and αMβ2receptors in iIC-induced ROS generation.

Freshly isolated neutrophils were pre-incubated with vehicle or blocking antibodies against FcγRI (10.1, 10 µg/ml), FcγRII (AT10, 5 µg/ml), FcγRIII (3G8, 5 µg/ml) and αMβ2 (Mac-1, ICRF44, 12.8 µg/ml) separately (**A-D**) or in combinations (**E** and **F**) for 30 min on ice prior to mixing with luminol to assay generation of internal ROS or luminol with HRP for analysing total ROS production. Cells were then treated with iICs (4 µg/ml) and luminescence was measured in a plate reader as detailed in **chapter 2**. The results presented are form three to seven separately conducted experiments. **A, B** show representative examples while **C-D** integrate the areas under the graph obtained in individual experiments, normalising the data to the iIC-stimulated condition. Each symbol represents the result obtained in one experiment. Raw data were subjected to statistical analysis with One way ANOVA test and Bonferroni post hoc analysis. ***, $p<0.001$; n.s., not significant. Figure adapted from Karmakar et al.,Cell death and disease, 2021.[326]

3.3. Discussion

In this chapter, I compared iIC-induced neutrophil apoptosis with PICD. In summary, my results suggested that iIC-induced apoptosis is mechanistically distinct from PICD (**Figure 3.14**). iICs activate the neutrophils by cross-linking FcγRII receptors. Moreover, iIC-induced apoptosis and PICD are regulated by different signalling pathways, and actin polymerisation was required for PICD but not for iIC-induced apoptosis. However, both processes were shown to be dependent on ROS production. Both iIC-induced apoptosis (**Figure 3.8**) and PICD (data not shown) were FcγRII dependent however other receptors such as FcγRI and αMβ2 were not studied in the context of neutrophil apoptosis.

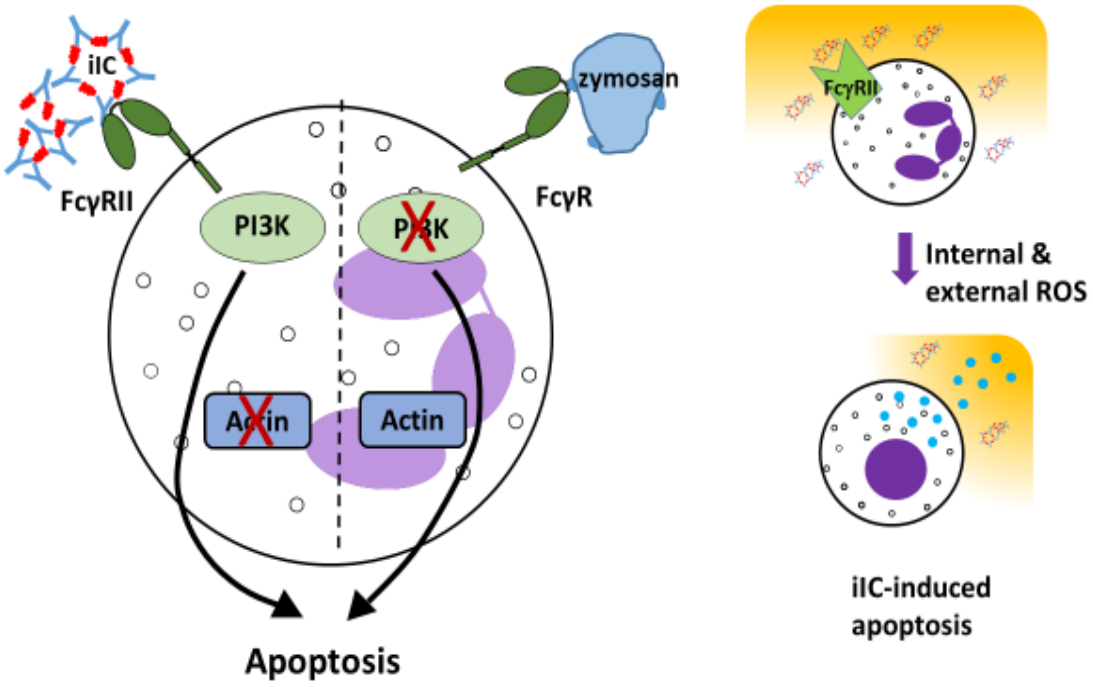


Figure 3.14: iIC-induced neutrophil apoptosis is mechanistically distinct from PICD.

FcγRII mediated iIC-induced apoptosis is PI3K-dependent whereas zymosan-induced apoptosis is not. Actin polymerisation is required for zymosan-induced apoptosis but not for iIC-induced apoptosis.

iICs induce the generation of internal and external ROS, both of which are required for the induction of apoptosis. Blocking actin polymerisation does not inhibit iIC-induced neutrophil apoptosis, instead more external ROS are generated. Interestingly, catalase is sufficient to interfere with iIC induced apoptosis by blocking the generation of external ROS. Since in this scenario, internal ROS should still be present, but was not adequate to induce apoptosis on its own, this points at a key role of external ROS. Altogether the results suggest that additional mechanisms, perhaps proteases are involved.[215,216] Involvement of proteases in apoptosis induction is discussed in **chapter 4**.

By blocking FcγRII receptors, both internal and total ROS were significantly reduced. However, to my surprise FcγRI and αMβ2 were also involved in regulating ROS production (**Figure 3.14**). Blocking these two receptors reduced specifically the internal, but not the external ROS. In my analysis, FcγRIII did not have any effect on iIC-induced ROS production or apoptosis contrasting with another report.[217] Since, the combination of all blocking antibodies did not completely wipe out the ROS production, it might be possible that additional factors are involved.

FcγRI is a high affinity Fcγ receptor (as described in the **chapter 1**) which is generally not expressed by resting neutrophils.[218,219] FcγRI has been found on neutrophils from patients with certain bacterial infections and leukocyte adhesion deficiency.[220] FcγRI can be induced *in vitro* after incubation with interferon-γ or recombinant human granulocyte colony-stimulating factor (rhG-CSF). Studies have shown that FcγRI was highly expressed in neutrophils in synovial fluid derived from rheumatoid arthritis patients however no overexpression was observed in blood derived neutrophils.[221] Moreover, Mayadas et al elucidated a novel mechanism by which FcγRI is expressed in neutrophils upon IC stimulation and they adopt antigen presenting abilities.[100]

Another study reported that upon successful FcγRII binding with heat aggregated IgGs, FcγRI expression is upregulated by mobilising it from intracellular stores.[106]

On the other hand, αMβ2 is a complement receptor, which binds to complement components[116] and also many other ligands including fibrinogen, factor X .[222,223] This leukocyte adhesion receptor has been shown to functionally interact with FcγR dependent IC-mediated neutrophil functions.[120,121] However, complement involvement is unlikely in my assay since the experiments were conducted in PBS^{++}, a serum-free condition.

Since FcγRI is not expressed in resting neutrophils and αMβ2 is complement dependent, involvement of these receptors in healthy donors' blood-derived neutrophils in serum-free conditions is surprising. Evidence suggests that the expression of certain receptors might be altered in disease conditions such as higher expression of FcγRI in neutrophils isolated from the synovial fluid of RA patients.[221] Further meticulous investigation is essential to address the alteration of receptor expression in healthy neutrophil as well as in rheumatoid arthritis patients.

Upon efficient cross-linking of Fcγ receptors neutrophils have been shown to degranulate, increase ROS production and trigger NET formation[224] and it has been shown to promote apoptosis in other cell types such as T cells and natural killer (NK) cells.[225] In the future, controlled cross-linking of the individual Fc receptors with specific subclasses of antibodies could be studied in detail in the context of iIC-induced neutrophil apoptosis for a better understanding of receptor involvement.

Another class of Fc receptor is present in neutrophils, FcRn (neonatal Fc receptor), that is a major histocompatibility class I (MHC-I) homolog.[226] Blocking FcRn-IgG interaction was shown to impair IgG mediated phagocytosis[110] and thus, exploiting this

mechanism to decrease circulating IgG levels is one strategy to treat auto-immune disease. In the absence of interaction with FcRn, IgG would be degraded more quickly.[103] Involvement of FcRn in iIC-mediated neutrophil functions could be evaluated to gain further knowledge.

In conclusion, I have shown in this chapter that iIC-induced neutrophil apoptosis and PICD are two distinct mechanisms, which led me to investigate the internalisation mechanism in more detail (**chapter 4**).

4. Results: iICs are internalised by macropinocytosis in a FcγRII dependent fashion prior to rapid degradation.

4.1. Introduction

The ability of the cells to internalise extracellular material was first reported in the year 1882 by Ilya Metchnikoff[227] referred as phagocytosis ("cell eating"). Later, in 1931, Warren H. Lewis discovered the uptake of extracellular fluid by cells,[228] referred as pinocytosis ("cell drinking").

Different modes of internalisation of particles by the cells are summarised in **Figure 4.1**. Ingestion of particles larger than 0.5 µm size typically occurs via triggered processes called phagocytosis or macropinocytosis while smaller particles are internalised by other modes of endocytosis. The size cut off for macropinocytosis has been described as 0.2 µm, and internalisation of particles < 0.2 µm therefore is termed as endocytosis.[229,230] Several endocytic pathways are involved in internalisation of small particles, including clathrin-mediated endocytosis, caveolin-dependent uptake and the CLIC/GEEC pathway which is clathrin- or caveolin-independent.[231–233]

The current chapter focuses on defining the mechanism of internalisation of iICs. I have discussed in the previous chapter that iIC-induced neutrophil apoptosis is distinct from PICD. The shape change and the fluorescent micrographs shown in **Figure 3.1** and **Figure 3.2** of the previous chapter provided evidence of neutrophils internalising iICs. This observation led to investigating whether iICs are internalised by phagocytosis.

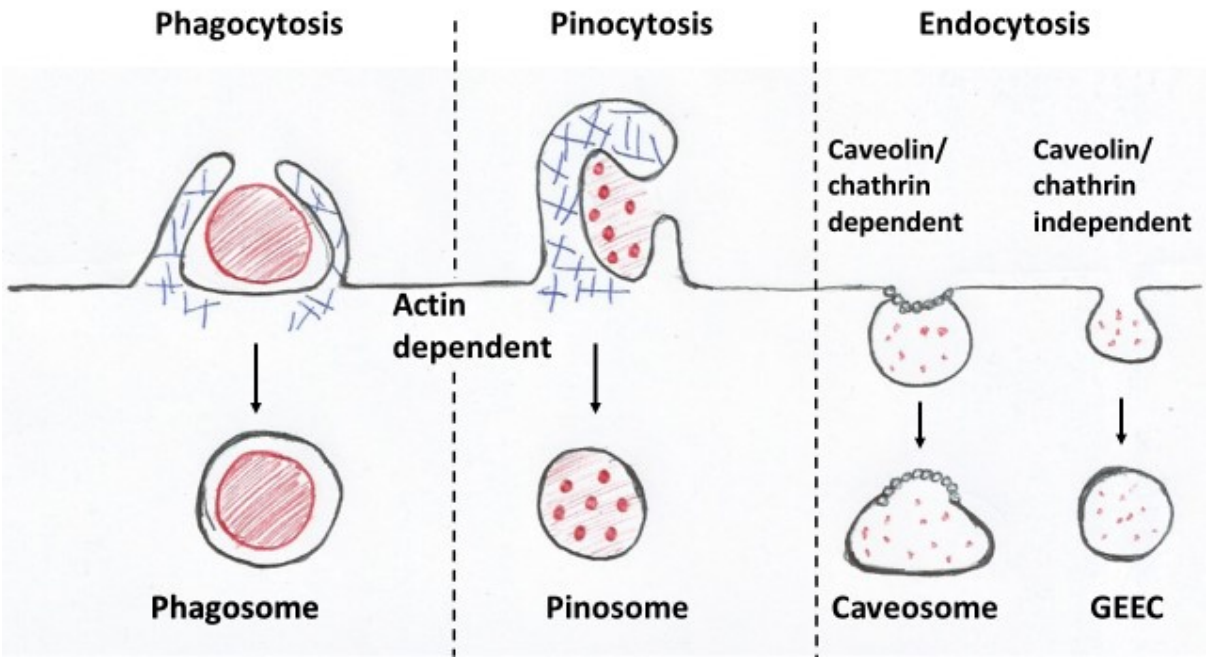


Figure 4.1. Different modes of internalisation by cells.

Actin-mediated (blue lines) uptake of larger solid particles (red circle) occurs via phagocytosis and involves the formation of a phagocytic cup whereas uptake of fluid with the solutes (larger red dots) occurs via macropinocytosis and involves the formation of dorsal ruffles. Smaller particles are internalised by endocytosis by mechanisms that are dependent on the coat protein clathrin, or caveolin, while others are clathrin- and caveolin-independent. The size of the vesicles, phagosomes and macropinosomes formed by phagocytosis and macropinocytosis respectively, is much larger than the endosomes formed during endocytosis.

GEEC = GPI-Enriched Endocytic Compartments.

4.1.1. Hypothesis

In view of the fact that iIC-induced apoptosis is not PICD, I hypothesised that iICs are not internalised by phagocytosis.

4.1.2. Aims

The abovementioned hypothesis was examined by addressing three aims

a) To compare the internalisation of iICs and IgG-beads by the neutrophils

b) To investigate the receptors involved in the internalisation process

c) To observe the fate of internalised iIC

4.2. Results

4.2.1. Neutrophils internalise iICs in a PI3K-dependent fashion

Experiments were carried out by labelling iICs with two different fluorescently conjugated antibodies to detect, and to differentiate intracellular iICs from their extracellular counterparts (**Figure 4.2.A**). Fixed cells with internalised iICs were counted visually using epifluorescence on a light microscope to establish the percentage of cells that had internalised iICs. Data with detailed quantification are shown in **Figure 4.4** later in this chapter.

Figure 4.2.A and **Figure 4.2.B** show that upon successful contact, iICs were internalised by neutrophils in a PI3K-dependent fashion. Treatment with wortmannin blocked the internalisation but the iICs were still found to be attached to the cells (**Figure 4.2.B**). To assess whether this phenomenon is dependent on cell adhesion to the substratum, experiments were replicated with cells kept in suspension, followed by quantification of internalisation using flow cytometry (**Figure 4.2.C** and **Figure 4.2.D**).

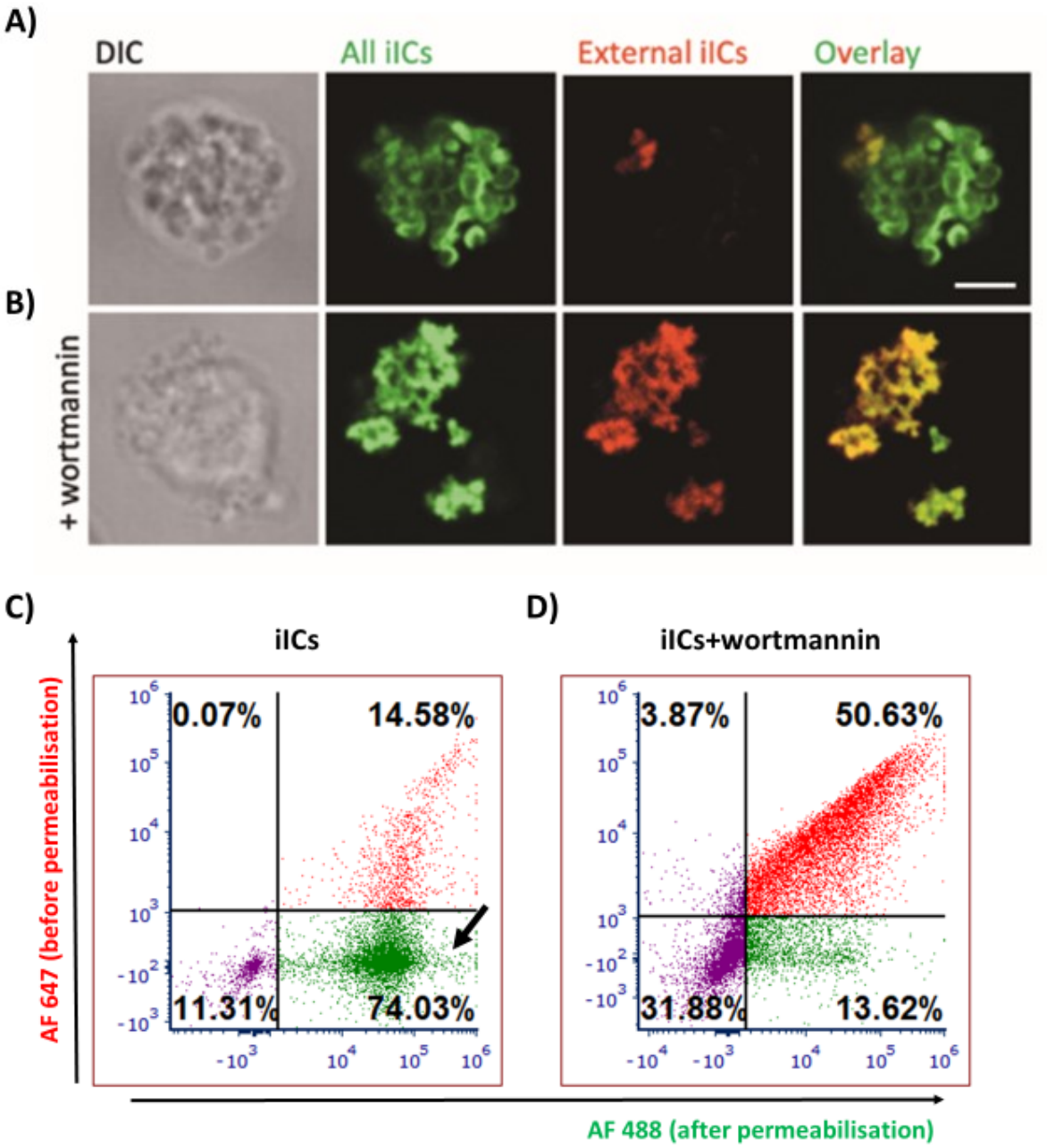


Figure 4.2. Neutrophils internalise iICs in a PI3K-dependent fashion.

Neutrophils were pre-incubated with vehicle (**A, C**) or wortmannin (**B, D**), for 10 min at 37 °C. Cells were then stimulated with iICs (2 µg/ml) for 30 min at 37°C in PBS^{++}. (**A, B**), neutrophils were allowed to adhere to a glass slide (see **section 2.8** in **chapter 2**) and, fixed with 2% PFA or (**C, D**) kept in suspension for flow cytometry. Attached, external iICs were labelled with AF 568 (**A, B**) and AF 647 (**C, D**) conjugated secondary antibodies, indicated by red colour. After permeabilisation, iICs were then labelled once more with an AF 488 conjugated secondary antibody (green). **A, B** images were obtained using a Zeiss LSM780 confocal microscope with 63x objective. Scale bar, 2 µm. **C** and **D** are flow plots showing the percentage of internalisation ($AF488^{+}$ $AF647^{-}$ cells, arrow). A representative example is shown from ≥ 5 independent experiments performed. Figure adapted from Karmakar et al.,Cell death and disease, 2021.[326]

4.2.2. Internalisation of iICs and IgG-beads occurs in a similar timeframe

For a comparative analysis, opsonised latex beads were used since latex beads are well-characterised particles for assessing phagocytosis.[203] To limit neutrophil receptor involvement to a single receptor class (FcγR) latex beads were opsonised with polyclonal rabbit IgG for the analysis of phagocytosis under serum-free conditions. In order to characterise the internalisation process, first a time-course was performed with both IgG-beads and iICs. Neutrophils were stimulated with iICs or IgG-beads for different durations up to 2 hours prior to fixation of the cells. The number of neutrophils with internalised particles was counted under the fluorescence microscope. IgG-bead numbers used in these experiments varied from 5-25 beads per cell, however, the percentage of internalisation was not significantly improved by altering the particle load. Similarly, increasing the amount of iICs did not increase the percentage of internalisation observed (data not shown).

Figure 4.3 shows the percentage of cells that had internalised iICs and IgG-beads at different time-points. The results obtained show that internalisation of iICs and also of IgG-beads is a fast process that commenced within minutes; a peak was reached after 30 minutes of stimulation, suggesting saturation of internalisation. Because of this, further analysis of internalisation was conducted after 30 minutes of stimulation with iICs or IgG-beads.

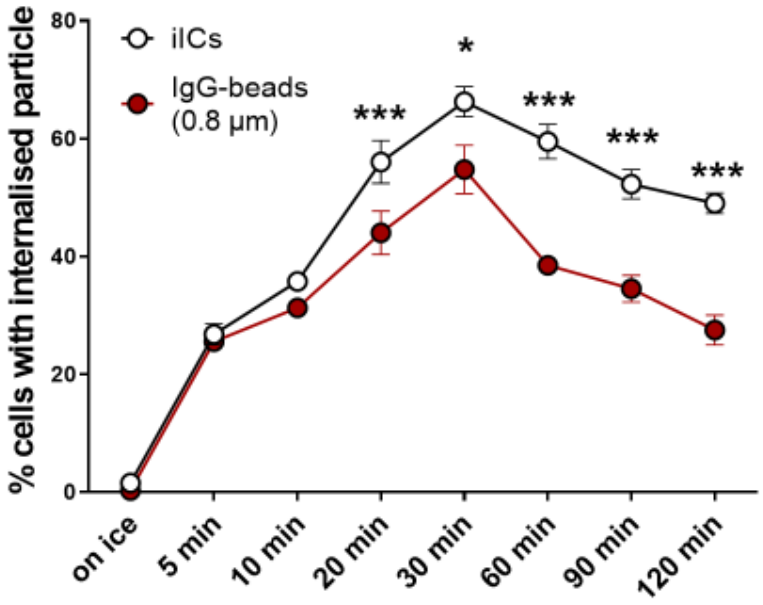


Figure 4.3. Both iIC- and IgG bead-internalisation reach peak at 30 min after stimulation.

Neutrophils were stimulated with iICs (2 µg/ml) or IgG-beads (5 beads per cell) followed by culture at 37 °C for up to 2 hours. A control sample was prepared by incubating the cells on ice where no internalisation is possible. Cells were then allowed to adhere to glass slides, fixed with 2% PFA, and iICs or IgG-beads were then labelled using fluorescently conjugated secondary antibodies followed by manual counting of internalised particles using an EVOS FI Auto 2 microscope. The circles represent mean values ± SEM obtained in four separately conducted experiments. The data were compared using two way ANOVA with Bonferroni post hoc test. * = p value < 0.5, *** = p value <0.001.

4.2.3. Internalisation of iICs and IgG-beads are regulated differently

Internalisation of particles larger than 1.5 μm is PI3K-independent during FcγR mediated phagocytosis by macrophages. Since, phagocytosis by macrophages is dependent on the particle size,[234–236] three different sizes (3 μm, 0.8 μm and 0.3 μm) of latex beads were used. Experiments were conducted to find out any difference between internalisation of iICs and IgG-beads, and also to identify regulators of internalisation.

Data from **Figure 4.2** showed earlier that internalisation of iICs is PI3K-dependent. Similarly, **Figure 4.4.A** show that inhibition of PI3K resulted in a significant decrease of iIC internalisation but uptake of 0.3 and 0.8 μm IgG-beads was not affected (**Figure 4.4.B** and **Figure 4.4.C**). The results suggest that PI3K dependency in phagocytosis by neutrophils is also size-dependent, just as previously shown with macrophages.[234–236] However, in the confocal images internalised iICs looked unlike the larger IgG-beads (**Figure 4.4.A** and **Figure 4.4.D**).

Moreover, actin polymerisation is a key regulatory step for internalisation of particles by the cells. As shown in **Figure 4.4**, blocking with actin polymerisation with latrunculin B inhibited internalisation of both IgG-beads and iICs, in line with the relevant literature.[208,237]

For simplicity, 0.8 μm IgG-beads were chosen for further analysis since they triggered similar response as iICs in terms of percentage of internalisation and similar size of the vacuoles that contained internalised particles.

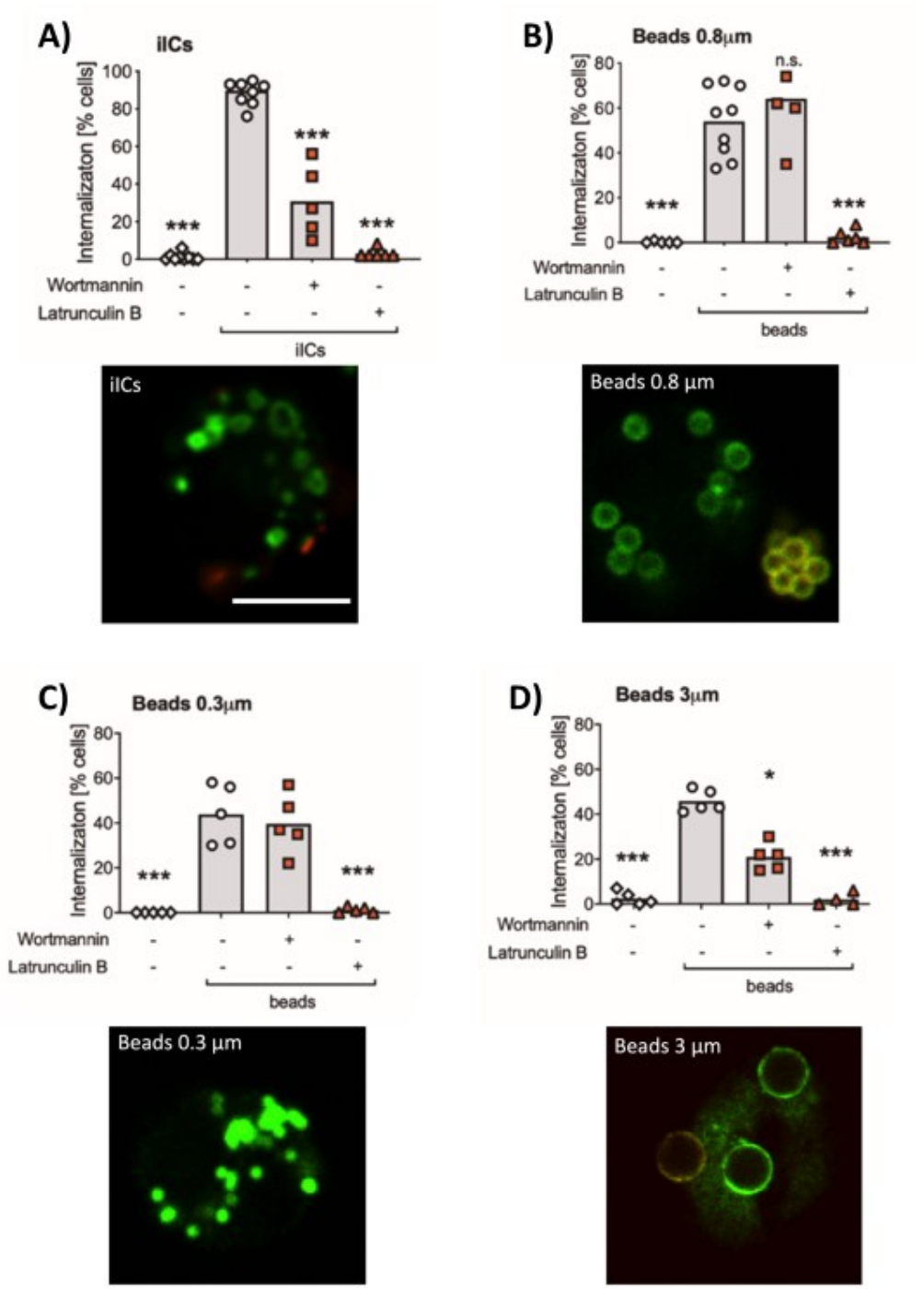


Figure 4.4. Actin polymerisation is required for internalisation and PI3K regulation is size dependent.

Neutrophils were pre-incubated with vehicle or inhibitors (Wortmannin and Latrunculin B), for 10 min at 37 °C. Cells were then stimulated with (**A**) iICs (2 μg/ml) or different sizes of IgG-beads (0.8 μm, **B**; 0.3 μm, **C** and 3 μm, **D**, 5 beads per cell) for 30 min at 37°C or on ice, in PBS^{++}. Representative images were obtained using a Leica SP8 (**A**) or Zeiss LSM780 (**B-D**) confocal microscope with 63x objective. Scale bar, 5 μm. A representative example is shown of ≥ 5 independent experiments performed. Graphs are showing the percentages of cells with internalised particles counted using an EVOS FI Auto 2 microscope. Each symbol in the graphs represents the value obtained in each experiment. The data were compared using one way ANOVA with Bonferroni post hoc test. * = p value < 0.5, *** = p value <0.001, n.s. = not significant. Figure adapted from Karmakar et al.,Cell death and disease, 2021.[326]

To investigate further and determine the location of internalised iICs or IgG-beads inside the neutrophil, co-stimulation experiments were designed where neutrophils were simultaneously stimulated with iICs and IgG-beads. Data from confocal images suggested that iICs and IgG-beads enter different compartments inside the cell (**Figure 4.5**). Importantly, pre-treatment of the cells with wortmannin interfered with the internalisation of iICs without affecting internalisation of IgG-beads. Together the results shown in **Figure 4.4** and **Figure 4.5** suggest strongly that iICs and IgG-beads are internalised by distinct mechanisms.

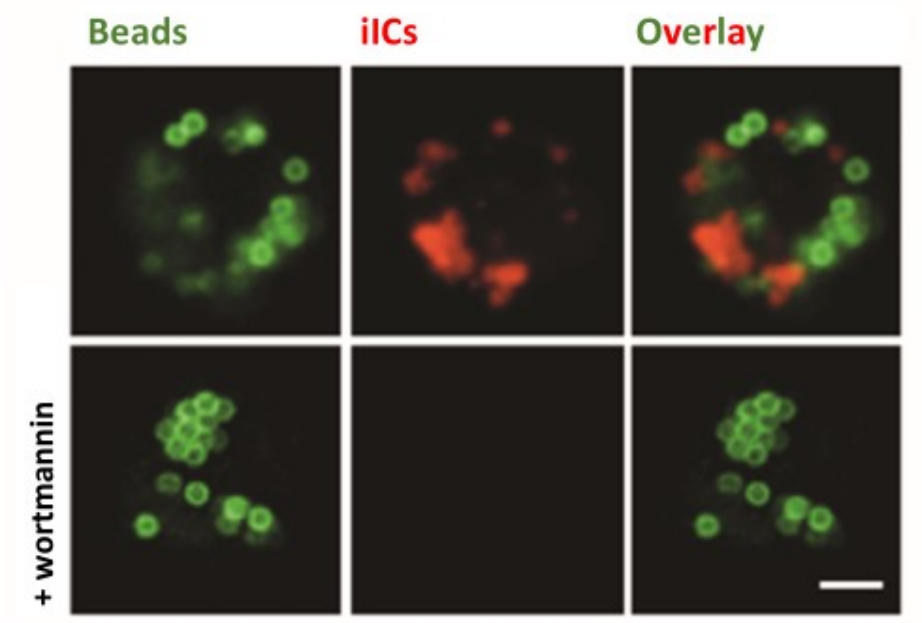


Figure 4.5. Blocking PI3K inhibits internalisation of iICs, but not of IgG-beads.

Freshly isolated neutrophils were pre-incubated with vehicle or wortmannin for 10 min at 37 °C. Cells were then stimulated with fluorescently labelled iICs (2 µg/ml, red) and IgG-beads (5 beads per cell, green) for 30 min at 37°C. Cells were viewed with a Leica SP8 confocal microscope with a 63x objective. A representative example of an optical section is shown here from three independent experiments performed. Scale bar, 2 µm. Figure adapted from Karmakar et al.,Cell death and disease, 2021.[326]

4.2.4. Neutrophil granules localise to IgG bead-containing phagosomes, but not to iIC-containing vacuoles

Neutrophils are characterised by specialised primary and secondary granules that contain cytotoxic components and proteolytic enzymes. In phagocytosis, granules fuse with the phagosome, directing cytotoxic cargo to appropriate granular and vesicular compartments in the cells for intracellular killing.[31,238–240] Such fusion processes of phagocytic vesicles and granules are in keeping with the formation of classical phagolysosome in macrophages.[30] I stained neutrophils that had or had not been allowed to internalise IgG-beads or iICs with CD63, a marker of primary azurophil granules. While distinct CD63 positive speckles were visualised in unstimulated neutrophils, they were reorganised to surround the ingested IgG-beads suggesting co-localisation with the phagosome on the top row of **Figure 4.6**. In contrast, CD63 signal did not neatly surround the vacuoles containing iICs, but instead showed some elongated structures that overlapped with iIC-containing vacuoles (**Figure 4.6**).

While the contents of azurophil granules are mainly secreted into the forming/formed phagosome, specific granules can fuse with the plasma membrane in a non-polarised way[241]. Moreover, in neutrophils LAMP proteins localise to the secretory vesicles.[242,243] I attempted performing similar experiments with a marker of specific granules, CD66b and a lysosomal membrane marker, LAMP1 that exhibited similar results. However, these experiments were unfortunately inconclusive.

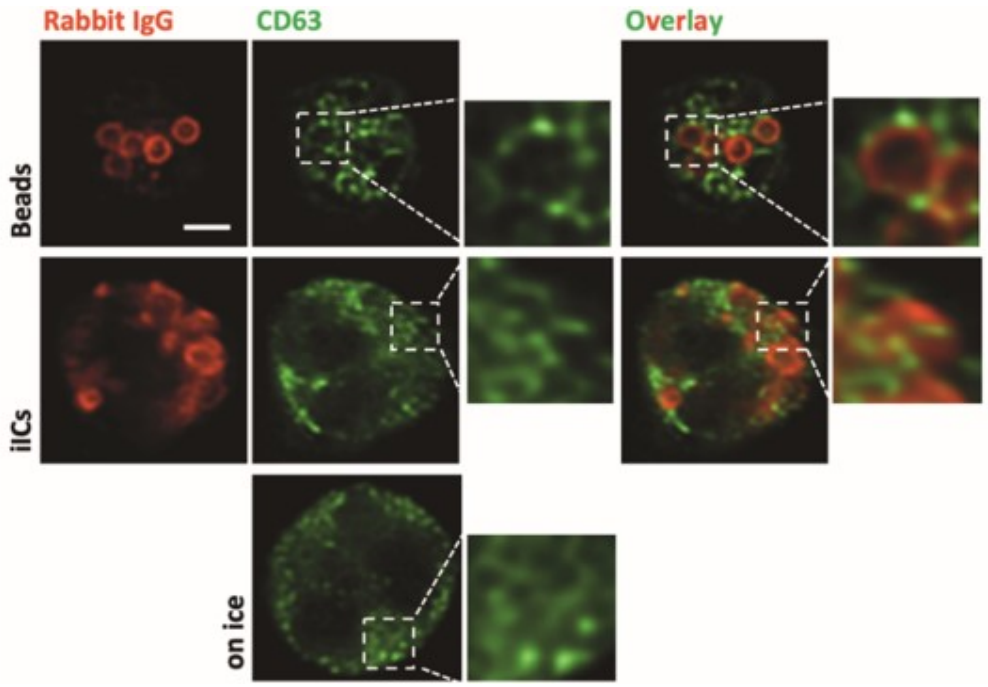


Figure 4.6. Neutrophil azurophil granules (CD63 marker) fuse with IgG-bead containing phagosomes, but not iIC containing vacuoles.

Neutrophils were subjected to stimulation with red fluorescently labelled iICs (2 µg/ml) or IgG-beads (5 beads per cell) for 30 min at 37°C, in PBS^{++}. Cells were then fixed, permeabilised and stained with anti-CD63 antibody (green) to label azurophil granules in neutrophils. A control sample with cells that had been kept on ice throughout. Images were obtained using a Leica SP8 confocal microscope with 63x objective. A representative example of an optical section shown from three independent experiments performed. Scale bar, 2 µm. Figure adapted from Karmakar et al.,Cell death and disease, 2021.[326]

4.2.5. iICs are internalised by macropinocytosis

Macropinocytosis is a comparatively poorly characterised process by which extracellular liquid is taken up by cells. It is distinct from phagocytosis and endocytosis. Importantly, pinocytosis can be either constitutive or receptor-dependent.[232,244] Internalisation of iICs and IgG-beads is regulated differently. I therefore sought to investigate whether internalisation of iICs could be due to either macropinocytosis or another form of endocytosis as depicted in **Figure 4.1**. I first investigated macropinocytosis, a PI3K-dependent internalisation mechanism[245,246] since internalisation of iICs was PI3K-dependent.

To test whether internalisation of fluid concurs with iIC internalisation, I supplemented the buffer with a dye, lucifer yellow, as a marker of the fluid phase[247] prior to feeding cells with iICs (**Figure 4.7.A** and **Figure 4.7.B**). A separate population was detected with positive lucifer yellow signal by flow cytometry (**Figure 4.7.B**), and the percentage of that cell population matched with the internalisation data presented earlier in **Figure 4.4.A**.

Additionally, I detected ample lucifer yellow stained extracellular fluid inside the cell which co-localised with internalised iICs by confocal microscopy (**Figure 4.8.A**). Since iIC (but not IgG-beads) internalisation is dependent on PI3K, pinocytosis was next assessed with wortmannin. In keeping with the observation in **Figure 4.2.B** and **Figure 4.4.A** earlier, wortmannin inhibited pinocytosis and no dye was detected inside the cell (**Figure 4.8.B**). In contrast, no or very little lucifer yellow was detected inside cells that had been fed with IgG-beads (**Figure 4.8.C**). These results suggest that iICs are internalised by neutrophils via a novel mechanism, macropinocytosis.

The same experiment was replicated with another dye, FITC-dextran,[248] in order to demonstrate that this observation is not dye specific. FITC-dextran was detected inside the cell and also co-localised with internalised iICs (**Figure 4.8.D** and **Figure 4.8.E**) confirming that iIC internalisation is indeed mediated through macropinocytosis.

In macrophages and dendritic cells, a major difference between macropinocytosis and other modes of endocytosis is the involvement of PI3K and actin, which regulate the macropinocytsis but not the latter.[249–251] Consistent with this notion, I concluded that the internalisation of iICs occurs via macropinocytosis.

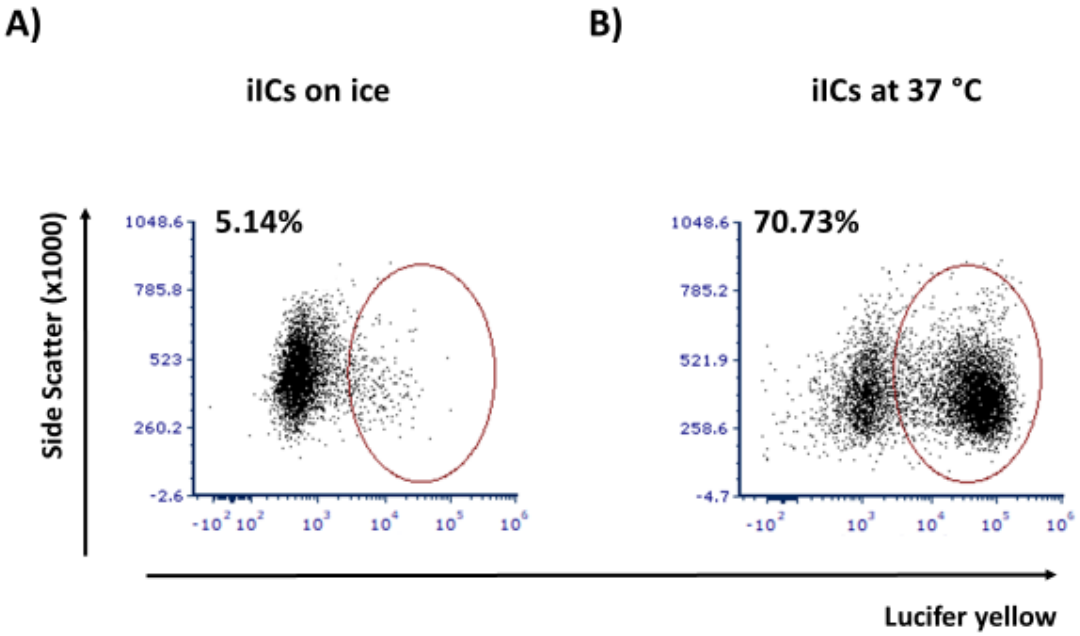


Figure 4.7. Pinocytosis of iICs detected by flow cytometry.

Freshly isolated neutrophils were stimulated with iICs (2 µg/ml) for 30 min on ice (**A**) or at 37°C (**B**), in the presence of Lucifer yellow in PBS^{++}. Lucifer yellow-positive cell populations were detected by flow cytometry identifying the percentage of pinocytosis of iICs in a quantitative fashion (oval gate) A representative example of flow plot shown from five independent experiments performed.

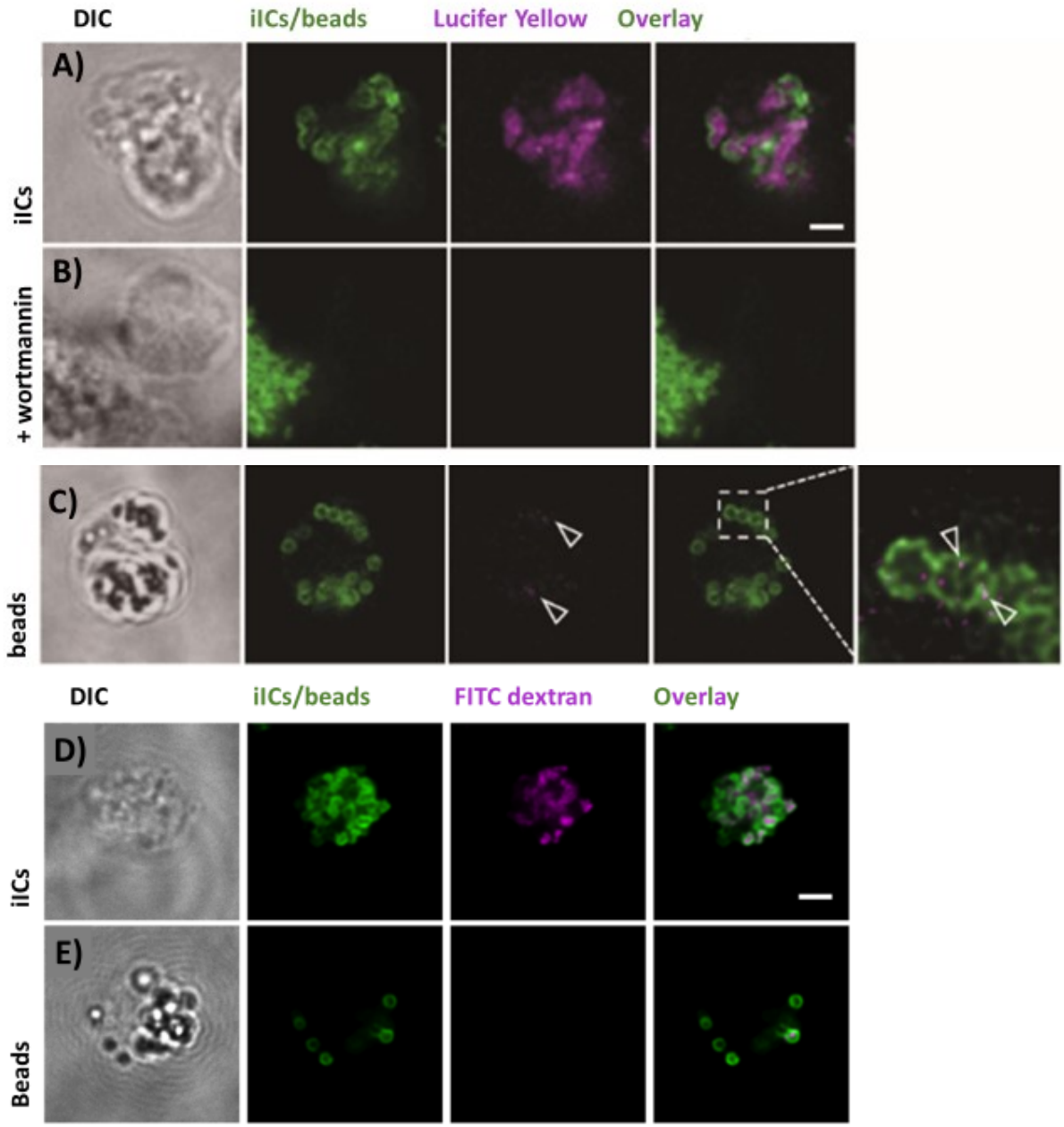


Figure 4.8. iICs are internalised by macropinocytosis.

Freshly isolated neutrophils were pre-incubated with vehicle or wortmannin for 10 min at 37 °C. Cells were then stimulated with green fluorescently labelled iICs (2 µg/ml) or IgG-beads (5 beads per cell) for 30 min at 37°C, in the presence of a fluid phase marker (magenta) Lucifer yellow (**A-C**) or FITC dextran (**D** and **E**) in PBS^{++}. Images were obtained using a Leica SP8 confocal microscope with 63x objective. The arrows indicate a small percentage of lucifer yellow accompanying IgG-bead phagocytosis. The examples shown are representative of three independent experiments performed. Scale bars, 2 µm. Figure taken from Karmakar et al.,Cell death and disease, 2021.[326]

4.2.6. iIC-pinocytosis is not constitutive but FcγRII dependent

Pinocytosis is either constitutive or receptor mediated. To test whether iIC internalisation occurs by receptor mediated macropinocytosis, further assessment of pinocytosis was next carried out that included a series of control conditions, such as individual components of iICs, HSA and anti-HSA. In contrast to iICs, neither of these components, nor FcγRIIa/RIIIb blocking antibodies alone were internalised by macropinocytosis (**Figure 4.9.A**) suggesting that iIC internalisation does not occur by constitutive macropinocytosis, but rather depends on a trigger that is specific to iICs.

A well-characterised pinocytosis inhibitor, 5-(N-ethyl-N-isopropyl)-amiloride (EIPA), was shown to inhibit pinocytosis at a very early stage by blocking the Na^+ -H^+ exchange at the plasma membrane.[252] EIPA selectively blocked macropinocytosis but not clathrin dependent endocytosis[230] confirming that iIC internalisation occurs via macropinocytosis (**Figure 4.9.B**). In contrast, autophagy inhibitors such as chloroquine[253] which also inhibits endocytosis did not inhibit macropinocytosis of iICs (**Figure 4.9.B**). Interestingly, blocking ROS production by DPI did not block pinocytosis of iICs, instead a slight increase was noticed (**Figure 4.9.B**) consistent with the previous observations in macrophages.[254]

Considering iIC macropinocytosis is not constitutive, further experiments were conducted to identify the responsible receptor(s), focussing on FcγRs. **Figure 4.10.A** and **Figure 4.10.B** show that FcγRII-blocking antibodies reduced internalisation of both iICs and IgG-beads significantly, confirming the receptor dependency of iIC pinocytosis. Since Fcγ receptors besides FcγRII, FcγRI and αMβ2, were involved in iIC-stimulated ROS production (**Figure 3.13**), internalisation of iICs was also tested by blocking the same receptors. Interestingly, use of blocking antibodies specific for these receptors alone or in combination did not further reduce iIC internalisation (**Figure**

4.10.C) indicating the existence of two distinct mechanistic processes for iIC-induced ROS production and uptake of iIC by macropinocytosis.

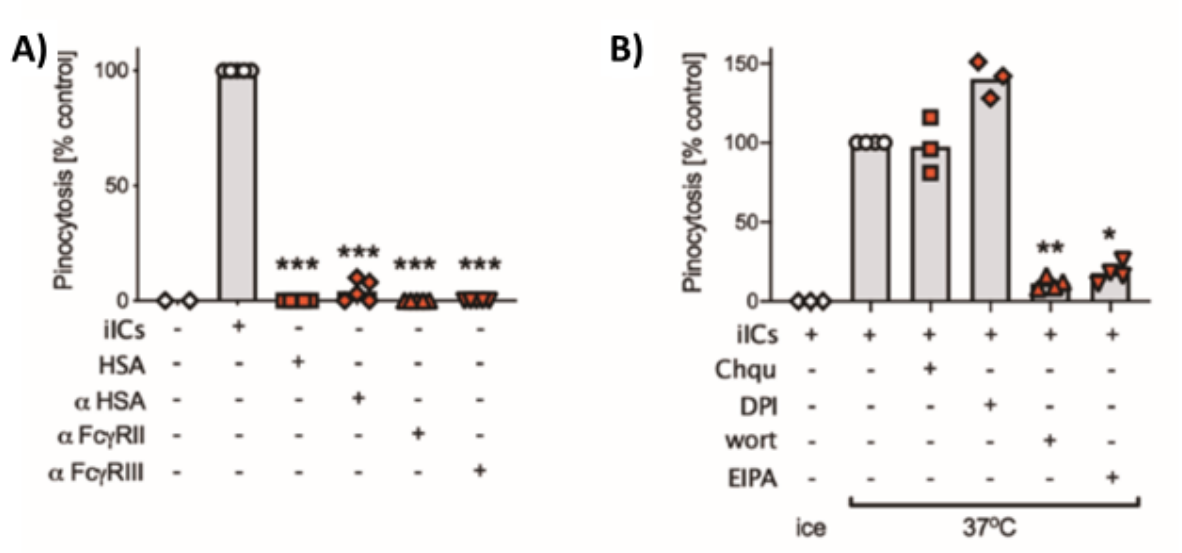


Figure 4.9. Internalisation of iICs does not occur by constitutive macropinocytosis.

A, neutrophils were subjected to stimulation with either iICs (2 µg/ml) or the components of iICs (HSA alone or anti-HSA alone) or vehicle (PBS) for 30 min at 37°C in the presence of lucifer yellow in PBS^{++}. In addition, blocking antibodies against Fcγ receptors (as indicated) were used as controls by incubating the cells in the absence of iICs for 30 min on ice prior to mock stimulation. **B** shows neutrophils that were incubated for 10-30 min with inhibitors (Chqu, chloroquine, 100 µM; DPI, diphenyleneiodonium, 10 µM; wort, Wortmannin 100 nM; and EIPA, ethylisopropylamiloride, 50 µM) at 37°C prior to being stimulated with iICs. A control sample was kept on ice throughout. Graphs depict the percentage of cells that internalised particles as counted by epifluorescence (EVOS FL Auto 2 microscope, 100x objective). Each symbol in the graphs represents the value obtained in each experiment. Data were analysed using RM one way ANOVA with Dunnett's multiple comparison test (**A**) and Mixed effect analysis followed by Tukey's multiple comparison test (**B**), * = p value < 0.05, ** = p value < 0.01 and, *** = p value < 0.001. Figure adapted from Karmakar et al.,Cell death and disease, 2021.[326]

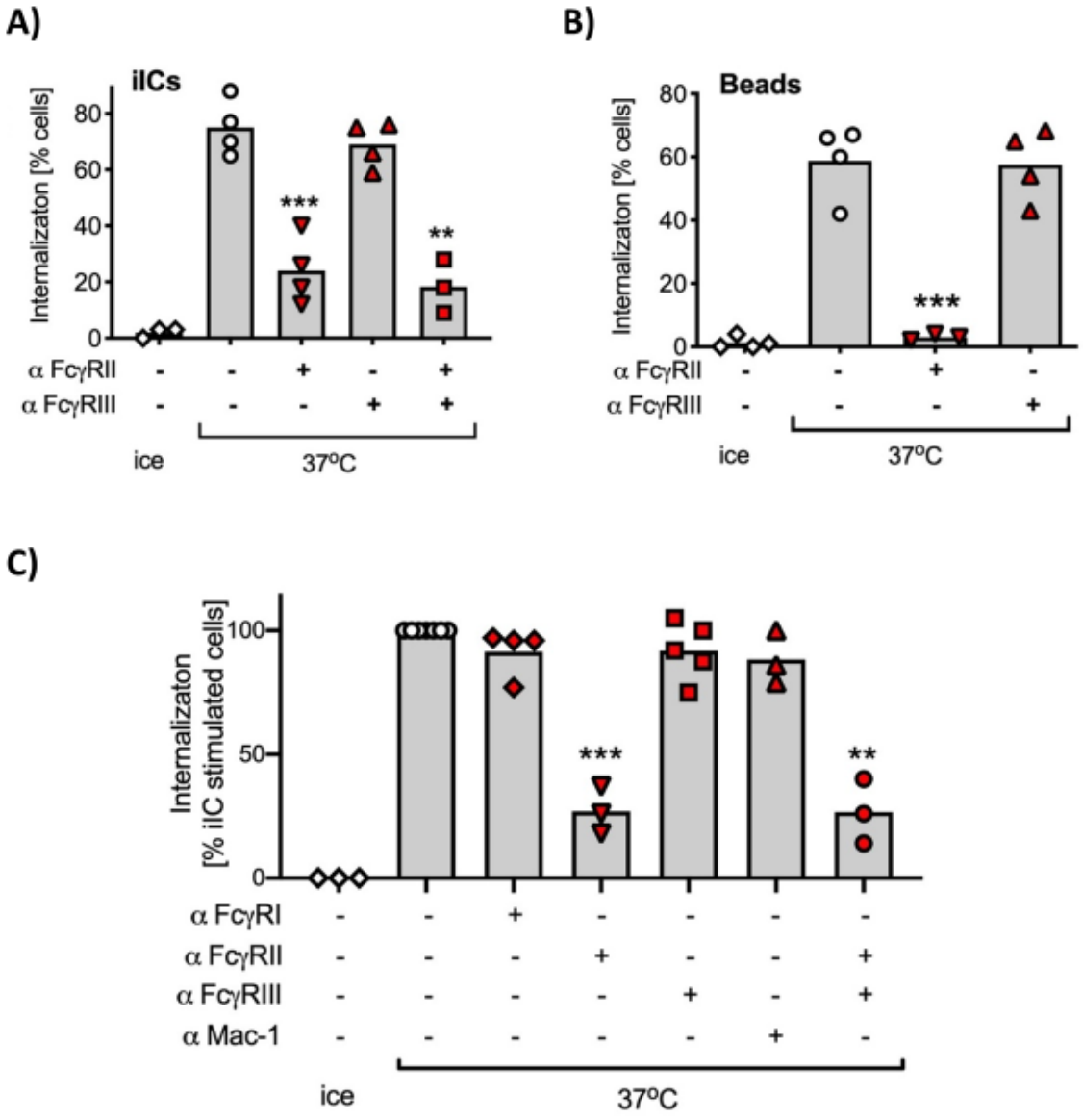


Figure 4.10. Pinocytosis of iICs is FcγRII dependent.

Neutrophils were pre-incubated with blocking antibodies against Fcγ receptors for 30 min on ice prior to being stimulated with iICs (2 µg/ml, **A**) or IgG-beads (5 beads per cell, **B**). A control sample was kept on ice throughout. Additional antibodies were used against FcγRI (10.1, 10 µg/ml) and αMβ2 (Mac-1/ICRF44, 12.8 µg/ml), in panel **C**. Graphs show the percentage of cells that had internalised particles as counted under the fluorescence microscope. Each symbol in the graphs represents the value obtained in each experiment. Data were analysed using one way ANOVA with Dunnett's multiple comparison test * = p value < 0.05, ** = p value < 0.01 & *** = p value < 0.001. Figure adapted from Karmakar et al.,Cell death and disease, 2021.[326]

4.2.7. iIC-pinocytosis is characterised by membrane ruffling and formation of macropinosomes

Macropinocytosis is initiated by the actin-driven extension of dorsal plasma membrane ruffles. Dorsal membrane ruffles form a cup like structure that seals distal tips to eventually form a relatively large vacuole termed the macropinosome. Depending on the cells (e.g. in macrophages), the size of these macropinosomes can be as large as 5 μm in diameter. Subsequent membrane fusion with various organelles leads to the formation of a mature, acidic macropinolysome.[230,232,255]

Bone marrow derived macrophages were shown to commence membrane ruffling within one minute of CSF-1 stimulation[230] following the stages of macropinosome maturation as shown in the schematic diagram (**Figure 4.11**). Neutrophils, in which macropinocytosis is not well characterised, are likely to be following a similar mechanism when pinocytosing iICs within minutes.

I therefore analysed iIC macropinocytosis by time-lapse video of neutrophils in which the plasma membrane had been labelled using a cell mask dye (still images in **Figure 4.12**). This showed plasma membrane ruffling and simultaneous rapid uptake of red fluorescently labelled iICs by a neutrophil. The intracellular compartment containing the iICs was formed by blue plasma membrane suggesting formation of a macropinosome.

In contrast with macrophages, which acidify their phagosomes, phagosomal acidification of neutrophils is a controversial subject. To test whether the neutrophil macropinosome acidifies, an experiment was attempted with neutrophils stimulated in medium containing a pH sensitive fluid phase marker, pHrodo Dextran. These pHrodo dyes show little to no fluorescent signal at neutral pH and fluoresce brightly in acidic

environments, making them ideal for use as pH indicators for a variety of applications.[256]

In a single experiment, I observed green pHrodo signal as scattered dots in a live movie (still images from the video are shown in **Figure 4.13**), however this signal did not co-localise with distinctly labelled iICs. This experiment was performed just before the lockdown, and the videos could only be analysed with the help of Dr Rolly Wiegand more than a year later, once the department had opened up once more with reduced distancing measures in place. Additional experiments with appropriate controls and detailed analysis would be required to validate this interesting pilot observation.

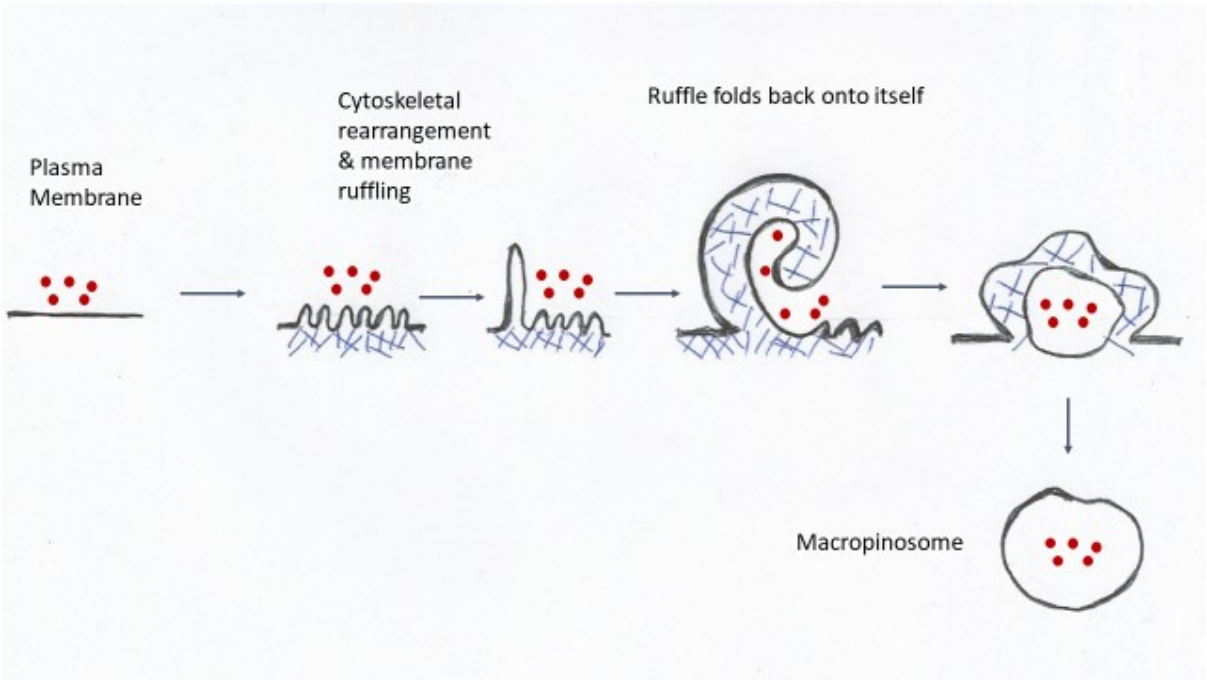


Figure 4.11. Stages of macropinocytosis.

Actin cytoskeleton (blue lines) rearrangement at the plasma membrane (black) forms membrane ruffles that fold back onto themselves. The ruffles then fuse at the base of plasma membrane forming macropinosomes and trap soluble substances or particles (red dots) with the solute.

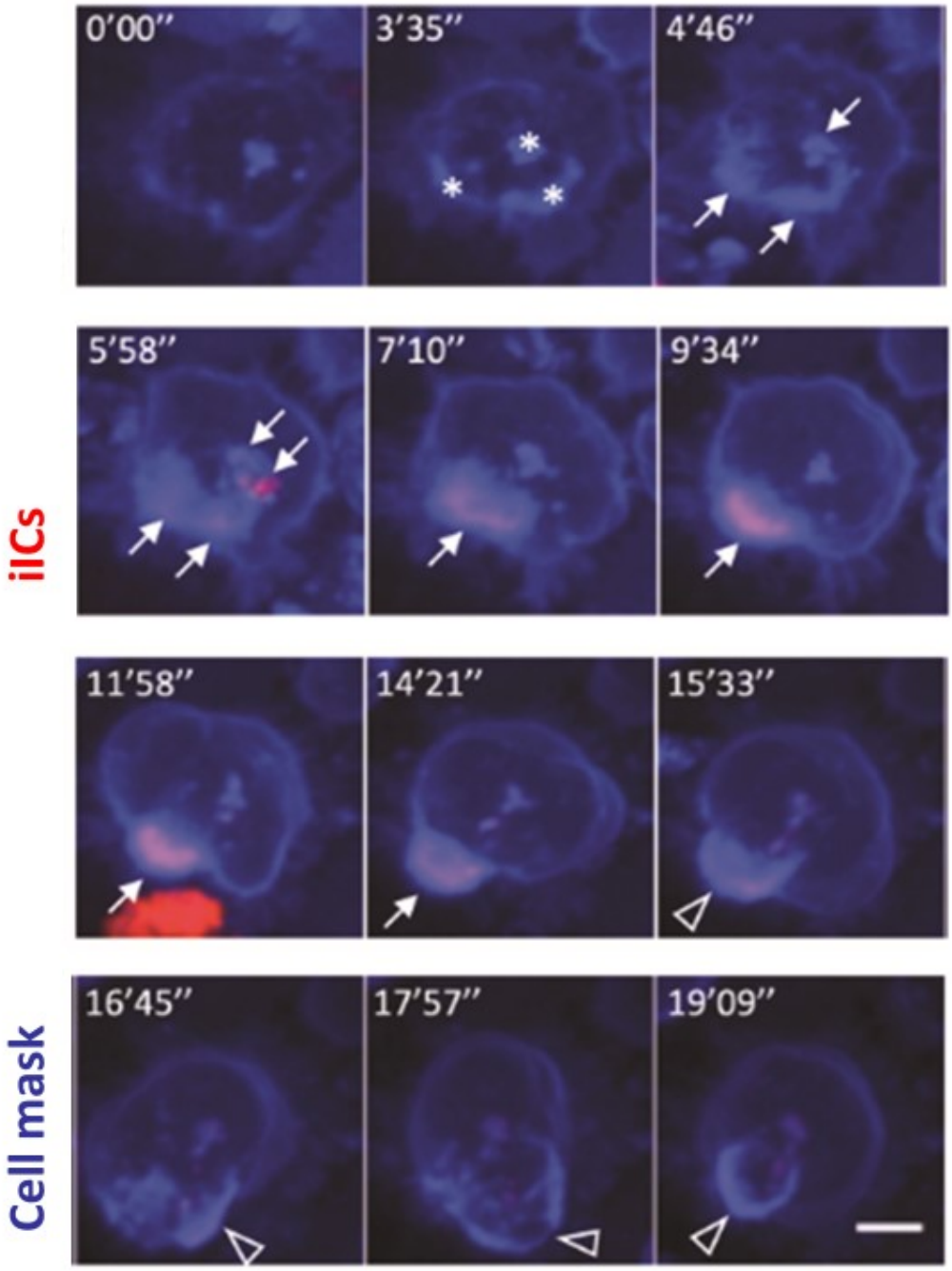


Figure 4.12. Neutrophils internalise iICs rapidly after stimulation.

Freshly isolated neutrophils stained with the plasma membrane dye cell mask deep red (pseudo coloured in blue) were allowed to adhere on a glass coverslip and then subjected to stimulation with AF 568-conjugated anti-rabbit secondary antibody labelled iICs (2 µg/ml, red) in a humidified chamber at 37 °C. Images were captured every 30 sec using an Andor Revolution XDi spinning disk confocal microscope with 60x objective. The asterisk symbols indicate dorsal membrane ruffling prior to/concurrent with iIC internalisation. White arrows indicate a vacuole, the macropinosome that is formed by folding the plasma membrane surrounding the internalised red iICs. Arrowheads, loss of internalised iIC-associated fluorescence in the macropinosome. Scale bar, 5 µm. Figure adapted from Karmakar et al.,Cell death and disease, 2021.[326]

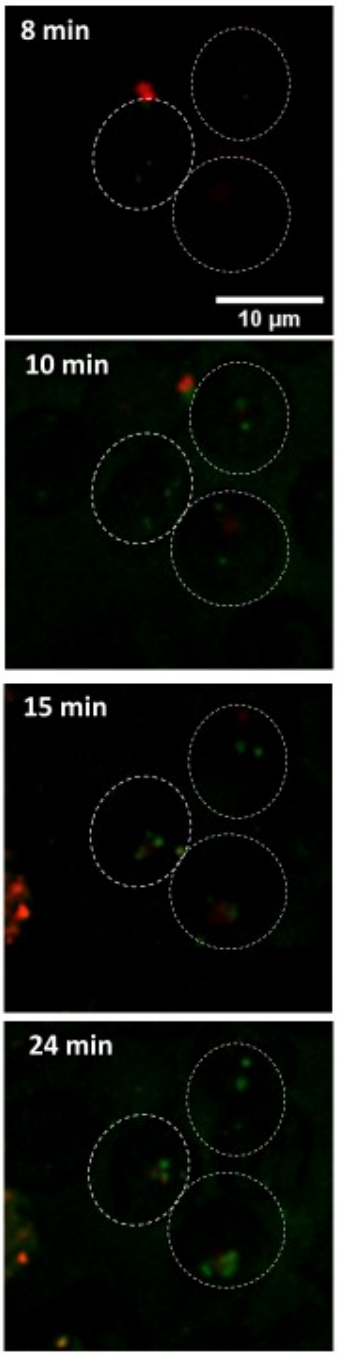


Figure 4.13. Acidification of iIC-induced pinosome is detected live with pHrodo dextran.

Freshly isolated neutrophils (indicated by white dotted circles) were incubated with pHrodo dextran (20 µg/ml, green) in PBS^{++} for 20 min at 37 °C, allowed to adhere on a glass coverslip and stimulated with fluorescently labelled iICs (red) in a humidified chamber at 37 °C. Live images were captured every 30 sec using an Andor Revolution XDi spinning disk confocal microscope with 60x objective. Each dotted circle represents a neutrophil.

4.2.8. iIC degradation is a fast, protease-dependent process

When assessing the internalisation of fluorescent iIC by neutrophils (**Figure 4.3**), it was noted that longer time points were associated with reduced fluorescence, raising the possibility of degradation of iICs. Neutrophils kill ingested pathogens using ROS, cytotoxic enzymes and powerful proteases.[31] To test whether iICs were subjected to degradation in the same way as IgG in internalised IgG-beads, I performed a second series of time-course experiments. I reasoned that both particles contained large amounts of IgG, the integrity of which could easily be analysed by Western blot. The IgG heavy chain runs at 55 kDa. The thickness of the 55 kDa bands in **Figure 4.14.A** reduces over time, indicating that degradation of internalised iICs occurred. Interestingly, IgG derived from iICs degraded faster (obvious from 5 min) than that of IgG-beads (from 20 min). The majority of IgG in either condition was degraded after 90 min. Therefore, further degradation experiments were conducted after 90 min of stimulation.

Blocking PI3K with LY294002 led to the appearance of a 34 kDa band. Since blocking PI3K interferes with iIC internalisation, but not binding, this is suggestive of the contribution of neutrophil surface-localised protease(s) in the degradation process (**Figure 4.14.B**). iIC degradation was further explored using inhibitors directed against proteases. iIC degradation was inhibited in cells that had been pre-incubated with DFP, a potent inhibitor of serine proteases, but not by E64, an inhibitor of cysteine proteases (**Figure 4.14.C**). Together this suggests that iIC degradation is likely to occur in a stepwise fashion and involves several proteases. Interestingly, DFP treatment, but not E64 also inhibited the induction of apoptosis (**Figure 4.14.D**) suggesting that iIC degradation, just like ROS production may be involved in the induction of apoptosis.

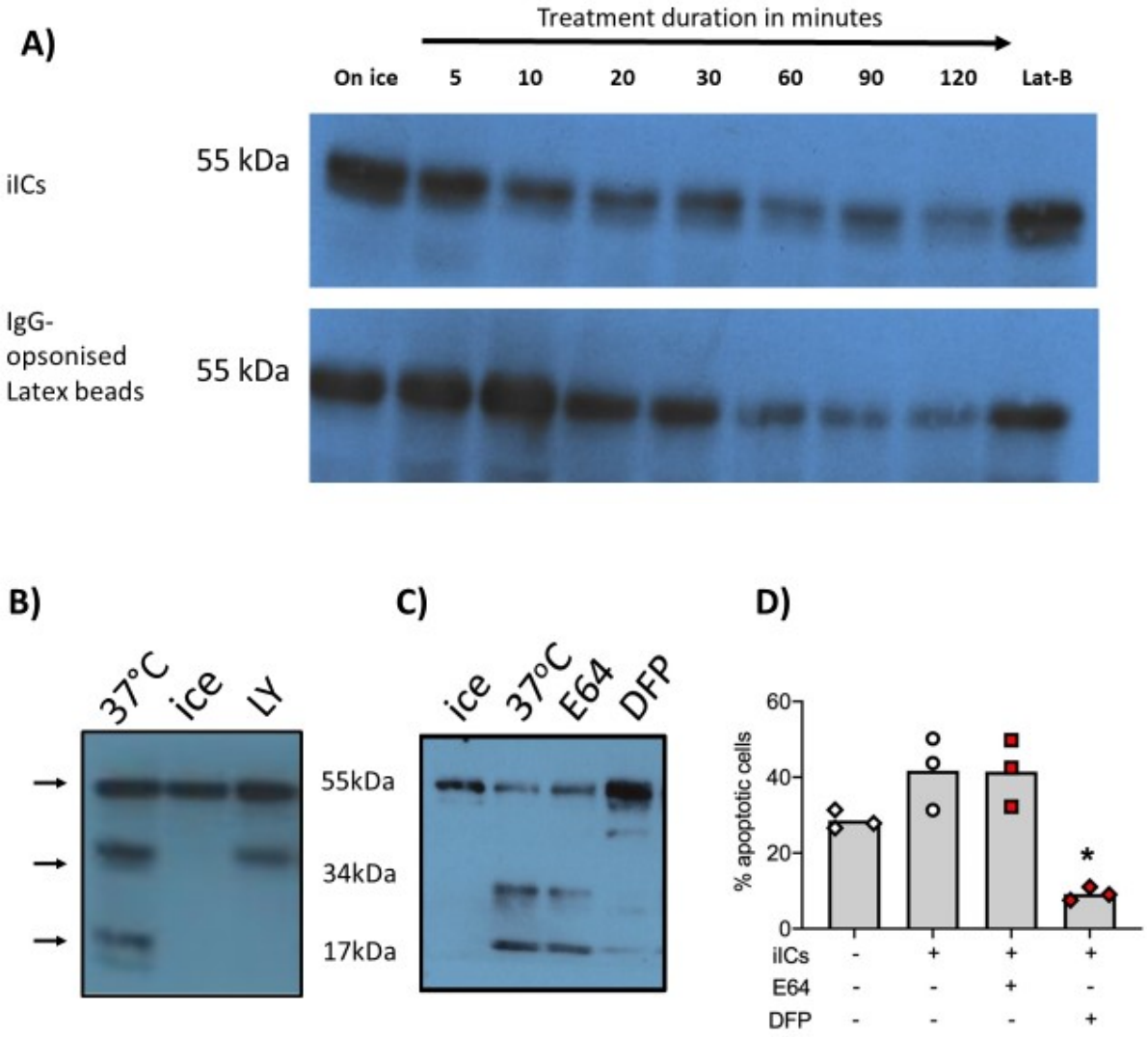


Figure 4.14. Internalised iICs degrade quickly and the IgG degradation depends on proteases.

A, Neutrophils were treated with iICs (2 µg/ml) or IgG-beads (5 beads per cell) in PBS^{++}, and incubated at 37 °C or, as a control, on ice for the indicated times. (**B** and **C)** Cells were pre-treated with inhibitors as indicated prior to being stimulated with iICs and incubated for 90 min at 37 °C. Cell lysates were prepared, proteins separated by SDS-PAGE and transferred to PVDF membrane. Rabbit IgG heavy chains (~55kDa) and degradation products were detected using a HRP-coupled secondary antibody and enhanced chemiluminescence. Representative blots of at least three independent experiments are shown. **D,** cells were or were not treated with E64 or DFP and stimulated with iICs as indicated prior to being cultured in IMDM supplemented with 10% autologous serum. The induction of apoptosis was determined by flow cytometry as detailed in **chapter 2**. Each symbol in the graphs represents the obtained in each experiment. Data were analysed using one way ANOVA with Dunnett's multiple comparison test * = p value < 0.05. Figure adapted from Karmakar et al.,Cell death and disease, 2021.[326]

4.2.9. Internalisation of iICs is not required for the induction of apoptosis

To acquire further mechanistic insight into the relationship of iIC pinocytosis and iIC-induced apoptosis, I asked whether neutrophil apoptosis is pinocytosis-induced, similar to the mechanism of PICD. To address this question, I compared the internalisation and apoptosis processes in more depth by performing a time-course of internalisation experiments for longer duration until induction of apoptosis is established.

Since iIC-induced apoptosis is dependent upon ROS production (as discussed in **chapter 3**, **Figure 3.7**) and proteolysis by serine proteases (**Figure 4.14.D**), additional inhibitors, DPI and DFP respectively were used in this internalisation experiment for longer duration. Blocking serine proteases and ROS production, both of which were shown to inhibit apoptosis, did not affect the internalisation of iICs (**Figure 4.15.A-C**). In contrast, internalisation of iICs remained negligible with latrunculin B treatment (**Figure 4.15.D**) even after 6 hours of iICs stimulation. Additionally, inhibiting apoptosis by QVD did not affect the internalisation even after prolonged incubation (**Figure 4.15.E**).

Moreover, **Figure 3.2** and **Figure 3.3** in the previous chapter showed that internalisation of iICs depends on PI3K but not on the downstream regulators of apoptosis. Altogether, these results confirm that two distinct pathways operate downstream of PI3K to regulate in parallel iIC-induced neutrophil apoptosis and iIC internalisation by neutrophils.

Table 4.1 summarises the contrasting effects of the inhibitors on the iIC internalisation and iIC-induced apoptosis confirming that the two processes are not dependent upon one another.

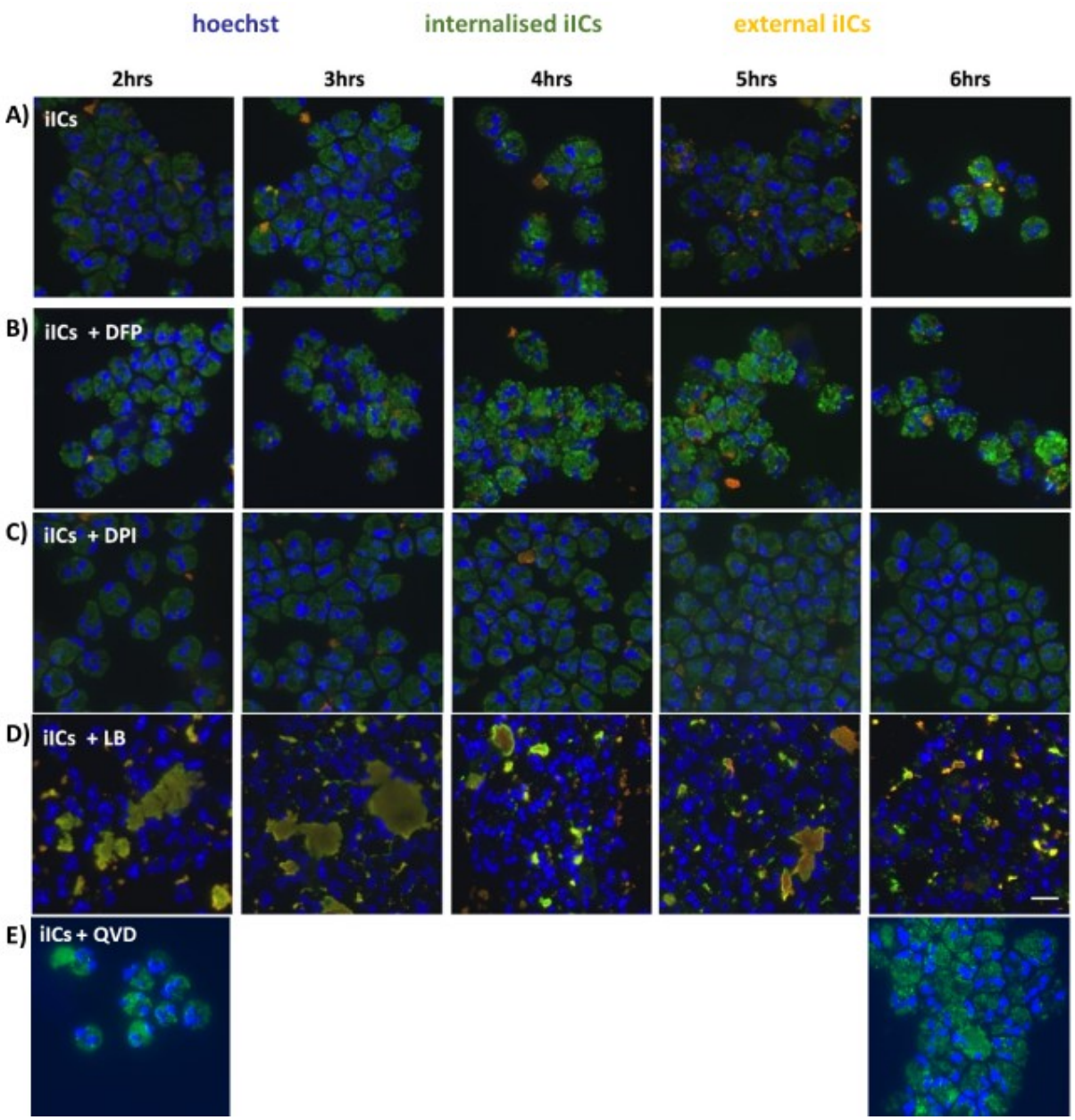


Figure 4.15. iIC-induced apoptosis is independent of iIC internalisation.

Freshly isolated neutrophils were pre-incubated with vehicle (**A**) or inhibitors (**B-E**, as indicated) prior to being stimulated with iICs (2 µg/ml) for up to 6 hour at 37 °C in IMDM with 10% autologous serum. Cells were allowed to adhere to glass slides at the indicated times, fixed and iICs were labelled before and after permeabilisation with AF 568 conjugated antibody (red) and AF 488 conjugated antibody (green) as detailed in **chapter 2** to visualise internalised and bound iICs. Nuclei of the neutrophils were labelled with Hoechst (blue). Images were captured using an EVOS FI Auto2 microscope with 40x objective. Representative examples are shown from a minimum of three independent experiments performed. Scale bar, 6 µm. Figure adapted from Karmakar et al.,Cell death and disease, 2021.[326]

Table 4.1. Contrasting effects of inhibitors on iIC-induced apoptosis and iIC-internalisation.

Inhibitors	Functions	Apoptosis	Internalisation	Reference
DFP	Serine protease inhibitor	Yes	No	Fig 4.14 & Fig 4.15
DPI	NADPH oxidase inhibitor	Yes	No	Fig 3.7 & Fig 4.15
Latrunculin B	Actin inhibitor	No	Yes	Fig 3.7 & Fig 4.15
QVD/z-VAD	Caspase inhibitor	Yes	No	Fig 3.1 & Fig 3.2 & Fig 4.15
PF3758309	Pak inhibitor	Yes	No	Fig 3.1, Fig 3.2 & Fig 3.3
Tramatinib	Mek inhibitor	Yes	No	Fig 3.1, Fig 3.2 & Fig 3.3
FR180204	Erk inhibitor	Yes	No	Fig 3.1, Fig 3.2 & Fig 3.3

4.3. Discussion

The results presented in this chapter showed that i) iICs are internalised by neutrophils via FcγRII-mediated macropinocytsis in a fashion that is dependent upon PI3K, ii) internalisation of IgG-beads and iICs can take place simultaneously without any preference for either particle in spite of occurring by two mechanistically distinct pathways and, iii) iICs are degraded in a proteases-mediated fashion prior to the induction of apoptosis (summarised in **Figure 4.16**). Interestingly, my work has identified that internalisation of iICs by macropinocytosis is not a pre-requisite for the induction of neutrophils apoptosis, unlike PICD.

The majority of pinocytosis studies were conducted with macrophages, dendritic cells and cancer cells, whereas studies on neutrophil macropinocytosis are only sparse. Macrophage and dendritic cell macropinocytosis occur either spontaneously or are induced by the engagement of growth factor, chemokine and toll like receptors. Being relatively large in size, macropinosomes facilitate non-selective entry of solute macromolecules and pathogens such as viruses, bacteria and prions. Moreover, macropinocytosis expedites MHC class II antigen presentation, especially in dendritic cells. Macropinocytosis has been shown to permit tumour cells to take up nutrients from the medium in a process that is thought to be relevant to tumour progression, and metastasis in cancer cells; as well as in the chemotactic response of neutrophils.[230,257] In another organism, *Dictyostelium* amoebae, PI3K dependent macropinocytosis has been shown to regulate chemotaxis.[245] Although an increasing number of regulators were identified, the exact sequence by which different components coordinate macropinocytosis remains to be discovered. The majority of the pinocytosis studies are conducted *in vitro* with cultured cells, and a complete understanding of this process in whole organisms or *in vivo* is not yet achieved. The

signal transduction pathways and maturation stages of iIC-induced macropinosome could be explored in details for a better understanding of this process in neutrophils.

Interestingly, FcγR mediated phagocytosis has earlier been shown to stimulate localised pinocytosis[258] that I also noted a very small amount of lucifer yellow signal as scattered dots after bead stimulation (arrowheads in **Figure 4.8.C**).

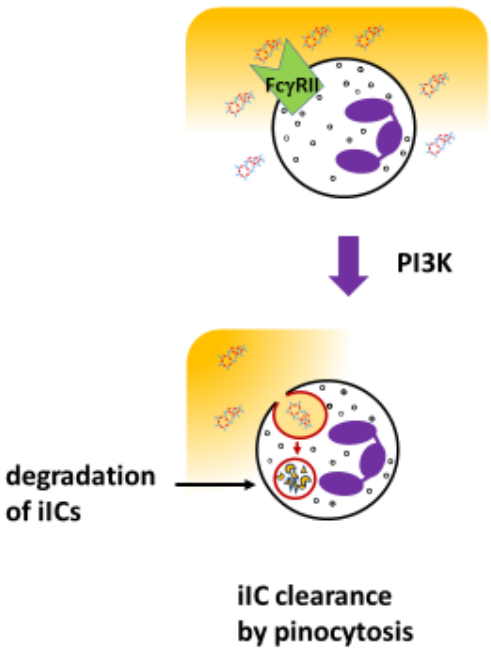


Figure 4.16. iIC stimulation occurs by FcγR mediated macropinocytosis.

iIC stimulation causes rapid internalisation of iICs via FcγRII that is PI3K dependent. iICs are degraded by proteases in the neutrophils. Neutrophil apoptosis requires degranulation (generation of both internal and external ROS as well as serine proteases) although it can occur independently of iIC internalisation.

Neutrophil phagosomes were shown to have neutral or a slightly alkaline pH (~pH 8), unlike the acidic macrophage phagosomes.[36] This could be due to robust ROS production by the neutrophils. The endosomal phagosome-maturation pathway in macrophages is replaced by the rapid delivery of granules to nonacidic phagosomes in the neutrophils.[29] Moreover, alkaline pH has been associated with enhanced membrane ruffling, thus facilitating micropinocytosis.[255] pH changes in neutrophil phagosomes remains highly controversial. Myeloperoxidase (MPO) appears to have optimal activity at pH 6, while for cathepsin G and elastase, these levels are pH 7-9 and pH 8-10, respectively.[259] Therefore, transient changes in phagosomal pH may provide optimal condition for different enzymes to function. Whether, and if so, how pH is involved in neutrophil microbial killing is still poorly understood. However, Jensen et al support the acidification theory of neutrophil phagosome down to the level of pH 6.[260,261] My contrasting pilot finding therefore requires more repeats and detailed analysis in the future.

In conclusion, I have shown that iICs are internalised by neutrophils via a novel mechanism, macropinocytosis. Since this process removes a powerful pro-inflammatory stimulus, I conclude that this is an anti-inflammatory neutrophil function, similar to bacterial killing via phagocytosis in PICD. This observation led me to investigate whether macrophages can clear the apoptotic neutrophil corpses (discussed in the **chapter 5**).

5. Results: iIC-mediated induction of neutrophil apoptosis promotes subsequent efferocytosis by macrophages.

5.1. Introduction

Efferocytosis literally means 'to carry to the grave' and it represents a mechanism for the disposal of apoptotic cells from the body as part of a homeostatic process for tissue maintenance and, also in development and during inflammatory or immune responses.[262] Professional phagocytes such as macrophages are very efficient at this although other cells like epithelial cells and dendritic cells can also efferocytose apoptotic cells. The phagocytic ability of macrophages to remove apoptotic cells was described in the late 19th century by Elie Metchnikoff, the father of phagocyte biology[263]. Apoptotic cells exhibit 'eat me' signals, the best characterised of which is externalisation of PtdSer, which is recognised by the macrophages. Macrophage receptors interact with externalised PtdSer either by direct recognition using receptors such as TIM-1, 4, BAI1 and stabilin-2 or, indirectly by engaging αvβ3 and αvβ5, Tyro-3, Axl, Mer with the help of bridging molecules such as DEL-1, Gal-3, Gas6 or protein S (as discussed in **chapter 1**).

Downstream, the efficient clearance of apoptotic neutrophils from circulation regulates granulopoiesis,[5] prevents secondary lysis and spillage of noxious neutrophil substances.[264] Efferocytosis is essential for maintenance of normal tissue homeostasis and a prerequisite for the resolution of inflammation. Moreover, apoptotic neutrophils directly trigger an anti-inflammatory and pro-resolving response in macrophages, and dendritic cells.[265] Several *in vitro* and *in vivo* studies demonstrated that following efferocytosis, macrophages produce high levels of IL-10, TGF-β and PGE_2 and secrete factors involved in tissue repair including vascular endothelial growth factor (VEGF). Moreover, specialised pro-resolving lipid mediators such as

resolvins, lipoxins, and maresins are released that further promote efferocytosis, potentiating resolution. At the same time, apoptotic neutrophils suppress the production of Toll-like receptor- (TLR) dependent cytokines including IL-6, IL-8 and TNF by macrophages.[128,266] Defective efferocytosis has the potential to perpetuate inflammation and may eventually lead to chronic inflammatory diseases or auto-immune disease.

5.1.1. Hypothesis

In this chapter, I examined whether iIC-induced apoptotic neutrophils are effectively efferocytosed by macrophages. I hypothesised that iIC-mediated induction of neutrophil apoptosis promotes efferocytosis by macrophages.

5.1.2. Aim

The aim to address the abovementioned hypothesis was to compare macrophage efferocytosis of neutrophils that had undergone constitutive and iIC-induced apoptosis, respectively.

5.2. Results

5.2.1. iIC-mediated induction of neutrophil apoptosis leads to increased efferocytosis by macrophages

I decided to use unstimulated, monocyte-derived macrophages (MDMs) in the initial experiments to eliminate the limitations of 'classically activated-alternatively activated' paradigm of macrophages.[267] A main limitation of the current view is, that the macrophage spectrum is commonly associated with properties of mature macrophages, but activation takes place in the extended macrophage family, including monocytes.[268]

I demonstrated in **chapter 3** that iICs induce neutrophil apoptosis. To test whether this results in efferocytosis, I generated MDMs; cultured with dexamethasone, latrunculin B or no treatment. I then co-cultured MDMs with pHrodo-labelled, apoptotic neutrophils that had been allowed to undergo i) constitutive apoptosis, ii) that had been treated with roscovitine or iii) that had been stimulated with iICs. The percentage of MDM efferocytosis of apoptotic targets was measured using flow cytometry based on separating the MDM cell population that was positive for pHrodo dye (as described in **chapter 2, section 2.12**). I observed that iIC stimulation of neutrophils resulted in significantly improved efferocytosis by MDMs (**Figure 5.1.A**). Some cells from each condition were also fixed for microscopic analysis to visualise the pHrodo signal by another technique. A representative confocal image of macrophage efferocytosis with pHrodo signal that was obtained following co-culture of MDMs with iIC-induced apoptotic neutrophils is shown in **Figure 5.1.B**.

Dexamethasone, a glucocorticoid used as an immune-suppressive drug, was shown to enhance phagocytic capacity of macrophages and also, to suppress the production of pro-inflammatory cytokines.[269–271] In my experiments, dexamethasone-treated macrophages displayed significantly increased efferocytosis when co-cultured with constitutively apoptotic neutrophils. The extent of efferocytosis was similar to that observed with iIC-induced neutrophils cultured with unstimulated MDMs (**Figure 5.1.A**). However, co-culturing dexamethasone-treated macrophages with iIC-induced apoptotic neutrophils did not induce any further increase in efferocytosis (data not shown), suggesting that a maximal efferocytosis was reached.

As shown in **Figure 3.4**, Roscovitine is more powerful at inducing neutrophil apoptosis than iIC stimulation. This was also true after prolonged neutrophil culture (21 hours), when I observed near complete neutrophil apoptosis with Roscovitine, and a little less with iIC-stimulated neutrophils (**Figure 5.1.C**). Interestingly, I observed a similar extent of efferocytosis of both of these neutrophil preparations by MDMs. This suggests that the increased macrophage efferocytosis observed with iIC-stimulated neutrophils is unlikely to be merely directly correlated with the percentage of neutrophil apoptosis.

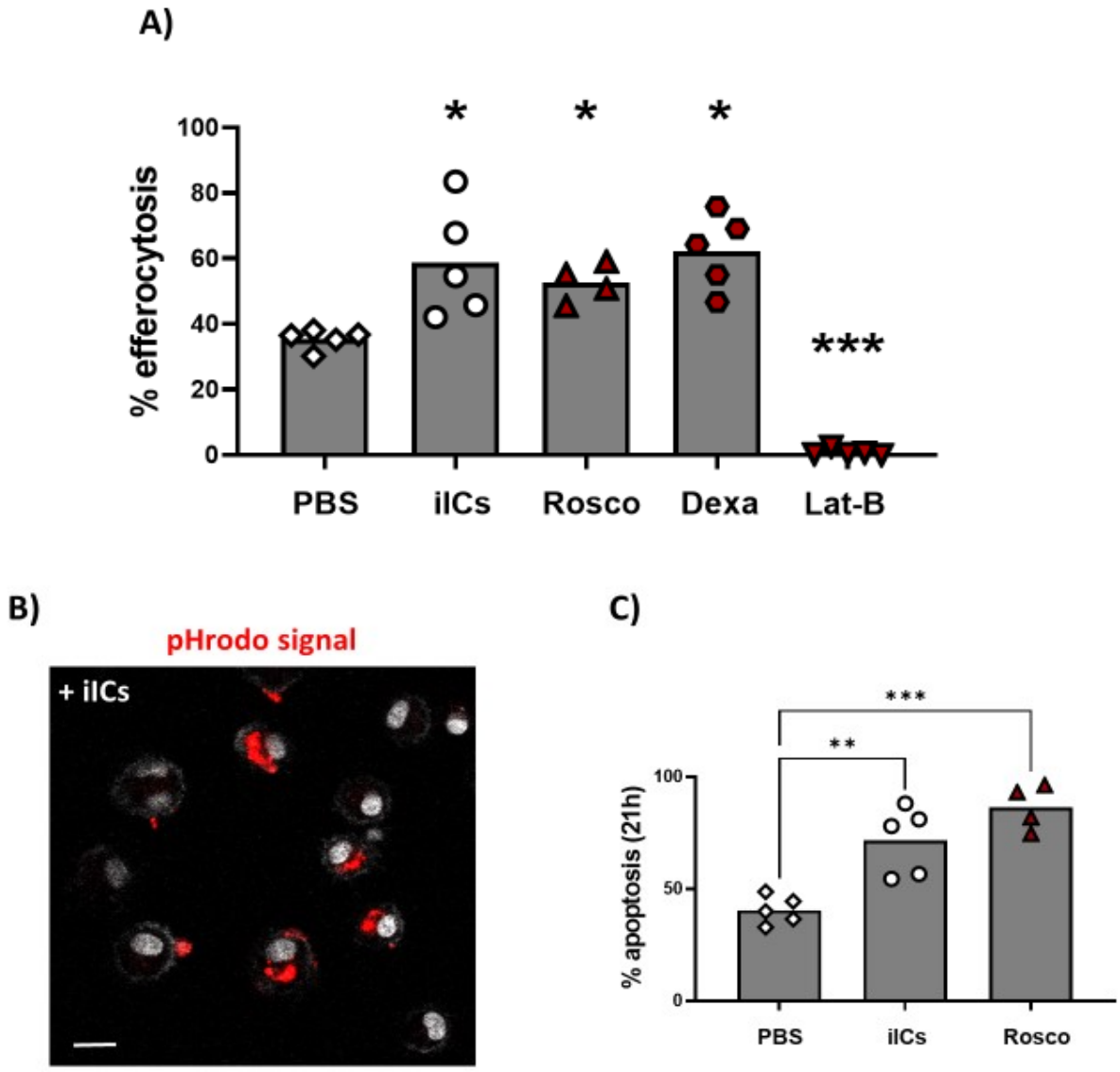


Figure 5.1. iIC-induced neutrophil apoptosis leads to increased efferocytosis by macrophages.

MDMs were co-cultured with apoptotic neutrophils for 45 min at 37 °C allowing efferocytosis. MDMs were treated with Dexa (Dexamethasone 250 nM) and Lat-B (Latrunculin B, 10 μM) as controls. Apoptotic neutrophils were prepared following stimulation with iICs (10 μg/ml) or Roscovitine (20 μM) for 21 hours in IMDM with 10% autologous serum. Vehicle (PBS) treated neutrophils underwent constitutive apoptosis. **A**, efferocytosis of pHrodo-labelled apoptotic neutrophils was analysed using flow cytometry as detailed in **chapter 2, section 2.12**. **B**, MDM efferocytosis is indicated by bright red pHrodo signal. Nuclei from both macrophages and apoptotic neutrophils were stained using Hoechst (gray). A representative field of fixed macrophages following co-culture with apoptotic neutrophils was imaged using Leica SP8 40x objective. Scale bar = 10 μm. **C**, the percentage of neutrophil apoptosis after 21 hour of stimulation was detected by annexin V-PI staining by flow cytometry. Data are displayed as the mean value with each symbol representing the value obtained from individual experiment. The groups were compared using ordinary one way ANOVA with Dunnett's post hoc test. * = p value <0.05, ** = p value <0.01 and *** = p value < 0.001.

5.2.2. Macrophages efferocytose apoptotic neutrophil blebs

To observe events in real-time, I performed a time lapse experiment to visually compare the MDM efferocytosis process of constitutively apoptotic neutrophils and iIC-induced apoptotic neutrophils (**Figure 5.2**). Interestingly, this video showed that macrophages do not need to internalise entire apoptotic neutrophils for pH changes to occur. Internalisation of apoptotic blebs is sufficient. This confirmed my observation in the confocal experiment (**Figure 5.1.C**) where most MDMs with bright pHrodo signal did not contain any Hoechst-stained neutrophil-derived apoptotic nuclei inside them. Further repeats and a more detailed investigation would be required to confirm this. In future experiments, nuclei derived from neutrophils and macrophages could be stained using different dyes to ascertain the origin of the nuclei.

In the **Figure 5.3.A** and **Figure 5.3.B**, a representative, single macrophage is shown from each condition in images obtained from the same time lapse experiment. Interestingly, more pHrodo signal was observed with MDMs cultured with iIC-treated neutrophils (**Figure 5.3.B**). This provides indirect evidence that the macrophages engulfed/nibbled more apoptotic neutrophils in the iIC-stimulated condition. Again, these two images are from a single experiment, hence more repeats and detailed analysis are required to validate this preliminary observation.

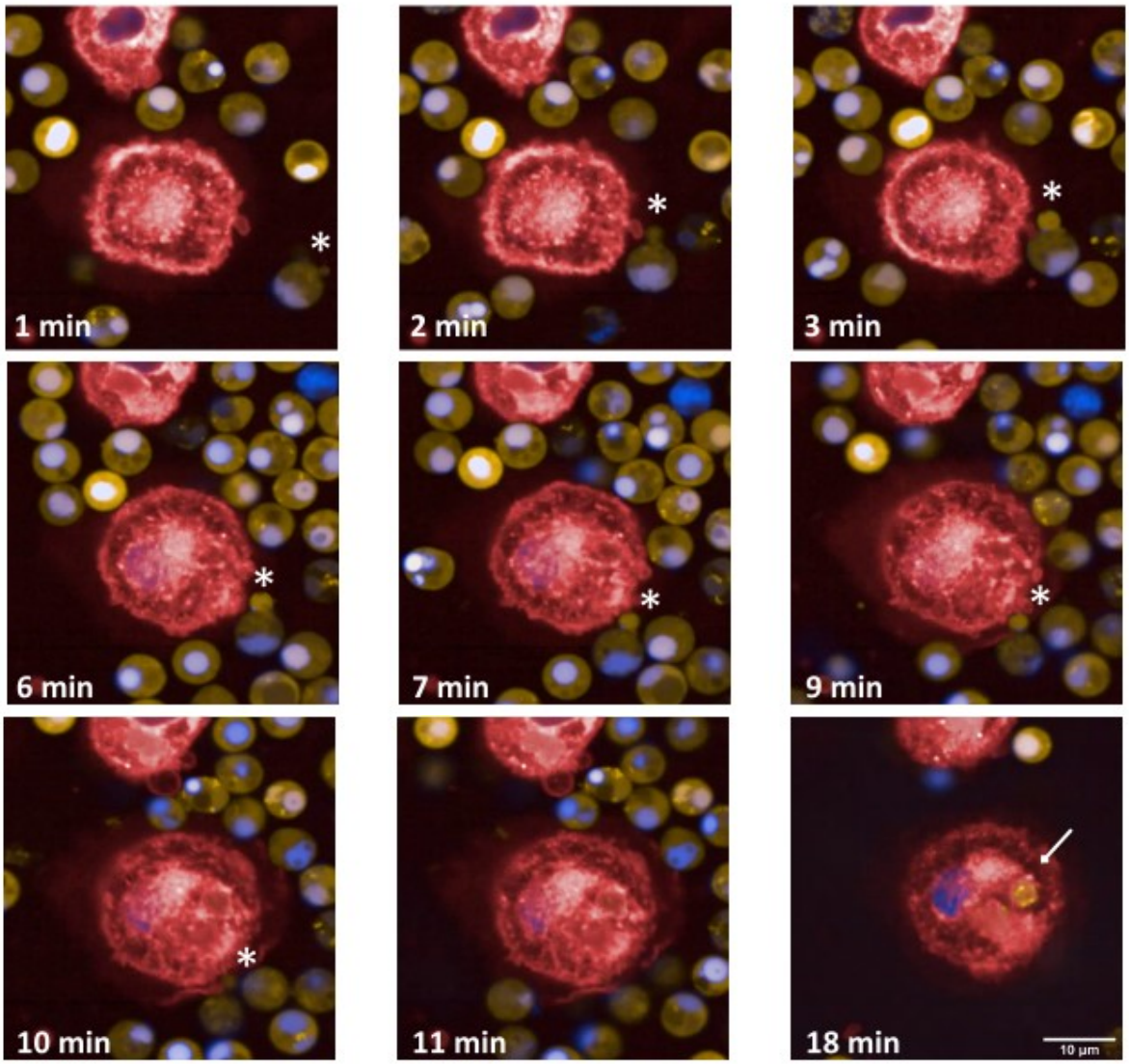


Figure 5.2. Time lapse showing internalisation of an apoptotic bleb by macrophages.

MDMs were co-cultured with apoptotic neutrophils at 37 °C allowing efferocytosis. Apoptotic neutrophils were prepared by stimulating with iICs (10 µg/ml) for 21 hours in IMDM with 10% autologous serum. Macrophages were stained with deep red cell mask and apoptotic neutrophils were stained with pHrodo red SE (yellow) and Hoechst (blue). Images were captured at indicated time points using Opera Phenix confocal microscope (60x objective). An apoptotic bleb is indicated by the white asterisk and pH change inside the macrophage is indicated by the white arrow. Scale bar = 10 µm.

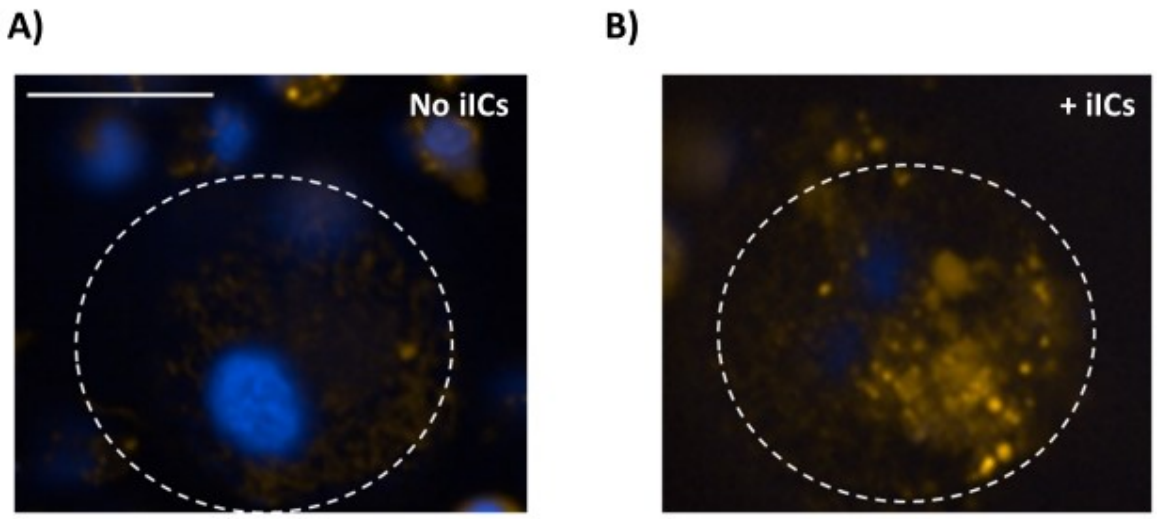


Figure 5.3. Macrophage phagosome becomes more acidic after phagocytosing iIC-induced apoptotic neutrophils.

MDMs were co-cultured with apoptotic neutrophils at 37 °C allowing efferocytosis. Neutrophils were stimulated without (**A**) or with iICs (**B**) for 21 hours in IMDM with 10% autologous serum to make them apoptotic. Macrophages were stained with or deep red cell mask (indicated by the dotted circle), and apoptotic neutrophils were stained with pHrodo red SE. Nuclei of both macrophages and apoptotic neutrophils were stained with Hoechst (blue). Images were obtained from a time lapse movie after 20 min of co-culture using Opera phenix 60 x objective. The presence of pHrodo red SE is indicated with yellow colour. Scale bar = 10 µm.

5.2.3. Increased engulfment or nibbling of iIC-induced apoptotic neutrophils is not due to FcγR engagement

Macrophage Fcγ receptors mediate the uptake and destruction of antibody-coated viruses, bacteria, and parasites by FcγR-mediated phagocytosis, which involves different receptors from efferocytosis.[272,273] FcR knock out animals have normal macrophage numbers, but their opsonic phagocytic capacity was highly impaired,[274] while in humans, genetic differences in FcRs contribute to autoimmune diseases, such as SLE, RA, and multiple sclerosis.[275–277] iIC-stimulated neutrophils do not internalise all iICs (**Figure 4.2**), and a small percentage of iICs remained bound to the cells even after repeated washing. Theoretically, these bound iICs might trigger FcγR-mediated phagocytosis by macrophages, as described by Allen et al.[278]

To address this possibility I first quantified the percentage of neutrophils which bound iICs after 21 hour of culture post stimulation (**Figure 5.4.A**). The percentage was similar to that I observed 30 min after neutrophil stimulation with iICs (**Figure 4.2** and **Figure 5.4.B**).

Next, I assessed internalisation with neutrophils that had been stimulated with iICs for 30 min to investigate whether phagocytosis of (living) neutrophils could take place exclusively due to macrophage FcγR engagement. **Figure 5.4.C** shows that (living) neutrophils were not taken up efficiently as a result of stimulation with iICs, even if they had been stimulated with iICs after PI3K inhibition, when they were able to bind, but not internalise iICs, effectively leading to neutrophil ‘opsonisation’ with iICs. Therefore involvement of FcγR-mediated phagocytosis was excluded in this context. Performing these experiments with FcγRII blocking antibodies could provide further support for the conclusions.

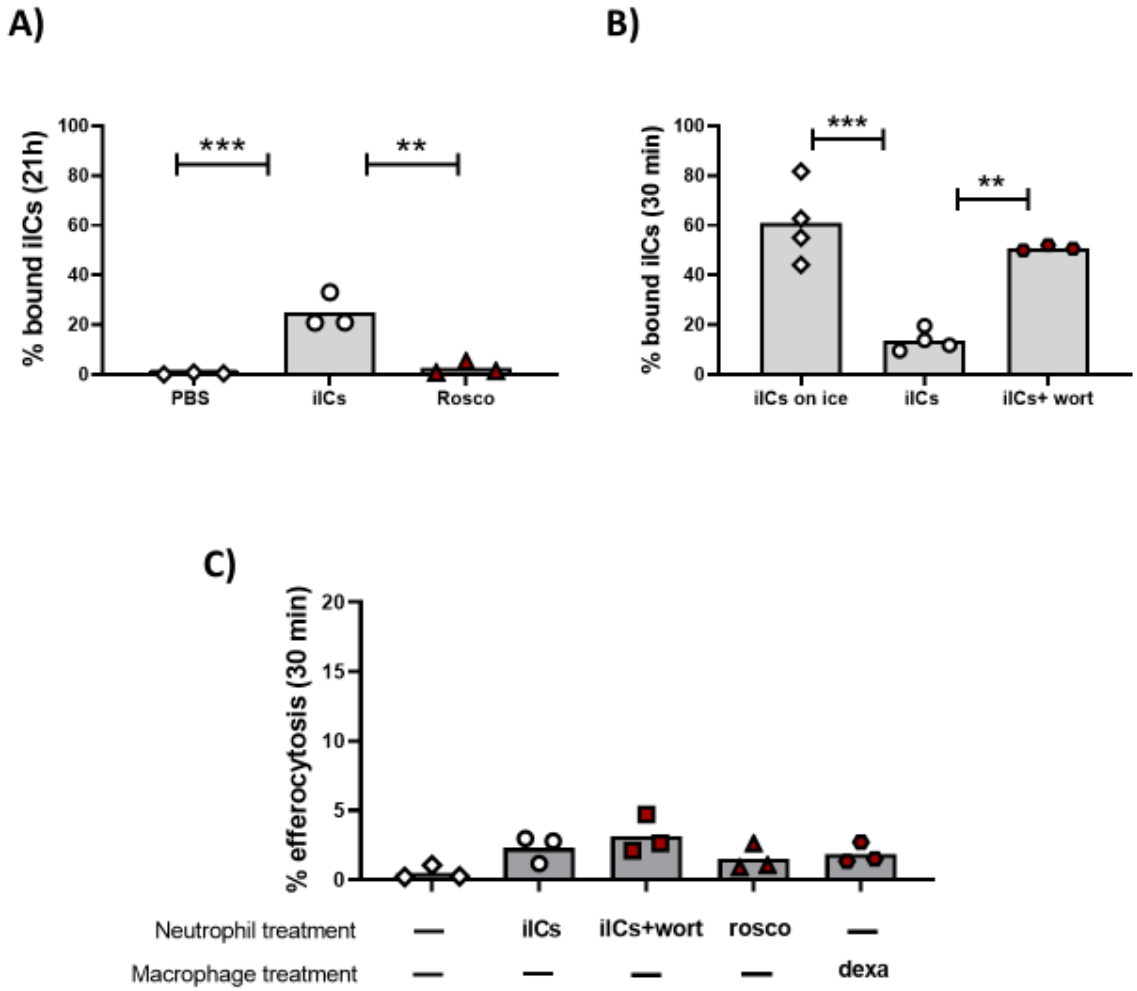


Figure 5.4. Increased engulfment/nibbling of iIC-induced apoptotic neutrophils is not due to FcγR engagement.

Freshly isolated neutrophils were subjected to stimulation with PBS, iICs (10 µg/ml) or Roscovitine (20 µM) for 21 hours **(A)** and for 30 min **(B)** in IMDM with 10% autologous serum. Neutrophils were treated with wortmannin (100 nM) for 10 min at 37 °C prior to iIC-stimulation in 'iICs+ wort' conditions. Cells were then washed to get rid of unbound iICs and bound iICs were detected by flow cytometry by staining the iICs with goat anti-rabbit alexa fluor 647 conjugated secondary antibody. After 30 min iICs stimulation, neutrophils were co-cultured with macrophages **(C)** and percentage of efferocytosis was detected by flow cytometry. Bars show the mean value obtained with each symbol representing the value obtained in one individual experiment. The groups were compared using ordinary one way ANOVA with Dunnett's posthoc test. ** = p value <0.01 and *** = p value < 0.001.

5.3. Discussion

In this chapter, I have shown how iIC-induced apoptotic neutrophils were more efficiently efferocytosed by MDMs (**Figure 5.5**). Interestingly, the increase in the efferocytosis observed was neither due to an increased percentage of iIC-induced neutrophil apoptosis, nor due to FcγR-mediated phagocytosis of neutrophils by macrophages. Rather this appears to be a true effect of iIC-stimulation of the neutrophils. I therefore concluded that stimulation of macrophage efferocytosis is a novel, iIC-induced anti-inflammatory neutrophil function.

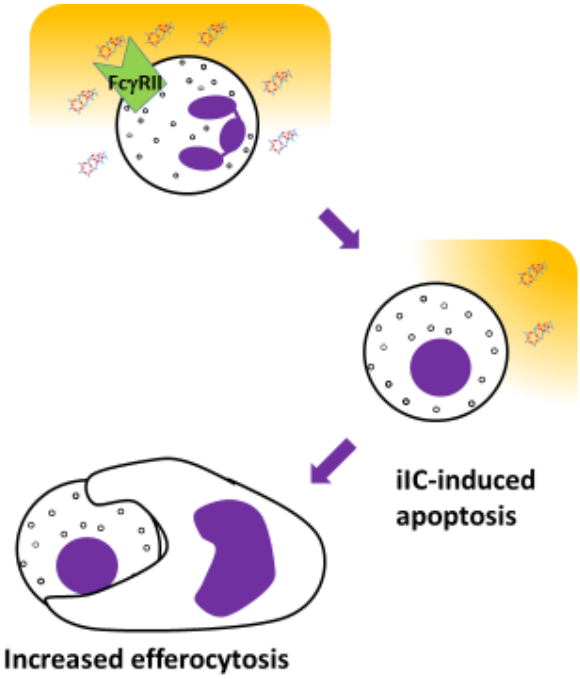


Figure 5.5. iIC-induced neutrophil apoptosis are cleared by macrophages.

A schematic diagram showing iICs bind to the FcγRII receptors of neutrophils which subsequently leads to neutrophil apoptosis. These apoptotic neutrophils are preferentially efferocytosed by the MDM-derived macrophages.

Although efferocytosis resembles phagocytosis, it is a distinct process, mediated by specific receptors, bridging molecules, and downstream signalling pathways.[127] Uptake of apoptotic cells by macrophages could depend on other receptors as well such as complement receptor.[278,279] It would be interesting to test if a different uptake mechanism by macrophages is used for internalisation of neutrophils that have undergone iIC induced apoptosis.

Mantovani and colleagues described two main functionally polarised groups of macrophages, based on their effects on selected markers and inflammation profile, as M1 (pro-inflammatory) and M2 (anti-inflammatory).[280–283] However, this old school concept of M1-M2 has now been replaced with a new spectrum of 'classically activated macrophages', 'wound-healing macrophages' and 'regulatory macrophages', which is more commonly observed in tissues[267,284,285].

Classically activated macrophages arise in response to interferon-γ (IFNγ), which can be produced during an adaptive immune response by TH1 cells or during an innate immune response by NK cells, and TNF, which is produced by antigen-presenting cells.[286] Pro-inflammatory cytokines, for example, IL-1, IL-6 and IL-23 are produced by classically activated macrophages that can cause extensive damage to the host.[287,288] Wound-healing (alternatively activated) macrophages arise in response to IL-4, which can be produced during an adaptive immune response by TH2 cells or during an innate immune response by granulocytes.[289,290] These cells secrete components of the extracellular matrix and their primary function seems to be related to wound healing, hence the name. Regulatory macrophages are generated in response to various stimuli, including immune complexes,[291] G-protein coupled receptor (GPCR) ligands, glucocorticoids, apoptotic cells[292] or IL-10[284]. The major cytokines produced by these macrophages are IL-10 and IL-12[293]. Each of these macrophage populations has a

distinct physiology. Classically activated macrophages have microbicidal activity, whereas regulatory macrophages produce high levels of IL-10 to suppress immune responses. Wound-healing macrophages have a role in tissue repair.[267]

Besides cytokines, regulatory macrophages were shown to produce different pro-resolving lipid mediators, resolvin and maresin, whereas pro-inflammatory mediators like LTB4 and PGE_2 are secreted by classically activated macrophages.[294] Interestingly, Fadok and colleagues showed that efferocytosis of IgG-opsonised apoptotic neutrophils led to the production of high levels of IL10 but also of IL-1β, IL-8 and TNF after 18 hour of co-culture.[128] With this in mind, a logical next step of the current project would be investigating the cytokine profile caused by efferocytosis of iIC-induced apoptotic neutrophils and comparing it to those induced by neutrophils that had undergone constitutive apoptosis. An array could be designed to screen different cytokines, chemokines and pro-resolving mediators followed by their quantitative analysis. Unstimulated or stimulated macrophages could be used to dissect the complexity of the response induced.

As mentioned in **chapter 1**, macrophage subtypes express different sets of cell surface receptors that direct their phagocytic ability. Unstimulated human macrophages primarily use the αvβ3 integrin (vitronectin receptor, CD61) system to recognise and engulf apoptotic neutrophils. Integrin αvβ3 recognises the bridging molecule thrombospondin, which in cooperation with CD36, tethers and engulfs the apoptotic neutrophil.[295,296] With this knowledge, macrophage receptors could be studied to understand whether there is any difference in receptor engagement when macrophages are co-cultured with constitutive or iIC-induced apoptotic neutrophils. As useful control, efferocytosis of dendritic cells that recognise the apoptotic cells through αvβ5 receptor could also be investigated.[297,298] Macrophages employ different

internalisation mechanisms to efferocytose apoptotic and necrotic cells. In a mouse cell line, apoptotic cells were shown to be internalised by phagocytosis whereas, necrotic cells were internalised by micropinocytosis.[299] This could be further explored to understand how iIC-induced apoptotic neutrophils are internalised by macrophages, and whether any difference lies in the internalisation process between constitutive and iIC-induced apoptotic neutrophils.

Impaired efferocytosis has been observed in many human inflammatory and autoimmune diseases, including cystic fibrosis, chronic obstructive pulmonary disease, asthma, idiopathic pulmonary fibrosis, RA, SLE, glomerulonephritis, and atherosclerosis[300] and understanding the mechanism of MDM efferocytosis following iIC-induced apoptotic neutrophils would set the foundations to exploring new avenues in this context.

6. Results: iIC-induced anti-inflammatory neutrophil functions are dysregulated in RA.

6.1. Introduction

Neutrophils play an important role in the pathogenesis of autoimmune diseases in several ways. First, they degranulate and generate highly toxic ROS at inflammatory sites that damage the tissues. Second, activated neutrophils release a range of pro-inflammatory cytokines and chemokines at the site of inflammation (shown in **chapter 1**, **Table 1.4)** although neutrophils are comparatively poor at producing cytokines. Third, they can serve as a source of autoantigen, e.g. via the release of neutrophil extracellular traps (NETs).[301–303]

So far, I have shown data obtained with peripheral healthy blood-derived neutrophils that contribute to the understanding of the effects of iIC-stimulation in neutrophils. In addition, the assessment of behavioural and functional properties of neutrophils derived from the patients with autoimmune diseases such as RA is immensely important. RA is characterised by the presence of autoantibodies such as rheumatoid factor (RF) and anti-citrullinated protein/peptide antibodies (ACPA) which form immune complexes that promotes chronic inflammation.[138,154,304] Synovial joint cavities are normally acellular, but in RA, they are heavily infiltrated by immune cells including neutrophils, possessing considerable potential to inflict tissue damage. RA synovial fluid (SF) contains both soluble and insoluble ICs, which stimulate infiltrating neutrophils. Neutrophils have been neglected for years, perhaps because of their short life span and more emphasis has been put onto other immune cell types.[154,305] However, current therapeutic agents used to treat RA have direct impacts on neutrophil functions. For example, non-steroidal anti-inflammatory drugs (NSAIDs) inhibit neutrophil adhesion, and methotrexate inhibits chemotaxis and induces

neutrophil apoptosis,[306] suggesting that more attention is required to explore the role of neutrophils in the pathogenesis of RA

Neutrophils from patients with RA are functionally different from those isolated from healthy individuals, with striking differences in gene and protein expression,[307] including higher levels of membrane-expressed TNFα and myeloblastin (also known as PR-3 or cANCA antigen).[308] Neutrophils derived from RA patient blood are in a primed state. This is thought to be due to presence of cytokines such as TNFα in the circulation.[309] Primed cells produce greater amounts of ROS, phagocytose more, and have greater cytotoxic activity (as laid out in **chapter 1**, **section 1.1.1**) than control cells as well as being characterised by increased *de novo* protein biosynthesis, which lead to generation and release of further cytokines forming a vicious cycle.[176,178,301,310]

Moreover, neutrophils derived from synovial fluid of the RA patients exhibit different profiles compared to their counterparts in the bloodstream. SF neutrophils have been shown to be activated with alteration in the surface receptor expression, such as higher expression of CR3 is observed on SF neutrophils compared to that on circulating neutrophils of RA patients.[175,311]

6.1.1. Hypothesis

Considering the differences between healthy and RA neutrophils laid out in the above section, I hypothesised that iIC-induced neutrophil effector functions are dysregulated in RA.

6.1.2. Aims

The following aims were addressed to test this hypothesis

a) To compare neutrophil functions (ROS production, macropinocytosis and apoptosis) derived from rheumatoid arthritis patients and healthy donors.

b) To analyse the abovementioned neutrophil functions derived from synovial fluid of RA patients.

6.2. Results

6.2.1. Demographic profiles of RA patients

This chapter explores the effects of iICs in peripheral blood derived as well as SF-derived neutrophils from RA patients. The details of the patients are summarised in **Table 6.1** that included nine patients in total with a diverse background in terms of age, sex, autoantibody status, comorbidities and medication. Some patients were recently diagnosed and others had had the disease for years. At the same time, neutrophils from age- and sex-matched heathy donors were also isolated and compared with the RA samples. In this chapter, neutrophils derived from peripheral blood of RA patients will be called RA neutrophils and, neutrophils derived from synovial fluid of RA patients will be termed as SF neutrophils while healthy donor derived neutrophils will be referred to as HD neutrophils.

Table 6.1. Demographic profiles of RA patients including their treatment plans.

No.	Disease	Age (years)	Sex	RF status	ACPA status	Treatment
1	RA-ILD	75	Female	+ve	Not known	Not on any RA medication, multiple medicines for ILD
2	RA	38	Female	+ve	+ve	Methotrexate and Hydroxychloroquine
3	RA-ILD	76	Female	+ve	Not known	Not on any RA medication
4	Early RA	66	Male	Not known	Not known	Not on any RA medication, aspirin for heart disease
5	RA	59	Male	+ve	+ve	Only Hydroxychloroquine
6	RA	65	Female	+ve	+ve	Had Rituximab 5 months ago, not on any DMARD
7	RA	54	Male	-ve	-ve	Did not respond to any treatment, now on Prednisolone 25 mg, responded to anti-TNF but had to stop 6 years ago
8	RA	36	Female	+ve	+ve	Only Hydroxychloroquine 400 mg/day
9	RA	58	Female	Not known	+ ve	On Sulphasalazine 3g once daily

Abbreviations: RA-ILD, Rheumatoid arthritis with interstitial lung disease; DMARD, disease modifying anti-rheumatic drugs; TNF, tumor necrosis factor;

6.2.2. RA neutrophils exhibited more ROS production following iIC treatment compared to those from healthy donor blood

No obvious difference was found in the total neutrophil number or morphology of RA compared to HD neutrophils (data not shown).

According to the literature, RA neutrophils are primed and thus produce more ROS after fMLF stimulation.[170,312] I investigated the effect of iIC stimulation on RA neutrophils compared with age and sex matched HD neutrophils. Although the RA patients that had been recruited were on distinct treatment regimes, their RA neutrophils behaved in the same pattern, generating more ROS in response to iIC-stimulation than those from HD controls (**Figure 6.1**). This greater production of ROS indicates that RA neutrophils might be already activated.

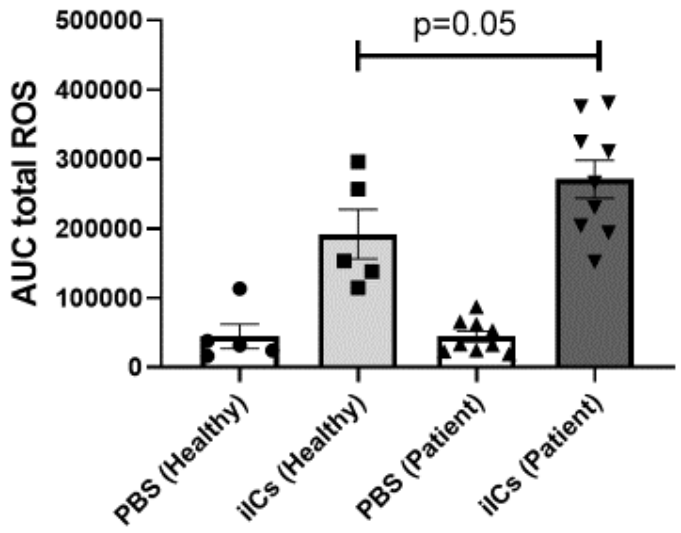


Figure 6.1. RA neutrophils generate more ROS.

Freshly isolated neutrophils from RA patients and respective age- & sex-matched healthy donors were or were not subjected to stimulation with iICs (4 µg/ml) in PBS. The total ROS production was analysed as detailed in the **chapter 2**, the area under the curve (AUC) is plotted. Data are displayed as the mean value with each symbol representing the value obtained from individual experiment. Data were compared with two-way ANOVA with Bonferroni's post hoc test.

6.2.3. Effects of iICs on the apoptosis of RA neutrophils were more variable

Previous studies have demonstrated delayed apoptosis in neutrophils derived from both blood and synovial fluid of RA patients.[171,306,313,314] I investigated the effect of iIC stimulation on RA neutrophil apoptosis without observing any significant delay in RA control samples without any stimulation compared to their HD counterparts. While, iICs induced apoptosis in HD neutrophils after 9 hours of stimulation (data not shown), RA neutrophils showed mixed effects (**Figure 6.2**) making the results inconclusive. Apoptosis was measured at 3 hour interval up to 24 hours of iIC stimulation

Patients' blood was collected either in citrated or heparinised tube. Although unfractionated heparin alone has been shown to induce neutrophil apoptosis and other anticoagulants have also effects on other neutrophil functions, no significant advantages or disadvantages were reported with any single anticoagulant.[315] Additionally, isolated neutrophils from heparinised blood of healthy donors did not show any obvious difference in neutrophil apoptosis (data not shown) excluding the effects of any particular anticoagulant on neutrophil apoptosis.

Since the analysis of induction of apoptosis involves culture of the neutrophils in autologous serum containing medium, any differences observed between the induction of apoptosis of HD and RA neutrophils could be due to a cell autonomous difference, or, alternatively due to factor(s) contained in the serum.[314,316] I therefore also analysed apoptosis of RA and HD neutrophils that had been cultured in RA and HD serum and vice versa. However, the results were again inconclusive (data not shown). To fully understand the effect of iICs on RA neutrophil apoptosis, the sample size needs to be increased to strengthen the statistical power and the patients need to be stratified based on coexisting complexities (medication, comorbidities etc.).

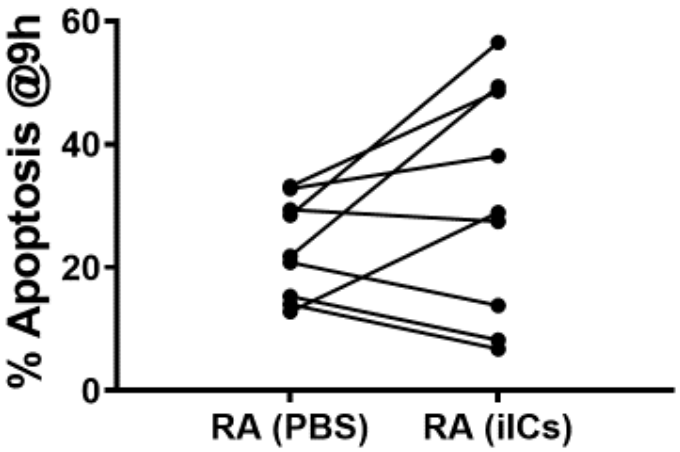


Figure 6.2. iIC stimulation has inconsistent effects on RA neutrophil apoptosis.

Freshly isolated neutrophils from RA patients and respective age & sex matched healthy donors were subjected to stimulation with PBS or iICs (10 µg/ml) in IMDM with 10% serum (as indicated). Apoptosis was measured using flow cytometry after annexin V-PI staining after 9 hours of iIC stimulation. Individual data points are shown from each experiment. Data points were compared using paired t-test, indicating no significant differences between the two groups.

6.2.4. RA neutrophils were equally able to pinocytose iICs

Deposition of ICs and complement proteins onto joint surfaces leads to cell attachment to these surfaces with incomplete phagosome closure and release of ROS and degranulation to the outside in a process known as frustrated phagocytosis.[312] As a consequence, neutrophils release more ROS and cytokines in the microenvironment of the open phagosome.

Since iICs are internalised by macropinocytosis (as discussed in **chapter 4**), assessment of pinocytosis was next conducted. HD neutrophils and RA neutrophils were subjected to iIC stimulation in serum-free conditions to exclude the involvement of complement proteins. Surprisingly, no difference was identified in iIC-pinocytosis by HD neutrophils and RA neutrophils (**Figure 6.3**), suggesting that RA neutrophils were equally capable of scavenging the iICs as HD neutrophils.

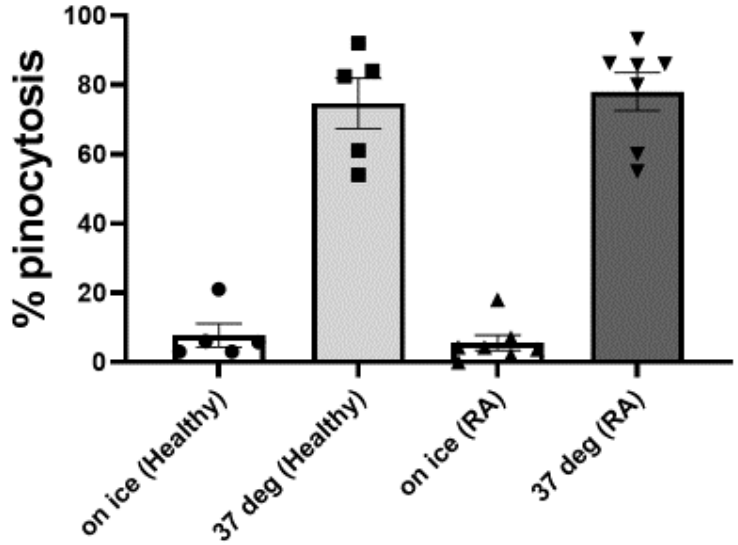


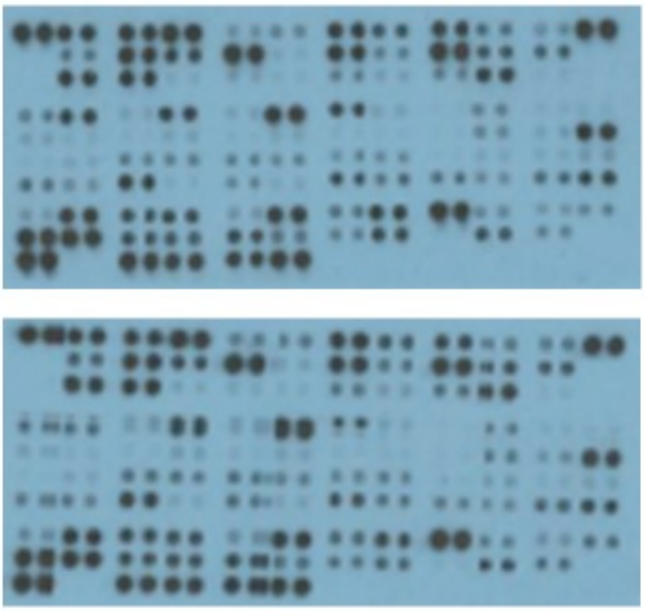
Figure 6.3. HD and RA neutrophils internalise iICs by macropinocytosis equally well.

Freshly isolated neutrophils from RA patients and respective age- & sex-matched healthy donors were subjected to stimulation with iICs (2 µg/ml) in PBS for 30 min on ice or at 37 °C. Internalised iICs were stained with alexa fluor conjugated secondary antibody in some samples. Lucifer yellow was added for 10 min at 37 °C prior to iIC-stimulation in the rest of the samples. Percentage of internalisation was calculated either by counting the cells with internalised iICs or by measuring the lucifer yellow signal using flow cytometry. Two way ANOVA analysis shows no significant difference between the healthy and diseased groups.

6.2.5. Comparative cytokine analysis of RA and HD serum was inconclusive

Numerous cytokines are expressed by and functionally active in synovial tissues. Cytokines represent an important therapeutic target group in RA (see **Table 1.3** in **chapter 1**). A wide range of cytokines including IL-1, IL-6, IL-12, and TNFα was reported in the peripheral blood and in the synovial tissues in RA[317]. Curiously, RA synovial fluid contains large quantities of cytokines that are secreted by macrophages and neutrophils (IL-6, TNF, TGF-13) and IL-8, but few T-cell-derived cytokines (IL-2, IL-3, IL-4 and IFNγ)[318]. Analysis of neutrophil cytokines thus becomes even more crucial, in the current era of treatment with biologics.[306]

Considering the involvement of a wide range of cytokines in RA, I asked whether particular cytokines are upregulated or downregulated in the peripheral blood of RA and whether I could identify a correlation between the cytokines and iIC-induced neutrophil effector functions. I used the serum samples obtained from RA patients and performed a cytokine array with age- and sex- matched healthy donor serum. One example of individual blots from each condition is shown in **Figure 6.4**. Unfortunately I failed to conclude anything from the cytokine array results, perhaps due to the small sample size, or because the patients' RA was effectively treated at the time these samples were taken.



RS= reference spots, NC= negative control, IL= interleukin, CCL/CXCL= chemokines

RS	Acrp30	ApoA1	Angiogenin	Ang-1	Ang-2	BAFF	BDNF	C5/C5a	CD14	CD30	RS
	CD40L	CHI3L1	CFD	CRP	Cripto-1	CST3	Dkk-1	CD26	EGF	CD147	
	CXCL5	CD105	CD178	FGF-2	KGF	FGF-19	FLT3LG	G-CSF	MIC-1	GM-CSF	
Groα	GH	HGF	ICAM-1	IFN-γ	IGFBP-2	IGFBP-3	IL-1α	IL-1β	IL-1ra	IL-2	IL-3
IL-4	IL-5	IL-6	IL-8	IL-10	IL-11	IL-12	IL-13	IL-15	IL-16	IL-17A	IL-18
IL-19	IL-22	IL-23	IL-24	IL-27	IL-31	IL-32	IL-33	IL-34	IP-10	ITAC	KLK3
Leptin	LIF	NGAL	CCL2	CCL7	CSF1	MIF	CXCL9	CCL3/4	CCL20	CCL19	CLG4B
MPO	OPN	PDGF-AA	PDGF-BB	PTX3	CXCL4	RAGE	CCL5	RBP-4	RLN2	ADSF	CXCL12
PAI-1	ABP	ST2	CCL17	TFF3	TfR	TGFα	TSP-1	TNFα	uPAR	VEGF	
RS		VDB	PECAM-1	TIM-3	VCAM-1						NC

Figure 6.4. Cytokine array analysis in serum derived from HD and RA patients.

Cytokine contents of RA and HD sera were determined using a cytokine array kit as detailed in **chapter 2**. The individual dot represents individual analyte (in duplicate) as shown in the table. Dots were visually compared. One representative blot from each condition is shown, obtained from four separately conducted experiments. RA= rheumatoid arthritis, HD= healthy donor

6.2.6. SF neutrophils showed an exaggerated response with iIC-stimulation, compared to RA neutrophils

Of all the cells that infiltrate diseased joints, the neutrophil has the greatest potential to inflict tissue damage. The involvement of neutrophils in RA pathogenesis requires a vicious cycle comprising: (i) migration from the circulation into joints; (ii) priming by local factors to permit efficient responsiveness to activating ligands; (iii) activation to secrete tissue damaging products; and (iv) subsequent generation of chemoattractants to stimulate further cycles of recruitment, priming and activation. Conditions within the synovial joint, such as hypoxia and the presence of anti-apoptotic cytokines (such as TNF, GM-CSF and IL-8) increase neutrophil survival for up to several days.[313,319,320]

Since SF neutrophils exhibit a different functional profile compared to blood derived neutrophils,[175,321] I also analysed the effect of iIC stimulation on SF-derived neutrophils. Unfortunately, I managed to obtain a single SF sample before the clinics were shut due to COVID-19 pandemic, making it impossible to draw any conclusions from this dataset. From the single RA patient with active disease, I obtained 7 ml of viscous synovial fluid containing 50 million cells. Morphologically, nearly 100% of the cells were neutrophils when observed under light microscope after Kwik-Diff staining. No patient history was available.

In the preliminary data shown in **Figure 6.5**, I noted accumulation of IgG inside the SF neutrophils (**Figure 6.5.A**), delayed apoptosis (**Figure 6.5.B**) and increased ROS production in response to iIC-stimulation (**Figure 6.5.C**), altogether suggestive of a more pro-inflammatory nature of these SF neutrophils. In particular, ROS production with iIC stimulation was ten times higher than that of HD blood-derived neutrophils (**Figure 6.1** and **Figure 6.5.C**) suggesting that the SF neutrophils were more primed.

Nurcombe et al. suggested, the SF neutrophils might be already at the optimum level of priming. The authors described that although stimulation with IFN-γ could prime blood derived RA neutrophils into a state of enhanced responsiveness, SF neutrophils could not be similarly further upregulated.[310] In IC-mediated diseases such as RA, extracellular release of granule content frequently occurs due to frustrated phagocytosis. Increased degranulation and delayed apoptosis of neutrophils correlate with the intensity of synovial inflammation and the destructive capacity of joint neutrophils in RA. For the same reasons, the SF neutrophils might struggle to digest ICs after internalisation[312] (as shown in **Figure 6.5.A**).

To validate the effect of iICs on SF neutrophil apoptosis additional repeats are required. However, if my pilot observation holds true, SF neutrophils and blood-derived neutrophils should be thought a separate entities due to their functional differences when designing a therapy targeting neutrophil apoptosis.

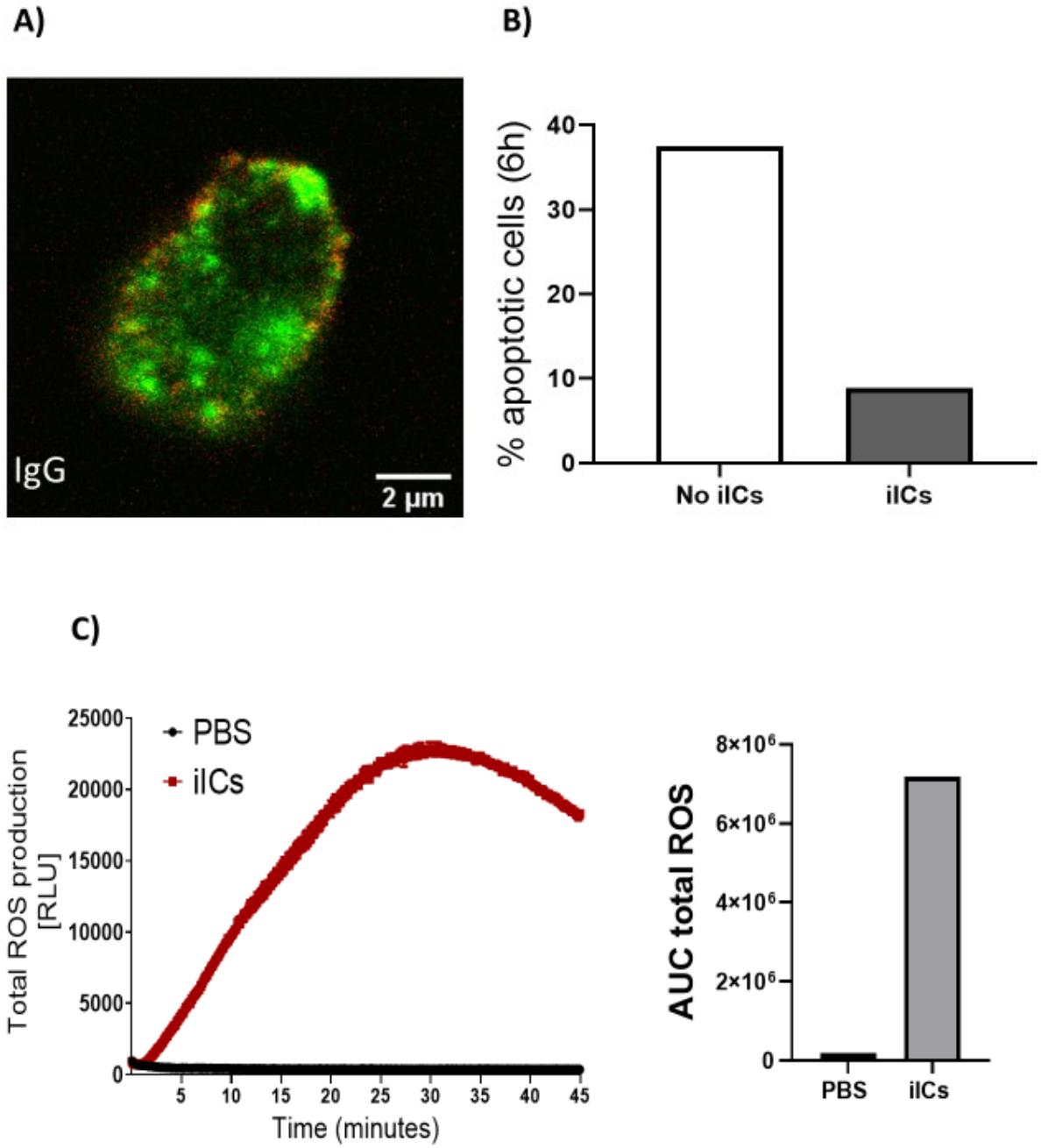


Figure 6.5. Synovial fluid derived neutrophil effector functions after ilC stimulation.

Neutrophils were separated from the synovial fluid after centrifugation and divided into three groups. First group of cells were adhered on slide and stained with alexa fluor conjugated anti-human IgG antibodies (red, before and green, after permeabilisation) in **A**, the second group was stimulated with PBS or ilCs (10 µg/ml) for 6 hour in IMDM with 10% of SF supernatant and apoptosis was measured using flow cytometry after annexin V-PI staining (**B**). The third group was subjected to PBS or ilC stimulation (4 µg/ml) followed by luminescence detection using a plate reader (**C**) and area under the curve (AUC) was measured.

6.3. Discussion

I did not come across any study where neutrophils derived from RA patients were subjected to iIC stimulation *in vitro*. Results from this chapter indicated that some iIC-induced neutrophil effector functions in RA, both in blood derived and SF derived, are either exaggerated (ROS) or altered (apoptosis). An exception is pinocytosis where RA neutrophils displayed no difference in the pinocytic capability. Importantly, the small sample size was not statistically powerful enough to obtain reliable results. Ideally, power calculation should be performed first to determine the sample size required for the proper assessment and interpret the results confidently. Moreover, samples from early RA patients before any treatment should be used to establish the baseline effect of iICs. This was not possible as the RA patients are being treated as soon as they are diagnosed. It is possible that effects of treatments or severity of the disease have had an impact on the data I obtained. With a sufficient large number, the patient data then could be stratified based on their treatment or comorbidities, age, sex and the stage of the disease etc.

One major issue I had was obtaining the patient samples. Access to RA patients was limited because i) NHS Lothian moved the care of RA patients from consultant-led to GP-led facility just prior to embarking on my PhD making it extremely difficult to recruit patients or making any arrangement for sample collection; ii) the next available option was to collect patient sample from RA-ILD patients from a respiratory fibrosis clinic. However, all these patients had interstitial lung diseases as a comorbidity; and finally, iii) due to COVID-19 all the clinics were shut for a long time and even after that in-person patient visits were replaced by tele-consultations except for particularly complicated cases such that the samples I obtained may not have been representative.

Pro-inflammatory cytokines represent potential targets for therapeutic intervention such as biologic drugs targeting TNFα (**Table 1.3** in **chapter 1**). The mechanisms by which anti-TNF-α improves disease are undefined. It is possible that this therapy both directly and indirectly downregulates neutrophil function by impairing cycles of neutrophil recruitment and activation within joints. Blocking TNFα inhibits ICAM1 on endothelial cells, thus interfering the leukocyte adhesion cascade. Anti-TNF therapy moreover blocks cytokine and chemokine synthesis in synovial tissue, lowers concentrations of MMPs, decreases influx of immune cells to synovial joints by downregulating adhesion molecule expression, induces apoptosis of monocytes and macrophages, and restores regulatory T-cell function.[322] Interestingly, TNF inhibitors did not modulate plasma cytokines although it reduced the SF cytokines.[175] Scientists have designed therapy targeting TNFα which reduces the expression of FcγRII.[323] Since we already know that iIC-pinocytosis is FcγRII dependent (**Figure 4.10** in **chapter 4**), the current anti-TNF therapy is potentially interfering with the internalisation capability of RA neutrophils. It would be interesting to test this possibility experimentally.

Other conventional drugs such as NSAIDs and corticosteroids also affect neutrophil functions. They decrease neutrophil adhesion, degranulation and ROS production, and promote neutrophil apoptosis. Additionally, corticosteroids decrease neutrophil production of chemokines, such as IL-8. Non-biologic DMARDs such as methotrexate induce neutrophil apoptosis and decrease neutrophil migration, LTB4 synthesis, ROS production and degranulation[302,306]

Another important aspect of RA pathogenesis is NET production and RA neutrophils have been shown to die by NETosis.[87] High number of apoptotic neutrophils (impaired efferocytsosis) or NETotic neutrophils are the reasons for further multi organ

involvement in other autoimmune diseases such as, lupus nephritis syndrome (impaired kidney functions in lupus patients). NETosis requires the NADPH oxidase which is followed by activation of PAD4, an enzyme that converts arginine residue to citrulline in histones. Elastase also moves to the nucleus and participates in histone modification and chromatin unfolding. NETs have microbiocidal activity but can also be a source of autoantigens in patients with autoimmune diseases.[176] NETs derived from RA patients present citrullinated histones and other granular proteins which subsequently help in formation of anti-citrullinated antibodies such as ACPA, potentiating further inflammation.[176–178] Interestingly, I have also observed NET formation in neutrophils derived from healthy donors after iIC-stimulation. An example is shown in **Figure 6.6**.

The difference between the blood and SF neutrophils add further complexities to the pathogenesis of RA. Interestingly, the image shown in **Figure 6.5.A** appeared very similar to those of IgG inclusion bodies found in the blood of patients with Felty's syndrome.[324] Felty's syndrome is a complication of rheumatoid arthritis associated with splenomegaly and neutropenia. The authors suggested that the neutropenia in Felty's syndrome is the result of excessive uptake of immune complex resulting in increased clearance of neutrophils from the system.[325]

In conclusion, I have discussed how iIC-stimulation could lead to different effects on diseased neutrophils. With proper validation, these results could help with better understanding of the role of iICs in RA pathogenesis.

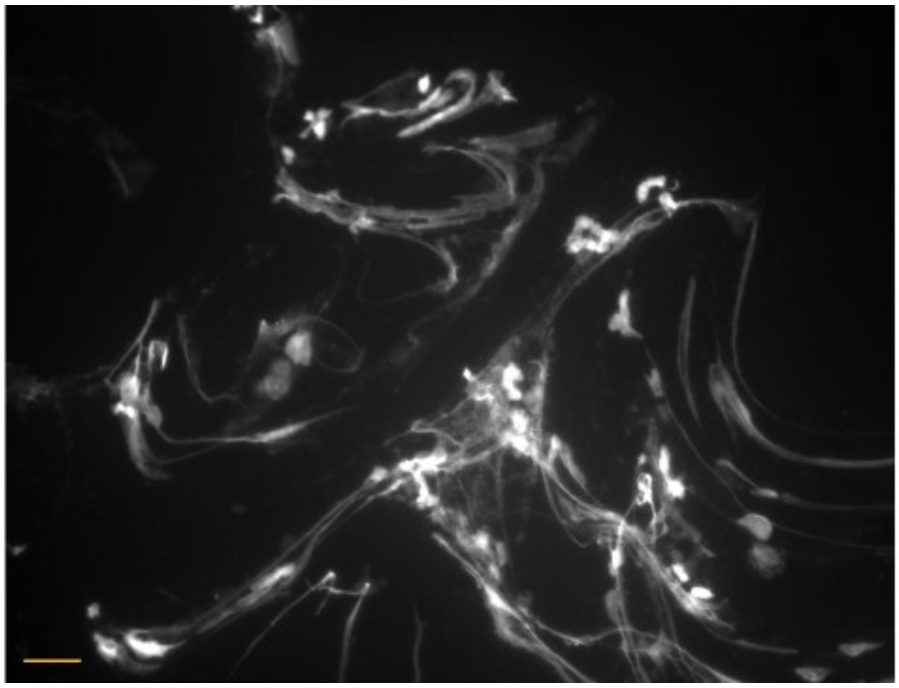


Figure 6.6. An example of NET production after stimulation of neutrophils with iICs.

Scale bar = 10 μm.

7. Final discussion and future directions

Summary

The aim of this thesis was to elucidate iIC-induced anti-inflammatory responses in unprimed neutrophils. Taken together all the data, I successfully demonstrated that iIC stimulation triggers underappreciated anti-inflammatory responses in neutrophils by three different mechanisms: i) iICs are internalised by neutrophils through PI3K dependent macropinocytosis and subsequently degraded by serine proteases, thereby eliminating the stimulus; ii) iICs induce neutrophil apoptosis through a PI3K dependent and actin independent mechanism which is also supported by other literature.[99,200]; and finally, iii) iIC-induced apoptotic neutrophils are efficiently cleared by macrophages. Eventually, enhanced efferocytosis of apoptotic neutrophils will possibly lead to resolution of inflammation (**Figure 7A**).

My results thereby revealed two distinct pathways downstream of PI3K in iIC-stimulated neutrophils (**Figure 7B**). This work has been published in March, 2021.[326]

I have identified a novel mechanism by which neutrophils internalise iICs, macropinocytosis, a process which has not yet been studied extensively in neutrophils. I have confirmed that iIC-induced neutrophil apoptosis is independent of iIC-macropinocytosis, unlike the situation with PICD. Another novel mechanism identified in this study is enhanced efferocytosis of the iIC-induced apoptotic neutrophils by macrophages. While iICs induce powerful pro-inflammatory responses including ROS and likely NET production, it might be feasible in the future to pharmacologically uncouple iIC-induced pro-inflammatory and anti-inflammatory responses in neutrophils. This strategy along with promoting efferocytosis of iIC-induced apoptotic neutrophils could be applied as a potential targeted therapy in autoimmune diseases.

I have also shown that RA blood neutrophils are able to successfully pinocytose iICs irrespective of patient comorbidities and different treatments. The impact of iICs on the other neutrophil functions that I analysed are not explicit. Due to the small sample size analysed stratification of results according to disease status, co-morbidities and drug treatment was not feasible. Doing this with a larger sample size will increase the statistical power and might identify clearer trends.

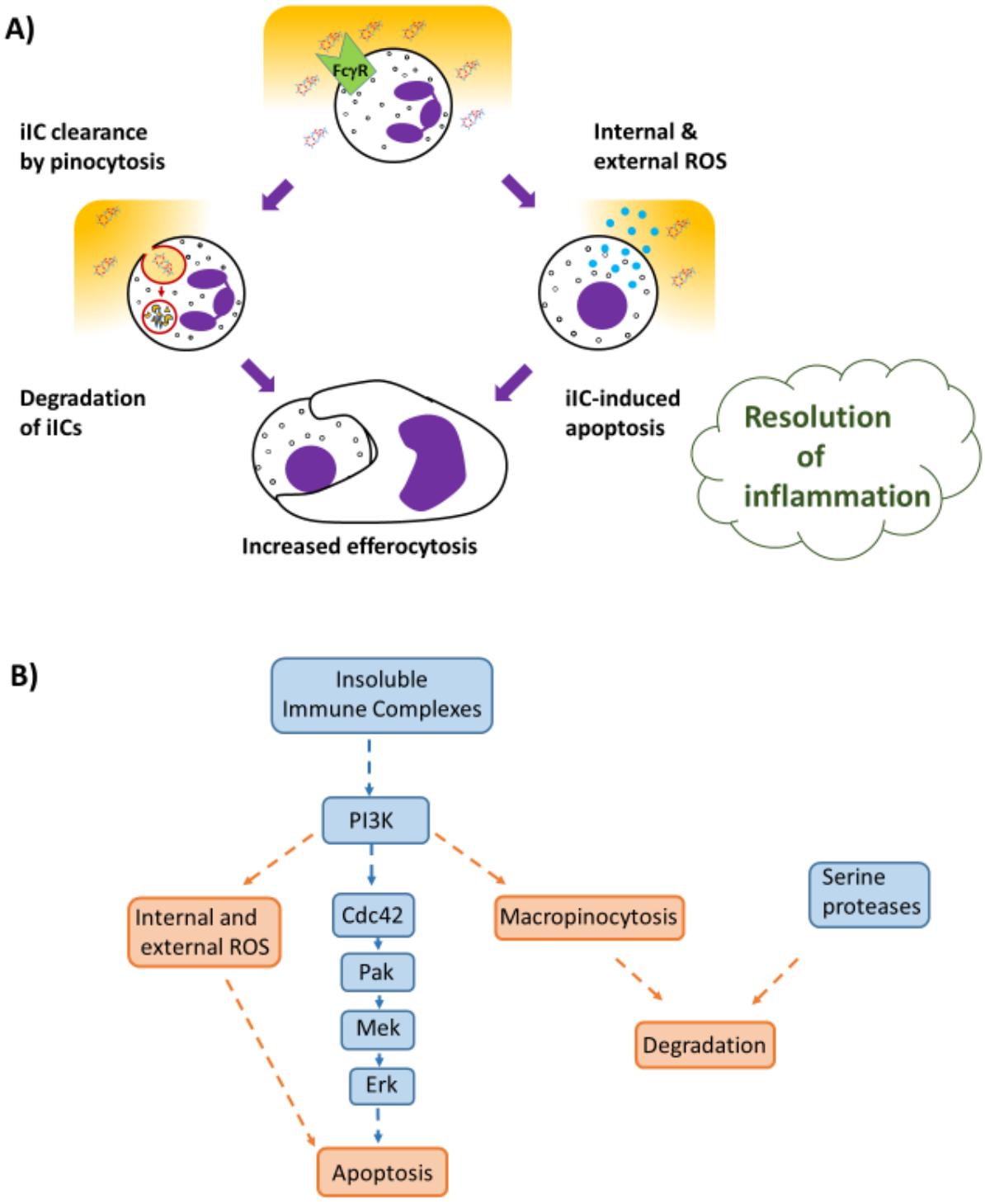


Figure 7. Summary of the results presented in this thesis.

A shows iICs bind to the FcγR to exert different neutrophil effector functions such as ROS production, pinocytosis and neutrophil apoptosis. Eventually, the apoptotic neutrophils are cleared by the macrophages that may further promote resolution of inflammation. **B** shows two distinct pathways by which i) iICs induce ROS dependent apoptosis and ii) iICs are internalised by pinocytosis. Both are PI3K dependent, however internalisation of iICs is not required for the induction of apoptosis. Altogether, iICs trigger anti-inflammatory functions in neutrophil.

Future directions

First, the effects of iICs upon the processes involved in the resolution of inflammation need further study. The obvious immediate next step to confirm whether iICs can indeed promote the resolution of inflammation would be an analysis of the cytokines released by macrophages after efferocytosing apoptotic neutrophils that had or had not ingested iICs. It would be enthralling to know whether this provokes a pro- or an anti-inflammatory macrophage cytokine signature.

Second, the sample size of patients' needs to be increased. Increasing the number of patient samples as well as stratification of results based on individual attributing factors would increase the statistical power. The latter would enable me to undertake a proper multi-variable analysis. Strengthening the patient dataset is of utmost importance to identify underlying functional differences. This would be an interesting avenue to explore, especially if the outcome of iIC stimulation is found to be dysregulated in RA patients. Including neutrophils from SLE patients and osteoarthritis would also be meaningful in this context, strengthen any observations made and allow us to conclude whether any functional differences are disease-specific or changes that occur as a consequence of neutrophil activation.

Third, NET production by iICs could be investigated further. I have shown an example of NET production after iIC stimulation in **chapter 6**. NET production could be a pro-inflammatory mechanism that acts to recruit more neutrophils to the site of inflammation[327] or it can represent an anti-inflammatory mechanism with its antibacterial properties by trapping and degrading toxic mediators.[328] It would be important to elucidate the mechanism of NET production induced by iICs. Cell disintegration in NETosis occurs via a morphologically distinct mechanism to the

classical non-inflammatory form of cell death, apoptosis.[329] Further experiments could be designed to study whether/to which extent iICs induce NETosis.

Fourth, the involvement of different receptors in regulation of apoptosis could be meticulously scrutinised. Due to time limitation, I could not explore the involvement of FcγRI and αMβ2 in iIC-induced neutrophil apoptosis. The role of αMβ2 was not initially considered while assessing iIC-induced apoptosis since these experiments were carried out in the presence of autologous serum and, complement receptor (CR) activation was unavoidable. However, more questions were raised after confirming its involvement in the ROS production where the experiments were carried out in a serum-free environment. The involvement of FcγRI in iIC-induced apoptosis was not studied in this thesis, since the expression of this receptor which was thought to only be expressed by activated neutrophils came as quite a surprise to us. Interestingly, while our paper was under revision, a report showed that iIC stimulation triggers FcγR1 expression by neutrophils.[106]

Finally, the effect of priming needs to be considered while extrapolating the iIC-data. It might be considered a potential limitation of the data presented here that unprimed neutrophils derived from healthy volunteers' blood were used throughout (with the exception of the experiments in RA disease model). This approach was taken because soluble and insoluble ICs are thought to promote different neutrophil functions. Given that iICs, but not sICs are able to stimulate unprimed neutrophils, absence of priming allowed us to differentiate between these responses. It is particularly meaningful in the context of iIC preparation, where residual sICs might remain despite washing steps taken to remove them. However, *in vivo*, a complex mixture of cytokines and chemokines might be expected in the circulation as well as in the inflammatory tissues in chronic inflammatory diseases representing a possible limitation of the data

presented here with unprimed neutrophils. It is not clear whether neutrophil priming might activate a separate iIC- driven intracellular signalling pathway. Additionally, differential priming conditions drive different neutrophil functions.[10] Thus, choosing a generic priming agent might not be similar to the ones causing specific disease pathology. To avoid this complication, I planned to screen the serum samples derived from RA patients to obtain one/more common agents that might be relevant for the pathogenesis in RA. I could use that particular cytokine to prime neutrophil to mimic more physiological condition. Unfortunately, however my analysis did not identify any positively or negatively associated cytokines that were specific to my patient pool.

Other immune complexes

Soluble immune complexes (sICs) have been extensively studied in neutrophils and shown to induce pro-inflammatory functions including delayed apoptosis. sICs have been reported to only activate primed neutrophils,[154] therefore any confusion regarding involvement of sICs in my results can be excluded (as discussed in the previous section). sICs and iICs activate neutrophils via different mechanisms, clearance of sICs is mainly dependent on FcγRIIIB whereas in this study, effects of iICs have been shown to be primarily FcγRII dependent. The ROS production by the sICs is extremely rapid and transient which contrasts with relatively slower release of ROS products by iICs.[120,154]

I have not explored the effects of immobilised immune complexes on neutrophil apoptosis. Working with adherent neutrophil represents a challenge since extensive crosstalk with integrin receptors and other adhesion receptors will add secondary complications.[118,120,330,331] However, analysing this phenomenon could add information towards understanding the disease pathogenesis which may be more

relevant in the inflammatory tissues such as synovial tissue in RA. It would be particularly interesting to analyse neutrophil functions in a scenario where different forms of immune complexes are present simultaneously, better mimicking the situation encountered in the arthritic joint, to identify which effector functions are overpowered, pro-inflammatory or anti-inflammatory.

In this thesis, rabbit polyclonal anti-HSA (IgG) was used throughout to make iICs because i) it is readily commercially available as a polyclonal purified antibody to HSA, ii) there is a single subclass of IgG in rabbit and, iii) rabbit IgG is able to activate human FcγRs,[332] contrasting with the situation in mouse which is a lot more complicated.[333] Nevertheless, other types of immune complexes (such as binding antigen with mouse IgG, goat IgG or even heat aggregated human IgG[334,335] as well as ICs generated from monoclonal antibodies could be tested to explore whether similar anti-inflammatory functions are observed. For meticulous observations, specific subclasses of human IgG could be tested.

Potential limitations to studies of iICs in human neutrophils

While it is desirable to perform research with primary human neutrophils, this line of research is hindered by the facts that (i) neutrophils are short-lived, and (ii) they are not amenable to genetic modifications. Several human leukaemia cell lines exist that can be chemically induced to become neutrophil-like. Widely used human myeloid cell lines such as HL-60 (established from an acute myeloid leukaemia[336]) and PLB-985 (human diploid myeloid leukemia cell line[337]) could also be studied. Nevertheless, each of these cell lines has its own limitation. These cell lines serve as primary model systems to investigate ROS production and chemotaxis, but, they do not fully recapitulate all neutrophil effector functions. Another model, HoxB8 cell lines, was

derived from murine myeloid progenitors that are conditionally immortalised with the homeobox oncoprotein HoxB8 driven by the estrogen receptor (ER-HoxB8). The advantages of this model include i) easily generated from mouse bone marrow stem cells and immortalised with ER-HoxB8, ii) they come from a non-cancerous background and follow the physiological neutrophil differentiation programme and iii) the cells can be efficiently transduced with lentiviral vectors to elucidate genetic information[338–340]. However, these cell lines do not replicate integrin and FcγR induced neutrophil functions,[341] making them unsuitable for the work described here. Much work in the neutrophil field has been elucidated with the help of genetically modified mice, and of course being murine in origin primary-like HoxB8 neutrophils[340] do behave much more akin to primary (mouse) neutrophils.

However, unfortunately, there are important differences between mouse and human neutrophils, including their FcγRs[102] and the signalling pathway employed to regulate iIC-induced neutrophil apoptosis.[99] I therefore relied on commercially available inhibitors to explore the involvement of different signalling pathways.

Due to these concerns, it could be asserted that the only reliable method to study neutrophil functions would be *in vivo* in animal models. However, in spite of widely used animal disease models, findings derived from animal experiments are extremely difficult to extrapolate in humans. For example, human neutrophils represent 40–70% of blood leucocytes, whereas mouse neutrophils represent much smaller fraction, perhaps partly because mice are kept under largely pathogen free conditions.[342]. Another layer of complexity is added by different levels and types of FcγR expression on mouse neutrophil. In mice, four different classes of Fcγ receptors comprising FcγRI, FcγRIIB, FcγRIII, and FcγRIV have been described that are different form the human FcRs laid out in **chapter 1**. Mouse FcγRIII is most similar to human FcγRIIA whereas

mouse FcγRIV is most closely related to human FcγRIIIA. FcγRIIIB is only found in humans, but both species have the inhibitory function of FcγRIIB in common.[343,344]

Regardless of the inherent limitations of any *in vivo* model, I undertook preliminary experiments with an arthritis disease model in mice, hoping that this might provide extra beneficial information regarding iIC-induced neutrophil effects.

In vivo RA model

Frequently, studies use rodent models to validate and investigate the efficacy of any treatment or to understand the pathophysiology of a disease. Chronic diseases have different stages, for example, as laid out in **chapter 1**, RA has distinct four stages (**Figure 1.13.A**); a) stage 1- susceptibility to RA, where the body mistakenly attacks its own joint tissues, b) stage 2- preclinical RA, where the body makes autoantibodies, c) stage 3- early RA, signs of inflammation are observed such as swelling around the joint, the joints are deformed and, bent joints press the nerves causing joint pain and, d) stage 4- established RA, progressive late stage disease when joints are fused and associated with chronic pain.[345]

Even without a global pandemic, and without consultant-led cases being moved to GP-led care, obtaining samples from each stage of RA patients would be very challenging, because the patients receive treatment as soon as the disease is diagnosed. In contrast, with an animal model, it is possible to have a perfect cohort in which disease progression can be studied and monitored longitudinally.

I therefore attempted establishing the reversible anti-collagen antibody induced arthritis model (CAIA) in mice,[346,347] induced by a combination of commercially available, monoclonal anti-collagen antibodies and LPS. I investigated iIC-triggered responses on different days, mimicking the different stages of arthritis. Because there

are only very few circulating neutrophils in the mouse, but there is a large reservoir of mature cells in the bone marrow,[348] neutrophils were isolated from the bone marrow of both control and arthritic mice for assessment of ROS production, internalisation/pinocytosis, and apoptosis. iICs did induce ROS production and neutrophil apoptosis similar to my observation with human neutrophils. After priming the mouse neutrophils I could also observe a comparable pinocytosis response to that observed with human neutrophils. However, in two preliminary experiments, no significant differences could be observed between control mice (which had been administered LPS) and arthritic mice (which had received the antibody cocktail as well as LPS) in terms of iIC-pinocytosis, iICs-induced ROS production, iIC-induced apoptosis or neutrophil counts. One possible explanation is that, the inflammatory response to the LPS may have exceeded the neutrophil response triggered by the antibody cocktail.

Although further experimentation would be required for a definitive study, I concluded that the CAIA model is not well suited to investigating iIC responses in bone-marrow derived mouse neutrophils. Other rodent arthritic models exist, including, spontaneous, antigen-induced, or antibody-induced and could be used even though every disease model comes with its own set of advantages and limitations.[349,350]

Relevance in auto-immune disease

Immune complexes deposit on the tissues forming immobilised immune complexes (on the joint surfaces in RA, on blood vessels in autoimmune vasculitis, on kidney in lupus nephritis). In SLE, clearance of iICs is impaired. Some diseases are presented with neutropenia such as in SLE and Felty's syndrome.[186,351]

In RA, the presence of neutrophils is deleterious and the neutrophil count is generally unaffected. The presence of neutrophils perpetuates the inflammatory process and enhances tissue destruction, leading to disability and a requirement for eventual surgical joint replacement. In line with this idea, neutrophils isolated from the synovial fluids of RA patients display delayed apoptosis. Preliminary studies exploring the use of apoptotic cells in the treatment of arthritis has found that mice injected with apoptotic cells prior to the onset of collagen-induced arthritis were protected from severe joint inflammation and bone destruction.[352] This protection was proposed to be the result of macrophage reprogramming towards an anti-inflammatory and pro-resolving state and subsequent modulation of other important cells including T and B lymphocytes, which also act to secrete pro-resolving factors, promote tolerance and inhibit chronic inflammation.[304,313,353,354]

In SLE, apoptosis and NETosis are dysregulated and overactive, providing an overload of self-antigens that under normal circumstances would not be available to be targeted by the immune system. In SLE patients, the clearance of apoptotic bodies is diminished, as is the capability to degrade NETs, related to the reduced DNase I activity.[187,355] The etiopathogenesis depends on autoantibody production (such as ANA and anti-dsDNA) and complement system activation triggered by the presence of immune complexes, leading to inflammation.[356–359] Studies revealed that binding of ICs to FcγRI contributed to the severity of arthritis and hypersensitivity responses in lupus nephritis.[360]

Neutrophils are believed to play a critical role in the pathogenesis of autoimmune vasculitis such as polyangiitis and granulomatous diseases. The autoantibodies found in these diseases, anti-neutrophil cytoplasmic antibodies[361] (ANCA), target two neutrophil granule proteins, proteinase 3 (PR3) and myeloperoxidase (MPO).

Autoimmune diseases may benefit from promoting the resolution of inflammation. According to the autoimmune diseases hypotheses, autoimmune diseases are induced as a result of a defect in apoptosis or removal of apoptotic cells. This leads to the immune system being exposed to fragments of the dying cell, which are able to induce a sustained humoral response to protein found on those fragments.[362–364] Additionally, apoptotic neutrophils expressing ANCA antigens on their surfaces can be opsonised by ANCA which enhances recognition and subsequent uptake by macrophage via FcR interaction. This leads to an increased synthesis of pro-inflammatory mediators such as IL-8 and IL-1 by macrophages, which results in the recruitment of more neutrophils and enhances the progression of inflammation.[365] Treatment with pro-apoptotic drugs has been proposed to remedy this.[366,367]

My findings provide further support for the development of drugs that are able to enhance neutrophil apoptosis, thereby promoting increased elimination of apoptotic neutrophils by macrophages while limiting the damage caused by prolonged activation of these cells. A counter argument lies in the increased susceptibility to infections that could result from the induction of significant neutrophil apoptosis.

Interaction with adaptive immunity

The pathophysiology of autoimmune diseases is closely connected to the adaptive immunity and involves the breakdown of T cell and B cell tolerance.

Interestingly, a recent study demonstrated that neutrophil endocytosis of ICs via FcγRs engagement rapidly converts them into fully immunogenic antigen presenting cells (nAPCs), and furthermore, the number of these nAPCs in SLE patients' blood correlates with disease outcomes[100]. Therefore, neutrophil FcγRs could provide a

direct link between innate and adaptive immunity and represent as a strategy to generate a large number of immunogenic nAPCs for T cell based immunotherapy.

Another study revealed a positive feedback loop whereby inefficient clearance of apoptotic blebs by macrophages results in positive selection of germinal centre B cells, which have self-reactivity against nuclear antigens exposed on these blebs.[188] These self-reactive B cells undergo T cell-dependent affinity maturation and isotype switching. They differentiate into long-lived, autoantibody producing plasma cells.[368] Importantly, the effector functions triggered by antibodies are markedly affected by Fc glycosylation,[369] which alone or in combination with Fc effector functions promoted research interest and progress in engineering therapeutic antibodies. ICs are also being explored in new approaches for prevention and therapy of diseases.[224,368] These observations may be useful in guiding the design of improved therapeutic avenues.

Final conclusion

My work suggests that ICs may in addition to their well-characterised properties in driving immune-pathological effects also potentiate beneficial effects. This is likely to depend on a range of factors, including the subclasses of the antibody, the ratio of antigen and antibody forming the IC, the biological characteristics of the IC components, the sites where the ICs were formed. While these factors remain to be addressed in more detail, the work presented in this thesis is suggestive of iIC-induced anti-inflammatory neutrophil functions which may act to dampen the degree of inflammation and promote the resolution of inflammation. Although further studies are required, my findings suggest that dysregulation of anti-inflammatory neutrophil functions would promote and extend autoimmunity.

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Appendix

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Immune complex-induced apoptosis and concurrent immune complex clearance are anti-inflammatory neutrophil functions

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Abstract

Persistent neutrophilic inflammation drives host damage in autoimmune diseases that are characterized by abundant immune complexes. Insoluble immune complexes (iICs) potently activate pro-inflammatory neutrophil effector functions. We and others have shown that iICs also promote resolution of inflammation via stimulation of neutrophil apoptosis. We demonstrate here that iICs trigger FcγRIIa-dependent neutrophil macropinocytosis, leading to the rapid uptake, and subsequent degradation of iICs. We provide evidence that concurrent iIC-induced neutrophil apoptosis is distinct from phagocytosis-induced cell death. First, uptake of iICs occurs by FcγRII-stimulated macropinocytosis, rather than phagocytosis. Second, production of reactive oxygen species, but not iIC-internalization is a pre-requisite for iIC-induced neutrophil apoptosis. Our findings identify a previously unknown mechanism by which neutrophils can remove pro-inflammatory iICs from the circulation. Together iIC clearance and iIC-induced neutrophil apoptosis may act to prevent the potential escalation of neutrophilic inflammation in response to iICs.

Introduction

Neutrophils, the most abundant circulating leukocytes are vital for host immunity, phagocytosing and killing bacteria and fungi[1,2]. The short-lived neutrophil originates in the bone marrow and is subsequently released into the circulation. Unless recruited to peripheral sites in response to specific stimuli, neutrophils circulate in the bloodstream for ~1 day before homing back to the bone marrow where they undergo apoptosis prior to clearance by resident macrophages[3,4]. Activating signals trigger neutrophil transition to a reversible, primed state which precedes full activation. Priming triggers heightened responsiveness to certain stimuli and physiological changes including delayed apoptosis[5]. Neutrophil apoptosis can be induced by diverse triggers; for example, having phagocytosed and killed microbes, neutrophils undergo phagocytosis-induced cell death [PICD[6–8]]. Apoptotic neutrophils generate 'find-me' and 'eat-me' signals that direct their efferocytosis by phagocytic macrophages and generates pro-resolution signals[4].

Neutrophils are key players of the inflammatory response, which is subject to tight control mechanisms. When these go awry, neutrophils are activated indiscriminately. Autoimmune diseases including rheumatoid arthritis are characterized by abundant autoantibodies that form immune complexes (ICs). IC-driven Fc receptor activation promotes neutrophil activation, contributing to host tissue damage[9–11]. Depending on antibody subclass and antigen–antibody ratio, soluble or insoluble immune complexes (iICs) form, which circulate in the bloodstream and are present in other bodily fluids, e.g., synovial fluid. Complement deposition on small soluble ICs enables efficient binding to erythrocyte complement receptor 1, which promotes efficient clearance of circulating soluble

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ICs by resident macrophages in the liver and spleen[12–15]. Larger ICs, which are cleared less efficiently, also circulate and are more likely to deposit in tissues and activate Fc receptor-expressing immune cells, e.g., neutrophils, enhancing inflammation and disease progression. Neutrophils constitutively express the low affinity receptors for IgG FcγRII (CD32) and FcγRIII (CD16); they also inducibly express the high affinity receptor FcγRI (CD64), which is expressed at very low levels on unprimed neutrophils[16]. Ligation of neutrophil FcγRs potently triggers pro-inflammatory functions, including the generation of reactive oxygen species (ROS), degranulation and cytokine production. Whereas soluble ICs stimulate only primed neutrophils, iICs also activate unprimed neutrophils[17]. We and others have previously shown that in addition to activating these pro-inflammatory neutrophil functions, iICs also promote neutrophil apoptosis[18,19].

We demonstrate here how iIC-induced neutrophil apoptosis coincides with neutrophil-mediated clearance of iICs. iIC-induced neutrophil apoptosis is distinct from PICD, and controlled by a different mechanism. Neutrophils do not phagocytose iICs, but rather internalize them by FcγRII-stimulated macropinocytosis. Our findings suggest that iIC-induced neutrophil apoptosis represents an efficient anti-inflammatory mechanism for clearance of iICs from the circulation.

Materials and methods

Reagents

Unless indicated otherwise, reagents of the lowest possible endotoxin level were from Sigma-Aldrich (Gillingham, UK). Tissue culture reagents were from Gibco (Thermo Fisher Scientific, Loughborough, UK) and percoll from GE Healthcare (Amersham, UK). For inhibitors and antibodies used see data supplement.

Isolation of human peripheral blood neutrophils

Neutrophils were prepared as described[18] to purity >95% according to Diff-Quik (Thermo Scientific) stained cytospins.

Insoluble immune complexes

iICs were prepared from human serum albumin (HSA) and rabbit polyclonal IgG to HSA as described[18,20]. Titration experiments determined iIC concentrations required to trigger substantial responses (not shown).

Analysis of neutrophil apoptosis

Neutrophil apoptosis and loss of plasma membrane integrity were analyzed by flow cytometry (FACSCalibur, BD Biosciences, Oxford, UK or Attune NxT, Thermo Fisher Scientific, Loughborough, UK) of FITC-annexin V (Roche, Welwyn Garden City, UK) and propidium iodide-stained neutrophils[18]. Activated caspase-3 was measured using an AF488-coupled antibody specific for cleaved human caspase-3 according to the manufacturer's instructions. Data were analyzed using FlowJo (TreeStar; version 6.4.7) or FCS Express 7 (De Novo).

Diisopropyl fluorophosphate (DFP) treatment of neutrophils

Neutrophils were incubated with 7 mM DFP for 5 min at room temperature in a fume hood, washed twice with PBS and used in experiments as detailed.

Western blotting

Neutrophils were lysed with ice-cold lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 0.1 mM PMSF, 10 µg/ml each of antipain, aprotinin, pepstatin A and leupeptin]. The detergent-soluble material was boiled in reducing sample buffer, subjected to SDS-PAGE and wet transferred to PVDF membrane (Millipore) for detection of proteins of interest using suitable antibodies. For IgG degradation assays, membranes were incubated with a HRP-coupled anti-rabbit secondary antibody. Blots were developed using chemoluminescence (Millipore).

Internalization assays

Neutrophils were resuspended in Dulbecco's PBS with $CaCl_2$ and $MgCl_2$, supplemented with 1 g/L glucose and 4 mM sodium bicarbonate (PBS^{++}), treated with inhibitor or vehicle as indicated and stimulated with iICs (sometimes fluorescently labelled) or opsonized latex beads at 37 °C. Negative controls were kept on ice. Cells were analyzed by flow cytometry (Attune Nxt; analysis by FCS Express 7), or allowed to adhere to (sometimes electrostatic) slides on ice prior to paraformaldehyde fixation and staining. For live imaging, freshly isolated neutrophils were incubated with Cell Mask Deep Red (Thermo Fisher Scientific) and allowed to attach to coverslips at 37 °C before stimulation with fluorescently labelled iICs.

Particles

Zymosan was opsonized with autologous serum for 30 min followed by PBS washes prior to use in experiments. Latex beads were opsonized with polyclonal rabbit IgG as per the manufacturer's instructions.

ROS production

ROS production was measured indirectly using chemiluminescence production by 5×10^5 neutrophils per well at 37 °C in luminescence-grade 96-well plates (Nunc, Thermo Fisher Scientific) in a Cytation plate reader (BioTek, Swindon, UK) as described[18,21], with neutrophils incubated with 150 µM luminol for the analysis of internal

ROS production, and with 150 μM luminol and 18.75 U/ml horseradish peroxidase for the analysis of total ROS. Data output was in light units/seconds.

Image processing

Image deconvolution of raw confocal images obtained with the Leica SP8 confocal was performed using Huygens Professional software (Scientific Volume Imaging). Confocal stacks were routinely obtained, suitable optical sections selected and pseudo colouring applied using ImageJ (NIH). Raw movie files were analyzed with Imaris (Bitplane) software without deconvolution.

Statistical analysis

Power calculations were not performed as part of this work. Statistical analysis was performed with Graph Pad Prism 8. Where data met the assumptions for parametric tests, one-way ANOVA or RM one-way ANOVA was performed with Dunnett's multiple comparison test; otherwise, Kruskal–Wallis test was performed. For kinetic experiments, the area under the curve was used for analysis. Individual values are plotted throughout to show the variance of each dataset. p values < 0.05 were deemed statistically significant. Comparisons shown relate to the activated control condition of each graph.

Results

As well as inducing neutrophil apoptosis, iICs are internalized by neutrophils in a PI3K-dependent fashion

We previously reported that iICs induce neutrophil apoptosis by engaging a non-canonical PI3K signalling cascade, altering the ratio of pro- and anti-apoptotic Bcl2 family proteins towards the pro-apoptotic Bax[18]. Specifically, iIC stimulation of neutrophils, and treatment with a positive control, the cyclin-dependent kinase inhibitor roscovitine[22,23] caused caspase-dependent plasma membrane phosphatidylserine exposure (Fig. 1A; Fig. S1A, B). Apoptotic neutrophils were characterized by chromatin condensation, resulting in loss of the characteristic multilobed neutrophil nuclear morphology (Fig. 1B; Fig. S1C). Stimulating neutrophils with iICs resulted moreover in activation of the executioner caspase-3 (Fig. 1C; Fig. S1D–F) and in gelsolin cleavage (Fig. 1D), further markers of apoptotic cell death[24,25]. The induction of apoptosis by iICs occurred over a timeframe of 6–12 h (Fig. 1E; ref. [18]) with cells beginning to lose plasma membrane integrity at later times (Fig. S1A).

When analyzing neutrophil apoptosis using flow cytometry, we observed that iIC stimulation caused a reproducible shift in the forward scatter properties of neutrophils (Fig. 2A). Increased forward scatter was not observed with iIC-stimulated neutrophils in which PI3K signalling had been inhibited by LY294002, while inhibiting other components of the pathway (Fig. 2A and not shown) or a pan-caspase inhibitor did not have this effect. Since an altered forward scatter is indicative of changes in neutrophil morphology[26], we hypothesized that neutrophils internalize iICs in a fashion that is dependent on PI3K but not Pak, Mek, Erk or caspases.

To test this possibility, we pre-incubated neutrophils with inhibitors specific for PI3K, Pak, Mek or Erk or a vehicle control prior to stimulation with fluorescently labelled iICs, and analyzed iICs internalization by flow cytometry (Fig. 2B; Fig. S2A, C, D). These experiments showed that iICs were internalized in a PI3K-, but not a Pak-, Mek- or Erk-dependent fashion. Inhibition of PI3K inhibited iIC internalization, but not iIC binding to neutrophils (Fig. 2C; Fig. S2C, D), with the concentration of the stable pan-PI3K inhibitor LY294002 used in the apoptosis assay causing significant, albeit sub-maximal inhibition of iIC internalization (Fig. S2B, C). We concluded that iIC internalization by neutrophils and iIC-dependent induction of neutrophil apoptosis are regulated by distinct pathways downstream of PI3K.

iIC-induced neutrophil apoptosis is distinct to phagocytosis-induced cell death

Internalization of serum and/or IgG-opsonized particles induces PICD[6,8,27]. We compared induction of neutrophil apoptosis by iICs with a potent activator of PICD, opsonized zymosan. In contrast to iIC-induced apoptosis and consistent with previous reports[18,28], PICD was PI3K- and Erk-independent (Fig. 3A, B). In keeping with previous observations[29,30], the NADPH oxidase inhibitor diphenyleneiodonium (DPI) blocked both iIC-induced apoptosis and PICD (Fig. 3C, D). In contrast, inhibiting actin polymerization with cytochalasin B or latrunculin B inhibited PICD, whereas iIC-induced neutrophil apoptosis was not significantly reduced (Fig. 3C, D).

Distinct internalization characteristics of iICs and IgG-opsonized beads

For further mechanistic insight into iIC-induced apoptosis, we compared the uptake of solid particles and iICs in more depth. To restrict neutrophil receptor involvement to a single receptor class, FcγR, we analyzed particle internalization with latex beads (0.3–3 μm diameter) that had been opsonized with polyclonal rabbit IgG in the absence of autologous serum (hereafter simply referred to as 'beads'). Disrupting actin polymerization abrogated internalization of both iICs and beads. However, inhibiting PI3K blocked iIC internalization, but not uptake of 0.3 and 0.8 μm diameter beads (Fig. 4A–D; Fig. S3A, B), consistent with a requirement for PI3K in phagocytosis depending on particle size in other cells[31].

These internalization experiments were performed 30 min after stimulation, while the induction of apoptosis occurs later (Fig. 1). We performed time courses,

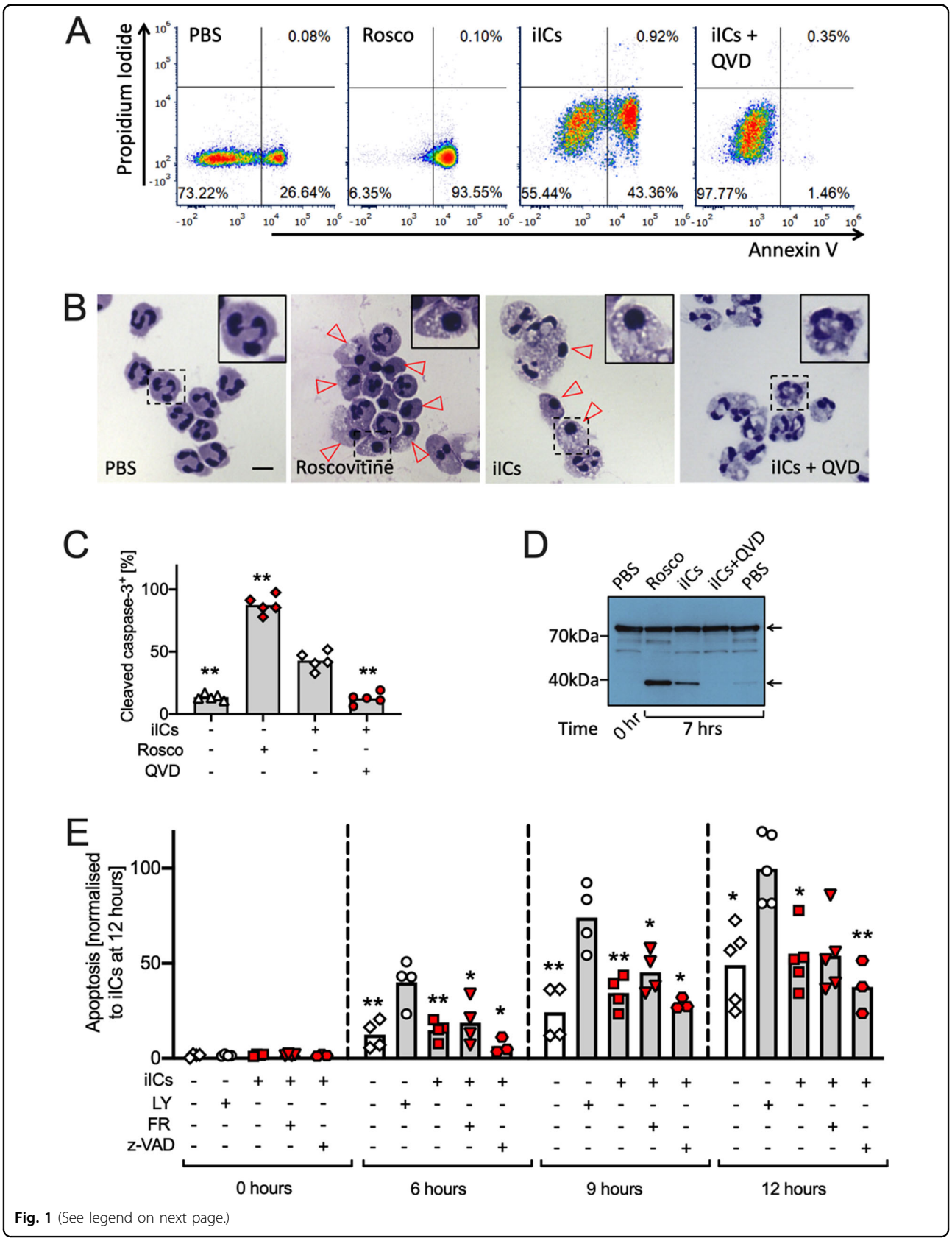

Fig. 1 (See legend on next page.)

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Fig. 1 iIC stimulation induces neutrophil apoptosis. Neutrophils were pre-incubated with inhibitors or vehicle as indicated (Rosco, roscovitine, CDK inhibitor; QVD, Q-VD-OPh hydrate and z-VAD, z-VAD-FMK, pan-caspase inhibitors; LY, LY294002, pan-PI3K inhibitor; FR, FR180204, Erk inhibitor) at 37 °C for 10 min, and stimulated with 10 µg/ml iICs (HSA anti-HSA) or vehicle in IMDM supplemented with 10% autologous serum and cultured at 37 °C. After 6 h **A** cells were stained with annexin V and propidium iodide and analyzed by flow cytometry (Fig. S1A for gating). **B** Cytospins were prepared and cytoplasm and nuclei stained. Brightfield images were taken (×40 magnification; Evos imaging system). Arrowheads identify some apoptotic cells with characteristic condensed nuclei. Boxed cells are shown enlarged in inset panels. Scale bar, 10 µm. **C** Cleaved caspase-3 was detected by flow cytometry in neutrophils that had been treated as indicated and cultured for 7 h (Fig. S1D, E for gating and additional controls). **D** Cell lysates were prepared and processed for Western blotting to detect full-length gelsolin and its cleavage product (arrows). **A**, **B**, **D** Representative examples from ≥ 3 separately performed experiments are presented. **E** Graphical representation of neutrophil apoptosis (as identified in **A**) under the indicated conditions and times. **C**, **E** Each symbol represents the value obtained in a separate experiment. Raw data were subjected to analysis by one-way ANOVA and multi comparisons post-hoc test, comparing all conditions to iIC-stimulated neutrophils. $*p < 0.05$, $**p < 0.01$.

analyzing iIC internalization also after longer incubation times. Even 6 h after stimulation, when iIC-induced apoptosis was already obvious, iIC internalization by latrunculin B pre-treated neutrophils remained negligible (Fig. S3C). We concluded that iIC-induced neutrophil apoptosis, unlike PICD, can occur independently of iIC internalization.

Neutrophils efficiently phagocytosed 0.8 µm diameter beads, which once phagocytosed appeared closest in size to the vacuoles that contained internalized iICs (Fig. S3D). Further experiments were therefore carried out with these beads. Neutrophils stimulated simultaneously with iICs and beads efficiently internalized both. Inhibiting PI3K prior to co-stimulation with beads and iICs interfered with iIC internalization, but not bead phagocytosis (Fig. 4E), indicating that neutrophils use distinct mechanisms to internalize beads and iICs.

iIC-induced neutrophil apoptosis and iIC internalization depend on FcγRII

We used blocking antibodies to decipher the regulation of neutrophil apoptosis by iICs, identifying a major role FcγRII, without significant contribution of FcγRIII (Fig. 5A). Internalization experiments identified that blocking FcγRII inhibited internalization of both beads and iICs (Fig. 5B, C; Fig. S4A, B), contrasting with the differential involvement of PI3K.

iICs signal through distinct cell surface receptors to trigger internal and external ROS production

In keeping with the observation that both iIC-induced apoptosis and PICD are ROS-dependent [Fig. 3C, D; (ref. [28–30])], inhibiting the NADPH oxidase or myeloperoxidase interfered with iIC-mediated induction of apoptosis. Incubating neutrophils with catalase, a scavenger of hydrogen peroxide that is not cell permeable also blocked the induction of iIC-induced apoptosis (Fig. 6A), suggesting a function of external ROS in this context. This contrasts with the predominant role of internal ROS reported in PICD[28].

We further analyzed ROS produced in response to neutrophil stimulation by iICs or beads. Internal and total iIC and bead-induced ROS production was PI3K-dependent (Fig. S5A–C). Inhibiting bead phagocytosis by blocking actin polymerization abrogated bead-induced ROS (Fig. 6B). In contrast, inhibiting iIC internalization reduced internal ROS but resulted in increased external ROS (Fig. 6C). Indeed, when comparing production of internal and total ROS by neutrophils that had been stimulated with iICs or beads, we noticed an early peak of external ROS with iIC- but not bead-stimulated neutrophils (Fig. 6D, E for representative examples).

We used blocking antibodies to decipher the FcγR dependency of the internal and total ROS response to neutrophil stimulation with iICs. Unexpectedly, this identified that the initial peak of external ROS was entirely dependent upon FcγRII, whereas internal ROS production was mediated by FcγRI, Mac-1 and FcγRII (Fig. 6F–I; Fig. S5D, E). This suggested a dual role of FcγRII in mediating iIC internalization and generating external iIC-induced ROS.

Neutrophils internalize iICs by receptor-dependent macropinocytosis

Our results suggested that iICs were internalized by a process other than phagocytosis. We used time-lapse imaging of neutrophils in which the plasma membrane had been dyed with cell mask to test whether iICs entered an intracellular compartment that was derived from the plasma membrane. We noted rapid uptake of fluorescently labelled iICs at several distinct locations within an area of bright cell mask suggestive of localized plasma membrane accumulation (Fig. 7A, asterisks). The distinct foci of internalized iICs subsequently merged into a vacuole, which was initially characterized by bright iIC-associated fluorescence, fading rapidly, leaving behind the circular, cell mask-positive vacuole (Fig. 7A and Supplemental Movie 1).

The prominent plasma membrane structures preceding iIC internalization (Fig. 7A) were suggestive of membrane ruffling. We therefore asked whether the internalization process was due to macropinocytosis, an endocytic process that is initiated by the formation of PI3K-dependent, circular membrane ruffles that initiate fluid internalization[32–34].

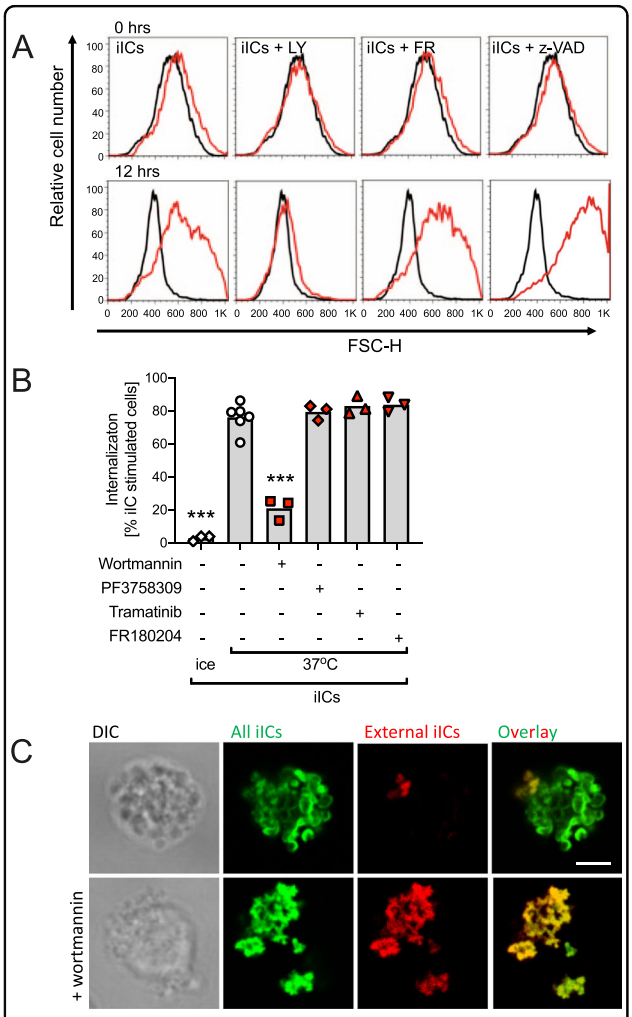

Fig. 2 Neutrophils internalize iICs in a PI3K-dependent fashion. Neutrophils were pre-incubated with small molecule inhibitors or vehicle (LY, LY274002, wortmannin, pan-PI3K inhibitors; FR, FR180204; z-VAD, z-VAD-FMK; PF3758309, Pak inhibitor; Tramatinib, Mek inhibitor) at 37 °C for 10 min and stimulated with 10 µg/ml iICs (HSA anti-HSA) in IMDM supplemented with 10% autologous serum and cultured at 37 °C. **A** Singlets were gated and analyzed by flow cytometry at the indicated times. Representative forward scatter histograms are plotted. For ease of viewing, plots of vehicle-treated cells obtained at the indicated times (black) are shown in all iIC-stimulated conditions (red). A representative example is shown of ≥3 independent experiments performed. (**B** + **C**) Following vehicle or inhibitor treatment, neutrophils were stimulated for 30 min with (**B**) AF488-labelled iICs on ice or at 37 °C in suspension. Neutrophils were then placed on ice and attached iICs labelled with an AF-647-conjugated secondary antibody, and cells analyzed by flow cytometry. Internalized iICs are plotted. Each symbol represents the average value obtained in a separate experiment. Raw data were subjected to analysis by one-way ANOVA with multi comparisons post-hoc test. ***$p < 0.001$; **C** neutrophils were stimulated with unlabelled iICs at 37 °C in suspension, allowed to adhere to glass slides on ice, and fixed. External iICs were labelled using an AF568-conjugated secondary antibody. Then, after permeabilization, all iICs were labelled with an AF488-conjugated secondary antibody. Representative optical sections obtained using a Zeiss LSM780 confocal microscope with 63× objective are shown. Scale bar, 2 µm.

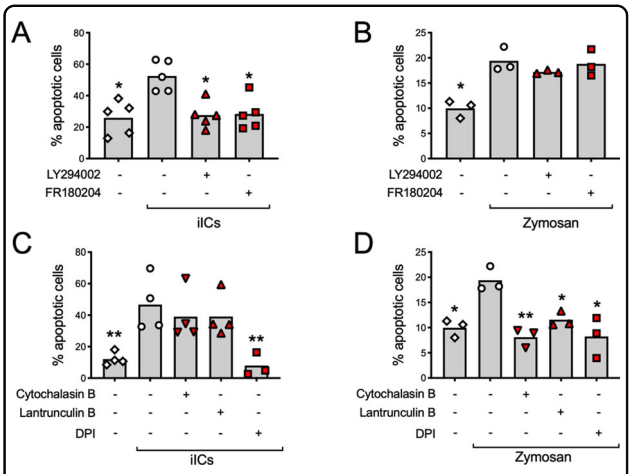

Fig. 3 iIC-induced neutrophil apoptosis is not PICD. Neutrophils were pre-incubated with small molecule inhibitors or vehicle at 37 °C for 10 min as indicated, stimulated with (**A**, **C**) 10 µg/ml iICs (HSA anti-HSA) or (**B**, **D**) 3 zymosan particles/neutrophil in IMDM supplemented with 10% autologous serum and cultured at 37 °C for 12 h in a humidified, CO_2-controlled incubator prior to labelling with annexin V and propidium iodide. The percentage of apoptotic cells is recorded. Each symbol represents the average value obtained in a separate experiment. Raw data were analyzed by Kruskal–Wallis test and multiple comparison post-hoc test. *$p < 0.05$; **$p < 0.01$.

Internalization experiments performed in media containing lucifer yellow (Fig. 7B) or FITC-dextran (Fig. S6) identified iIC-lined macropinosomes filled with fluid phase markers; internalization of the fluid phase markers was PI3K-dependent. In contrast, fluid uptake as defined by lucifer yellow signal was minimal in neutrophils phagocytosing beads (Fig. 7B, arrowheads). Neutrophil macropinocytosis did not occur constitutively, but was dependent upon the presence of iICs. Neither iIC constituents, HSA, anti-HSA, nor FcγRII/III blocking antibodies alone were able to induce macropinocytosis (Fig. 7C). Macropinocytosis was inhibited by pre-treating neutrophils with the pinocytosis inhibitor EIPA, but not with an autophagy inhibitor (chloroquine) or DPI (Fig. 7D).

Degradation of iICs

To examine the kinetics of iIC internalization, we performed time courses, labelling internalized iICs or beads after fixation. Internalization was observed as early as 5 min after stimulation, with peak uptake recorded at 30 min (Fig. 8A). At later timepoints we noted reduced fluorescence, suggesting that the internalized iICs were being degraded. Western blotting confirmed proteolysis of the IgG heavy chain associated with both iICs and beads (Fig. 8B). On overexposed blots IgG degradation products were clearly visible (Fig. 8C, arrows). We noted a degradation band ~34 kDa, and a doublet ~17 kDa with cells that had been stimulated with iICs at 37 °C. Blocking iIC internalization by inhibiting PI3K or actin

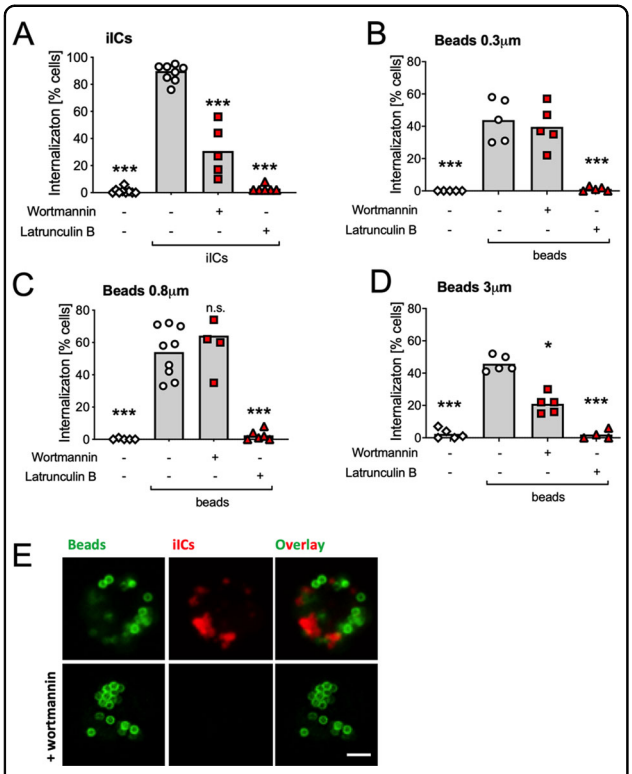

Fig. 4 Internalization of iICs and beads is controlled by distinct pathways. Neutrophils were incubated with small molecule inhibitors or vehicle at 37 °C for 10 min as indicated to inhibit PI3Ks and actin polymerization, respectively, and stimulated by mixing with **A** 2 μg/ml iICs, **B–D** 5 IgG-opsonized beads/neutrophil of the indicated size or **E** a mixture of the iIC and 0.8 μm beads for a further 30 min. Neutrophils were then allowed to adhere to electrostatically charged glass slides on ice and fixed. Internalized and external iICs were labelled as detailed in 'Materials and methods'. **A–D** The percentage of cells that had internalized particles was counted and plotted. Each symbol represents the average value obtained in a separate experiment. Raw data were analyzed by one-way ANOVA and multiple comparison post-hoc test; ***$p < 0.001$; *$p < 0.05$. **E** Representative confocal images taken with a Leica SP8 with 63× oil immersion objective of neutrophils that had or had not been pre-incubated with wortmannin prior to being simultaneously stimulated with pre-labelled beads (green) and iICs (red). Scale bar, 2 μm.

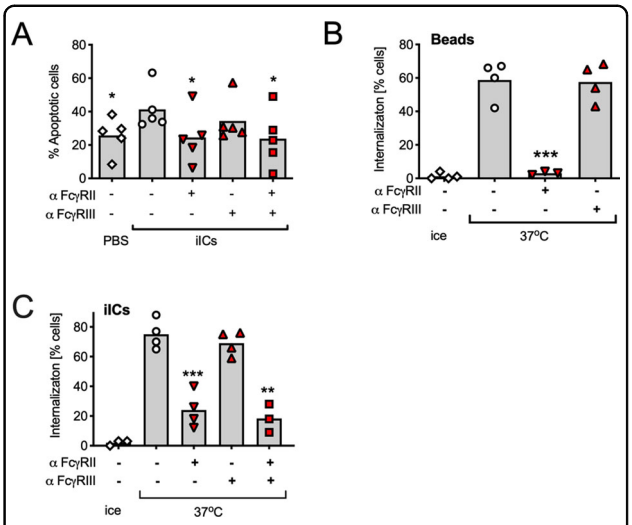

Fig. 5 FcγR dependency of iIC-induced neutrophil apoptosis and iIC internalization. A Following incubation on ice with FcγR blocking antibodies (3G8, IV.3) or vehicle as indicated neutrophils were stimulated with 10 μg/ml iICs for analysis of neutrophil apoptosis after 12 h. Internalization of beads (**B**) and iICs (**C**) by neutrophils that had been incubated with blocking antibodies (3G8, AT10) or vehicle as indicated. Each symbol represents the average value obtained in a separate experiment. Data were subjected to analysis by one-way ANOVA with multiple comparisons post-hoc test; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

polymerization caused the appearance of a prominent ~34 kDa band, suggestive of degradation even of non-internalized iICs by a surface protease. With neutrophils treated with the NADPH oxidase inhibitor DPI or the pan-caspase inhibitor z-VAD-FMK, differential banding patterns and smaller degradation products were observed. iIC degradation was inhibited in cells that had been incubated with DFP, a potent inhibitor of serine proteases, but not by E64, an inhibitor of cysteine proteases (Fig. S7A); DFP treatment, but not E64 also inhibited the induction of apoptosis (Fig. S7B). Together our observations suggested that iIC degradation occurred in a stepwise fashion involving several proteases.

Neutrophils are characterized by specialized granules that contain cytotoxic components and proteolytic enzymes. In phagocytosis, granules fuse with the phagosome, emptying their cargo into it for intracellular killing. We stained neutrophils that had or had not been allowed to internalize beads or iICs with CD63, a marker of azurophil granules[35]. CD63 localized to distinct speckles in unstimulated neutrophils, which reorganized in cells that had ingested beads such that CD63-positive spots surrounded beads. In contrast, CD63 signal did not neatly surround the vacuoles containing iICs (Fig. 8D), but instead showed some elongated structures which overlapped with iIC-containing vacuoles.

Altogether, our results demonstrate two separate FcγR-dependent pathways in iIC-stimulated neutrophils, (i) the induction of apoptosis in a PI3K- and ROS-dependent, but actin-independent fashion, and (ii) the actin- and PI3K-dependent internalization by macropinocytosis and subsequent digestion of iICs (Fig. 8E).

Discussion

Prompted by the observation that iIC-induced neutrophil apoptosis occurs concomitantly with iIC internalization, we compared iIC-induced neutrophil apoptosis and PICD. Our findings suggest that these 2 specialized types of apoptosis are regulated by different signalling

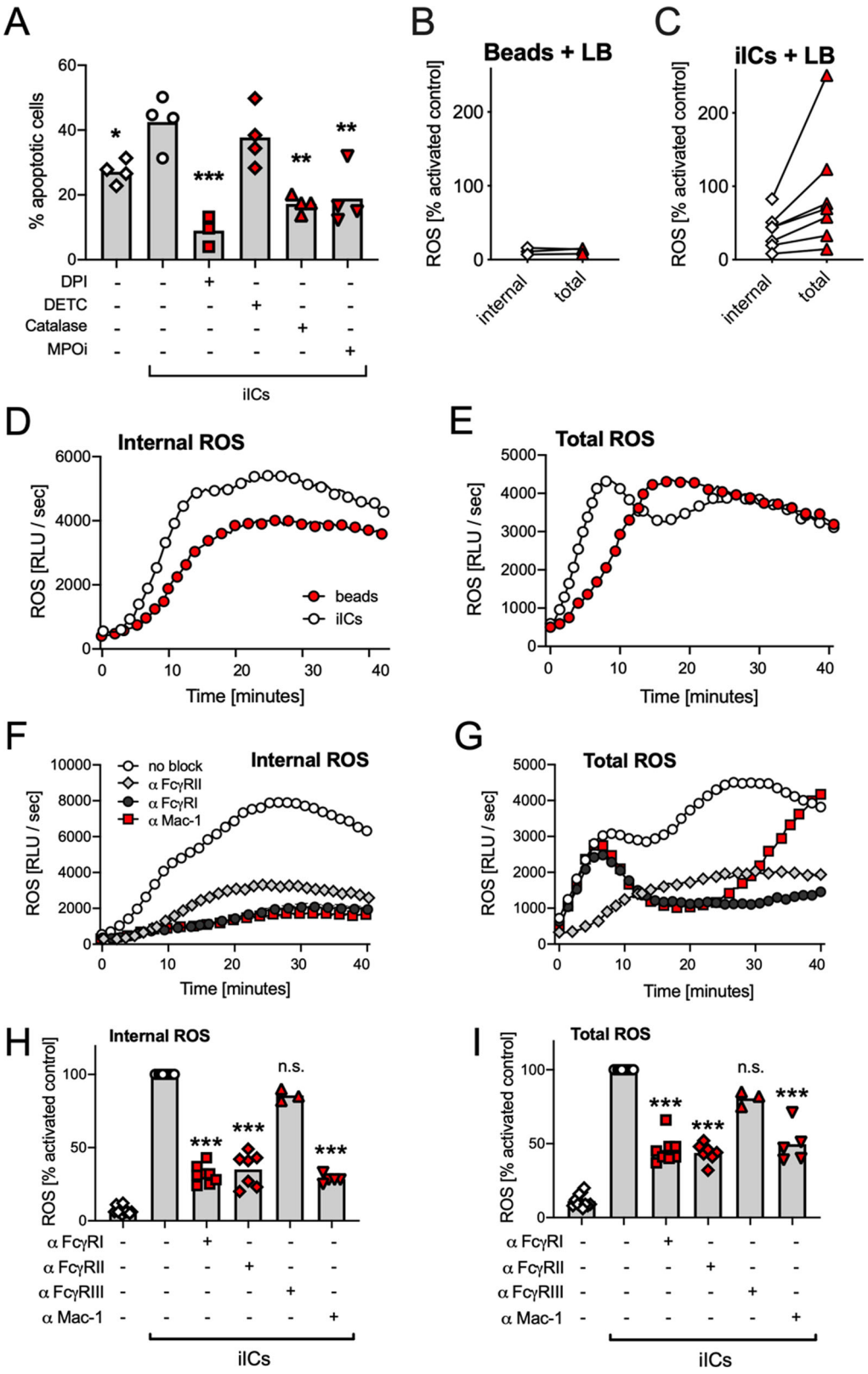

Fig. 6 (See legend on next page.)

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Fig. 6 iIC-stimulated neutrophils produce FcμRII-dependent external ROS. A Following incubation with inhibitors, neutrophils were or were not stimulated with 10 μg/ml iICs for analysis of neutrophil apoptosis after 9 h. **B–I** Internal and total ROS production was analyzed in neutrophils that had been stimulated with 4 μg/ml iICs (**B**, **D-I**) or 25 beads/neutrophil (**C**) as detailed in 'Materials and methods'. **B**, **C** Neutrophils were pre-treated for 10 min with inhibitor or vehicle as indicated prior to performing ROS assays. Internal (**D**, **F**, **H**) and total (**E**, **G**, **I**) ROS were analyzed with cells that had been pre-incubated for 30 min on ice with blocking antibodies (10.1, AT10, 3G8, ICRF44) or vehicle as indicated prior to stimulation with iICs. **D–G** Representative examples are shown; **H**, **I** Integrated total ROS production. **A-C**, **H, I** Each symbol represents the average value obtained in a separate experiment. **A**, **H**, **I** Raw data were analyzed by one-way ANOVA with multiple comparisons post-hoc test; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; n.s. not significant.

cascades, with iIC-induced neutrophil apoptosis, but not PICD promoted by PI3K and Erk [Fig. 3 (ref. [18,28])].

A second difference between the two cell death processes is that PICD, but not iIC-induced neutrophil apoptosis depends upon particle internalization. We found that iICs and IgG-opsonized beads are internalized by two different mechanisms which can take place simultaneously within the same cell (Fig. 4E). iIC internalization occurs by class I PI3K-dependent macropinocytosis rather than phagocytosis (Fig. 7B), with iIC internalization sensitive to pan-PI3K inhibition (Fig. S2C) at inhibitor concentrations previously described for macrophage pinocytosis[32]. Macropinocytosis is a comparatively poorly defined process that is initiated by circular ruffles, which close around extracellular fluid, engulfing it. Unlike constitutive macropinocytosis, which professional antigen presenting cells (e.g. dendritic cells, macrophages) employ to sample extracellular antigen for presentation of peptides[36–39], our data demonstrate that neutrophil macropinocytosis of iICs is FcγR mediated (Fig. 5C). FcγR stimulation during phagocytosis was previously shown to induce concurrent receptor-mediated focal pinocytosis in the neutrophil[40] in a process that correlated with the secretion of primary granules. We also noticed a small amount of fluid phase marker uptake by neutrophils that were phagocytosing beads (arrowheads in Fig. 7B). Unlike the localized pinocytosis accompanying phagocytosis[40], iIC-induced macropinocytosis was dependent upon the intact actin cytoskeleton.

Both iIC-induced neutrophil apoptosis and PICD share their dependency on ROS production (Fig. 6A). A closer examination identified that ROS induced by beads and iICs exhibited key differences. We observed an early, FcγRII-mediated peak of external ROS with iIC-stimulated neutrophils, and while inhibiting bead internalization abolished PICD-associated ROS production, inhibition of iIC internalization induced a switch from internal to external ROS. Interestingly, incubating neutrophils with an FcγRII blocking antibody inhibited iIC internalization and the induction of apoptosis (Fig. 5A, C), whereas blocking iIC internalization by inhibiting actin polymerization was not sufficient to interfere with the induction of apoptosis (Fig. 3C). The observation that the cell-impermeable ROS scavenger catalase inhibits iIC-induced apoptosis (Fig. 6A) suggests that the initial wave of external ROS that occurs upon stimulation of neutrophils with iICs (Fig. 6D–G) may be sufficient to induce apoptosis. Similarly, the increased external ROS generated upon iIC stimulation of neutrophils in which actin polymerization has been inhibited (Fig. 6C) are likely to promote the induction of neutrophil apoptosis even in the absence of iIC internalization.

A second unexpected feature of iIC-induced ROS production was the cell surface receptor involvement. FcγRII ligation was found to be responsible for the initial wave of external ROS, while the subsequently produced internal ROS were largely due to FcγRI and Mac-1 (Fig. 6F, G). This suggests that low levels of FcγRI on circulating human neutrophils are sufficient to make a marked contribution to the generation of ROS released into macropinosomes of neutrophils stimulated with iICs. Mac-1 is an integrin that has been shown to bind to multiple ligands, including ICAM1, fibrinogen and complement components[41–43]. In our experiments iICs were prepared from lyophilized HSA and affinity purified polyclonal rabbit anti-HSA IgG. Internalization occurred in the absence of serum, suggesting that involvement of complement in this context is unlikely. However, extensive cross-talk between β2 integrins and FcγRs has previously been documented by many groups. With immobilized ICs, for example, FcγRs were found to be sufficient for initial interaction of neutrophils with immobilized ICs, but Mac-1 activity was required for sustained binding[44,45].

Both internalization mechanisms we have examined here, FcγR-mediated phagocytosis and macropinocytosis, lead to the rapid degradation of internalized IgG (Fig. 8; Fig. S6). Our results suggest that iICs are degraded in a stepwise fashion by several proteases and that some extracellular digestion of IgG occurs prior to internalization. This might be mediated by proteinase-3, a plasma membrane-localized serine protease that colocalises with FcγRIII[46,47], while intracellular serine proteases, e.g., those stored in azurophil granules may be responsible for intracellular iIC degradation. While we were unable to identify individual proteases involved, the powerful serine protease inhibitor DFP interfered with iIC degradation as

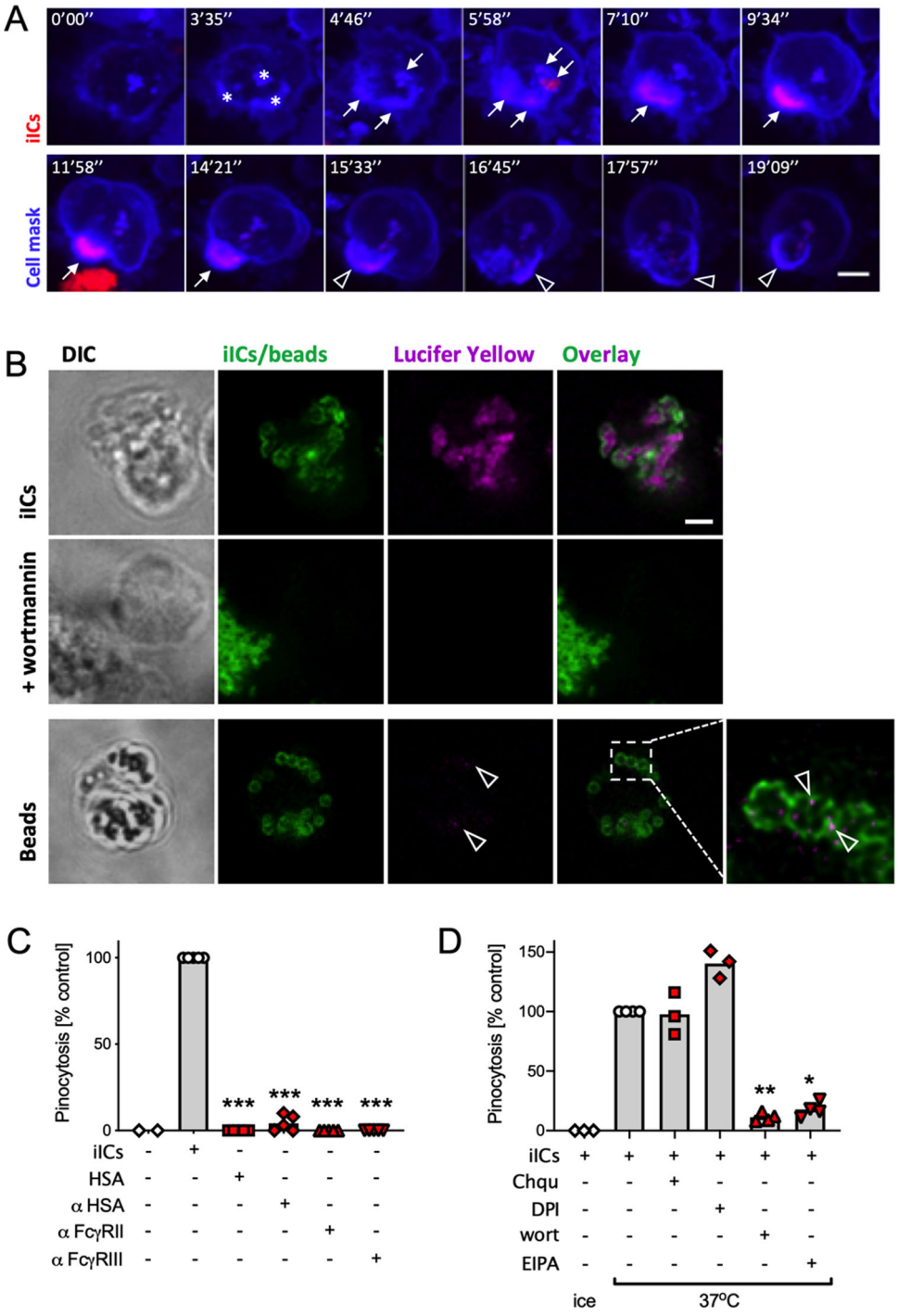

Fig. 7 (See legend on next page.)

well as neutrophil apoptosis (Fig. S6), consistent with previous reports which showed that DFP inhibits constitutive neutrophil apoptosis[48,49].

In the context of iIC-induced neutrophilic inflammation, our observation of iIC degradation following their internalization by neutrophils suggests that neutrophils make an important contribution to the clearance of iICs. Given that several studies have described ICs localized to vacuoles in human patient neutrophils [e.g. (ref. [50–53])], we propose that the mechanism elucidated here is of

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Fig. 7 Neutrophils internalize iICs by macropinocytosis. A Neutrophils were incubated with a Cell Mask plasma membrane dye, allowed to adhere to glass coverslips and imaged using an Andor Revolution XDi spinning disc confocal microscope with 60× objective, acquiring roughly 2 images per minute. At time 0 cells were stimulated with fluorescently labelled iICs. Stills depict a single cell taken at the indicated timepoint are shown (see also Supplemental Movie 1). * denotes areas of abundant plasma membrane stain (likely ruffling) following stimulation; arrows identify internalized iICs. Arrowheads, loss of internalized iIC-associated fluorescence in the plasma membrane-derived vacuole. **B** Neutrophils were stimulated with 2 μg/ml iICs or 5 beads/neutrophil in the presence of the fluid phase marker lucifer yellow. Representative confocal images of neutrophils that had ingested iICs (top and middle) or beads (bottom) and in which PI3K had (middle) or had not (top, bottom) been inhibited. Arrowheads, lucifer yellow internalization that accompanied phagocytosis. **A**, **B** Scale bars, 2 μm. Pinocytosis was assessed by uptake of lucifer yellow with neutrophils (**C**) that had been stimulated with vehicle, iICs, components thereof (HSA and anti-HSA) or FcγR blocking antibodies (AT10, 3G8) or (**D**) that had been pre-incubated with small molecule inhibitors as indicated (Chqu chloroquine, DPI diphenyleneiodonium, wort wortmannin, EIPA ethylisopropylamiloride) prior to stimulation with iICs. **C**, **D** Cells that had undergone macropinocytosis (i.e., taken up lucifer yellow) were counted. Each symbol represents the average value obtained in a separate experiment. Raw data were subjected to analysis by one-way ANOVA with multi comparison post-hoc test; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

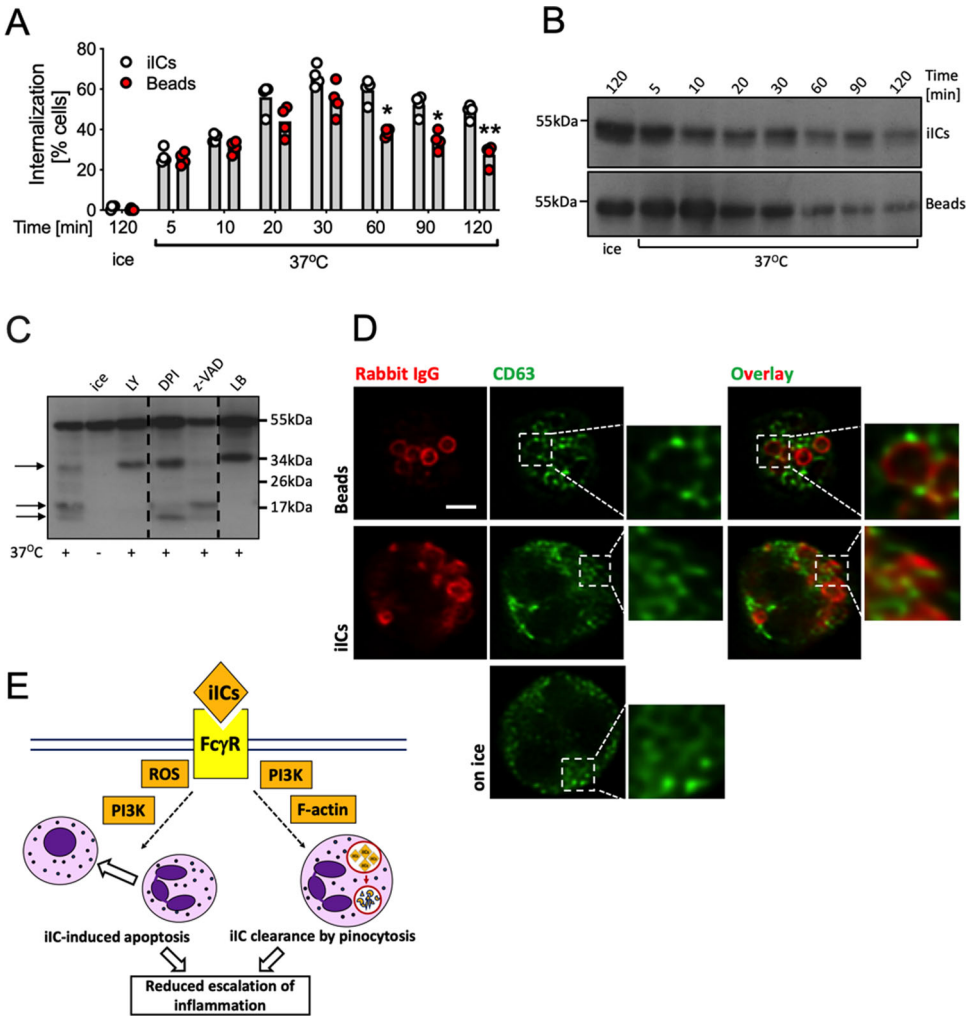

Fig. 8 Internalized iICs are subject to rapid degradation. A Suspension neutrophils at 37 °C or on ice were stimulated with 2 μg/ml iICs or 5 IgG-opsonized beads/neutrophil for the indicated times, allowed to attach to glass slides on ice, fixed, and labelled for external and total particles. Cells containing internalized particles are plotted. Each symbol represents the average value obtained in a separate experiment. Data were analyzed by two-way ANOVA with multiple comparisons post-hoc test; *$p < 0.05$; **$p < 0.01$. Neutrophils were stimulated with 2 μg/ml iICs for the indicated time at 37 °C or on ice (**B**), or after being pre-incubated with inhibitors or vehicle as indicated to inhibit PI3K, ROS production, caspases and actin polymerization (**C**; LY, LY294002; DPI diphenyleneiodonium, z-VAD z-VAD-FMK, LB latrunculin B, respectively). Cell lysates were subjected to SDS-PAGE, transferred to PVDF membrane and probed for the rabbit IgG heavy chain. Representative blots are shown from ≥3 separately performed experiments. **D** Neutrophils were or were not stimulated with iICs or IgG-opsonized beads as in (**A**), and allowed to adhere to glass slides prior to labelling CD63 and rabbit IgG. Samples were viewed on a Leica SP8 confocal microscope with 63× objective. Representative examples are shown. Scale bar, 2 μm. **E** Schematic diagram illustrating the two concomitant iIC-induced neutrophil anti-inflammatory pathways, iIC macropinocytosis and iIC-induced neutrophil apoptosis.

pathophysiological relevance. Circulating iICs are highly prone to being deposited on host tissue surfaces where they potently stimulate neutrophilic inflammation resulting in host tissue injury as happens in autoimmune diseases, including rheumatoid arthritis. The fact that neutrophils are involved in clearance of these large ICs suggests hitherto unappreciated anti-inflammatory function aimed at preventing host tissue injury. Intriguingly, iICs are not only degraded by neutrophils, but independently and concurrently also induce neutrophil apoptosis[18,30]. It is well-documented that the induction of neutrophil apoptosis coincides with the release of 'find-me' and 'eat-me' signals[54] and ultimately efferocytosis by tissue macrophages, dampening inflammation and promoting resolution and the release of pro-resolving mediators[4,55]. These iIC-induced neutrophil anti-inflammatory functions essentially operate as a safeguard, promoting iIC clearance, as well as neutrophil clearance by apoptosis. In this way neutrophils contribute to attenuating the escalation of iIC-induced inflammation.

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Author contributions
Experimentation—U.K., J.Y.C., K.S., H.G.; data analysis—U.K., J.Y.C., K.S., H.G., A.L. A., S.V.; reagents and advice—C.G.H., I.D.; funding acquisition—S.V., I.D.; wrote the draft—U.K., S.V., I.D.; all authors edited and concur with the paper.

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Data availability
All data generate or analyzed during this study are included in this published article and its supplementary information files.

Conflict of interest
The authors declare no competing interests.

Ethics statement
Ethics approval was obtained from the local Lothian Research Ethics Committee (AMREC 15-HV-013 and CIR 20-HV-069) and all blood donors gave informed consent.

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REVIEW

NEUTROPHIL INFLUENCE ON ADAPTIVE IMMUNITY
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Crosstalk between B cells and neutrophils in rheumatoid arthritis

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Abstract

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease without known cure that primarily affects synovial joints. RA has a prevalence of approximately 1% of the population worldwide. A vicious circle between two critical immune cell types, B cells and neutrophils, develops and promotes disease. Pathogenic anti-citrullinated protein antibodies (ACPA) directed against a range of citrullinated epitopes are abundant in both plasma and synovial fluid of RA patients. In addition to stimulating numerous cell types, ACPA and other autoantibodies, notably rheumatoid factor, form immune complexes (ICs) that potently activate neutrophils. Attracted to the synovium by abundant chemokines, neutrophils are locally stimulated by ICs. They generate cytokines and release cytotoxic compounds including neutrophil extracellular traps (NETs), strands of decondensed chromatin decorated with citrullinated histones and granule-derived neutrophil proteins, which are particularly abundant in the synovial fluid. In this way, neutrophils generate citrullinated epitopes and release peptidylarginine deiminase (PAD) enzymes capable of citrullinating extracellular proteins in the rheumatic joint, contributing to renewed ACPA generation. This review article focusses on the central function of citrullination, a post-translational modification of arginine residues in RA. The discussion includes ACPA and related autoantibodies, somatic hypermutation-mediated escape from negative selection by autoreactive B cells, promotion of the dominance of

Abbreviations: ACPA, anti-citrullinated protein antibodies; BAFF, B-cell-activating factor; CAIA, collagen antibody-induced arthritis; CarP, carbamylated peptide; CCP, cyclic citrullinated peptide; CIA, collagen-induced arthritis; MPO, myeloperoxidase; NE, neutrophil elastase; NET, neutrophil extracellular trap; PAD, peptidylarginine deiminase; PTPN22, protein tyrosine phosphatase nonreceptor 22; RA, rheumatoid arthritis; RF, rheumatoid factor; SE, shared epitope; SF, synovial fluid; SHM, somatic hypermutation; SNP, single nucleotide polymorphism.

citrullinated antigens by genetic and lifestyle susceptibility factors and the vicious circle between ACPA-producing pathogenic B cells and NET-producing neutrophils in RA.

KEYWORDS

anti-citrullinated protein antibodies, B cells, citrullination, dysbiosis, immune complexes, neutrophil extracellular traps, neutrophils, peptidylarginine deiminase, rheumatoid arthritis

RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease (reviewed in Ref. [1]). This most common form of inflammatory arthritis affects ~1% of the population worldwide and is more prevalent in women than men. RA is a disabling condition characterized by symmetrical inflammation of synovial joints, with small, peripheral joints most commonly affected. The synovial fluid (SF) becomes enriched in leucocytes and cytokines, and the inflamed synovial membrane develops into an inflammatory pannus, an abnormal layer of blood vessel-containing tissue which invades the space between the bones, covering bones and cartilage. Unless treated, RA erodes the joint cartilage and bone, causing chronic pain, stiffness, progressive loss of function, disability and, once fusion of bones has occurred, lasting deformities. Up to 40% of patients develop extraarticular RA, which ranges from systemic features, such as vasculitis, to affecting individual organs, for example the lung (e.g., interstitial lung disease) or heart (e.g., pericarditis). With the advent of improved and increasingly sophisticated disease-modifying anti-rheumatic drugs, RA has become more manageable in recent years. Although it remains incurable, a combination of early intervention, control of inflammation and prevention of joint damage can culminate in reaching a sustained state of remission.

Rheumatoid arthritis is sometimes regarded not as a single disease but a group of related diseases. Although the pathogenesis of RA is complex and remains incompletely understood, it is clear that this is a long, stepwise process which involves the dysregulation of many cell types, all of which make contributions to this disease. Despite their important contributions, cell types, including T cells, osteoclasts, macrophages and fibroblast-like synoviocytes, are not discussed here. Instead, this review focusses on the interplay of B cells and neutrophils in autoantibody-driven (seropositive) RA. Following on from the introduction to these two important cell types, and their critical role in the formation of autoantibodies and protein citrullination, which drive RA, we will discuss risk factors and how they link into RA pathogenesis by promoting citrullination and autoantibody formation.

B CELLS

Anti-citrullinated protein antibodies (ACPA; see below for a detailed discussion) are present in serum of >80% of patients with established RA and in ~50% of those with early RA. These autoantibodies can present as much as a decade prior to the onset of any clinical disease [2–4] indicative of an early loss of tolerance that initiates disease pathogenesis. During B-cell development, the antibody repertoire is developed. Tolerance is regulated at the central and peripheral checkpoints, when autoreactive B cells are eliminated, become anergic or undergo B-cell receptor editing [5]. These processes are, however, less strict than those applying to T cells, and some autoreactive B cells escape. Survival of autoreactive B cells may be aided by their genetic predisposition (see below), and self-reactive low-affinity antibodies may be masked on anergic B cells. Moreover, somatic hypermutation (SHM) leading to N-linked glycosylation of the variable region may permit escape from negative selection [6]. Indeed, glycosylation of the ACPA Fc changes during the transition from pre-arthritis to arthritis with the appearance of a more pro-inflammatory glycoform of these autoantibodies [7,8]. In the context of collagen-induced arthritis (CIA) in the mouse, differential glycosylation of ACPA was shown to affect their pathogenicity [9], with IL-23 and Th17 cells having key roles in promoting pathogenicity [10].

In addition to pathological autoantibodies, the success of B-cell depletion therapies targeting CD20 and BAFF [11–14] identified a critical function of B cells in promoting chronic inflammation in RA. The curious observation that good responsiveness to B-cell depletion therapy did not correlate with reduced ACPA titres, prompted studies into B cells as drivers of chronic inflammation. Such studies identified continuous antigen-driven B-cell activation, proliferating ACPA-positive memory B cells in both circulation and SF, as well as the production of pro-inflammatory mediators, notably the neutrophil chemokine IL-8 by synovial $ACPA^+$ B cells [15], suggestive of crosstalk between pathogenic B cells and neutrophils (Figure 1).

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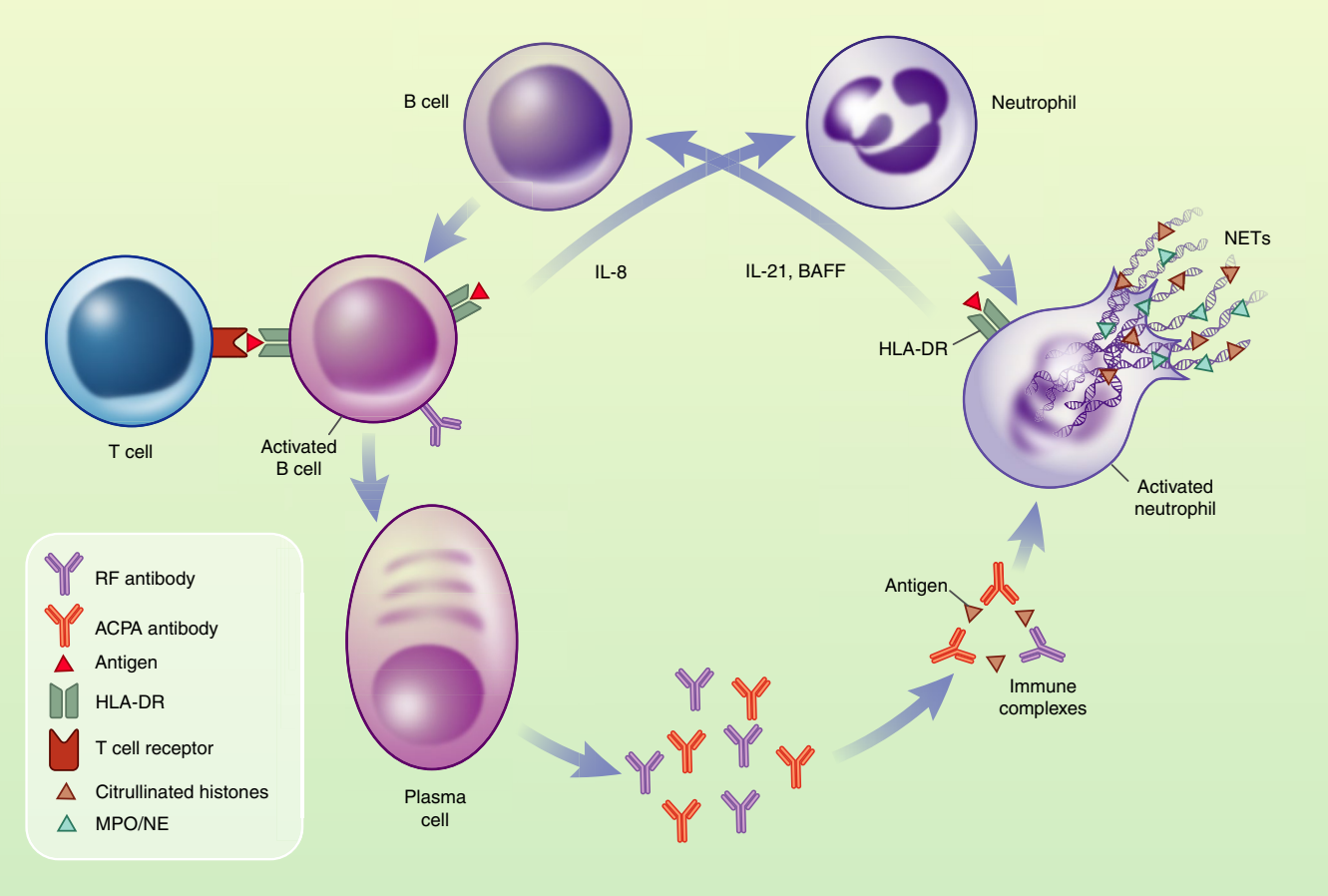

FIGURE 1 B cells and neutrophils form a vicious circle in RA. Activated B cells release cytokines to crosstalk with other immune cells, with B-cell-derived IL-8 recruiting neutrophils to the synovium [15]. B cells receive T-cell help with class switching and somatic hypermutation, promoting the development of autoantibodies in a HLA-DR SE-dependent fashion. Local plasma cells produce large amounts of autoantibodies including RF and ACPA; these form ICs which activate the complement pathway and promote inflammation, for example by stimulating neutrophils both in the circulation and also in the synovium. Amongst other events, this results in the release of NETs, which are particularly abundant in RA. Myeloperoxidase (MPO), neutrophil elastase (NE) and citrullinated histones are amongst the proteins that decorate NETs [22,141]. Citrullinated histones are thought to act as a continuous source of fresh antigen to B cells, promoting the production of new IgM ACPA. In the synovium, this is promoted by HLA-DR expressing, activated neutrophils which release cytokines including BAFF and IL-21, activating B cells [38–41]. In the interest of clarity, RF and ICs are simplified in this cartoon drawing

NEUTROPHILS

Neutrophils, the most abundant circulating leucocyte in humans, play a key role in host defence in killing bacteria and fungi either intracellularly, following phagocytosis, or extracellularly [16,17]. Unstimulated neutrophils circulate for only up to 1 day before homing back to the bone marrow where they undergo apoptosis to be cleared by resident macrophages in an anti-inflammatory process termed efferocytosis. In contrast, upon activation, the short-lived neutrophil leaves the blood stream and travels to inflammatory sites, such as the inflamed synovium, following gradients of chemo-attractants and chemokines [17,18].

Neutrophils are armed with granules loaded with powerful proteases and highly toxic antimicrobial peptides and possess the ability to generate cytotoxic reactive oxygen species (ROS), which can be released into a phagosome or to the outside of the cell. Neutrophils can moreover release their chromatin as ‘neutrophil extracellular traps’ (NETs; see also below), strands of granule protein-decorated chromatin that have important functions in host defence and that are highly inflammatory [16,17]. Neutrophilic inflammation can be triggered by microbes and sterile stimuli and can impart serious host tissue injury. Immune complexes (ICs) are powerful pro-inflammatory stimuli of neutrophils that ligate Fc receptors, and trigger effector functions, including, ROS production, degranulation, NETs, chemokine and cytokine generation [19–22], all of which are thought to contribute to tissue damage incurred in RA. ICs are key to RA. Depending on their ratio of antibody and antigen, ICs can be soluble or insoluble. Both

types are abundant in the SF, with further ICs precipitated onto synovial surfaces.

The SF in RA is characteristically sterile, though containing chemokines and cytokines and infiltrated by a large number of leucocytes (>5000/μl), the majority of which are neutrophils [23,24]. At early stages of (clinical) disease, neutrophils are also recruited into the synovial tissue [25,26], providing indirect clues about the important role of neutrophils in RA.

Circulating neutrophils from RA patients are characterized by an activated phenotype that is characterized by increased ROS, cytokine, protease and NET production as well as delayed apoptosis [27–30]. Intriguingly, patients with RA, as well as rats in an RA model, were found to harbour circulating low-density granulocytes, with differential cell surface marker and gene expression signatures that are the subject of ongoing investigation [31–33]. SF neutrophils were reported to be more activated still and having a differential gene expression signature compared to circulating cells from the same patient [34]. SF neutrophils are longer-lived than circulating neutrophils [35,36], secrete proteases, and release cytokines and chemokines to activate and recruit further neutrophils [29,37]. SF neutrophils also crosstalk with adaptive immune cells, for example by production of B-cell activating factor (BAFF), inducing B-cell proliferation and directly contributing to autoantibody production [38,39]. SF neutrophils moreover acquire the ability to present antigen in an MHC-II-dependent fashion and drive $CD4^+$ T-cell proliferation [40,41]. This is in keeping with observations that the SF containing neutrophil-derived cytotoxic products, NETs, cytokines and chemokines produced by neutrophils further inflammation [36,42,43].

While experiments with laboratory animals and disease models need to be interpreted with caution, mouse models of RA suggest that neutrophils play a key role in this disease. Antibody-mediated neutrophil depletion abolished development of K/BxN serum transfer arthritis and also of collagen antibody-induced arthritis (CAIA) [44,45], and neutrophil depletion after disease onset resulted in steep decline of CIA [46]. In a series of extensive and elegant investigations, the recruitment of neutrophils to the rheumatic joint in the K/BxN serum transfer model was shown to depend on FcγR as well as a cascade of chemokines, cytokines and chemo-attractants generated by a variety of cells and their respective receptors on neutrophils [47–50]. Meanwhile, IC-mediated neutrophil gene expression, including that of pro-inflammatory mediators, is dependent on CARD9-dependent regulation of the NFκB pathway downstream of FcγR, Src family kinases and Syk [51–53]. Elegant, very recent work with FcγR-humanized mouse neutrophils revealed that internalization of ICs permits these neutrophils to become antigen-presenting cells in a FcγR-dependent fashion [54]. The resulting neutrophils combined dendritic cell (antigen presentation, T-cell activation, cytokine production) and neutrophil functions (ROS production, phagocytosis) [54].

NETS

Neutrophil extracellular traps are web-like structures that are released by neutrophils. NETs consist of decondensed chromatin decorated with cytotoxic proteins, citrullinated histones, granule-derived proteins including neutrophilic proteases, myeloperoxidase (MPO), and antimicrobial peptides. Initially described as a pro-inflammatory cell death mechanism (NETosis), vital NET release was since also demonstrated [55], with the pathway employed being stimulus-dependent and varying in its NADPH oxidase dependency as well as cleavage of N-terminal histone tails [56]. Apart from their crucial function in host immunity, trapping and killing pathogens, NETs have important functions in autoimmune diseases including RA [57,58]. Elevated levels of NETs were identified in RA serum and SF [56,59,60].

Peptidyl-arginine deiminase 4 (PAD4), a leucocyte-restricted nuclear PAD, mediates histone citrullination, a post-translational modification of arginine residues during NET formation (Figure 2). NETs represent an important source of citrullinated (and homocitrullinated) epitopes in RA. Indeed, NET-associated citrullinated histones represent a continuous source of antigen for B cells and promote the localized generation of ACPA (see below for a detailed section on ACPA) that are able to cross-react with citrullinated histones in ectopic germinal centres in the inflamed synovial joint [59,61,62]. Plasma cell differentiation and antibody production are promoted in situ by pathogenic, IL-21-producing T peripheral helper cells [63]. Not only are ACPA pathogenic by themselves. Potentially aided by RF (see below for a detailed section on RF), which has the capacity to bind several IgG molecules, ACPA form ICs. These ICs fix complement, accumulate in SF and/or deposit on synovial surfaces to amplify inflammation [64–66]. ICs are powerful stimuli of NET release (e.g., [22,67,68]). In addition, NETs stimulate neutrophils to produce further NETs and to secrete IL-8 and BAFF [69], promoting the vicious circle between B cells and neutrophils (Figure 1).

Having introduced neutrophils and B cells, and how they activate one another in established disease, the next section of this review article will discuss genetic and lifestyle factors that predispose to RA. The focus lies on how these factors promote post-translational modification such as citrullination and/or the NET generation by neutrophils, in turn promoting B-cell generation of autoantibodies, in particular ACPA, promoting RA pathogenesis.

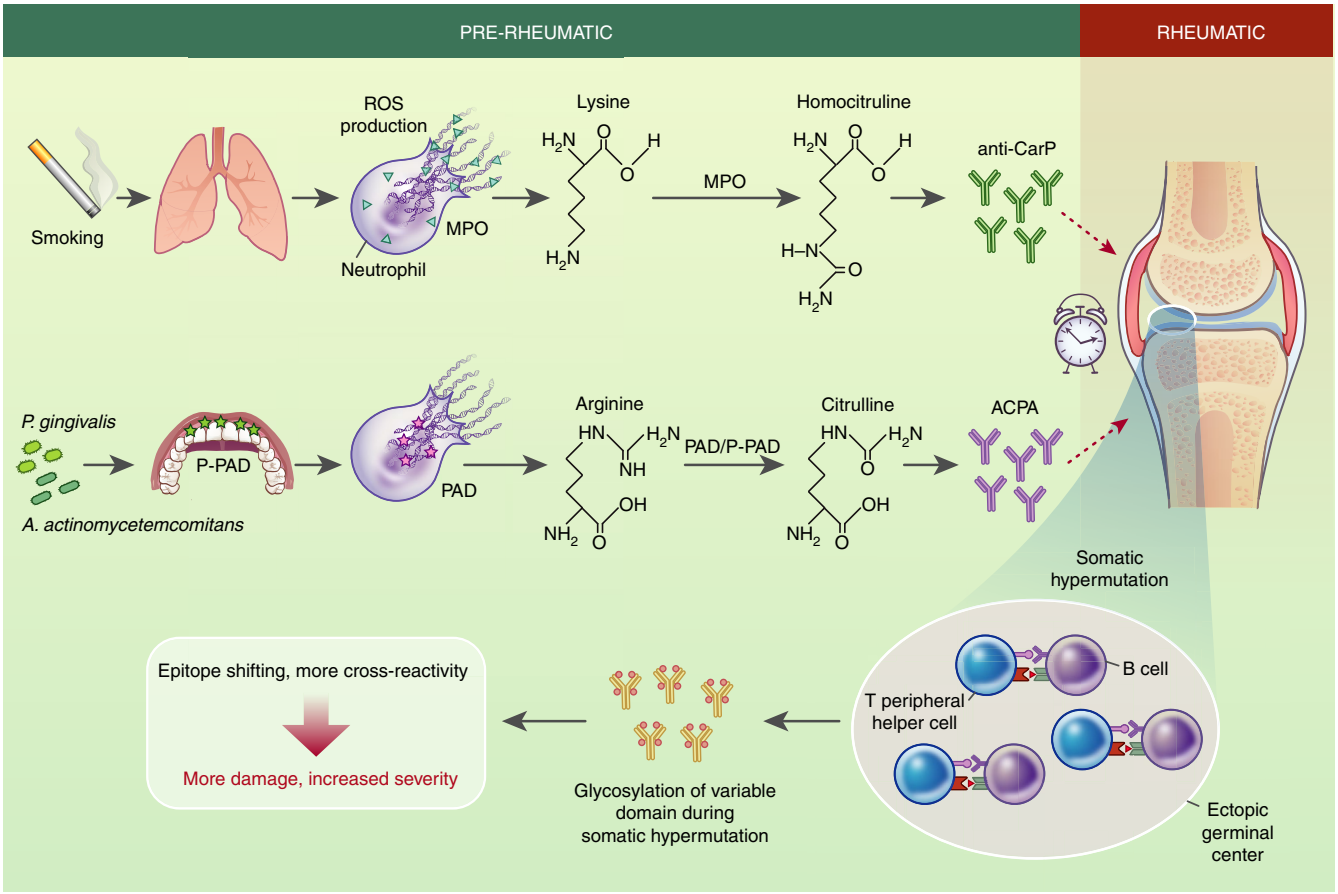

FIGURE 2 Citrullination and homocitrullination underpin the pathogenesis of RA. Pathogenic autoantibodies including ACPA and anti-CarP in pre-rheumatic patients precede the onset of clinical symptoms in RA by up to a decade. In addition to a genetic disposition, triggers at mucosal sites are thought to play a key role in these early events. Smoking-induced lung inflammation can promote neutrophil-derived NETs in the lung form and exteriorization of myeloperoxidase (MPO). Together with smoke-derived cyanate, homocitrullination (also known as carbamylation) of lysine causes the generation of homocitrulline, indirectly promoting the generation of anti-carP antibodies [86]. Alternative scenarios involve microbial dysbiosis at mucosal surfaces, such as the gingival tissues. Two causative pathogens in gingivitis, *P. gingivalis* and *A. actinomycetemcomitans* can promote citrullination of antigens by employing bacterial P-PAD and neutrophil PAD4, respectively [95,100]. Citrullination is a process by which arginine is enzymatically converted to citrulline, generating a highly immunogenic antigen [62]. The events leading to the onset of symptomatic disease years later remain obscure, with possibilities including infection and/or local trauma. In the rheumatic phase, autoantibody maturation occurs locally in the inflamed synovial tissue. B-cell SHM involving crosstalk of pathogenic B cells and pathogenic peripheral T helper cells occurs in ectopic germinal centres [63]. Strikingly, SHM results in characteristic glycosylation of the antibodies variable domain. This unusual form of SHM does not result in affinity maturation but instead drives epitope shifting and cross-reactivity of ACPA [110,115], ultimately resulting in more tissue damage and increased disease severity

GENETIC FACTORS

While it is still unclear why some people ultimately develop RA, there are well-documented genetic and environmental risk factors. The most significant genetic risk factor identified to date is the class II major histocompatibility locus, with so-called shared epitope (SE) containing alleles increasing the risk of developing seropositive RA according to epidemiological studies [70,71]. SE sequences ^{70}QKRAA^{74}, ^{70}QRRAA^{74} or ^{70}RRRAA^{74} within HLA-DRB1 are involved in shaping the peptide-binding pocket of the HLA molecule. The presence of two positively charged residue (lysine, arginine) in residues 71–73 increases the binding capacity of citrullinated peptides over native peptides [72,73]. In addition, citrullinated peptides were also shown to display enhanced binding to HLA-DQ [74]. Altogether, these mechanisms achieve that citrullinated peptides are preferentially presented, and activate CD4$^+$ T-cell responses, which in turn promote the immune response by helping ACPA-producing B-cell antibody maturation (class switching and somatic hypermutation).

Additional genetic risk factors have been attributed to single nucleotide polymorphisms (SNPs) in a range of genes. The most prominent of these is a SNP, C1858T, in the leucocyte-restricted protein tyrosine phosphatase non-receptor 22 (PTPN22) which encodes the R620W variant

[75]. This allele and especially C1858T homozygosity cause an elevated risk of developing RA, earlier disease onset and more aggressive disease, with RF positivity conferring increased odds. R620W PTPN22 was reported to drive blunted BCR signalling and reduced B-cell apoptosis, resulting in increased escape of poly- and autoreactive B cells from central and peripheral tolerance in humans and mouse models [76–78]. PTPN22 is most highly expressed in neutrophils, with R620W reported to promote transendothelial migration and ROS production in human neutrophils [79], while in a mouse model *Ptpn22* deficiency caused decreased pro-inflammatory responses to IC stimulation without affecting neutrophil recruitment to inflammatory sites [80]. PTPN22 was moreover shown to physically interact with and inhibit PAD4 in a phosphatase-independent fashion. Indeed, R620W PTPN22 promoted enhanced citrullination which resulted in increased NET production by neutrophils and in defective Th2 and Th17 cytokine production by peripheral blood-derived mononuclear cells [81,82].

LIFESTYLE: SMOKING

The major environmental factor associated with developing RA is smoking, which in combination with the HLA-DR SE confers a significantly increased susceptibility to ACPA-positive RA according to epidemiological studies (e.g., [83,84]). This suggests that smoking may provide an external trigger for those already carrying genetic risk factors to develop RA, and identifies the lung as an important mucosal site in RA development (Figure 2). Indeed smokers' lung biopsies were characterized by upregulated PAD2/4 expression and increased protein citrullination [85,86]. Recently, enhanced carbamylation and anti-CarP (see below) were also found in smokers and smoke-exposed laboratory mice [87,88], directly implying neutrophils. Interestingly, neutrophils isolated from smokers were moreover shown to be more prone to NET production in a nicotine-dependent fashion. In experimental mice, too, nicotine promoted NET production and caused more severe disease scores in CIA [89,90].

DYSBIOSIS: EXAMPLE PERIODONTITIS

Links between the disruption of the beneficial relationship between commensal bacteria and the host at mucosal surfaces were documented in inflammation, with dysbiosis of the oral microbiota and periodontitis most clearly associated with RA pathogenesis [91] (Figure 2). A statistically significant association between RA and periodontitis was shown in a number of clinical studies, with RA patients with severe periodontitis suffering from more severe RA [92]. Associations between ACPA and severity of periodontitis in RA and pre-RA patients were also described [93,94]. A possible explanation for this observation rests with two key periodontal pathogens. *Porphyromonas gingivalis* expresses a bacterial deiminase, PPAD, which citrullinates C-terminal arginines of bacterial and host origin in a calcium-independent fashion, potentially contributing to the breaking of tolerance [95]. In the context of CIA in the mouse, *P. gingivalis* PPAD activity could increase inflammatory arthritis [96,97]. Notably, *P. gingivalis* also affects the cytokine response of gingival epithelial cells, driving recruitment of Th17 cells and neutrophils via CCL20 and CXCL8. *P. gingivalis* was further shown to trigger release of non-bactericidal NET production by neutrophils, encouraging microbial growth and increasing citrullinated antigens in the periodontal space [98,99]. In a separate mechanism, *Aggregatibacter actinomycetemcomitans* was shown to use its pore-forming leucotoxins to induce hypercitrullination and NET formation by neutrophils, which in turn increased ACPA in the periodontal space [100].

AUTOANTIBODIES

As laid out above, the earliest and perhaps most conspicuous feature of RA are autoantibodies, which can be present years or even decades prior to onset of clinical symptoms (pre-RA), with epitope spreading and expansion of autoantibodies occurring prior to the onset of clinical disease.

Rheumatoid factor

Rheumatoid factor refers to antibodies directed against the Fc region of IgG and was described in the 1940s. Despite its high prevalence in RA (up to 80% of patients) and positive association with more severe disease progression, RF is not restricted to RA, making it an unreliable diagnostic marker (reviewed in Ref. [1]). RF can undergo class switching, with IgM and IgA RF most commonly observed in RA. RF moreover undergoes somatic hypermutation and affinity maturation in RA. By recognizing IgG, RF is perfectly suited to forming large ICs, and to promoting deposition of complement to improve clearance of excess antibodies, the likely function of natural RF [101]. However, as laid out above, ICs also play a key role in promoting persistent inflammation including via neutrophils.

Anti-citrullinated protein antibodies

Anti-citrullinated protein antibodies represent a second class of highly prevalent autoantibodies found in RA.

ACPAs are present in the serum of 80–90% of patients with established RA and in up to 20% of their first-degree relatives [102]. For diagnostic purposes, presence of ACPA in the serum is detected by using cyclic citrullinated peptide (CCP2/CCP3) assays, where synthetic CCPs are used that were optimized for optimal ACPA capture. Serum ACPA react with peptides derived from a range of citrullinated protein antigens that are found in the rheumatic joints, including fibrin vimentin, α-enolase and histones [62,103–106], with a high degree of cross-reactivity between substrates observed for individual monoclonal ACPAs [107]. Interestingly, it was recently suggested that improved screening might detect autoantibodies in seronegative RA patients that do not cross-react well with the CCPs used in current clinical testing [108]. Although the presence of ACPA in a person without clinical symptoms does not predict that they will be developing RA, contrasting with RF, the presence of ACPA is highly specific to RA, making them a useful diagnostic tool. ACPA is moreover indicative of more severe disease progression [109]. In RA, ACPA associate with RF and the HLA SE, as laid out above.

Anti-citrullinated protein antibodies undergo class switching, extensive somatic hypermutation as well as conspicuous variable region glycosylation and epitope spreading, leading to cross-reactive antibodies (Figure 2). However, affinity maturation is limited and even serum IgG ACPA is characterized by low binding affinity for citrullinated protein [110–115]. Interestingly, despite the short half-life of 5.9 days of IgM in RA [116], and the fact that long-lived IgM-secreting plasma cells are not described in humans, IgM ACPA continues to be present in the serum of RA patients, or can occur at later stages in previously IgM ACPA-negative patients [117,118]. This suggests that new citrullinated antigen-specific B cells continuously generate new ACPA, implying the continued generation of fresh citrullinated antigens, for example due to neutrophil-mediated production of NETs [57,59,60].

Anti-CarP

Processes related to citrullination also lead to secondary modification of protein epitopes that are similarly antigenic. Carbamylation, also known as homocitrullination, is the post-translational modification of lysine to homocitrulline (Figure 2). Unlike citrullination, carbamylation is a chemical modification that does not rely on an enzyme, but occurs on the presence of cyanate as a myeloperoxidase-dependent oxidation of thiocyanate which is abundant in smokers [86]. Carbamylated peptides were predicted to bind SE alleles [119]. Anti-CarP (carbamylated peptide) antibodies are directed against carbamylated proteins and frequently cross-react with citrullinated proteins [119]. Anti-CarP are present in 45% of RA patients including some of those who are ACPA-negative. Like ACPA, anti-CarP antibodies are very specific to RA, present in the serum up to 10 years before the onset of clinical symptoms, and are associated with NETs [58,120].

Anti-PAD antibodies

Neutrophils express three PAD enzymes, PAD2/3/4. Extracellular, active PAD was observed in cell-free SF of RA patients [121]. Neutrophil NETosis was found to result in release of free PAD2/4 in vitro, raising the possibility that dying neutrophils may be the source of free PAD2/4 [122]. However, neutrophils were also found to spontaneously secrete or expose PAD2/4 [123], providing an alternative explanation for the citrullination of extracellular targets in the rheumatic joint. Interestingly, PADs themselves also serve as RA autoantigens. Indeed, ~30% of RA patient, but not control sera, were found to be anti-PAD4 positive, with anti-PAD4 positivity occurring prior to (clinical) disease onset and being a marker of severe disease [124–126]. PAD4 requires >100 μM Ca^{2+} to display any catalytic activity in vitro and mM Ca^{2+} concentrations for optimal activity. This vastly exceeds the Ca^{2+} concentration of the resting cell [127], but can be achieved following activation and opening of Ca^{2+} channels [128]. The fact that histone citrullination occurs physiologically to regulate gene expression [129] moreover suggests the existence of additional mechanisms that allow PAD enzymes to be active at lower Ca^{2+}. Fascinatingly, a subset of cross-reactive anti-PAD3/4 antibodies were shown to inducing a conformational change into PAD4 when binding to it, rendering it hyperactive by which vastly reduced its Ca^{2+} requirement to a physiological level [130,131]. Presence of these truly pathogenic anti-PAD3/4 antibodies correlates with particularly aggressive disease.

CONCLUDING REMARKS

Despite decades of research into RA, the trigger that precipitates the original break of tolerance and the nature of additional events that initiate clinical disease remain obscure. It is clear, however, that a combination of genetic predisposition, lifestyle choices and dysbiosis can all contribute to culminate in clinical disease. Citrullination, much of it likely to be neutrophil-derived, and ACPA lie at the heart of the initial break of tolerance, and subsequently, a vicious circle between neutrophils and B-cell-derived autoantibodies/ICs plays out. Neutrophils are often portrayed as uniquely pro-inflammatory; however, this is likely not the whole truth. Not only do neutrophils clear insoluble ICs from biological

fluids, thereby reducing these highly pro-inflammatory stimuli, but the same ICs also induce neutrophil apoptosis [132–134], a cell death that promotes the resolution of inflammation [135]. A separate intriguing notion is moreover that physiologically citrullination may occur in order to limit excessive inflammation. Free histones are extremely cytotoxic [136]; however, their citrullination in NETs renders them more susceptible to proteolytic degradation. It also reduces their ability to stimulate further NET production [137,138]. In a similar way, citrullination may confer the host with a degree of protection from the highly toxic antibacterial peptide LL37 [139,140].

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CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTION

UK and SV wrote the manuscript and designed the figures.

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REVIEW

WILEY

Immune complex-induced neutrophil functions: A focus on cell death

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Abstract

Neutrophils are amongst the first cells to be recruited to sites of infection or trauma. Neutrophil functional responsiveness is tightly regulated by many agents including immune complexes. These immune cells can generate reactive oxygen species and degranulate to release abundant cytotoxic products, making them efficient at killing invading microorganisms. If neutrophil function is dysregulated, however, these cells have the potential to cause unwanted host tissue damage as exemplified by pathological and chronic inflammatory conditions. In physiological inflammation, once the initial insult has efficiently been dealt with, neutrophils are thought to leave the tissues or undergo programmed cells death, especially apoptosis. Apoptotic neutrophils are then rapidly removed by other phagocytes, primarily macrophages, by mechanisms that do not elicit a pro-inflammatory response. In this review, we discuss the interesting observations and consequences that immune complexes have on neutrophil cell death processes such as apoptosis.

KEYWORDS
apoptosis, immune complexes, neutrophil

1 | BACKGROUND

Terminally differentiated neutrophils represent up to 70% of all peripheral blood leucocytes in humans. By fighting bacterial and fungal infections, these leucocytes form an essential compartment of the innate immune response.[1,2] Circulating neutrophils have a short lifespan, which was determined to be <24 hours in the past,[3] although a recent study calculated that human neutrophils can circulate in the blood for up to 5 days[4] (See also the review by Koenderman et al in this issue). At the end of their sojourn in the blood, circulating neutrophils that were not recruited to any site of inflammation, home to the liver, spleen or bone marrow where they undergo constitutive apoptosis to be cleared by resident macrophages.[5-7] This represents a major mechanism of neutrophil clearance which is covered in depth in a separate review by De Filippo & Rankin in this issue. Tissue migrated neutrophils, once their function has been completed, are thought to undergo apoptosis and clearance by "professional" phagocytes such as macrophages or other "semi-professional" cells such as epithelial cells. Evidence is mounting to suggest that extravasated neutrophils can move away from the tissues by a process termed "reverse migration" and home to other tissues such as the lungs under certain circumstances.[8] Approximately 5-10 $\times$ 10^{10} new neutrophils are generated each day in the bone marrow from promyelocytic progenitors.[9] Aged neutrophils are continuously replaced by fresh cells from reserves in the bone marrow. Additional neutrophils can be recruited into the circulation from the bone marrow if required, for instance in acute inflammation. When there is great demand, some of the recruited neutrophils can be immature (banded).[10] There is evidence to suggest that neutrophils that have lodged themselves in marginated pools in certain vascular beds can be liberated upon demand.[11]

Upon appropriate stimulation, circulating neutrophils initially adhere to and then cross the vessel wall making

use of adhesion molecules (eg, selectins, integrins and immunoglobulin family members; see also the review by Gomez-Moreno et al in this issue). They leave the blood stream and follow gradients of chemokines (eg, IL-8, etc.), mediators (eg, LTB_4, C5a, etc.) and pathogen-derived chemoattractants (eg, formylated peptides) to be recruited to sites of inflammation or infection.[12] The lives of activated neutrophils are lengthened compared with the short lifespan of circulating (unprimed) neutrophils. This is due to exposure to a range of factors that induce pro-survival signalling (eg, hypoxic environments, G-CSF, GM-CSF, TNF, LPS[13-15]). By promoting cell survival, usually by delaying apoptosis, the viability of neutrophils that have been recruited into infected or inflamed tissues is ensured.

At sites of infection, neutrophils phagocytose pathogens. They generate reactive oxygen species (ROS) and degranulate intracellularly into phagolysosomes, producing a host of powerful cytocidal agents designed to kill and digest internalised bacterial and/or fungal pathogens (see the review by Vogt et al in this issue). They also produce mediators to recruit and cross-talk with other immune cells, thus shaping the immune response. Failing to tightly control and limit neutrophilic inflammation can result in significant host damage, both acute and chronic. Therefore, once pathogens have been destroyed and the neutrophils' function has been fulfilled, the recruited neutrophils die and/or are removed.

Several programmed neutrophil death programmes exist (reviewed in Refs 16-18; Figure 1). Apoptosis is likely the main form of physiological neutrophil death that does not trigger a pro-inflammatory response. However, neutrophils can also "die" by necroptosis, a RIP kinase 3-dependent form of programmed necrosis,[19] and pyroptosis, a caspase 1 and inflammasome-dependent cell death that is induced intracellular antimicrobial response.[20] Both are regarded as highly pro-inflammatory forms of programmed cell death.

The release of neutrophil extracellular traps (NETs), granule protein-rich chromatin, is often referred to as NETosis. NET release serves as a mechanism of extracellular killing, for example of pathogens that are too large to be ingested (see also the review by Van Avondt & Hartl in this issue). NETs are often associated with infections and autoimmune disease.[21,22] NETosis is often investigated and induced with nonphysiological agents in vitro and has been described as a separate form of programmed cell death that involves rupture of the plasma membrane.[23] NETs have been described in many pathophysiological situations in situ; yet, the existence of NETosis as a physiological cell death programme remains somewhat controversial.[24,25] Alternative mechanisms proposed for the generation of NETs include the release of mitochondrial (or indeed nuclear) DNA by viable granulocytes.[26-28] Whilst the NETs per se are generally regarded as pro-inflammatory, there is evidence to suggest that they may limit inflammation under certain circumstances.[29]

2 | NEUTROPHIL APOPTOSIS

Apoptosis, caspase-dependent cellular suicide, represents the best understood programmed cell death mechanism. Apoptotic neutrophils are characterised by their distinctive shrunken bodies, condensed nuclei, DNA fragmentation, membrane blebbing as well as accumulation of phosphatidylserine (PtdSer) on their plasma membranes (Figure 1).[30]

3 | NEUTROPHIL APOPTOSIS AS A PRO-RESOLUTION MECHANISM

The induction of neutrophil apoptosis at sites of inflammation is seen as an important pro-resolution mechanism that limits the extent of neutrophilic inflammation and associated bystander injury.[31-34] By displaying "find-me" and "eat-me" signals, apoptosing neutrophils induce their own clearance by pro-resolution macrophages in a specialised phagocytosis mechanism known as "efferocytosis" or "phagoptosis." The best understood "eat-me" signal is phosphatidylserine (PtdSer) that is exposed to the outside by plasma membranes of apoptosing cells. This permits the recognition of apoptotic cells by PtdSer receptors and/or bridging molecules on phagocytes, triggering apoptotic neutrophil uptake.[34,35] Efferocytosis occurs before the plasma membrane of apoptotic cells becomes leaky and thereby prevents the display of pro-inflammatory neutrophil debris (eg, chromatin or damage-associated molecular patterns) or the occurrence of secondary necrosis. The process is immunologically silent, that is it does not induce the recruitment of additional inflammatory cells and trigger further inflammation. Rather, the engulfment of apoptotic neutrophils by macrophages promotes the production of pro-resolving lipid mediators, for example resolvins, protectins and lipoxins. Pro-resolving mediators are thought to lessen further neutrophil recruitment and promote increased uptake of apoptotic cells by tissue resident macrophages. The mechanisms by which such mediators act is undergoing intense investigation, and it appears that specific receptors are responsible for the recognition of many of the pro-resolution lipids.[36,37]

In-line with the pro-resolution function of neutrophil apoptosis and the clearance of apoptotic neutrophils by efferocytosis, delayed neutrophil apoptosis has been linked to many inflammatory and autoimmune diseases (eg, COPD[38] and rheumatoid arthritis[39]) and also to disease severity. Therapeutic strategies aimed at enhancing

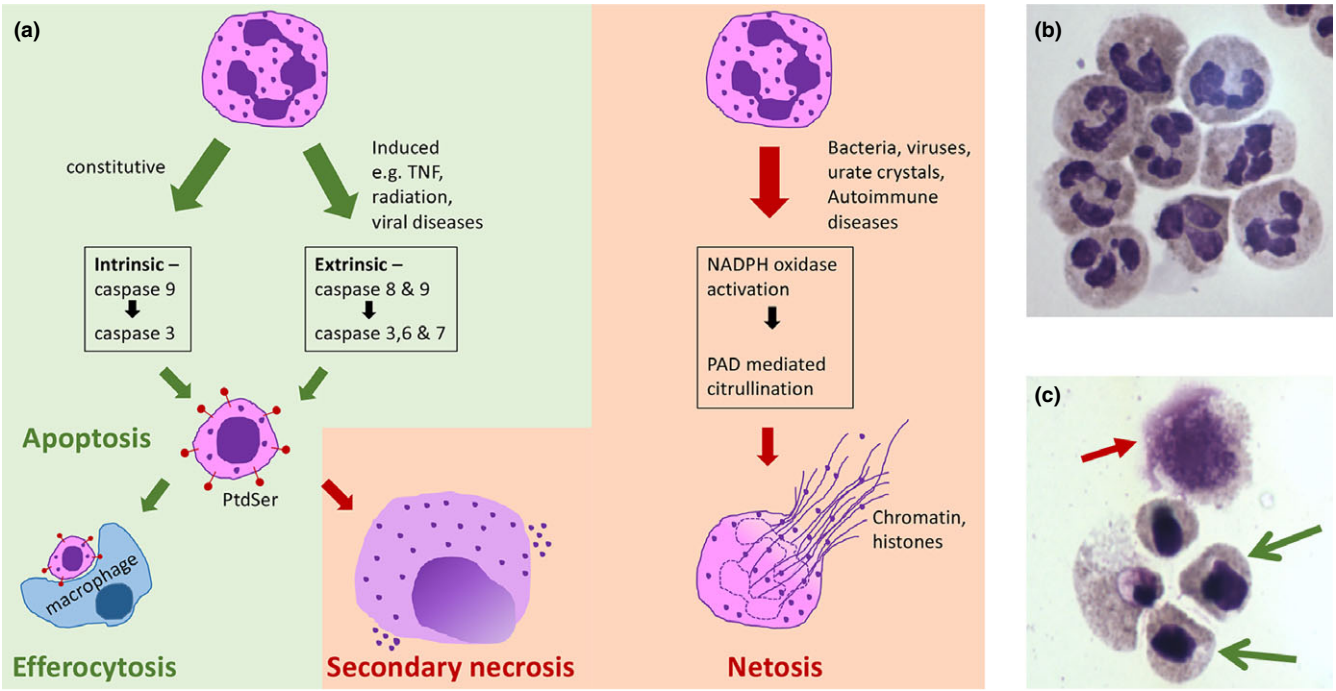

FIGURE 1 Cell death programmes in neutrophils. (A) A schematic overview of neutrophil cell death by apoptosis, secondary necrosis of apoptotic neutrophils or NETosis. Pro-resolution processes comprising apoptosis, and efferocytosis are colour coded in green, whilst peach indicates pro-inflammatory processes as encountered in secondary necrosis and NETosis. (B, C) Cytocentrifuge preparations of human neutrophils that had been solated from bloods of healthy human volunteers with approval from the local research ethics committee (AMREC 15-HV-013). Shown are viable neutrophils with characteristic polymorphonuclear morphology (B) and neutrophils that have undergone constitutive apoptotic (green arrows) and undergone secondary necrosis (red arrow) following culture for 24 h (C). Note the condensed nuclei and small cell size of the apoptotic cell compared to the swollen body of the necrotic neutrophil

granulocyte apoptosis at sites of inflammation are being investigated.[34]

4 | MOLECULAR EVENTS REGULATING NEUTROPHIL APOPTOSIS

The decision whether to undergo apoptosis or not depends upon the balance of pro- and anti-apoptotic factors, in particular Bcl-2 family members, within the cell. A major anti-apoptotic protein in the neutrophil is the short-lived Bcl-2 family member Mcl-1, whilst pro-apoptotic counterparts (eg, Bax and Bak) have longer half-lives. Discontinued synthesis of Mcl-1 or enhanced proteolytic degradation of Mcl-1 is sufficient for the induction of neutrophil apoptosis. Apoptosis is characterised by the controlled demolition of cellular components by caspases, which act in an activation cascade. These specialised cysteine proteases exist as inactive pro-enzymes in the cytosol and are themselves processed by proteolytic cleavage to generate active enzymes. Cleavage of pro-caspases is inhibited by anti-apoptotic IAP family proteins. XIAP, the most important neutrophil IAP protein, binds pro-caspases to protect them from activation until its own cleavage during apoptosis frees the caspases for processing.[18,30]

5 | PATHWAYS REGULATING NEUTROPHIL APOPTOSIS

In common with most other cell types, neutrophils utilise 2 major apoptosis pathways, intrinsic and extrinsic.[18,30] The intrinsic pathway mediates constitutive neutrophil apoptosis, which is driven by the permeabilisation of the mitochondrial outer membrane. Dimers of the 2 pro-apoptotic Bcl-2 family members Bax and Bak insert themselves into the mitochondrial membrane, causing loss of mitochondrial outer membrane potential and triggering the release of pro-apoptotic factors, chiefly cytochrome *c*, into the cytosol.[40] This mediates the activation of the initiator caspase 9, which in turn causes the activation of the executioner caspase 3. Interestingly, neutrophils use glycolysis rather than oxidative phosphorylation for ATP generation, suggesting that the induction of apoptosis may represent a major function of neutrophil mitochondria.[41]

The extrinsic pathway of neutrophil apoptosis is induced by ligation of TNF receptor superfamily cell surface death

receptors (eg, Fas, TNFR1 and Trail receptor). This triggers downstream activation of the initiator caspases-8 and eventual activation of the executioner caspase 3. Extrinsic neutrophil apoptosis has been shown to be dependent on NADPH oxidase-derived ROS (indeed many neutrophil processes are dependent on or involve NADPH oxidases, please see Gómez-Moreno et al, in this issue), as demonstrated by the absence of this pathway in neutrophils isolated from chronic granulomatous disease (CGD) patients, who lack a functional NADPH oxidase.[42] Considerable overlap exists between the 2 apoptosis pathways. For example, an amplification loop downstream of initiator caspase 8 in extrinsic apoptosis involves Bax/Bak-mediated disruption of the mitochondrial membrane potential as summarised for the intrinsic mechanism.[43]

Neutrophil apoptosis has also been observed to be induced downstream of receptors outwith the classical death receptors. One example is phagocytosis-induced cell death (PICD),[44-46] which is mediated by Fc and/or complement receptors and which specifically couples microbial ingestion and killing to the induction of apoptosis. PICD is triggered by phagocytosis of complement and/or antibody coated particles (living or heat-killed bacteria, yeast, beads or drops of oil) following binding to their cognate receptors.[46-48] As with death receptor-induced neutrophil apoptosis, PICD has been shown to be dependent on (intracellular) ROS, with neutrophils from CGD patients protected from PICD.[45,49] PICD actively links pathogen killing to the resolution of inflammation, using differential gene expression programmes within the apoptosing neutrophil.[48] Considering the numbers of pathogen immune evasion strategies that have evolved to evade killing following phagocytosis (reviewed in Ref. 50), a further benefit of PICD might lie in the reduced likelihood of intracellular microbial survival in a dead host cell.

6 | IMMUNE COMPLEXES

Immune complexes are antigen-antibody aggregates that are usually smaller than antibody-opsonised cells and powerful activators that stimulate neutrophils following binding of the neutrophil Fc receptors to the invariable Fc tail of the antibody (Table 1 for Fc receptor expression in human and mouse neutrophils). Fc receptor binding induces tyrosine phosphorylation of immunoreceptor tyrosine-based activating (or inhibitory) motifs, receptor-proximal activation of Syk and Src family tyrosine kinases (SFKs) and further downstream activation of multiple signalling cascades including regulators phosphoinositide 3-kinases (PI3Ks), protein kinases (eg, MAP kinases), other lipid modifying enzymes and small GTPases (reviewed in Refs 51,52).

Insoluble and soluble immune complexes exist in vivo and can be generated in vitro. They are characterised by different ratios of antibody to antigen, and can or cannot be precipitated by centrifugation, respectively. In vitro experimentation with human and mouse neutrophils established that soluble immune complexes cause predominantly external ROS production and degranulation only of primed neutrophils, whilst insoluble immune complexes cause predominantly intracellular ROS and degranulation even in the absence of priming.[53]

Immune complexes form even under healthy conditions, when they are efficiently cleared from the circulation without causing any harm. If clearance is insufficient, however,

TABLE 1 Fc receptor expression in human (A) and mouse (B) neutrophils

(A)				
Name	**FcαR1**	**FcγRI**	**FcγRIIA**	**FcγRIIIB**
CD name	CD89	CD64	CD32A	CD16B
Affinity	Low	High	Low	Low
Activity	Activating/inhibitory	Activating	Activating	Activating
Expression	Constitutive	Postmigrated	Constitutive	Constitutive

(B)			
Name	**FcγRIIB**	**FcγRIII**	**FcγRIV**
CD name	CD32B	CD16	CD16.2
Affinity	Low	Low	High
Activity	Inhibitory	Activating/Inhibitory	Activating
Expression	Constitutive	Constitutive	Constitutive

Notable differences are that (i) human neutrophils express the IgA-binding FcαR, whilst mouse neutrophils only express IgG-binding FcγRs; (ii) mouse neutrophils constitutively express a high affinity FcγR (FcγRIV), whereas human neutrophils express their high affinity FcγRI inducible when activated; (iii) mouse neutrophils constitutively express an inhibitory FcγR (FcγRIIB) whilst the inhibitory FcγRIIB is thought to only be expressed by a subset of human neutrophils.[52]

immune complexes persist and accumulate in the blood stream and in other bodily fluids, such as the synovial space in rheumatoid arthritis. Immune complexes are prone to precipitating onto biological surfaces (eg, the synovial wall) when they are referred to as immobilised immune complexes, further contributing to neutrophilic inflammation.[54] Immobilised immune complexes cause predominantly external ROS, degranulation and cytokine production even of unprimed neutrophils.[55]

As immune complexes induce neutrophil production of many inflammatory mediators, including the potent neutrophil chemoattractants such as IL-8 and LTB_4, this causes recruitment of further neutrophils and promotes additional neutrophilic inflammation.[56,57] Not surprisingly, immune complexes have been shown to act as major drivers of neutrophilic inflammation in a range of (autoimmune) diseases that include rheumatoid arthritis, lupus erythematosus, glomerulonephritis and vasculitis. The important role of immune complexes in autoinflammatory diseases has also been shown in mouse models of such conditions.[58]

7 | APOPTOSIS INDUCED BY INSOLUBLE IMMUNE COMPLEXES

In addition to the stimulatory effects on neutrophils that are discussed above, immune complexes have been linked to neutrophil death, by modulating neutrophil apoptosis and/or inducing NETosis (Table 2—IC-induced neutrophil responses).

Insoluble immune complexes were shown to be powerful inducers of caspase-dependent neutrophil death, consistent with apoptosis.[59,60] By employing blocking antibodies, the investigators identified that FcγRIIa but not FcγRIIIb or the β2 integrin Mac1 was required for immune complex-induced neutrophil apoptosis, and that antibody-mediated cross-linking of FcγRIIa was sufficient for induction of apoptosis. Interfering with the generation of reactive oxygen species significantly reduced insoluble immune complex-induced neutrophil apoptosis, and the induction of apoptosis of neutrophils isolated from CGD patients, who lack a functional NADPH oxidase was lower, albeit not abolished, in-line with a ROS-dependent apoptosis pathway.[59,60] Co-incubation of the neutrophils with the survival factor GM-CSF (but not with several other survival factors tested) interfered with immune complex-induced apoptosis.[61] Insoluble immune complex-induced apoptosis was regulated by a pathway involving the initiator caspase 8, but not 9, suggesting that this is a type of extrinsic neutrophil apoptosis.[62] Further upstream, the pathway was shown to depend on pro-apoptotic PI3Kβ/δ-Cdc42-Pak-Mek-Erk signalling downstream of the FcγR to regulate the balance of pro-and anti-apoptotic Bcl-2 family members.[56] Although not unheard of,[63,64] pro-apoptotic functions of Erk and PI3K are somewhat atypical. In contrast, PICD was shown to involve anti-apoptotic Erk signalling,[47] suggesting that 2 separate mechanisms of neutrophils apoptosis exist downstream of FcγR engagement.

8 | NEUTROPHIL DEATH INDUCED BY IMMOBILISED IMMUNOGLOBULINS/IMMUNE COMPLEXES

No analysis of the induction of neutrophil apoptosis by immobilised immune complexes is available; however, immobilised IgG and IgA stimulate neutrophil apoptosis in a ROS-dependent fashion. The effect of immobilised IgG depended on FcγRIIa/IIIb,[65] whereas immobilised IgA-induced apoptosis was dependent on FcαRI.[66] The major neutrophil integrin Mac-1 is involved in the induction of neutrophil apoptosis in this adhesion-dependent context, in keeping with the observation that immune complexes drive Mac-1 activation.[67] Immobilised IgA was more efficient at inducing apoptosis of primed, than of unprimed neutrophils, in-line with the upregulation in surface FcαRI observed with primed neutrophils. Cross-linking neutrophil FcαRI also resulted in the induction of apoptosis. In parallel with apoptosis, cross-linking FcαRI of primed neutrophils, or those from rheumatoid arthritis or sepsis patients (who also display upregulated surface FcαRI),

TABLE 2 Overview of neutrophil effector functions induced by different types of immune complexes

	Soluble ICs	Insoluble ICs	Immobilised ICs
ROS production	Predominately external; priming required[48]	Predominately internal; even in absence of priming[48]	Predominately external; even without priming[50]
Induction of NET production	Yes[65]	Unknown	Yes[63]
Induction of cytokine/lipid mediator release	Yes[49]	Yes (IL-8, LTB_4)[51,68]	Yes[50]
Induction of apoptosis	Delayed[54]	Promoted[54,56,60]	Unknown
Degranulation	Yes, priming required[48]	Yes, even in absence of priming[48]	Yes, even without priming[50]

induced PI3Kβ/δ and p38/JNK-dependent but Erk- and caspase-independent death that involved neutrophil vacuolisation. Interestingly, and in keeping with exhibiting low annexin V (which binds to PtdSer) binding and plasma membrane integrity, neutrophils that had undergone the alternative death pathway were efferocytosed as efficiently by macrophages as their apoptotic counterparts, suggesting that this alternative neutrophil death programme was not pro-inflammatory but apoptosis-like.[66]

In addition to the induction of "anti-inflammatory" neutrophil death, immobilised immune complexes have also been linked to the induction of neutrophil NETosis.[68] Mechanistically, this NETosis occurs in a ROS-dependent fashion, where it was mediated by FcγRIIIb in combination with Mac-1. Immobilised immune complex-induced NETosis was further shown to depend on Src/Syk, PI3K/Akt, Erk and p38 MAPK signalling.

9 | SOLUBLE IMMUNE COMPLEX-INDUCED NEUTROPHIL DEATH

Contrasting with insoluble immune complexes, soluble immune complexes (and heat aggregated IgG) were found to delay the induction of apoptosis of (unprimed) neutrophils.[59] Soluble synthetic or human plasma derived IL8-anti IL8 immune complexes were also shown to delay constitutive neutrophil apoptosis via FcγRIIa. Interestingly, inhibition of Src, Syk, PI3K and Erk signalling all interfered with this anti-apoptotic pathway,[69] directly opposing these regulators of pro-apoptotic functions in neutrophils that were stimulated with insoluble immune complexes. Given that soluble immune complexes only drive pro-inflammatory effector functions of primed neutrophils, it would be interesting to investigate their effect on neutrophil apoptosis following priming.

Soluble (but not immobilised) immune complexes were found to induce NETosis in neutrophils from mice that expressed human rather than mouse FcγRs in vitro and also in vivo following intravascular deposition of immune complexes.[70] Interestingly, in this context, FcγRIIa rather than FcγRIIIb was required for soluble immune complex-induced NETosis. The process was proposed to be ROS independent, as NET production was unchanged in mice that were deficient in the NADPH oxidase protein $gp91^{phox}$, myeloperoxidase or neutrophil elastase. Rather, induction of NETosis was dependent on endocytic internalisation of the immune complexes.[70] Contrasting with this careful analysis of transgenic mice, a separate report with human neutrophils identified that ribonucleoprotein immune complex uptake was equally dependent on both FcγRIIa and FcγRIIIb.[71] Interestingly, the induction of NETosis by ribonucleoprotein immune complexes was dependent on mitochondrial ROS. This is not affected in CGD patients who lack a functional NADPH oxidase,[72] providing an explanation for the intact NETosis observed with neutrophils from mice mutants unable to generate a respiratory burst.

10 | SUMMARY

Immune complexes stimulate neutrophil degranulation and ROS production and, by inducing inflammatory mediator production, also promote the recruitment of further neutrophils. In some circumstances, immune complexes can induce NETosis. All of these would generally indicate a pro-inflammatory event. Yet the data summarised above show, that immune complexes also induce their own uptake, thereby reducing any pro-inflammatory stimulus. Immune complexes moreover induce neutrophil apoptosis, a function associated with the resolution of inflammation. Compared with the pro-inflammatory effects of immune complexes, our understanding of their pro-resolution effects remains very limited. It will be interesting to analyse these facets further in future. How is the balance between neutrophilic pro-resolution and pro-inflammatory functions induced by immune complexes disturbed in autoimmune disease? Could it be possible to re-adjust this balance therapeutically?

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