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The Translation Potential of Harnessing the Resolution of Inflammation

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13 June 2023

THE TRANSLATIONAL POTENTIAL OF HARNESSING THE RESOLUTION OF INFLAMMATION.

Dear Dr Galipeau,

I hope this find you well.

Please find attached our re-vamped manuscript in line with the referee's comments and editorial requirements, which are summarised below:

Reviewer 1. Manuscript has been improved.
Response: Thank you.

Reviewer 2. *Major concern – differentiating pro-resolution processes communicable and non-communicable diseases.*

Response: Please alterations made to sentences 124-126. Highlighted in red.

Minor concern - mentioning pandemics.
Response: We prefer to leave this in as it's a real and present danger and has highlighted, more than ever, the role immunology and pharmacology plays in society today. Given the scale of the last pandemic, we feel that it also impresses upon the reader the need to invest in developing new drugs/treatments for (the likely) next one.

Minor concern – line 33 and line 37

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Response: Clarified, highlighted in red (lines 33 and 37). "*To name but a few"* has been deleted.

Minor concern – Line 160-161 (point i).

Response. Clarified now on line 164 in red.

Editorial requirement - Endnote

Response. Corrected as requested.

Finally, we thank you very much for asking us to write this article.

All the very best

Derek W Gilroy

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THE TRANSLATION POTENTIAL OF HARNESSING THE RESOLUTION OF INFLAMMATION

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These are heady times in inflammation research. While immunology and pharmacology have always been important, rarely have they been so popular. The pandemic caused by *SARS-CoV-2* is a reminder of this, and with four such outbreaks in the past 20 years, including SARS (2002), Swine flu (2009), Middle East Respiratory Syndrome (2015) and COVID-19, it is not unreasonable to expect another pandemic soon.

Another problem is the impact of age on our immune systems. This is often referred to as "inflammaging" or immunosenescence. Complications of ageing include multimorbidities (two or more illnesses or diseases occurring in the same person at the same time), prolonged wound healing, vaccine inefficacy and frailty (1, 2). With persons aged 60 or above expected to rise from 962 million globally in 2017 to 2.1 billion in 2050, and 3.1 billion in 2100, this will result in financial strain on society and increased pressure being placed on national healthcare systems (https://www.who.int/news-room/fact-sheets/detail/ageing-and-health). In addition, we are yet to find cures for non-communicable diseases such as rheumatoid arthritis, cancer, psoriasis, asthma, systemic lupus erythematosus, multiple sclerosis, and atherosclerosis~~, to name but a few~~. In short, there is a great deal to do.

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Understanding the pathogenesis of communicable and non-communicable ~~these~~ diseases has been a Herculean effort that has borne comparatively little fruit despite the financial, academic, creative, and technological input. In addition to playing "safe science", there are significant experimental hurdles in the way, including an overreliance on rodent models of inflammation. Certainly, experiments on humans offers the advantage of being directly relevant to human physiology and medicine. However, human models of inflammation are limited by ethical and practical considerations, such as the difficulty of obtaining samples, a limited number of patients suitable for study, aging, multimorbidity, polypharmacy and the inability to glean mechanistic proof of a hypothesis in the definitive manner afforded by rodents.

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Nonetheless, we have been very good at treating the symptoms of chronic inflammatory diseases by blocking pathways that drive inflammation, namely heat, swelling, redness and pain. Such treatments primarily include non-steroidal anti-inflammatory drugs, biologics, and steroids. However, these medicines do not bring about a cure, are ineffective in subsets of patients, and have side effects. Thus, there is a need to identify more effective and safer therapeutics to treat chronic inflammatory diseases. Consequently, attention has turned to the other end of the inflammatory spectrum, resolution, to understand the endogenous processes involved in switching off inflammation. The idea is to identify novel internal counter-regulatory systems that can be harnessed for therapeutic gain, and that have fewer side effects compared to current anti-inflammatory medicines (3).

Inflammatory resolution is being extensively studied and is demonstrated to be an active process with quantifiable indices and specific physiological requirements (4). Key determinants of resolution include clearing pathogenic stimuli through the phagocytosis-mediated generation of reactive oxygen species in the pathogen-containing vacuole, fusion with intracellular phagocyte granules or NETosis. Once the pathogen is neutralised, there is a catabolism of pro-inflammatory mediators *via* adsorption onto the surface of apoptotic bodies or scavenging by D6 - a decoy receptor expressed at low levels on leukocytes, but at high levels on trophoblasts and on endothelial cells of lymphatic afferent vessels in the skin, gut and lung. Deletion of D6 in mice predisposes to the development of chronic inflammation. The resolution cascade continues with pro-inflammatory signalling pathways being silenced, a surprisingly understudied aspect of resolution biology. These include LRRC33, which inhibits TLR4/NF-κB activation or tristetraprolin, which destabilises mRNA transcripts that encode several diverse pro-inflammatory modulators. These have all contributed to the evidence that resolution is both tractable and druggable, a process that is ripe for drug discovery, and the opportunity to understand the aetiology of chronic inflammatory diseases (5).

Before a discussion on cell death and clearance, in some tissues - the colon, for instance -

hypoxia is a requirement for resolution through several mechanisms, including extracellular acidification, purine/adenosine biosynthesis and the generation of pro-resolving lipid mediators, driven by phagocyte oxygen consumption and stabilisation of HIF-1α. In this regard, the phagocyte effectors of host response are also key regulators of resolution. Consequently, disruptions in the microenvironment can prevent the resolution of acute inflammation and favour chronic inflammatory lesions. The pharmacologic stabilisation of HIF is already showing promise as an anti-inflammatory therapy in human patients (ClinicalTrials.gov: NCT04353791).

When these phagocytes perform their primary function, they must be cleared from the inflamed site. For these cells, death comes in many guises with apoptosis being the most studied, a process that parcels effete cells up for non-phlogistic removal by phagocytes, notably macrophages (6). On this note, efferocytosis is the process by which phagocytes clear and recycle cellular debris and apoptotic cells. The molecular pathways involved include (i) recognition and binding to surface markers on apoptotic cells such as phosphatidylserine *via* TIM4 expressed on the surface of phagocytes, which we found to be downregulated on the phagocytes of elderly people *via* tonically elevated p38 mapkinase activity (7), (ii) engulfment; (iii) signal transduction, where upon binding and engulfing the apoptotic cell, MerTK and STAT 3 dampen pro-inflammatory signalling followed by (iv) degradation, where the phagocyte then uses lysosomal enzymes to degrade the apoptotic cell, after which the remaining fragments are recycled or excreted. On this theme, recent research has shown that apoptotic cell-derived methionine is used by phagocytes to drive pro-resolution pathways and the secretion of immune-dampening signals such as TGFβ (8), **FIGURE 1.**

Taken together, not only does programmed cell death of granulocytes safely remove cellular entities with a great capacity to cause harm - through the release of cytotoxic agents and secretion of antigen from their membrane blebs - but they also programme phagocytes down

a pro-resolution pathway. So important are these events that defects in efferocytosis are believed to underpin the pathogenesis of systemic lupus erythematosus(9) and prolonged inflammation in the elderly(7). Indeed, a significant source of tissue damage in inflammatory conditions, including asthma, rheumatoid arthritis, and inflammatory bowel disease, is attributed to neutrophil non-clearance.

However, pro-resolution processes do not stop at clearing up dead cells and immune debris. Pain must be switched off, and the microvascular hyperreactivity that caused redness must be reversed by mechanisms yet to be fully defined. It is, essentially, dampening down Celsus' four cardinal signs in a manner that was considered, for many years, to be passive in nature. It's around this time that regulatory T cells (Tregs) accumulate in the resolved tissue to maintain immune tolerance. Moreover, we increasingly appreciate that resolution of immune responses to infection is followed by a prolonged phase of immune activity that imprints long-term tissue immunity (10).

So far, so good - by understanding the mechanisms that switch off inflammation, we can develop new therapies that promote diseases driven by chronic inflammation down a pro-resolution pathway. This could lead to the development of more effective and targeted treatments for these conditions, which could improve patient outcomes, and reduce adverse effects. However, pro-resolution pathways are likely species, tissue, stimulus, sex, and perhaps ethnicity specific. For instance, the processes required to resolve inflammation driven by infectious diseases and their post-resolution sequalae (10) are not the same as those required to switch off inflammation driven by, say, non-communicable diseases. Indeed, there is little point in making resolving inflammation resolve faster. Our next step is to understand how immune responses become dysregulated, leading to chronic non-resolving inflammation. In other words, how pro-resolution pathways become silenced, leading to maladaptation to the point of no return. Here, tolerance is broken, endogenous antigens feed the development

Commented [MOU1]: – *differentiating pro-resolution processes communicable and non-communicable diseases.*

of autoimmunity, while epigenetic changes occur in both leukocytes and stromal cells - the latter turning from innocent bystanders into pathogenic drivers of disease.

Hence, inflammatory responses do not always follow the prescribed resolution script. For instance, failed pathogen clearance is the hallmark of Chronic Granulomatous Disease, where neutrophils have impaired production of superoxide due to defects in NADPH oxidase resulting in recurrent and severe infections. Autoimmune Lymphoproliferative Syndrome (ALPS) arises from a mutation in the cell death receptor Fas (CD95), dysregulating lymphocyte homeostasis and hence increasing the risk of autoimmune diseases. Age-acquired defects in efferocytosis in humans lead to immune cell debris accumulation of M1-like macrophage in tissues(7). Besides apoptosis as the preferred mode of death leading to resolution, neutrophils can also die by necroptosis, ferroptosis, pyroptosis, parthanatos, necrosis and NETosis, resulting in more complicated outcomes. While apoptosis takes hours, necrosis, for example, is faster. In necrosis, intracellular damage-associated molecular patterns (DAMPs) leak out of the damaged cell, triggering inflammation *via* TLR2, TLR4, TLR9 and RAGE.

NETosis represents a unique form of cell death resulting in neutrophil extracellular trap (NET) formation. Nuclear chromatin decorated with nuclear proteins, elastase, high-mobility-group protein B1, myeloperoxidase, proteinase-3, glycolytic enzymes and cytoskeletal proteins are released from neutrophils into the extracellular environment. NETs help eradicate infections and promote the resolution of neutrophilic inflammation by degrading cytokines and chemokines, as well as sterile crystal-mediated inflammation. NETs, however, can also promote the post-translational modification of proteins and other macromolecules, whilst revealing autoantigens such as DNA and histones to the immune system, increasing the risk of autoimmune disease. Consequently, a variety of inhibitors that prevent NET formation as well as molecules that degrade NETs are under investigation for the treatment of inflammatory diseases. However, as with most modulators of host defence, NET inhibition in animal models

increases susceptibility to infections and decreases neutrophil functions involved in innate immunity.

Hence, besides inherent or acquired defects in resolution, there are many factors that determine whether inflamed tissues resolve or progress to chronicity, including (i) the pathogenic nature of the infection, (ii) severity of tissue injury, (ii) immune cell infiltrate veering from hypoxia to "inflammatory acidification" triggering glycolysis and lactic acid secretion, (iv~~ii~~) the magnitude of tissue damage, and (~~i~~v) modes of immune cell death, **FIGURE 2**. On this note, when a neutrophil switches from apoptosis to other forms of unwanted cell death in the evolution of chronic inflammatory disease remains unknown. Investigations have demonstrated that only a subpopulation of neutrophils undergoes NETosis, suggesting heterogeneity within neutrophil populations. In systemic lupus erythematosus, for instance, low-density granulocytes have a greater tendency to form NETs compared to normal density granulocytes, such that an expansion in low-density granulocytes in lupus might explain a link between this disease and NET formation. This would suggest that a chronically activated immune system "enriches" for cells and pathways that drive towards maladaptation and multimorbidities.

This is the "real life" challenge of curing chronic inflammatory diseases - resetting immunity, invoking tissue homeostasis, and restoring proper function. Moreover, we need to remember that this is a challenge of the human condition, complete with multimorbidities, immunosenescence and polypharmacy, which do not beset rodents. Therefore, we need more appropriate experimental human models of disease and approaches to identify pro-inflammatory pathways that suppress resolution in a context-specific manner. Additionally, early diagnosis and activation of pro-resolution pathways will be more effective than trying to cure maladapted chronic inflammation.

In summary, the reality is that we are faced with the challenge of driving chronic inflammation down a pro-resolution pathway. Whether engaging pro-resolution mechanisms is sufficient to do so - should they be active and amenable to manipulation in the chronic disease of interest - has yet to be fully proven. Perhaps inhibiting pro-inflammatory signals is sufficient to trigger spontaneous resolution. However, it is also possible that some diseases may be too advanced and maladapted, such that pro-resolution pathways are permanently repressed or dysregulated, and that some other novel approach is required. That notwithstanding, for those in the field of resolution biology, there is much to do, and much to learn. It seems that while we need to continue identifying the factors that drive acute resolution, we also need to identify those that subvert it.

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Figure 1, Classic Resolution. Following infection, locally released chemokines and cytokines as well as upregulated cell adhesion molecules facilitate granulocyte accumulation into tissues.

Granulocytes such as neutrophils phagocytose pathogens and die by apoptosis. Phosphatidylserine, expressed on the surface of apoptotic cells, engages with macrophage (M_{φ}) TIM-4 in preparation for efferocytosis. This has the dual effect of clearing dangerous granulocytes along with their dangerous cargo of histotoxic agents and endogenous antigens within their cell surface blebs, in a manner that programs M_{φ}s down a pro-resolution/wound healing pathway.

The resolution cascade continues with pro-inflammatory signalling pathways being silenced, a surprisingly understudied aspect of resolution biology. These include LRRC33, which inhibits TLR4/NF-κB activation or tristetraprolin (TTP), which destabilises mRNA transcripts that encode several diverse pro-inflammatory modulators

This is followed by the infiltration of Tregs, which maintain immune tolerance.

M_{φ}s clear immune debris, drive wound healing, maintain tolerance & resolve inflammatory responses.

Figure 2. Inflammation clears infection, heals wounds & restores homeostasis - **Resolution.** But, it can be over-exuberant from the start & fail to resolve leading to **chronic inflammation**. The reasons for this are many and varied - Inherent or acquired defects in pathogen clearance or efferocytosis, respectively. In addition, there is (i) the pathogenic nature of the infection, (ii) immune cell infiltrate veering from hypoxia to “inflammatory acidification” triggering glycolysis and lactic acid secretion, (iii) the magnitude of tissue damage, and (iv) modes of immune cell death leading to DAMPs, Nets and tissue stress.

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Figure 1, Classic Resolution. Following infection, locally released chemokines and cytokines as well as upregulated cell adhesion molecules facilitate granulocyte accumulation into tissues.

Granulocytes such as neutrophils phagocytose pathogens and die by apoptosis. Phosphatidylserine, expressed on the surface of apoptotic cells, engages with macrophage (M_φ) TIM-4 in preparation for efferocytosis. This has the dual effect of clearing dangerous granulocytes along with their dangerous cargo of histotoxic agents and endogenous antigens within their cell surface blebs, in a manner that programs M_φs down a pro-resolution/wound healing pathway.

The resolution cascade continues with pro-inflammatory signalling pathways being silenced, a surprisingly understudied aspect of resolution biology. These include LRRC33, which inhibits TLR4/NF-κB activation or tristetraprolin (TTP), which destabilises mRNA transcripts that encode several diverse pro-inflammatory modulators

This is followed by the infiltration of Tregs, which maintain immune tolerance.

$M\varphi$s clear immune debris, drive wound healing, maintain tolerance & resolve inflammatory responses.

Figure 2. Inflammation clears infection, heals wounds & restores homeostasis - **Resolution.** But, it can be over-exuberant from the start & fail to resolve leading to **chronic inflammation**. The reasons for this are many and varied - Inherent or acquired defects in pathogen clearance or efferocytosis, respectively. In addition, there is (i) the pathogenic nature of the infection, (ii) immune cell infiltrate veering from hypoxia to “inflammatory acidification” triggering glycolysis and lactic acid secretion, (iii) the magnitude of tissue damage, and (iv) modes of immune cell death leading to DAMPs, Nets and tissue stress.

Figure 1

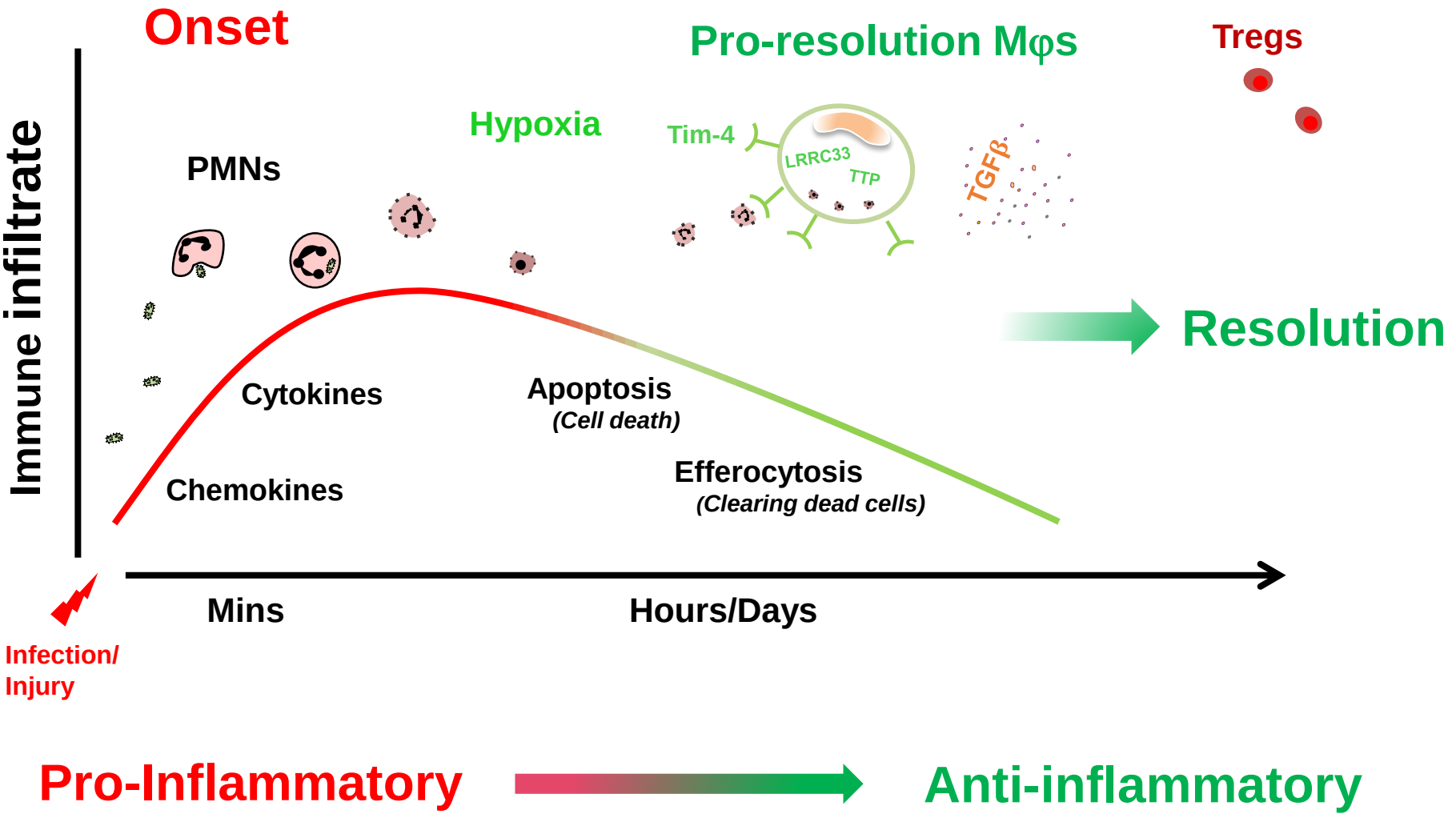

Figure No. 2

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Figure 2