Innate Immune Memory in Fibroblasts

by

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Abstract

The innate immune system is a generic response to infection or injury. Evidence shows the innate response has immunological memory capable of altering subsequent responses to stimuli. Fibroblasts are ubiquitous stromal cells capable of responding to inflammatory triggers, and of orchestrating endothelial cell and leukocyte behaviour during inflammation.

Repeated challenge with cytokines (such as tumour necrosis factor (TNF) α) induced an augmented second response to stimulation. Fibroblasts from multiple anatomical locales significantly increased cytokine secretion upon second challenge with TNFα. The precise mediators augmented depended on fibroblast site of origin. Depending on site, memory was inherent, or only present in fibroblasts from chronically-inflamed tissue. This suggests a phenomenon intrinsic to some sites but pathological in others.

The secreted mediators from the fibroblast initial or memory responses exerted differing effects on leukocytes, dependent upon fibroblast site of origin. Finally, examination of intracellular signalling showed the augmented response was at least partly due to prolonged activity of nuclear factor (NF) κB during the memory response.

Innate immune memory exists in fibroblasts from multiple tissues, but may be pathologically acquired in some. The altered response to second challenge may represent a fibroblast mechanism for altering the recruitment and behaviour of the inflammatory infiltrate.

Acknowledgements

This PhD was, without doubt, the hardest thing I've ever done. Luckily, I had a great group of colleagues, family and friends, without whom I wouldn't have reached this stage.

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All work and no play makes Jack a dull boy, and I have certainly proven that plenty of times. Fortunately, I have very understanding friends. Thanks to Rob, Laura, Mooney, Kate and Thewlis, who I don't see enough, and the Woodleigh Wolfpack, who made living in Birmingham so fun. I would also like to thank Martin and Janet Lester, who generously offered me their attic for my hermit hole during my write up.

I don't know how to express my thanks to my parents. They have encouraged, supported and loved me through the good times and the bad. Their common sense resolved so many problems I couldn't see through, and when my motivation failed, remembering that they are proud of me has been all I needed to get back on my feet.

My final thanks must go to Tamsin, who has supported me in every way possible. She helped me remember there's a world outside of the lab, and has been the most important part of it for me.

It isn't in my nature to be earnest. There are, however, few occasions more deserving of gravity than this. So, to Tamsin, my parents, and everyone listed above, I would like to offer my most sincere thanks.

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Abbreviations

ACPA anti-citrullinated protein antibody

AP-1 activator protein 1

APC antigen presenting cell

ARE AU-rich elements

BAFF B cell activator of TNF family

BCG Bacillus Calmette–Guérin

BCR B cell receptor

BMDF bone marrow-derived fibroblast

BMX Bone marrow tyrosine kinase on chromosome X

CCL C-C motif ligand

CD cluster of differentiation

CIA collagen-induced arthritis

CM conditioned medium

COPD chronic obstructive pulmonary disease

CTLA4 cytotoxic T lymphocyte antigen 4

CXCL C-X-C motif ligand

DABCO 1,4-diazabicyclo [2.2.2] octane

DAMP damage-associated molecular pattern

DAPI 4', 6-diamidino-2-phenylindole

DNA deoxyribose nucleic acid

DNMT DNA methyl transferase

DTT dithiothreitol

EC endothelial cell

ECL enhanced chemi luminescence

ECM extra cellular matrix

ERK extracellular signal-regulated kinase

ET endotoxin tolerance

FAP fibroblast activating protein

Fc fragment crystallisable

FLS fibroblast-like synoviocyte

GM-CSF granulocyte macrophage-colony stimulating factor

gp130 glycoprotein 130

gp130-RAPS gp130 rheumatoid arthritis antigenic peptide-bearing soluble form

HAT histone acetyl transferase

HDAC histone deactelyase

HDF human dermal fibroblast

HGF human gingival fibroblast

HLA human leukocyte antigen

HLF human lung fibroblast

HTF human tonsil fibroblast

HuR Human antigen R

HUVEC human umbilical vein endothelial cell

ICOS include T cell co-stimulator

IFN interferon

Ig immunoglobulin

IKK inducer of κB kinase

IL interleukin

IL-1RA interleukin 1 receptor antagonist

IL-1-RAP interleukin 1 receptor accessory protein

IL-6R interleukin 6 receptor

iNOS inducible nitric oxide synthase

IPF idiopathic pulmonary fibrosis

IκB inhibitor of NFκB

JNK c-Jun N terminal kinase

LIF leukocyte inhibiting factor

LPS lipopolysaccharide

M1 type 1 macrophage

M2 type 2 macrophage

MAP2K MAPK kinase

MAP3K MAPK kinase kinase

MAPK mitogen-activated protein kinase

M-CSF macrophage-colony stimulating factor

MHC major histocompatibility complex

miR micro RNA

MLS macrophage-like synoviocyte

MMP matrix metalloproteinase

NEMO NFκB essential modulator

NET neutrophil extracellular trap

NFκB nuclear factor κ B

NFκBIA NFκB inhibitor A

NIK NFκB inducing kinase

PAMP pathogen-associated molecular pattern

PD periodontitis

PDPN podoplanin

Ps psoriasis

PTPN tyrosine-protein phosphatase non-receptor

RA rheumatoid arthritis

RANKL receptor-activated nuclear factor κ B ligand

ROS reactive oxygen species

SAR systemic acquired response

SCID severe combined immune deficiency

sIL-6R soluble interleukin 6 receptor

STAT Signal transducer and activator of transcription

TB tuberculosis

TCR T cell receptor

TF transcription factor

TGF transforming growth factor

Th helper T cell

TIMP tissue inhibitors of metalloproteinase

TL1A Tumour necrosis factor-like ligand 1A

TLR toll-like receptor

TNF tumour necrosis factor

TNFR tumour necrosis factor receptor

Treg regulatory T cell

TRIF TIR-domain-containing adapter-inducing interferon-β

TTP Tristetraprolin

UTR untranslated region

VCAM vascular cell adhesion molecule

1 Introduction

1.1 Inflammation

Inflammation is the healthy response of a multicellular organism during infection or injury. Both of these scenarios involve perturbations from the resting state, and their resolution is required in order to return to this state (repaired tissue or non-infected status). In mammals, the inflammatory response is highly complex, and intricately detailed. Moreover, it is finely tuned to respond to the precise event that induced the inflammatory episode.

Despite decades of research, our understanding of inflammation is still incomplete. What we do know would take more space than I have, even summarised as concisely as possible. In this chapter, I will describe some of the main cells and mediators involved in the inflammatory response, and how that inflammatory response may go awry, resulting in disease.

1.1.1 The innate and adaptive immune system

The simplest division of the mammalian immune system is into innate and adaptive arms. The innate immune response is a generic inflammatory response occurring rapidly following an insult or in the early stages of an infection. It is conserved throughout vertebrates, invertebrates, and even exists in some form in plants. The innate immune cell types discussed herein are monocytes, macrophages and neutrophils. Whilst these cells are described briefly below, the concept of innate memory is examined to a greater extent in chapter 3.

A more evolutionarily advanced mechanism is the adaptive response. This is formed by lymphocytes: The T and B cells. Adaptive memory evolved in jawed

fish, some 450 million years ago, and is perceived as the more sophisticated immune response. Adaptive immunity is antigen-specific, allowing a greater degree of specificity in the immune response to inflammatory triggers. A crucial aspect of the adaptive arm of the immune system is the ability to store ‘memory’: an increased response to second challenge with the exact same antigen. This concept is described briefly below, but chapter 3 will show that the concept of memory is not so strong a delineator of adaptive versus innate immune systems.

1.1.2 T cells

T cells are lymphocytes produced and educated in the thymus. Their signature surface marker is cluster of differentiation (CD) 3, part of the T cell receptor (TCR) complex used to recognize epitopes on self and non-self- antigens. The selection of T cells (which is not discussed, for the sake of brevity) is a multi-step process in which the cell must undergo binding of antigens, and it is assessed for strength of affinity. If it fails any of the tests during its development, it is destroyed, to avoid the circulation of useless or self-reactive T cells.

Activation of T cells (reviewed in [1]) occurs by three signals. The first is the TCR, which is ligated by antigen presented by major histocompatibility complex (MHC). Antigens are usually offered by professional antigen presenting cells (APC) although all nucleated cells express MHC. MHC class I present intracellular antigens. Whilst these are often self, and therefore not reacted to by the T cell, virally-infected cells produce viral proteins, and as such foreign proteins will be presented in MHC-I. Alternatively, MHC-II presents extracellular antigens to T cells. These are gained by endocytosing extracellular proteins and digesting them, before trafficking to the cell surface to be presented with MHC II.

Recognition of a cognate antigen induces activation in the T cell, but this is very short lived. If T cells only receive signal 1, they become anergic: a non-responsive state with no effector function. If however, all three signals are received but the TCR is chronically-stimulated, the T cell becomes exhausted, and loses its effector function too [2].

The second signal is induced by co-stimulatory proteins like CD80 or CD86 on the APC. Lymphocyte CD28 is bound by CD80/86 and is the best studied signal 2 (reviewed in [3]). $CD28^{-/-}$ mice have decreased T cell proliferation after TCR activation [4]. This is not a complete abrogation of T cell function however. This may be because other ligand receptor pairings exist (like inducible T cell co-stimulator (ICOS), which can also be inhibited to reduce T cell responsiveness [5]), or because some suggest that signal 2 modulates or enhances T cell activation, rather than acting as a second ‘on switch’ [3].

Finally, signal three is stimulation by cytokines, which has been shown as required for optimal gene expression [6-8] and memory T cell production [7]. The sequence of signals is structured, as signal 1 is required before signal 2, and signal 3 must be provided within 24h of signal 2 in order to induce an augmented gene expression [7].

There are multiple subsets of T cells which perform different roles. Whilst these roles are tightly linked to signature transcription factors, the supposed terminal differentiation of effector T cells is now being disproved, and evidence is growing to demonstrate the plasticity of T cells [9].

CD8+ T cells are cytotoxic, and respond to MHC class I with its associated antigens. These antigens often signal cell infection by viruses, and recognition of

MHC I-loaded antigen by CD8+ T cells induces rapid and massive proliferation to facilitate the cytotoxic response [10], and the production of memory CD8+ T cells. Depending on the infection, CD8+ T cells can migrate to the infection site in a CD4+ T cell-dependent or independent manner (discussed in [11]). CD8+ T cells can release IL-10, suggesting a regulatory role. This only occurs in the early stage of inflammation, compared to CD4+ IL-10-producing T cells can be found throughout the inflammatory episode [12].

CD4+ T cells form the ‘helper T cells’, so called because of their support for B cells. Th1 and Th2 cells have been known for decades, and are antagonistic. Th1 release interferon (IFN) γ and interleukin (IL) 12 in order to combat intracellular pathogens. They also induce other cell types to respond to pathogens, such as the IFNγ-induced production of inducible nitric oxide synthase (iNOS) by macrophages. Th2 cell secreted products include IL-4, IL5, IL-10 and IL-13, which combat extracellular infections but drive allergic responses.

Th17 were discovered much later than the classic dichotomy of effector T cells. Their signature cytokine is IL-17 (IL-17A and IL-17F), but also secrete CCL20 and IL-21. They are used in the response to extracellular bacteria and fungi, but have been implicated in multiple autoimmune conditions [13]. IL-17 is found at high concentrations in inflamed sites, and can not only stimulate resident cells, but recruit other cell types, such as monocytes and neutrophils [14].

Regulatory T cells (Tregs) are suppressors of the T cell response, and come in several forms. Natural Tregs are produced in the thymus, whilst inducible Tregs are converted into a regulatory phenotype by micro environmental factors in the peripheral tissue [15]. Their suppressive role is both via cell contact and secreted

mediators. Cytotoxic T lymphocyte antigen (CTLA) 4 antagonizes the co-stimulator receptors by binding and thus removing CD80/86, therefore inhibiting prolonged T cell activation [16] and proliferation [17]. They also secrete anti-inflammatory mediators such as IL-10 to suppress inflammatory functions of T cells and other cell types. Tregs therefore play an important role in suppressing the T cell response [18, 19], and suppressing autoimmunity when T cell responses are against self-antigens [20].

1.1.3 B cells

B cells are the other cell type of the adaptive immune system. Developed in the bone marrow, they share several features with T cells, such as a highly specific and almost infinitely diverse B cell receptor (BCR) and co-stimulator receptors. Development of B cells from pro-B, to pre-B, to immature B cell all occurs in the bone marrow, and the advance through development is linked to distinct stages of BCR production (reviewed in [21]).

Once immature B cells leave the bone marrow they circulate in blood, and can migrate to secondary lymphoid organs to mature. Mature B cells may reside in tissue (such as the splenic marginal zone) or egress back into the circulation, and intermittently ingress back into tissue to form germinal centres for the assessment of antigens. Movement between tissue and circulation is carefully balanced by relative abundances of chemo attractants. CXCL12 is produced by stromal cells to recruit B cells into the tissue [22], whilst sphingosine-1 phosphate is released by blood born cells and draws B cells back into circulation [23].

B cells can have regulatory roles, as proven by the regulatory B cell, which can limit inflammatory responses through anti-inflammatory cytokines like IL-10 [24].

The classical role of B cells is to produce antibodies against innumerable antigens. Activation of the BCR results in clonal expansion of B cells, which develop into memory B cells and effector cells called plasma cells. Plasma cells produce antibodies capable of binding the antigen that triggered their specific BCR. Antibodies can be membrane bound or secreted, and play several roles in the immune response (reviewed in [25]). Briefly, they may inhibit pathogen motility, adhesion or cell entry, and may activate complement to be lytic or act as chemo attractants for immune cells. They also function in contact with immune cells, such as in the formation of immune complexes, wherein the Fc portions of immunoglobulins (Ig) are bound by cellular Fc receptors to trigger or modulate inflammatory responses. They also include phagocytosis, or antibody-dependent cellular cytotoxicity, which can induce cell death in opsonized pathogens or infected host cells.

Unsurprisingly, the BCR is necessary for survival of B cells, but can be inhibited either directly or through inhibiting signalling molecules downstream of it. This is a useful treatment in cancer [26] or autoimmune diseases [27, 28], wherein antibodies against self-antigens can elicit pathologic immune responses against self. Selection of B cells is via the BCR during development [29], and whilst auto-reactive cells are usually removed, self-reactive B cells exist in an anergic state even in healthy individuals [30]. This is a possible ‘leak’ in the selection system, and may be the root of auto-antibodies arising in various chronic inflammatory diseases. B cells reactive to DNA are one example [31], and it has been shown that anti-DNA antibodies exist in autoimmune diseases like rheumatoid arthritis [32].

As with T cells, B cells have co-stimulatory ligands and receptors. Genetic deletion of costimulatory receptors on B cells results in decreased antibody responses and greater susceptibility to infections [21]. Inhibition of costimulatory signals on B cells can however increase antibody production, suggesting a negative regulatory roll [25].

This discrepancy may be due to different signalling requirements of different forms of B cells. B cell activator of TNF family (BAFF) is a crucial survival signal for immature and mature B cells [33, 34], but not plasma cells [35, 36]. Plasma cells and memory B cells are two long-lived cell types, which form from effector B cells following T cell-dependent antigen stimulation. Plasma cells may be especially dangerous in autoimmunity, as they are long-lived, can survive the B cell-specific toxicity of Rituximab (an anti-CD20 antibody), and receive survival signals from IL-6 and hyaluronic acid [37] (both of which are high in chronic inflammatory diseases).

1.1.4 Monocyte/ Macrophages

Macrophages were recognized in 1893 as a tissue-resident ‘big eater’. This referred to their capacity for phagocytosis. Macrophages have been described as the bridge between innate and adaptive immunity because of their roles in both arms of the immune system. As innate cells, they patrol tissue to detect damage via danger-associated molecular patterns (DAMPs) and infection via pathogen-associated molecular patterns (PAMPs). They can respond to these via numerous receptors, such as toll-like receptors (TLRs). Responses include the release of cytokines, chemokines, reactive oxygen species (ROS), and the phagocytosis of debris or cells (whether foreign, infected or dead).

Phagocytosis is crucial in facilitating resolution of inflammation (by removing dead cells and debris from the tissue, and altering macrophage phenotypes towards pro-resolving cells [38]) but also to their role in the adaptive immune system. Macrophages and dendritic cells are APCs. Once endocytosed products are degraded in the cytotoxic phagolysosome, they are trafficked to the surface to be presented on MHC to be scrutinised by lymphocytes. As already discussed, recognition of a cognate antigen induces a lymphoproliferative reaction and thence an adaptive response by effector lymphocytes, and the production of memory lymphocytes for future defence.

Macrophages were originally thought to become tissue-resident following bone marrow haematopoiesis and circulation of monocytes, which would then settle in tissue. However macrophages have now been shown to reside in tissue from a time preceding bone marrow haematopoiesis [39]. This means tissue-resident macrophages can be yolk sac-derived, although they are certainly replenished by infiltrating monocytes, both through homeostasis and during inflammatory reactions [40].

Macrophages *in vitro* can be differentiated into distinct subsets. Monocyte-colony stimulating factor (M-CSF) induces M2, or alternatively-activated macrophages, whilst granulocyte macrophage colony stimulating factor (GM-CSF) induce M1 ‘classical macrophages’ [41]. These are seen as pro-wound healing and proinflammatory. Tissue-resident macrophages have been suggested to be akin to M2 in their normal state [42], although a large degree of plasticity exists [43-45]. M1 macrophages are characterized by TNFα, IL-1β, IL-12, iNOS, and surface expression of MHCII. M2 macrophages are characterized by surface expression of mannose receptor (CD206) and secretion of IL-10 and TGFβ.

The M1-M2 differentiation was traditionally described following colony stimulating factors and IFNγ for the former or IL-4 for the latter. This matched the Th1 and Th2 T cell responses, providing a useful parallel between the intracellular and extracellular pathogen responses of T cells and macrophages. The M1-M2 paradigm should be treated with caution, as further categorization is well recognized. Indeed, in our own laboratory ‘M1-like’ and ‘M2-like’ are used as descriptive short hand terms, rather than in their strict definitions. The field now looks at macrophage polarization as a spectrum rather than a binary action. This is simply illustrated in figure 1.1.

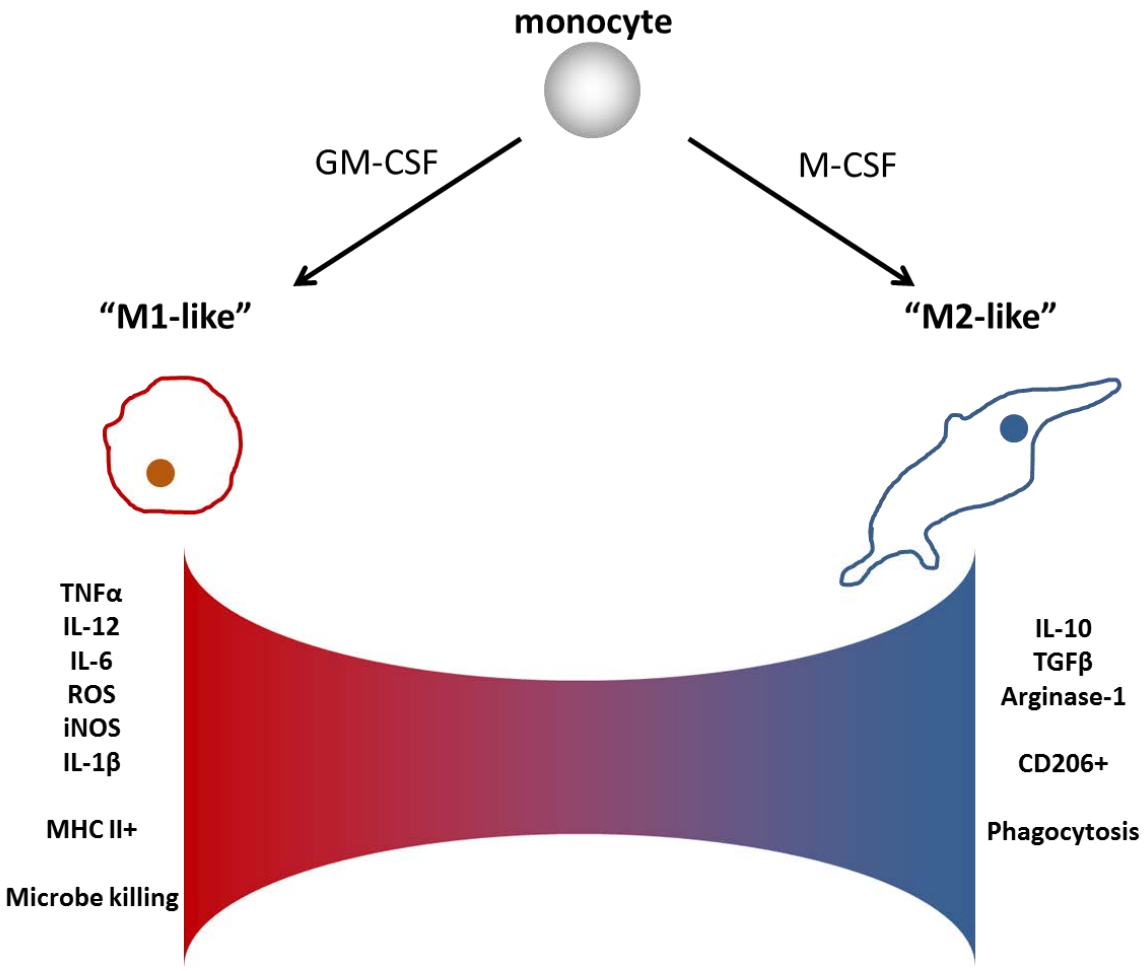

Figure 1.1: The differentiation of monocytes into polarized macrophages.

The polarization of macrophages is simplified into M1 and M2 subsets, with known characteristics. These are artificially induced *in vitro*, whilst a spectrum (indicated by the graduated bar) exists *in vivo*. The secreted mediators, markers and functions shown are not exhaustive.

Monocytes are formed in bone marrow haematopoiesis and circulate in the blood, responding to chemokine gradients in times of inflammatory need. They extravasate in to tissue, where they subsequently differentiate into macrophages, but also act as effector cells in their own right.

Monocytes are largely defined by surface expression of CD14 (LPS co-receptor) and CD16 (FcγIII receptor). 'Classical' $CD14^{hi}$ CD16- cells account for the larger percentage of circulating monocytes. CD14+ $CD16^{hi}$ cells are supposedly the 'inflammatory' subset, and are expanded during inflammatory episodes [46]. This subset produces the highest concentrations of IL-6, IL-1 and TNF [47], and induce Th1 and Th17 differentiation [46, 48, 49]. They are also the best APC monocyte population [50], and the best at inducing Th1 responses [50, 51]. Further, macrophages originating from CD14+ $CD16^{hi}$ monocytes maintain a distinct phenotype from those differentiated from classical monocytes, including an enhanced ability to phagocytose [52]. An intermediate population of $CD14^{hi}$ CD16+ monocytes (classified as a distinct population by [53]) are found to secrete the highest concentration of IL-10 [47, 54] and IL-1receptor antagonist (IL-1RA) [47]. The three populations are shown in figure 1.2 below.

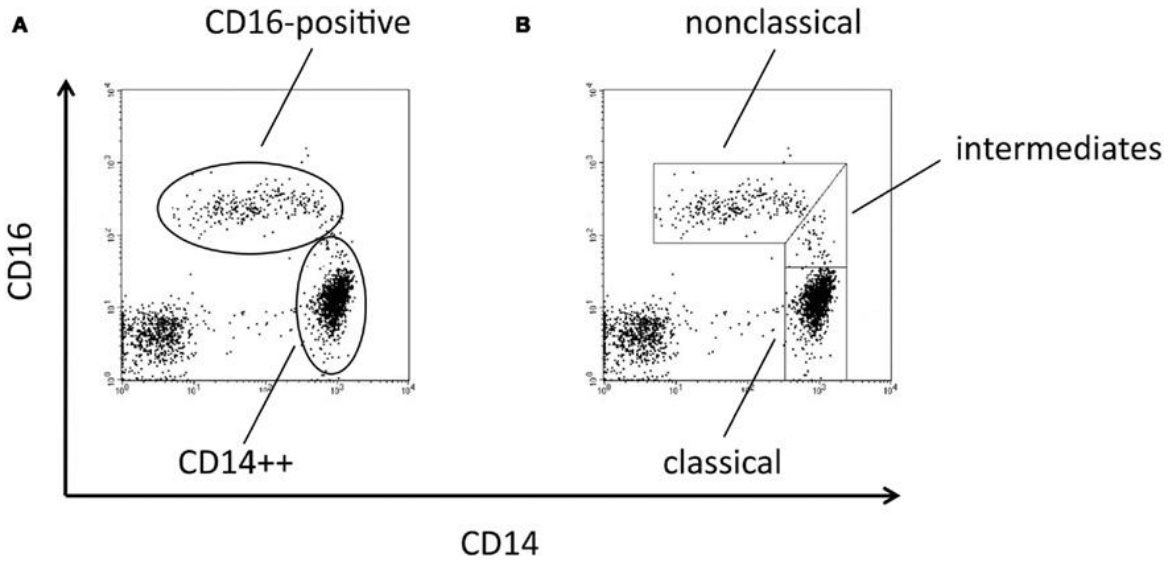

Figure 1.2: A two-subset, and three-subset model of monocyte subsets. classification. Monocytes are identified by CD14 and CD16 surface expression. **A** Traditionally they have been viewed as classical and non-classical (CD14hi CD16- and CD14+ CD16hi, respectively). **B** the new categorization accounts for an intermediate population of CD14hi CD16+ cells. Take from Zietler-Heitbrock et al (2013) [53].

1.1.5 Dendritic cells

Dendritic cells (DC) are professional APCs, and are the best cells for promoting T activation [55]. They do this via collection of antigens for presentation of MHCII, which they express at high levels. DC then home to lymphoid organs to present said antigens to T cells for immune surveillance and induction of the adaptive response.

Whilst their role sounds similar to that of macrophages, the two are distinct cells. One example is the efficient lymphoid homing of DC, compared to the competent phagocytic capability of macrophages. These two cells haves traditionally been viewed as separate products of monocyte differentiation, and indeed recent publications still describe monocyte-derived DC, or DC-like monocyte-derived cells [56, 57].

The difference between macrophages and DC was recognized as far back as 1973, with morphological distinctions [58]. As technology has advanced, we can now distinguish monocyte/macrophages from DCs with transcriptional profiling and cyTOF technologies [59, 60]. It is now largely accepted that macrophages and DC are distinct [61]. The difficulty in distinguishing between them can be forgiven however, as surface markers such as CD64 (a macrophages marker) have now been shown on certain subsets of DC [57].

DC derive from a progenitor distinct from that of monocytes. This common DC progenitor gives rise to plasmacytoid DC (which terminally differentiate in the bone marrow [62]) and classic DC (cDC), which can in turn be divided into cDC_1 and cDC_2. The pre-cDC migrate via the bloodstream into lymphoid and non-lymphoid tissue before terminally-differentiating.

1.1.6 Neutrophils

Neutrophils are polymorphonuclear cells, so called for their irregular nucleus shape. They are the predominant inflammatory cell in the circulation, and the first cell to extravasate in most inflammatory episodes. Their lifespan in circulation is less than 24h, after which they undergo controlled apoptotic cell death. Once in tissue, however, longevity is increased [63].

They are recruited by numerous mediators, the archetypal one being IL-8. Neutrophils stimulated in tissue also release IL-8, thus establishing a feedback loop of recruitment to inflamed tissue. Alongside chemokines, neutrophils secrete a number of factors which are mostly proinflammatory, as befits a rapid-response inflammatory cell. They do not produce anti-inflammatory IL-10 [64], but do release cytokines, ROS and degradative mediators, such as matrix metalloproteinases (MMP), neutrophil elastase and cathepsin G (reviewed in [65]). They also upregulate receptor-associated nuclear factor κ B (NFκB) ligand (RANKL) expression [66], thereby facilitating osteoclastogenesis.

Neutrophils have several other roles in inflammation. Upon entry into tissue and during apoptosis neutrophils shed IL-6 receptor (IL-6R), an important signalling event that is discussed later. They also provide survival factors for B cells in tissue [67] and (in a non-inflammatory process) to follicular B cells in the marginal zone [68]. They can also stimulate tissue stroma and infiltrating T cells [69].

Phagocytosis plays an important part in neutrophils' function and death. They have strong phagocytic abilities, and are highly cytotoxic to endocytosed cells. When aged, neutrophils undergo carefully orchestrated apoptosis, and then are rapidly cleared, as the highly inflammatory granzymes they disgorge would be

released indiscriminately upon necrosis. Upon apoptosis, neutrophils flip out their inner membrane, exposing phosphatidylserine, which acts as a marker for macrophages to phagocytose the neutrophils and thus remove the danger of tissue damage [70]. This process of removing apoptotic cells is called efferocytosis [71], and is an interactive process between dying cells and phagocytes. 'Find me' signals are released by apoptotic cells to recruit phagocytes, and 'eat me' signals (such as phosphatidyl serine) are expressed on the surface of the cell for recognition, followed by subsequent engulfment in efferocytosis [72].

In a more dramatic form of cell death, neutrophils can also undergo NETosis, whereby they release their DNA in neutrophil extracellular traps (NETs) to trap pathogens [73]. Whilst an ingenious mechanism for prolonging the anti-microbial effects of older neutrophils, this does have proinflammatory consequences, which will be discussed later.

1.2 Inflammatory mediators

1.2.1 Tumour necrosis factor (TNF) α

First recognized for its cytotoxicity when administered to tumour cells, TNFα has since progressed to hold a premiere position in our view of inflammatory reactions [74]. It can induce cytokines and growth factors [75-77], adhesion molecules [78], and vasodilatory factors [79], along with mediating a balance between apoptosis and proliferation [80].

TNFα can bind to TNFR1 or TNFR2. The former is largely seen as an inducer of inflammatory responses, whilst the latter appears to have roles in regeneration and T cell proliferation. Whilst they induce distinct pathways [81], it has been

shown that TNFR2 can ‘pass on’ TNFα to TNFR1, suggesting interplay between the two [82]. Perhaps because of the different receptor pathways, and certainly because of the array of consequences of TNFα-release, models and clinical trials have found both therapeutic and pathologic effects of neutralizing TNFα (reviewed in [74]).

Nevertheless, its role in inflammatory conditions is largely seen as pathologic. Over expression of TNFα results in mice spontaneously developing arthritis and inflammatory bowel disease [83], whilst inhibiting TNFα leads to resistance to collagen-induced arthritis (CIA) [84]. Addition of anti-TNFα therapy on a population of cells removed from the rheumatoid arthritis (RA) synovium decreased inflammatory mediators [85], providing further evidence to its contribution to inflammatory diseases.

1.2.2 IL-1

IL-1 is a cytokine with clear links to acute and chronic inflammation. In the same study as above it was seen to be reduced by anti-TNF therapy, whilst inhibiting IL-1 reduced all cytokines except TNFα [85]. This suggests that in RA, a hierarchy exists with TNF as a master regulator, and IL-1 as the next step down the ladder.

Whilst perhaps not preeminent in RA, IL-1 is certainly preeminent in a number of other conditions. Belonging to a family of 11 members, the main ligands are IL-1 are α and β. The latter was found to be the major IL-1 protein in RA [85]. Both can bind receptors to elicit responses, but IL-1α is also capable of acting within its cell of origin, to facilitate inflammatory gene transcription [86]. IL-1β must be cleaved from pro-IL-1β before it can induce its functional effects. This occurs by inflammatory triggers inducing the formation of the inflammasome, which cleaves

and therefore activates caspase-1, which in turn cleaves pro-IL-1β into biologically active IL-1β [87]. Pro-IL-1α however, can act as an alarmin when released by necrotic cells [88]. Despite some differences, the downstream signalling following IL-1α or β signalling are very similar [89].

IL-1 can function to increase neutrophil survival [90], and elicit production of cytokines [85] and matrix degrading enzymes form a wide range of cell types [91, 92]. Beyond this, IL-1 may be necessary for Th17 differentiation [93, 94], and can also fine tune the Th17 response to the microbe being responded to [95].

IL-1 binds to several receptors. IL-1R1 is the main receptor, but requires assistance from IL-1R-accessory protein (IL-1RAP) to induce signal transduction. In comparison, IL-1R2 is capable of binding IL-1, but does not induce signal transduction. It binds IL-1 with greater affinity than ILR1, and acts as a decoy receptor.

Another decoy is IL-1 receptor antagonist (IL-1RA). This circulating mediator binds IL-1R to inhibit IL-1 from doing so. The role for IL-1RA in modulating the inflammatory effects of IL-1 are clear, as neonates with defective IL-1RA develop sepsis-like symptoms but in a sterile manner. This includes pustular psoriasis, vasculitis and destructive bone abnormalities [96, 97]. On a less extreme scale, a list in the review by Dinarello [98] showed deficits in IL-1RA to result in a whole host of inflammatory disorders.

1.2.3 IL-6

IL-6 is a pleiotropic cytokine with proinflammatory and regenerative roles. It is secreted by a number of stromal, leukocytic and epithelial cells, but is only capable of signalling into a small number of them. It signals through a receptor

complex comprised of the ubiquitously expressed gp130, and the IL-6R, which is only expressed by leukocytes, hepatocytes and epithelial cells. Classic signalling through membrane-bound IL-6R plays roles in epithelial regeneration [99].

IL-6R is shed during neutrophil apoptosis or by neutrophils [100] or T cells [101] upon activation. Soluble IL-6R (sIL-6R) binds IL-6, and the complex then binds surface gp130, inducing signal transduction in cells normally incapable of responding to IL-6. This process (illustrated in figure 1.3) is called trans-signalling, and is reviewed extensively [102, 103].

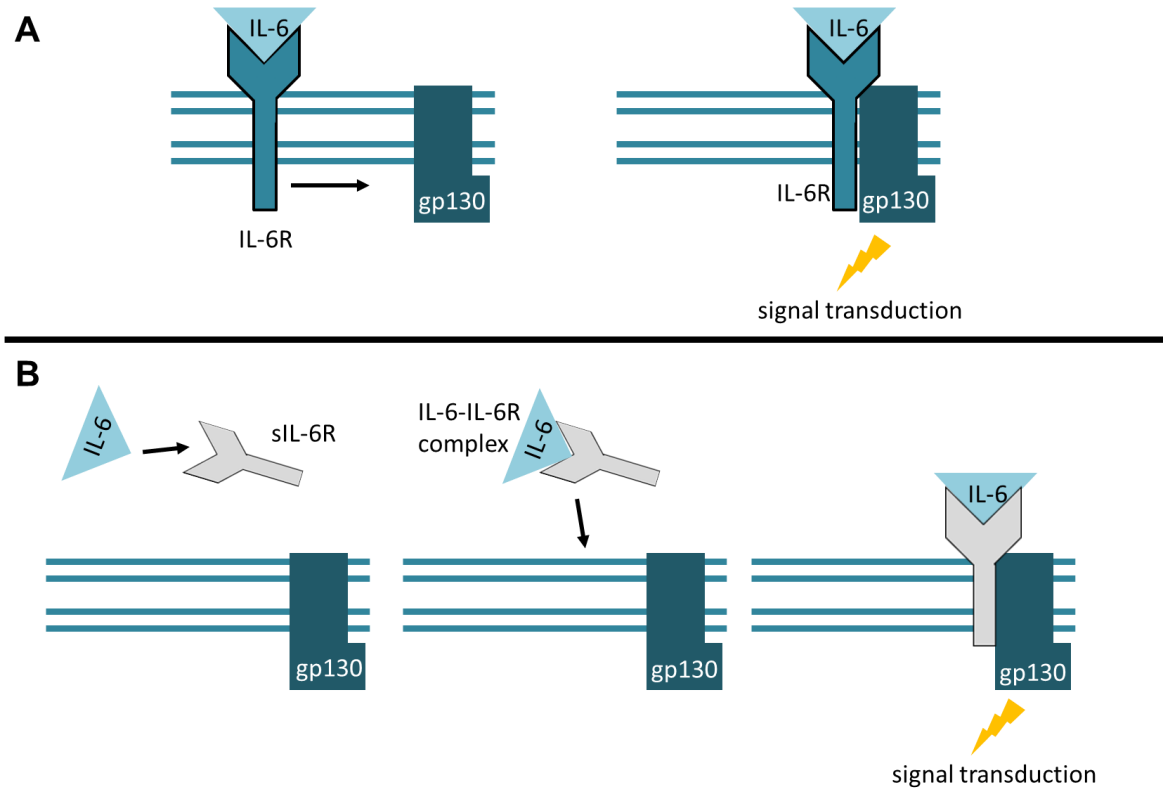

Figure 1.3: Classic and trans signalling through IL-6R and gp130. A Classic signalling occurs by IL-6 binding membrane-bound IL-6R, and this complex binding gp130, thus facilitating signal transduction from gp130. **B** Trans signalling requires soluble IL-6R (sIL-6R) to bind IL-6 in the extracellular space, before the complex can bind gp130 in the cell membrane and induce signal transduction.

IL-6, whether by classic or trans-signalling, plays numerous roles in inflammation. These include recruitment of leukocytes via activation of epithelial cells [104], rescue of T cells from apoptosis [105], and induction of differentiation into Th17 cells rather than Tregs [106].

A defence against the proinflammatory effects of IL-6 is to induce a refractory state in cells by degradation of the receptors [107]. However, this cannot halt trans-signalling, which is thought to be how Th17 cells are maintained in the RA synovium [101].

Fibroblasts and endothelial cells can only respond to IL-6 through trans-signalling, and many studies have been conducted on this interaction. Trans-signalling increases endothelial cell adhesion molecule expression [108], induces fibroblast secretion of IL-6 in an IL-1-induced feedback loop [109], and also induces secretion of CCL2 [104], a mononuclear cell chemoattractant. It does not induce secretion of IL-8 [110].

This latter fact is important, as the dogma for healthy inflammation is an initial neutrophil infiltrate, followed by apoptosis (and thus release of sIL-6R). This is followed by infiltrating mononuclear cells, including monocytes, which (as macrophages) efferocytose apoptotic neutrophils and thus clear the cellular debris to allow inflammation to resolve [111, 112]. Inhibition of trans-signalling does not affect neutrophil infiltration, but severely impairs monocyte influx [112].

In this regard, trans-signalling appears to be a useful mechanism in inflammation, and it induces fibroblast release of mediators capable of inhibiting collagen-degradation [113]. On the other hand, trans-signalling also induces osteoclastogenesis [114] (at least partly through induction of fibroblast RANKL

[115]). Because of this, a well-cited theory suggests that trans-signalling drives proinflammatory IL-6 functions [112]. This is evidenced by the fact that inhibiting IL-6 is detrimental in many diseases, whilst inhibiting IL-6R (via tocilizumab) is beneficial in several [103, 116]. It is not, however, beneficial in all diseases, suggesting the dichotomy of 'classic=good, trans=bad' is an over simplification [117].

1.3 Rheumatoid arthritis

RA is a chronic inflammatory disease, predominantly affecting the joints. It affects approximately 1% of the UK population [118]. Before menopause, women are approximately three times more likely than men to develop RA. After menopause, reports differ, with some claiming menopause contributes to the imbalance in incidence between sexes (reviewed in [119, and see 120]), whilst others claim the incidence of RA is equal after age of menopause [121].

Despite the sex imbalance, causative links to sex hormones are still contentious. Pre-menopausal females are more likely to have RA than age-matched males, but pregnancy (associated with increases in female sex hormones) alleviates symptoms [122]. In opposition, some studies found RA to be more common immediately post-menopause [123], suggesting female sex hormones were protective. Another suggestion is that male sex hormones are protective, as male RA patients have been found to be hypogonadic [124], yet androgen-replacement therapy has mixed results [125]. Thus, whilst the sex imbalance is clear, the relationship between RA and sex hormones remains to be properly elucidated.

1.3.1 Aetiology

RA is a complex disease, and its aetiology comprises a incompletely understood interaction between environmental triggers and genetic susceptibility alleles. The precise causative stressors are unlikely to be the same in all patients, and no clear causative agent has been found. Cigarette smoking is the strongest associated environmental factor [126], and first-degree relative of RA patients who smoke and are under 50 years of age are the most at risk demographic [127].

Various microorganisms and viruses have been proposed as infectious triggers [128]. The joint is a sterile environment, and as such does not harbour a microbiome like that of the gut, lung or oral cavity. The concept of microorganismal triggers is therefore linked to the prevailing theory that the breakdown of tolerance and establishment of autoimmunity in RA occurs away from the joint. This theory is strengthened by the link between RA and inflammatory bowel disease [129] and periodontitis [130]. The link between oral health and RA has been understood for decades. The removal of teeth from patients with rheumatism was once a logical but ineffective standard practise (as the link with oral health is gingival, not dental).

One of the best recognised auto-antibody groups in RA recognizes citrullinated peptides. Citrullination is a normal post-translational modification of arginine residues, but it can also occur through pathological means. For example, *Porphyrus gingivalis* (the major causative microbe in periodontitis) is capable of citrullinating host proteins [131]. Cigarette smoking too, induces protein citrullination [132]. It is therefore logical that these environmental triggers may be linked to autoimmunity in RA.

Twin studies have shown a clear role for genetics in the development of RA. To date, over 100 polymorphisms have been associated with the disease [133], but many are shared with most other autoimmune disorders (the strongest associations are with the human leukocyte antigen (HLA) loci), and do not explain the tropism of RA [134].

Beyond the HLA loci, some polymorphisms (such as in micro RNA (miR) 146a and tyrosine-protein phosphatase non-receptor (PTPN) 2 [135]) have been found to associate with more aggressive RA, and as such may represent more specific susceptibility alleles. PTPN genes arise more than once in the association studies, as PTPN11 (which encodes SHP2) has an RA-associated variant, which contributes to RA patient fibroblast-like synoviocyte (FLS) invasiveness [136].

Those cited above are merely a sample of the preponderance of studies into the aetiology of RA. Whilst population and twin studies have provided abundant candidates, the precise causative environmental and genetic instigators of RA are still open to debate. This is in part due to the complexity of the disease, and also due to variations between ethnicities (discussed in [137]). However, some genetic variations lead to specific transcriptional variations [138], and this may help to join the aetiology to the immune and inflammatory observations seen in the laboratory and clinic.

1.3.2 Clinical symptoms

1.3.2.1 Extra articular

Whilst RA is first and foremost a disease of the joints, there are numerous co-morbidities (reviewed in [139, 140]). These can affect a range of organs, and to

differing extents. Joint damage is a reducer of the quality of life, and yet RA has long been recognized to also lower life expectancy.

The leading causes of RA-associated mortality are pulmonary and cardiovascular [141, 142]. Pulmonary conditions are common, estimated as affecting 19-44% of RA patients, with poor prognosis once symptomatic. Various cardiovascular problems (i.e. myocardial infarction and stroke) are associated to different extents with RA.

Cancer as an overarching diagnosis is not significantly increased in RA patients, but certain types are; lung and breast cancer displaying the strongest correlation [143, 144]. The former is not especially surprising given that cigarette smoking is the leading environmental risk factor for both lung cancer and RA [126]. Both incidence and severity of infection are both higher in RA patients than the general population.

An important distinction should be made at this stage between the increased probably due to RA, and due to RA therapies. RA patients are not, for instance more likely to develop tuberculosis than the general population, but those being treated with anti-TNF therapy are [141]. Similarly, gastrointestinal disorders and ulceration, skin thinning and concomitant bruising are all associated with RA therapies, rather than with the disease itself (reviewed in [139]).

Cataracts and uveitis are both associated with RA (although the latter occurs more commonly in childhood arthritis [145]), and depression is associated with RA compared to the general population, but no more than for chronic inflammatory diseases in general [146].

Conditions that may appear more obviously linked to RA include carpal tunnel syndrome due to synovial inflammation [147], and osteoporosis [148, 149]. This latter is found in elderly, but also pre-menopausal patients, suggesting a correlation beyond that of hormonal changes.

1.3.3 Articular manifestations

Articular joints (where two bones meet) are the primary focus for RA, amongst many other arthropathies. Not all joints are affected however, with the most commonly affected being the proximal-interphalangeal and metacarpophalangeal joints of the hand, and metatarsophalangeal joints of the feet [150]. Interestingly, the cervical, but not thoracic, lumbar or sacral joints, can also be affected, especially in older patients with longer disease progression [151]. Similarly, larger joints such as the knee and hip tend to be affected later in disease than digital joints.

1.3.3.1 The normal synovium

The synovium is the main tissue of pathology in RA. In healthy individuals, the synovium is a thin membrane encapsulating the joint, providing a supportive yet flexible tissue to facilitate the movement of bones around their axis. The joint space is filled with hyaluronic acid, produced by the cells of the synovium and suitably viscous to provide a cushioning and (along with lubricin) lubricating role for smooth joint flexing.

As shown in figure 1.4, the normal synovial membrane (described in [152]) is organized into the lining and sub lining layers. The lining takes the place of the epithelium, which (along with the basement membrane [153]) is absent from the

joint. It is usually two to three cells deep [154], and comprises macrophage-like synoviocytes (MLS) and FLS [155].

The sub lining is more acellular, with the majority of cells being FLS [152]. It also includes extracellular matrix proteins including various collagens. Blood [156] and lymphoid [157] vessels increase in size and abundance deeper into the sub lining. Nerves are also present [158], apparently following the blood vessels. Whilst the number of leukocytes is limited, there are reportedly small numbers of lymphocytes found in the sub lining of healthy, non-inflamed joints [159].

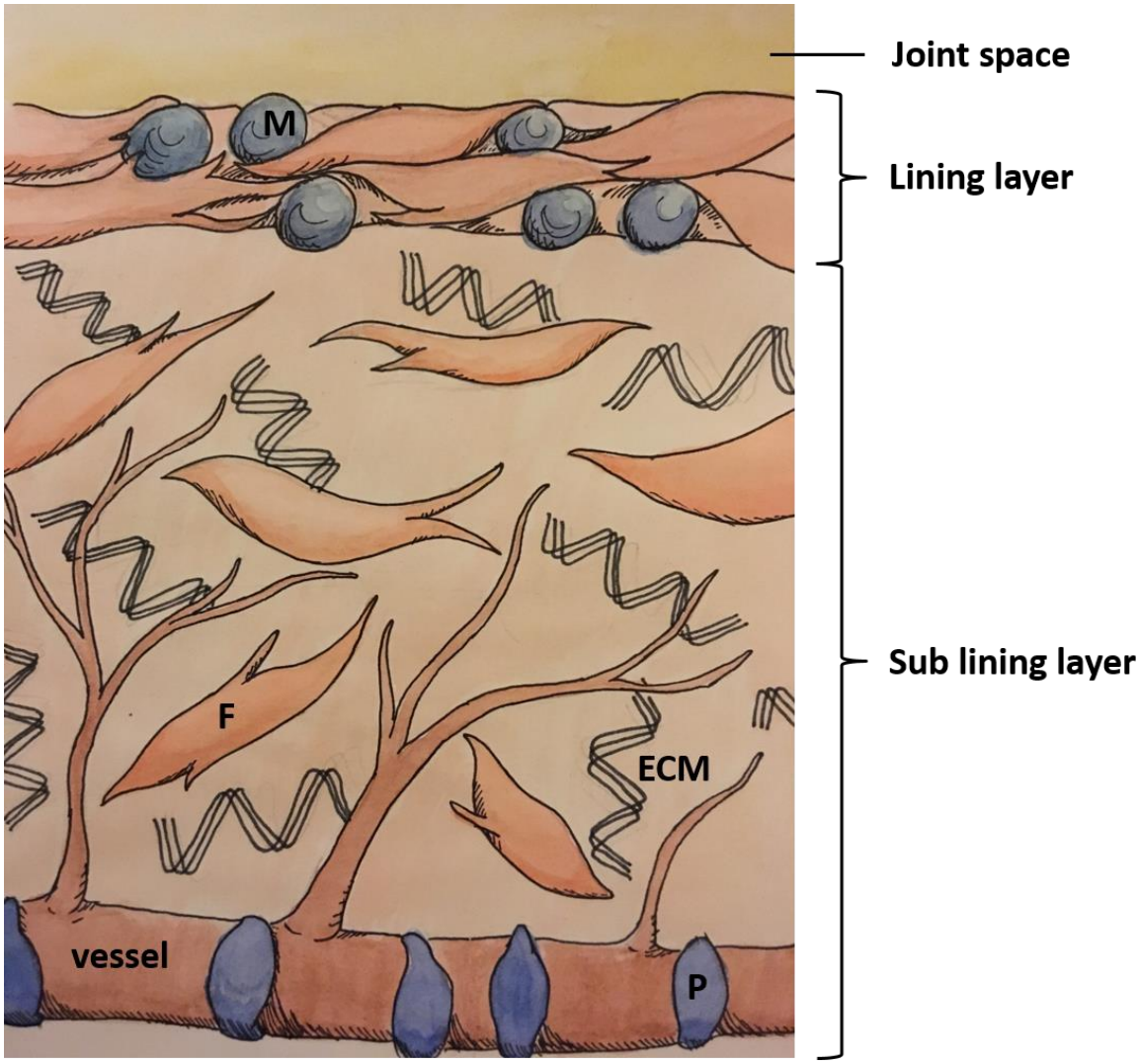

Figure 1.4: The healthy synovium. Simplified cartoon illustrating the basic structures and cellular components of the healthy synovium. M macrophage, F fibroblasts, ECM extra cellular matrix, P pericyte. Not to scale.

1.3.3.2 The RA synovium

In RA, a number of architectural changes occur. The synovial tissue becomes grossly enlarged, forming an invasive pannus early in disease progression [160]. This pannus expands outwards (thus causing problems like carpal tunnel [147]), into the joint space, and into the articular cartilage and bone by way of invasion and degradation. It is the pannus that forms the pathological structure in RA, and the radiological damage it causes can often be seen within three months of diagnosis [161].

The lining layer expands by several methods. The MLS proliferate and are replenished by peripheral blood monocytes [152, 162]. The FLS also proliferate and become hyperplastic, and infiltrating monocytes and other leukocyte subsets further expand the lining layer to around 10-15 cells deep [163]. There is an increased resistance to apoptosis [164], meaning cell turnover is low. This increase in tissue-resident and infiltrating cells, and decreased turnover, leads to hypoxia ([165], and reviewed in [166]), forcing cells into a glycolytic state [167]. Hypoxia normally induces apoptosis [168], which does not occur in the RA synovium (examples in fibroblasts, T cells and neutrophils given in [166]). Combined with the release of glycolysis waste products like lactate [167], the synovium becomes increasingly inhospitable. The alterations in the synovium during RA can be seen in figure 1.5.

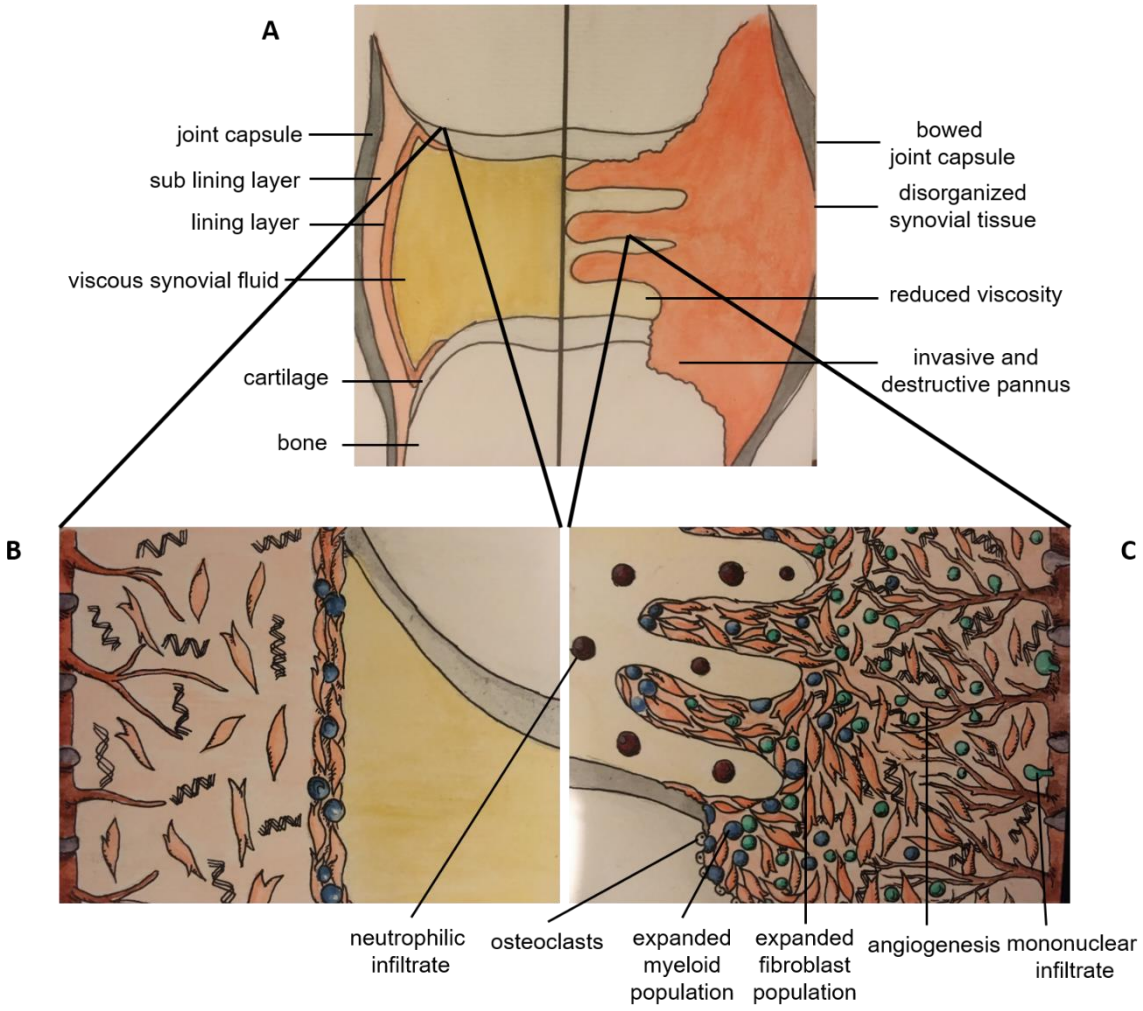

Figure 1.5: Comparison of the healthy and RA synovium. Illustrated comparison of the healthy and RA synovium. **A** Comparison of the structural changes that occur in the joint during RA. **B** Healthy synovium, with an organized lining and relatively acellular sub lining layer. **C** RA synovium, with loss of organization, an invasive pannus, reduced viscosity of the synovial fluid and increased angiogenesis and cellular infiltrate. Colouring of cells: fibroblasts pink, neutrophils brown, myeloid cells blue, lymphocytes green, pericytes purple. Size and cellular proportions not to scale.

During inflammation hyaluronan and lubricin production decreases, so despite oedema increasing fluid volume, the levels of lubrication go down in the joint [169], subsequently increasing friction. Perhaps because of this, extra cellular matrix proteins like hyaluronan and fibronectin are fragmented, and act as TLR ligands to stimulate resident and infiltrating cells [170, 171]. These alterations in the synovial tissue and fluid are profound, and the synovial fluid of RA patients has been shown to alter the behaviour of infiltrating cells. T cells upregulate IL-6R in response [164], and $CD14^{hi}$ CD16- peripheral blood monocytes upregulate CD16+ to form an 'inflammatory' monocyte phenotype [46].

1.3.3.3 Cellular drivers

Infiltrating cells are highly heterogeneous, and there is no predominant leukocyte type in the RA synovium. Smeets et al reported an increase in T cells, monocytes, and plasma cells in the RA synovium compared to controls [172], and high concentrations of neutrophils are also reported [173].

1.3.3.3.1 Neutrophils

Neutrophils play several roles in RA (reviewed in [65, 173]), and their deletion renders mice immune to the K/BxN serum transfer arthritis model [174]. They are found in high numbers in both synovial tissue and fluid, though more prominently in the latter [175]. Their lifespan is prolonged in tissue, and neutrophils survive several days in the RA synovium [176-178]. This is due to activation by immune complexes [179], but also by stromal cells, partly via GM-CSF [176, 179].

Neutrophils respond more strongly to immune complexes if 'primed' with cytokines [180]. In RA, the circulating neutrophils are already primed [181], inducing a faster and more prolonged inflammatory response in tissue. This includes release of

degradative mediators and reactive oxygen species, which (given the large influx) leads to tissue damage. They also release numerous proinflammatory cytokines such as TNFα, and disgorge their DNA in suicidal NETosis. This occurs spontaneously in RA patients [182], with inflammatory consequences.

1.3.3.3.2 B cells

One of those consequences is the exposure of self-antigens. As cited above, plasma cells are increased in the RA synovium, and 40% of those matured in tertiary lymphoid structures in the joint are capable of reacting to citrullinated histones released by NETosis [183]. Last year it was reported that there are over 80 over-citrullinated antigens in the RA joint compared to that of healthy counterparts [184]. This means ACPA+ RA patients have abundant autoimmune triggers in their joint. These are not only responded to by an increased number of plasma cells [172], but also an apoptosis-resistant population (due to the high concentration of IL-6 and BAFF).

Alongside citrullinated proteins, RA patients may have any of a number of auto-antigens (reviewed in [185]). The oldest example is rheumatoid factor, which reacts to the Fc element of IgG, thus forming immune complexes which trigger inflammation, partly by activating neutrophils [186]. RA patients may also have antibodies against the gp130 rheumatoid arthritis antigenic peptide-bearing soluble form (gp130-RAPS) [187]. Soluble gp130 and gp130-RAPs act to inhibit the IL-6R-IL-6 complex. Inhibiting them with auto-antibodies causes an increase in inflammation [103, 188].

The role of B cells in RA is strongly evidenced by the auto-antibodies used to initiate mouse models, and the efficacy of Rituximab in treating RA patients.

However, whilst ACPA and rheumatoid factor are diagnostic tools, they can be present decades before clinical symptoms. RA patients may alternatively be sero-negative for auto-antibodies against ACPA and rheumatoid factor, yet still develop the disease. This implies a pathological driver beyond the adaptive system (initially proposed by Firestein and Zvaifler in 1990 [189]).

1.3.3.3.3 T cells

T cells exist in small numbers in the healthy joint, but are multiplied manifold during RA. They are recruited by numerous chemokines, such as CCL5, CCL2, CXC10 and CXCL12, and Th1, Th0, Th2 cells are recruited in that order of preference [190]. The traditional dichotomy of Th1 and Th2 cells has been superseded by an increase in new subsets, such as Tregs and Th17 cells which have roles to play in RA, and even younger subsets, such as Th9 cells, which were only discovered recently [191]. A large number of T cells in the RA joint are found to be anergic, but this doesn't stop their stimulation of FLS [192, 193].

When T helper cells were classified as Th1 or Th2, it was well recognized that Th1 cells were predominant in the joint, and played pathogenic roles [75, 194]. This is largely due to their release of IFNγ [195], which inhibits IL-10's anti-inflammatory effect on macrophages [196], and induces inflammatory responses from fibroblasts. Further research however has yielded contradictory evidence, suggesting that IFNγ may be protective in the joint and other sites [197-199].

Th0 cells have the potential to differentiate based on microenvironmental cues. In the inflammatory milieu of the RA synovium T cell differentiation and plasticity is towards pathogenic subsets [200]. The Th2 response is minimal in RA, and

attempts to skew the immune response to a Th2 phenotype has been posited as a therapeutic strategy [201, 202].

Th17 cells and Tregs, play roles important in RA. Differentiation into Tregs or Th17 cells from a $CD25^{lo}$ progenitor is biased in favour of Th17 cells by RA FLS IL-6 production [200]. This results in a feedback loop of Th17 IL-17 production inducing RA FLS to release more IL-6.

Despite this, Tregs are reportedly abundant in the synovium [203], and donation of Tregs can protect recipient mice from CIA [204]. Contrasting studies suggest they are not necessarily efficacious however, showing retarded CTLA4 movement to the immunological synapse in RA Tregs [205]. Further, whilst thymic Tregs are unaffected, induced Tregs are inhibited by high concentration of IL-6 [206]. Due to the high concentration of IL-6, Tregs in the RA synovium (but not blood) can be converted into Th17 cells [207], further exacerbating the imbalance between pro and anti-inflammatory lymphocytes.

The roles of γδT cells are not properly elucidated in RA, however they are ‘involved’ in a number of autoimmune conditions [208]. Abrogation of γδT cells before induction of CIA resulted in a delayed onset, but abrogation as the arthritis began to subside led to an immediate increase in severity [209]. The authors posited pro and anti-inflammatory subsets, and this has been agreed in other publications [210, 211]. These cells are, however, a major source of IL-17 (already shown to be a key player in RA [93]) and were found to be the main producers of IL-17 in psoriasis [212].

1.3.3.3.4 Myeloid cells

Myeloid cells are both resident (MLS) and infiltrating (monocytes), and both populations play roles in RA. They are strong responders to both DAMPs and PAMPs, which are abundant in the RA synovium [170, 171]. It has been reported that the expansion of the pannus is largely due to monocytes and macrophages (proliferation of the resident and infiltration of the circulating) [152]. Their role is clearly important, as macrophage numbers correlate with disease activity and response to therapy [213, 214].

Macrophages are the main producers of TNFα in the RA joint [215]. This cytokine is the key mediator of RA, and mice with constitutively stable TNF mRNA develop severe arthritis [83]. The classical 'M1' predominates in the RA joint, and its signature cytokines are more abundant than those of 'M2' macrophages.

At the pannus leading edge monocytes receive RANKL from a number of resident and infiltrated cell types, inducing their osteoclastogenesis. This contributes to the bone production: resorption imbalance, along with high concentrations of MMPs and limited protective features like tissue inhibitor of metalloproteinases (TIMPs) and osteoprotegrin). Macrophages also stimulate FLS [216] and T cells [48, 217], both by secreted mediators and direct cell contact.

Infiltrating monocytes not only replenish the synovial tissue stock of macrophages and osteoclasts, but have pathogenic roles in T cell differentiation. $CD14^{hi}$ CD16- monocytes upregulate CD16 in the RA synovial fluid [46], and this CD16+ population is raised in blood and synovium of RA patients. CD14+ $CD16^{hi}$ monocytes have also been shown to drive differentiation of Th1 [46, 48] and Th17 [46, 49] cells linked to pathology in RA.

1.4 Fibroblasts

1.4.1 Origins and markers

The origin and surface markers of fibroblasts are many and varied. Even within the same tissue fibroblasts arise from different precursors. In the heart, the populations of fibroblasts differ according to embryological stage [218]. An example of this (and of fibroblast origin in general) is epithelial-to-mesenchymal transition. Cells from the epicardium undergo transition and migrate as a fibroblast population to form the myocardium [219]. Fibroblasts of other sites arise from different sources, such as head and neck development from the neural crest, whilst those of the synovium develop from mesenchymal stem cells.

During inflammation, fibroblasts, and particularly the activated myofibroblast, can arise from various populations (examples in ischemic heart defects are reviewed in [218]). Perhaps because of the wide variety of origins, both embryological and during inflammatory responses, pan-fibroblasts surface markers have not been reported. Many publications define fibroblasts as adherent cells that are non-haematopoietic, non-epithelial and non-endothelial. Whilst this negative selection of markers does indeed give rise to fibroblasts, it does not define them as a population.

Markers also fluctuate based on inflammatory state, freshly-isolated compared to cultured, anatomical locale, and even populations within the same tissue. CD90 is used to mark fibroblasts in both humans and mice, but both $CD90^+$ and $CD90^-$ populations have been described, and even compared, in different tissues [220, 221]. CD90 and vimentin mark most fibroblasts, but also certain endothelial cell

populations [222]. Efforts have been made to develop clear marker panels in synovial fibroblasts, and some of these will be addressed later. Other populations have been recognized with an array of surface markers, but the preponderance of fibroblasts populations means any panel is limited to specific populations.

1.4.2 General functions

There are general functions of fibroblasts in all tissues, which are the traditional roles ascribed to them. These are structural support and extra cellular matrix (ECM) production. ECM is a complex network of proteins and glycoproteins that act as scaffolding, barriers and signalling molecules. Roles as supporting cells can be illustrated with the increased proliferation and deposition of ECM by cardiac fibroblasts at birth, in order to stiffen the ventricles against their increased workload [223].

Other roles as ‘supporting’ cells can be seen in recruitment of cells during development (recruitment of T cell precursors to the embryonic thymus [224] and cardiomycoytes to the developing heart [225]). This recruitment role is also crucial during inflammation, and tissue-resident fibroblasts release a host of chemoattractant mediators to recruit appropriate leukocyte subsets in an appropriate sequence.

Fibroblasts also interact with other tissue-resident cells to mediate homeostasis or inflammatory responses. This occurs in all sites. Cardiac fibroblasts electronically couple cardiomyocytes [226], and dermal fibroblasts regulate epidermal proliferation [227]. During inflammation, fibroblasts in the joint and skin can interact with endothelial cells to modulate leukocyte infiltration into the tissue [228].

Wound healing is another important role for fibroblasts, and has been researched extensively, particularly in the skin. Wound healing is a fibrotic response, and aberration leads to fibrotic diseases such as chronic tendinopathy, pulmonary fibrosis and heart failure. Wound healing is driven by transforming growth factor (TGF)β, which is responded to (and produced) by fibroblasts, amongst other populations.

Fibroblasts become activated to myofibroblasts, which proliferate and express α smooth muscle actin. These cells are contractile, and can therefore pull a wound closed [229-231]. Myofibroblasts migrate into the wound site and secrete a preliminary ECM matrix of hyaluronan and fibronectin [232]. They are subsequently replaced by a second wave of non-myofibroblasts, which replace this with a disorganized collagen ECM (its disorganization produces a scar) [230]. If this process is perturbed, it leads to over-production of ECM, increased stiffening of tissue and loss of function in the organ. Fibrosis is seen as an inflammatory disease.

Different populations of fibroblasts have different surface markers, and these translate into different functional roles. The $CD90^-$ population of mouse cardiac fibroblasts respond strongly to IFNγ, releasing more proinflammatory cytokines and expressing more MHCII surface molecules [233]. It is theorised that this population is therefore responsible for the fibroblast inflammatory response in the heart, supported by evidence that the CD90+ population is less responsive to IL-1 in humans [234, 235].

Different populations exist in many sites (i.e. different populations in gingiva and periodontal ligaments [235, 236], and subsets within these different groups [235,

237]). Populations have been shown to respond differently to inflammatory triggers in the heart, lung, skin and gingiva [233, 238, 239], which may prove important in therapeutic targeting of fibroblasts in inflammatory diseases.

1.4.3 Functions in the joint

Fibroblasts in the joint have been identified with many markers. The distinct populations seen in the lining and sub lining layers are demonstrable by the markers used to describe FLS. CD55 [240], fibroblast activating protein (FAP) [241], PDPN [242, 243] and vascular cell adherence molecule (VCAM) 1 all mark the healthy synovium during inflammation, whilst the sub lining is denoted by the vascular cell adherence molecule, CD248 [244] and CD90.

As with other tissues, a main role of fibroblasts in the joint is structural support, whether directly or by production of ECM. The synovial fluid which provides lubrication and support is produced by FLS, which release various proteins and glycoproteins. FLS produce hyaluronan [245], and the size of the product dictates the viscosity and thus the support provided by the synovial fluid. Fibroblasts also provide lubricin [246] another mediator of joint lubrication.

Beyond homeostatic functions, FLS perform multiple roles in inflammation [247], shown in figure 1.6. They can respond to exogenous and endogenous danger signals via TLRs and cytokine receptors, and perform antigen presentation [248] and phagocytosis/ efferocytosis [249], albeit to a lesser extent than macrophages.

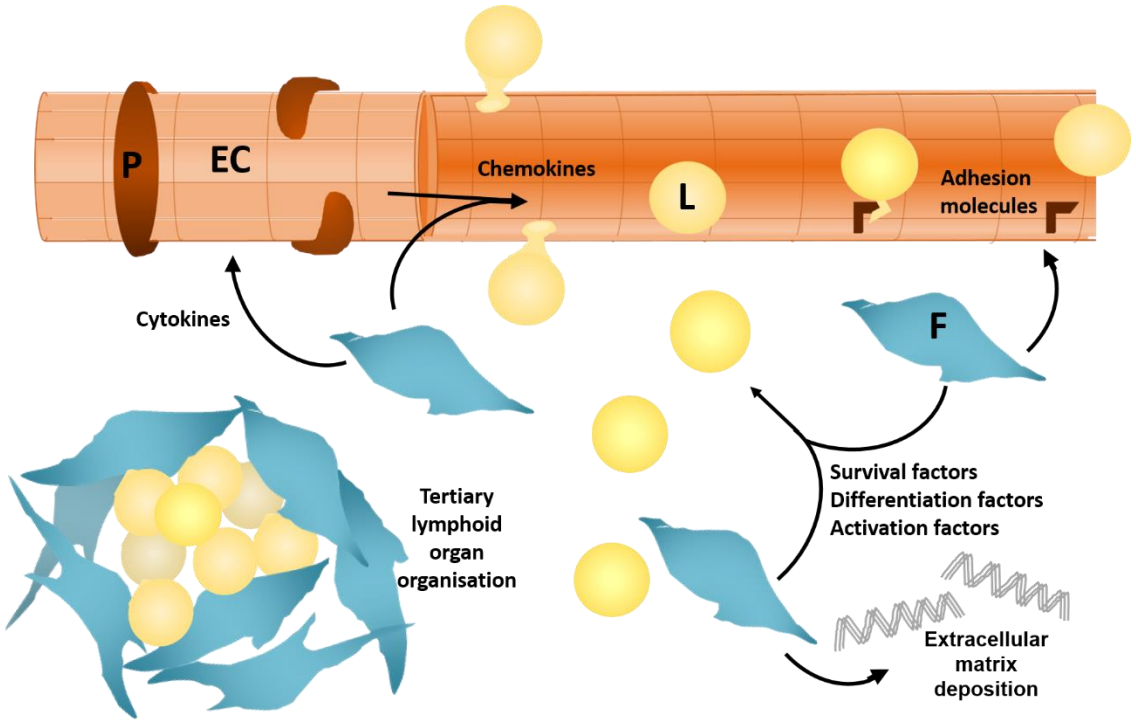

Figure 1.6: Illustration of the roles fibroblasts play in inflammation. Cartoon illustrating a number of roles fibroblasts play in an inflammatory response. These are shown with arrows, not distinguishing between stimulatory and inhibitory effects, as these differ according to site and disease state. P pericyte, EC endothelial cell, L leukocyte, F fibroblast.

FLS responses include a wide range of cytokines and chemokines. These have roles in directly recruiting [190, 250] and inducing the transmigration [251] of leukocytes, but can also modulate the EC response to stimulation (reviewed in [252]). FLS from healthy tissue have been found to suppress EC recruitment of leukocytes through a combination of IL-6 and TGFβ [228, 253].

Once leukocytes reach the tissue, fibroblasts interact with them to provide pro-survival factors [176], retention factors, and dictate activation or differentiation by both secreted and cell contact-dependant mechanisms. FLS can also modulate the inflammatory responses of leukocytes. This wide array of roles helps to explain why perturbation of FLS behaviour can cause such wide-reaching and destructive consequences.

1.4.4 Functions in the RA synovium

The pathologic role of FLS in RA has been reviewed extensively [254]. Their role as initiators or responders is still debated [255] after being initially proposed nearly 30 years ago [189]. Nevertheless, it is well recognized that they are fundamentally changed in RA.

FLS are stated as the main contributors to the invasive pannus, by becoming proliferative [163], hyperplastic [256], and apoptosis-resistant [257]. The intrinsic changes to FLS in RA are largely believed to be epigenetic (reviewed in [258], and properly discussed in chapter 4). The changes are maintained through *in vitro* passage. Once co-implanted into a severe combined immune deficient (SCID) mouse with human cartilage, RA FLS will degrade cartilage in the absence of immune cells, showing an autonomous inflammatory nature [259]. Injected human

RA, but not control, FLS have been shown in mouse studies to be capable of circulating in the blood to induce a polyarticular pathology [260].

Much of the pathological phenotype is ascribed to the lining layer. RA FLS co-implanted with cartilage into SCID mice self-assemble into lining and sub lining layers. The lining layer is PDPN+ (as in the joint) and cartilage adjacent. It is this leading edge PDPN+ population that degrades cartilage, and is also the first population to migrate from the primary implant site to a secondary piece of cartilage [242]. Cadherin-11 is another lining layer marker, and its abrogation results in protection from inflammatory arthritis by stopping the lining layer becoming hyperplastic [256].

RA FLS mount increased production of a number of proinflammatory mediators such as IL-6 [261], GM-CSF [177], MMPs [262], and CXCL12 [250]. The combination of RANKL and MMPs means RA FLS are extremely destructive in the articular joint.

Interestingly, despite the obvious role in inflammation, FLS (even in RA) produce limited quantities of TNFα [263-266] and IL-1 [265, 266]. Whilst they do not produce these mediators in meaningful amounts, they respond strongly to them, both in health and RA. The release of cytokines and chemokines in response to TNF and IL-1 can act in feedback loops, as fibroblast IL-6 and IL-8 are induced by a number of chemokines [267].

Feedback responses are important, as FLS secrete IL-6 which they cannot respond to (due to lack of IL-6R). FLS release robust quantities of IL-8, which recruits neutrophils (as do other cytokines abundant in the RA joint, such as IL-

17). Neutrophil shedding of IL-6R has already been covered, but many trans-signalling studies have focussed on RA FLS.

Trans-signalling in RA FLS has differing effects. It induces the secretion of RANKL [115], CCL2 [268] and IL-6 [197] but not IL-8 [110], and has been shown to both induce [269] and inhibit [270] RA FLS proliferation. It also induces TIMPs [113], suggesting an anti-inflammatory role. Targeting trans-signalling rather than all IL-6 signalling has been shown to be effective in treating RA (Tocilizumab review [117]) [105, 268], suggesting trans-signalling in RA FLS is predominantly inflammatory.

As with their healthy counterparts, RA FLS can interact with neutrophils [176, 243], macrophages [271], and lymphocytes [176, 200], often with the effect of increasing their survival. A previously cited proinflammatory role of RA FLS is the induction of $CD25^{lo}$ progenitors to differentiate into Th17 cells rather than Tregs, establishing a proinflammatory feedback loop of FLS and Th17 cells [200]. The relationship between FLS and EC also changes in RA, as RA FLS were shown to encourage endothelial-leukocyte interactions and leukocyte trans migration, rather than inhibiting the process [228].

1.5 Project aim

1.5.1 Directly relevant concepts

The roles of FLS in both healthy and RA joints following an inflammatory trigger are clearly crucial to the fate of that episode. The difference between healthy and RA FLS is a large focus for our department. Attempts to understand the underlying causes and their physiological effects have taken many guises.

In the year this project started, Lee et al reported that RA FLS mount an unremitting response to TNFα, involving continued transcription and secretion of inflammatory mediators. This was maintained for at least four days (the end of the observation period) as long as TNFα was still present [272]. This suggests a pathological inability to self-regulate, a concept supported by the finding that RA FLS express negligible mRNA for a host of negative regulators of the macrophage inflammatory response.

This raised the possibility that in RA, FLS lose the ability to switch off their inflammatory mediators. This would fit well with the ‘delinquent’ phenotype ascribed to them.

Macrophage negative regulation is multi-faceted (discussed in chapter 4), and one particularly dramatic form is endotoxin tolerance [273]. This is properly introduced in chapter 3, but at its simplest: macrophages undergo an inflammatory response to TLR stimuli, but are refractory to the next stimulation. This mechanism defends the host from tissue damage, and is a dramatic form of negative feedback.

Given the hypothesis of a failure to self-regulate, modelling this system in RA FLS (with non-inflamed FLS as controls) was the main basis for this thesis.

1.5.2 Chapter 3 (results 1)

In this chapter, I initially conducted experiments to confirm and progress the data published by Lee et al regarding the unremitting response. This involved stimulating cells for several days, or stimulating for 24h and then removing the stimulus.

Following this, the second half of the chapter was used to examine the concept of negative regulation upon second exposure, as seen in macrophage endotoxin tolerance. I examined the FLS inflammatory response to an initial and secondary challenge.

The overarching aim of this chapter was to characterize the inflammatory response of FLS to chronic or repeated stimulation to answer the following questions:

1. Do FLS form unremitting inflammatory responses during and following proinflammatory triggers?
2. Do FLS alter their inflammatory response to stimulus if they have already received an inflammatory challenge?

1.5.3 Chapter 4 (results 2)

Once the FLS response to first and second challenge was characterized, I wished to assess the mechanistic differences between the two responses. This chapter therefore included experiments designed to assess cellular response and the consequence on secreted products.

The questions under scrutiny were:

1. Which intracellular mechanisms are altered in the memory response, as compared to the initial response, to stimulation?

2. Is it possible to manipulate any altered mechanisms in order to render them similar to the initial response?

1.5.4 Chapter 5 (results 3)

As discussed in detail in chapter 5, fibroblasts from different anatomical locales differ in gene expression and function. This chapter begins with an examination of the response to repeat challenge by fibroblasts from different locations. Given the literature comparing healthy and chronically-inflamed tissues in multiple diseases, this chapter includes assessment of the effects of disease state on the fibroblasts memory response.

The role of fibroblast in orchestrating leukocyte functions is beyond question, particularly in RA. The second half of this chapter is therefore used to examine how (if at all) fibroblast memory can change leukocyte function. This was conducted by comparing the response of leukocytes to fibroblasts' initial or second inflammatory response to challenge.

The two rather different sections were brought into the same chapter as both involve comparison of different fibroblast populations, whether by site or disease state. With that in mind, the overarching questions addressed are as follows:

1. Does site of origin influence the inflammatory response of fibroblasts undergoing initial or second challenge?
2. Does disease state influence the inflammatory response of fibroblasts undergoing initial or second challenge?
3. Do fibroblasts undergoing initial or memory response to stimulus have different effects on leukocyte behaviour?

1.5.5 Overarching aim

The overarching hypothesis for this thesis encompasses the questions posed above and addressed in subsequent chapters. This hypothesis is:

Previous inflammatory triggers will alter the response of fibroblasts to subsequent inflammatory stimuli.

The treatments used in RA (and other chronic inflammatory diseases) have progressed over the last decades with an increasing appreciation for molecular mechanisms. Despite this, even successful clinical trials achieve relatively low remission rates. Even in RA, where the role of fibroblasts is so apparent, there is no treatment specifically targeting these cells.

The overarching aim of this project was therefore to assess the inflammatory functions of FLS, both healthy and RA, in the hope of establishing new lines of therapeutic potential. If RA FLS do indeed lose the ability to switch off their inflammatory secretions, then this could be a useful new avenue of research. If the ‘off switch’ could be reapplied, it may help to inhibit the self-propagating, autologous inflammation due to RA FLS.

At the end of this thesis will be presented an overarching discussion (chapter 6). In this chapter, I will return to the questions presented here and addressed in their relevant chapters, and discuss whether and to what extent they have been fulfilled.

2 Methods

2.1 Reagents

2.1.1 Cell isolation and culture

Tissue culture flasks were purchased from Corning: T25cm^2 #430639, T75cm^2 #4306410, and T150cm^2 #430825. Six and 10 cm^2 dishes were also purchased from Corning (#430196 and #430167 respectively).

Tissue culture wells were purchased from Costar: 96 well flat bottom (#3596), round bottom (#3799), 48 well flat bottoms (#3548), 24 well flat bottoms (#3526), 12 well flat bottoms (#3513), and 6 well flat bottoms (#3516).

Glass chamber slides were purchased from Flacon (#354108).

Pipette tips were purchased from StarLab, with filter tips and non-filter tips suitable for p2-10 (#51111-3000 and #51121-3810), p20-200 non-filter (#51113-1006), p20 filter tips (#51120-1810), p200 filter tips(#51120-8810) and p1000 (#51111-6001 and #51122-1830). Glass stripettes were purchased from Costar: 5ml (#4487), 10ml (#4488) and 25ml (#4489). Micro centrifuge tubes were purchased from Sarstedt (1.5ml #72.690, 0.5ml #72.699). Bevelled cell lifters were purchased from Fisher (#08.100.240).

Phosphate-buffered saline (PBS) was purchased from Sigma (#C5914) as pellets, used dissolved in sterile water at 10 pellets per litre. Trypsin-EDTA was purchased from Sigma (#T4174), and used at 1:5 or 1:10 v/v in sterile PBS. Cell-dissociation solution was purchased from Sigma (#C5914) and used neat. Trypan blue was purchased from Sigma #T8154). Ficoll-Plaque Plus was purchased from GE

Healthcare (#17-1440-03). Human CD4+ T cell enrichment cocktail was purchased from Stem Cell (#15062).

Growth medium was dependant on the cells cultured, but media were purchased from Sigma unless otherwise stated. Fibroblasts and T cells were cultured in RPMI-1640 (#R0883), Monocytes were cultured in RMPI-1640 (#R8758), the former lacking glutamine whilst the latter included glutamine. BJ fibroblasts were grown in EMEM (ATCC #30-2003). Doubelcco's modified Eagle's medium (DMEM) for mouse fibroblast was purchased from Sigma (#D6546). Hanks' Buffered Salt Solution (HBSS) was used for washing monocytes, and purchased from x (#14170-088).

Supplements to basic media included foetal bovine serum (FBS) (Labtech.com #FCS-SA), sodium pyruvate (SOP) (Sigma #S8636), non-essential amino acids (NEAA), (Sigma #M7145), and glutamine, penicillin and streptomycin (GPS) (Sigma #G1146). β Mercaptoethanol was purchased from Sigma (#M6250). Hepes was purchased from Sigma (#H4034).

The supplements added to these basic media are explained in table 2.1 below.

Table 2.1: Constituents of complete culture media for different cell types. HI-FBS= heat inactivated FBS. Other abbreviations are explained above.

Fibroblasts	Outgrowth fibroblasts	T cells	Macrophages	Mouse fibroblasts
RPMI 1640	RPMI 1640	RPMI 1640	RPMI 1640	DMEM
FBS 10%	FBS 10%		FBS 5%	HI-FBS 10%
SOP	SOP			Hepes
GPS	GPS x2			β Mercaptoethanol
NEAA	NEAA			GPS
				NEAA

2.1.2 Stimuli and inhibitors

Human recombinant TNFα (Peprotech #300-01), human recombinant IL-1α (Peprotech #200-01), *E.coli* LPS serotype EH100 (Enzo Life Sciences #ALX-581-010-L002), human recombinant IL-10 (Peprotech #200-10), human recombinant IL-1RA (Peprotech #2001-01RA), human recombinant soluble IL-6R (Peprotech #200-06R), human recombinant TGFβ. Adalimumab (gift from Chris Buckley).

2.1.3 Cell product isolation

2.1.3.1 RNA

RNA isolation kits were purchased from QIAgen: RNeasy mini kit (#74106), and micro kit (#74004). QIAshredders were also from QIAgen (#79656). β mercaptoethanol were purchased from Sigma #. Ethanol (EtOH) was purchased

from ProLab (#20821.330). qPCR tubes were purchased from FisherBrand (#14230210).

2.1.3.2 Intracellular protein

Total intracellular protein isolation by radio immunoprecipitation assay (RIPA) buffer:

Sodium chloride	150mM	Fisher Scientific	#S/3120/63
NP-40	1%	Sigma	#I3021
Sodium deoxycholate	0.5%	Sigma	#D6750
Sodium dodecyl sulphate	0.1%	Sigma	#71727
Tris pH8	50 mM	Sigma	#T1503

Cytosolic fraction of intracellular protein isolated by hypotonic buffer:

HEPES pH7	20mM	Sigma	#H4034
Potassium chloride	10mM	Fison	#P/4280
Magnesium chloride	1mM	Fison	#M/0600
Dithiothreitol (DTT)	0.5mM	Roche	#10-708-984-001
Triton X-100	0.1% v/v	Sigma	#X100
Glycerol	20% v/v	Sigma	#G5516

Nuclear fraction of intracellular protein isolated by nuclear buffer:

HEPES pH7	20mM	Sigma	# H4034
Potassium chloride	10mM	Fison	#P/4280
Magnesium chloride	1mM	Fison	#M/0600

DTT	0.5mM	Roche	#10-708-984-001
Triton X-100	0.1% v/v	Sigma	#X100
Glycerol	20% v/v	Sigma	#G5516
Sodium chloride	420mM	Fisher	#S/3120/63

All above solutions have Roche phosphatase (#04906837001) and proteinase (04693124001) inhibitors added before being used on cells.

2.1.4 Analysis

2.1.4.1 ELISA

Human IL-6 (#88-7066-88), IL-8 (#88-8086-88), CCL2 (#BMS-287/2(INST)) were purchased from EBioscience. CCL5 (#BMS-287/2INST) was purchased from ThermoFisher. A composite anti-human TNFα ELISA was made in house from BD Pharminogen mouse anti-human TNFα (#55120), biotinylated mouse anti-human TNFα (#554511) and KPL's TMB Peroxidase substrate solution (#50-76-00).

Tween was purchased from Fisher (#BP337).

2.1.4.2 Luminex

Human 6-plex from R&D (#LXSAHM-06).

2.1.4.3 qPCR

cDNA synthesis was conducted using the BioRad IScript reverse transcription kit (#170-8891). Sybr green was purchased from Takara (#RR820L). Primers were ordered from Eurofins. Primers are displayed below in Table 2.2.

Table 2.2: Primer sequences used for qPCR experiments. Pt= primary transcript. Mat = mature transcript.

Target	Forward primer	Reverse primer
UBC	5'CGGGATTTGGGTCGCAGTTCTTG3'	5'CGATGGTGTCACTGGGCTCAAC3'
GAPDH	5'GTCAGCCGCATCTTCTTTTGC3'	5'AATCCGTTGACTCCGACCTTCC3'
pt IL-6	5'CCACTCCACTGGAATTTG3'	5'TTTTCTGCCAGTGCCTCTTT3'
mat IL-6	5'CAATGAGGAGACTTGCCTGGT3'	5'TGGCATTTGTGGTTGGGTCA3'
pt IL-8	5'CAGTTTTGCCAAGGAGTGCT3'	5'ACTGTAATCCTAACACCTGGAAC3'
mat IL-8	5'GGTGCAGTTTTGCCAAGGAG3'	5'TTCCTTGGGGTCCAGACAGA3'

2.1.4.4 Western blotting

0.1% Tris-buffered saline (TBS) tween (100ml 10xTBS to 900mL water, then 1mL Tween to 1L TBS). Powdered milk was manufactured by Marvel. Bovine Serum Albumin (BSA) was purchased from Melford Bio laboratories (#A1302). ECL Western blotting substrate solution (used 1:1 solution A: B) was purchased from ThermoScientific (#32106). Reblot plus mild solution (10x) was purchased from Temecula California (#2502).

Table 2.3 displays the antibodies used to assess internal protein abundance in both total protein Western blotting and intracellular fraction Western blotting. All were purchased from Cell Signal. Secondary antibodies used against the primary antibodies shown below were Cell signal anti-mouse (#70765) and anti-rabbit (#70745) IgG.

Table 2.3: Primary antibodies used in Western blotting experiments.

Target	Size/ KDa	Dilution	Species	Catalogue #
NFκB p65	65	1:2000	Rabbit	8242P
NFκB p65-P (S536)	65	1:1000	Rabbit	3033S
p38-P (T180/Y185)	38	1:1000	Rabbit	9211S
JNK-P (T183/Y185)	46/54	1:1000	Rabbit	46685
ERK1/2-P (T202/Y204)	42/44	1:1000	Rabbit	4370P
αTubulin	52	1:2000	Mouse	2144
Lamin A/C	63/74	1:1000	Rabbit	2032

2.1.4.5 Immunofluorescence

Chamber slides were purchased from Falcon (#354108), paraformaldehyde (#), Triton X-100 (Sigma #X100), horse serum (Sigma #H0146), anti-NFκB p65 (Santa-Cruz #H0714), 4′,6-diamidino-2-phenylindole (DAPI) was from Sigma (#D9542). pHrodo Red E. coli BioParticles were purchased from ThermoFisher (#P35361).

2.1.4.6 Flow cytometry

FACs buffer was comprised of: sterile PBS with 2% w/v BSA.

The solution used for fixing and permeabilizing cells was Cytofix/Cytoperm from BD Bioscience (#51-2090KZ). When beads were required for compensation, I used eBioscience OneComp eBeads (#4317277).

Zombie yellow (BioLegend #423104) was used to assess cell viability by flow cytometry. It was diluted 1:200 in DMSO, and aliquots were further diluted in FACS buffer 1:100 at the time of analysis. Zombie fluorescence was read by analysis of Bv605.

The antibodies used for flow cytometry are displayed in table 2.4.

Table 2.4: Antibodies used in flow cytometry experiments. *H.s*= human, *M.m* = mouse.

Target	Fluorophore	Dilution	Company	Catalogue #
H.s CD206	APC	1:50	BioLegend	321110
H.s TNFα	AlexaFluor700	1:50	BD Pharmingen	557996
H.s RORγ	FITC	1:50	Abcam	Ab104906
H.s Tbet	PerCP/Cy5.5	1:50	BioLegend	644806
H.s GATA3	AlexaFluor647	1:50	BD Bioscience	560068
H.s FOXP3	eFluor450	1:50	eBioscience	48-4777/E7
H.s IL-17A	PE	1:50	BioLegend	512306
H.s IL-4	FITC	1:50	BioLegend	500806
H.s IFNγ	Pacific Blue	1:50	BioLegend	502522
H.s IL-10	PE/Cy7	1:50	BioLegend	501420
M.m CD3	APC/Cy7	1:50	BioLegend	100222
M.m B220	eFluor450	1:100	BioLegend	48-0452-82
M.m Gr1	Bv510	1:100	BioLegend	127633
M.m F4/80	APC	1:50	eBioscience	17-4801-82
M.m CD11b	FITC	1:100	eBioscience	11-0112-82
M.m γδTCR	PE	1:100	BioLegend	118018

2.2 Patients

2.2.1 Rheumatoid arthritis and synovial controls

Fibroblast-like synoviocytes were collected from the joints of patients suffering RA or arthralgia (joint point without discernible synovitis). Patients suffering arthralgia

had exploratory surgery at the Royal Orthopaedic Hospital, Birmingham. Patients donated small biopsies of their synovium after giving fully-infirmed written consent. These acted as our synovial controls. Whilst not ‘healthy’ *per se*, these patients lacked synovitis, and therefore are the most appropriate control available for RA samples.

The classification of RA is based on the American Rheumatism Association’s 1987 criteria for RA. This classification requires a minimum of four of the following seven criteria.

1. Morning stiffness in and around the joints lasting at least 1h before reaching maximal improvement.
2. Soft tissue swelling of three or more joints as observed by a physician.
3. Swelling of the proximal interphalangeal, metacarpophalangeal, or wrist joints.
4. Symmetrical joint swelling.
5. Rheumatoid nodules.
6. The presence of rheumatoid factor
7. Radiological erosions and/or periarticular osteopenia in hand and/or wrist joint.

The first 4 symptoms must have been present for at least six weeks.

Fibroblasts from RA patients are taken at all stages of disease progression from the Birmingham Early Arthritis Cohort (BEACON) by ultrasound guided biopsy. This allows our department to longitudinally follow each patient’s disease progression from earliest diagnosis of arthritis, during arthritis differentiation

(inflammatory, reactive, psoriatic, rheumatoid etc.) and during the patients' progression through treatment.

Samples from 'end stage' RA were used. These are named 'JREPS', meaning the sample was taken during joint replacement due to advanced disease. In my thesis, JREPs are referred to as RA FLS, as samples from other stages of disease were not employed.

Table 2.5 Patient details from donors of FLS used in this study. Years are represented as mean± standard deviation.

Samples	Control Synovium n=9	RA synovium n=8
age	38± 8.5	47± 15.5
disease duration	NA	15.7± 8.7
auto-Ab	0	7 of 8
sex	male 7, female 2	male 1 female 7
joint	knee	knee 5, hip 2, elbow 1

2.2.2 Psoriasis and dermal controls

Patients donating skin samples were recruited in Birmingham, UK and Aarhus, Denmark. Those in Birmingham were RA or OA patients undergoing joint replacement surgery at the Royal Orthopaedic Hospital. They had no known skin involvement in their disease. These patients were aged 69± 5.2 years, with 14.7± 8.2 years of disease. RA patients (but not OA) patients in this group were rheumatoid factor positive. Three were male, three were female, and five were from knee joints, one from a hip joint.

The dermal samples collected in Aarhus were of healthy and Psoriasis skin. No clinical details other than disease diagnosis were available. Healthy samples were taken at the time of breast of abdominal cosmetic surgery. Psoriasis samples were taken from active lesions of plaque psoriasis. Samples were also taken from non-involved skin from the same patients, but these were not used in our study, therefore ‘psoriasis skin’ refers only to active psoriatic plaques.

To my knowledge there are no psoriasis diagnostic criteria. This may make sense in that psoriasis is almost as broad a term as arthritis and arthritis is also not diagnosed by criteria. The patients are therefore diagnosed as having psoriasis by a dermatologist. The distinction between forms of psoriasis is discussed elsewhere, but the cells used in this study are from plaque psoriasis.

Skin fibroblasts (other than those from the BEACON cohort) were provided by Tue Kragstrup of Aarhus University, Denmark.

2.2.3 Tonsillitis

Patients undergo tonsillectomy at the discretion of their physician, as such the number of bouts of acute tonsillitis suffered before removal of the tonsils vary. Nevertheless, recurrent tonsillitis is a prerequisite for tonsillectomy.

An example of departmental guidelines is as follows:

1. Recurrent infection of the tonsils (five to seven events), characterized by fever, antibiotic use and medical assistance.
2. Tonsil hypertrophy causing swallowing, respiratory or phonological disorder.
3. Peri-tonsil abscess which depends on the patient’s age and family history.

Tonsil fibroblasts were provided by Anne Fletcher and Kostas Knoblich, of the Institute for Immunotherapy at the University of Birmingham. No clinical details were provided for these samples.

2.2.4 Pulmonary disease

Patients suffering lung pathologies provided samples for the isolation of lung fibroblasts after fully informed written consent was given. The predominant pathology was cancer. With regard to this particular study, the details of the pathology were not revealed as the aim was to isolate fibroblasts from chronically inflamed tissue, not to differentiate based on pathology.

That being said, as chronic obstructive pulmonary disorder is a disease discussed elsewhere in this thesis it is worth noting the classification for it.

The Gold classification is a global initiative for study of lung pathology, and suggests physicians should be aware of risk factors, particularly in those over the age of 40. These include: dyspnoea (difficulty breathing) which is temporally progressive and worsened by exercise, chronic cough and/or sputum production, recurrent lower respiratory tract infection, and a history of risk factors such as genetic susceptibility or childhood respiratory infections.

A spirometry test produces quantitative values for forced vital capacity (FVC: the volume exhaled after maximal inhalation) and forced expiratory volume (FEV_1: the volume exhaled in the first second of FVC exhalation). The ratio of FVC: FEV_1. A value of ≤0.70 is considered an indicator of airflow limitation. The FVC: FEV_1 ratio is however a sensitive but not specific indicator of disease.

A questionnaire assess the patient's perception of symptoms during, for example, walking up a slight hill. These occur in different forms, but the COPD Assessment Test (CAT) for example offers a 0-5 scale of agreement with eight statements pertaining to COPD symptoms. As with spirometry, a cut off threshold is employed.

Pulmonary samples were provided by the thoracic surgical team at the Heartlands hospital, Birmingham.

Table 2.6: Clinical details of patients used in the study of lung fibroblasts. Values are represented as mean± standard deviation.

Samples	Lung samples n=12
age	60.5± 14.5
forced expiratory volume (FEV1)	2.2± 0.7
forced vital capacity (FVC)	3.1 ±0.8
pack years (pack/day for one year)	28.8± 15.3

2.2.5 Periodontitis and gingival controls

It is debated in the field as to whether periodontitis (PD) and gingivitis are separate diseases, or whether the former develops from the latter. In both, the gingiva is affected, whilst only PD results in damage to the periodontal ligament and alveolar bone.

PD is diagnosed generally by probing the gingival sulcus (gap between the gum and tooth). A 'pocket' deeper than 3mm, and probe-induced gingival bleeding, are

indicators of PD. Radiological examination may provide secondary confirmation, but often not until later in disease progression.

The 1999 meeting of American Association of Periodontists provided eight subcategories of PD, which may also be sub classified in some cases into localized or generalized. For the purpose of this study the exact form of PD was not determined. Rather, patients diagnosed with PD by their dentist gave fully informed written consent for 'waste' tissue removed at the point of dental surgery to be used to isolate gingival fibroblasts from. Control gingival tissue is not healthy, rather it was removed from a patient undergoing gingival surgery who had no sign of chronic inflammation in that area of the mouth.

These samples were provided by Iain Chappele at the School of dentistry, University of Birmingham. Clinical details were not available for these samples, other than diagnosis of periodontitis.

2.3 Cell isolation

2.3.1 Fibroblasts

The fibroblasts collected in the Rheumatology Research Group at the University of Birmingham were isolated via the outgrowth method. Tissue samples were cut into small cubes (approximately 0.5-1cm^3) and placed evenly across a 10cm plate or T25 flask. Fibroblast growth medium was added to the cells. Medium was changed every seven days. When fibroblasts visibly colonized a large percentage of the plate, surface tissue was removed. Cells were kept in this plate until approximately 70-80% confluent. This method is illustrated by figure 2.1.

Fibroblasts received from collaborators were isolated differently. Tonsil fibroblasts for example, were digested from tonsils using dispase, collagenase and DNase I in RPMI1640 for 1h, before sorting the fibroblasts by flow cytometry (excluding the CD45- population, then gating based on PDPN and CD31). Once placed under my care all fibroblasts were treated in the same way throughout culture.

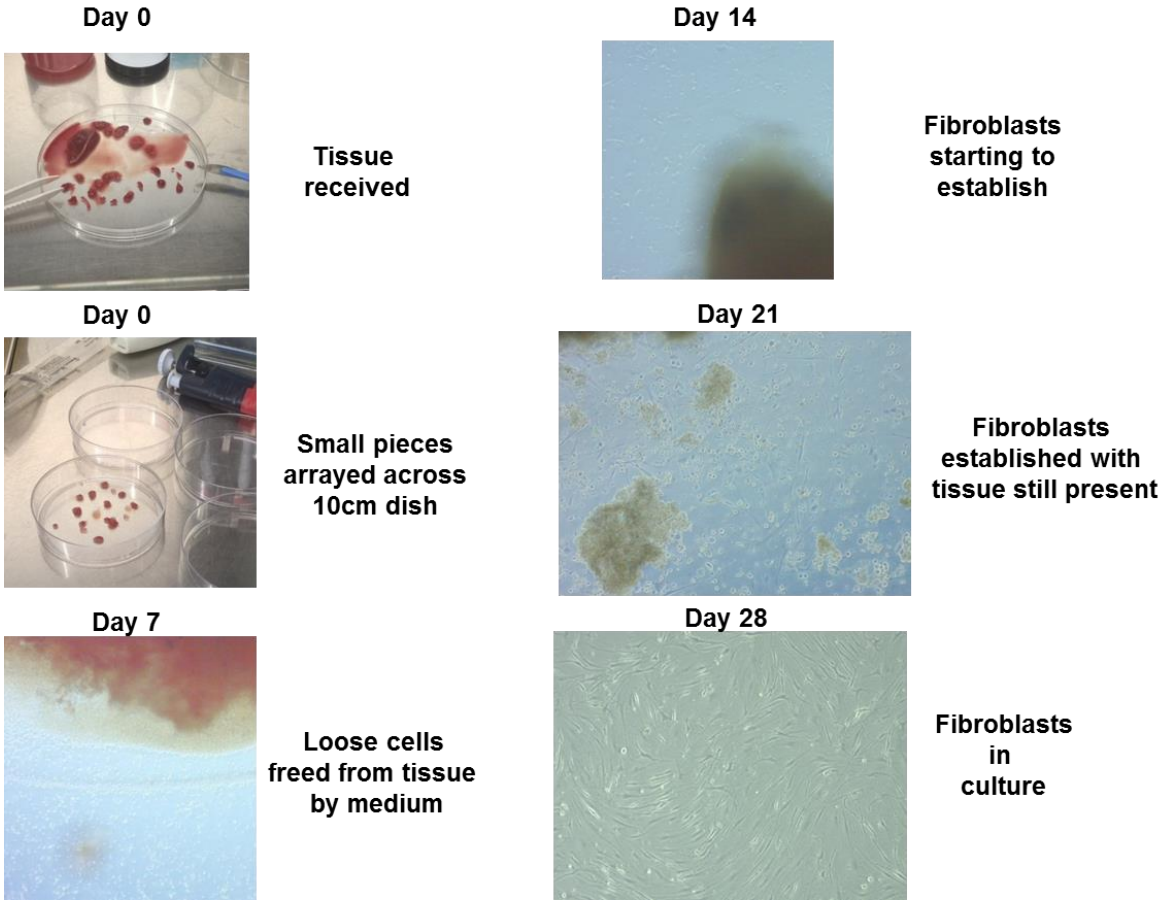

Figure 2.1: Illustration of the outgrowth technique for isolating fibroblasts from tissue. Tissue was cut into small pieces and arrayed on a 10cm dish (or a number of 10cm dishes). The tissue is left for 30 minutes to encourage adherence to the plastic, before one drop of outgrowth medium is added to submerge each piece. The next day 7ml outgrowth medium is added. The tissue is left for 7 days before loose cells are washed away and medium is replaced, with care to avoid losing the tissue. As weeks progress the fibroblast begin to establish themselves by adhering to the plastic under the tissue, and then beyond it. Once the fibroblasts have clearly established populations the tissue is removed and cells washed, then cultured in outgrowth medium until passaged.

2.3.2 Monocytes

Peripheral blood was taken from healthy volunteers by a qualified phlebotomist. This blood was transferred to a 50ml Falcon tube and made up to 40ml with HBSS. This was layered onto an equal volume of Ficoll, and the falcon was centrifuged for 2200RPM for 20minutes without break or acceleration. The erythrocytes and polymorphonuclear cell pellet, with serum, buffy coat (containing mononuclear cells) and Ficoll layered above them in discrete levels.

The buffy coat and mononuclear cells were carefully removed and transferred to a fresh Falcon tube, whereupon it was made up to 40ml final volume with HBSS. This was centrifuged at 1400RPM for 5 minutes with full break and acceleration. The HBSS was removed leaving a mononuclear cell pellet and the process was repeated twice more. The final resuspension was in serum free RPMI 1640. Cells were counted using haemocytometer, and $2x10^7$ cells were plated in 10ml of RPMI 1640 for 1 hour, before medium and non-adherent mononuclear cells were removed, leaving a population of adherent mononuclear cells (monocytes).

2.3.3 CD4+ T cells

T cells were kindly isolated by Kalvin Sahota, a PhD student in our department. His method was to isolate peripheral blood from blood cones (blood with the serum removed, stored and provided by the blood bank). The cells from the blood pack were incubated with CD4+ T cell enrichment cocktail (75µl/ 1ml of blood cone) for 30minutes, then diluted 1:3 with PBS supplemented with 1% FBS.

This was layered onto Ficoll (15ml Ficoll and 25ml blood solution) before centrifugation at room temperature at 900xg for 30minutes with no break or acceleration. The cell layer was removed, and due to the enrichment cocktail

comprised only CD4+ T cells (validated by Kalvin). The T cells were washed with PBS and centrifuged at room temperature at 600xg for 8 minutes, and then twice with T cell medium at room temperature at 300xg for 8 minutes (these three spins were with full break and acceleration).

2.4 Cell culture

2.4.1 Fibroblasts

BJ neonate dermal foreskin fibroblasts were cultured in EMEM with the supplements listed in table 2.1. BJ cells are not transformed, but undergo a rapid rate of proliferation and may be passaged higher than adult fibroblasts without undergoing replicative crisis and senescence. Passage was therefore not an issue, although cells were never used higher than passage 15.

Fibroblasts isolated from tissue samples were initially grown in 'outgrowth media' (see table 1.1). Once debris and other cells were removed and fibroblasts were transferred into culture flasks these cells moved onto 'fibroblasts growth medium' ('Fibroblasts' in table 1.1). These fibroblasts were used between passages 3-8.

Cells were incubated at 37^{o}C with a 5% CO_2 atmosphere. Depending on initial number of cells and proliferation levels cells were cultured in T75 or T150 flasks in 12ml or 24ml of medium, respectively. Every seven days, two thirds of the medium was removed and replaced with fresh complete growth medium.

Once cells reached 70-80% confluency, medium was removed and cells were washed with sterile PBS, then treated with trypsin to end their adherence to the culture plastic. BJ cells received 7ml 10% trypsin (90% PBS) according to manufacturer recommendation, whilst non-BJ fibroblasts received 7ml 20% trypsin

(80% PBS) as per standard practise in our department. After approximately 10 minutes cells were agitated to dislodge them into suspension.

The suspended cells was mixed with 13ml of fresh medium and centrifuged at 1500 RPM for 6 minutes at room temperature. The solution was removed and cells were agitated to separate the pellet. The pellet was then suspended in 1ml of medium before 10µl was taken and mixed 1:1 with trypan blue for haemocytometer cell counting. If cells were required for experiments the appropriate volume of cells was removed. For passaging, one third of cells were returned to a culture flask and two thirds were frozen for storage.

For freezing cells were centrifuged at 1500 RPM for 6 minutes at room temperature, before medium was removed and the pellet was agitated. 1ml freezer medium was added (10% DMSO in FBS) for each cryovial and cells in freezer medium were transferred into cryovials, appropriately labelled with identification, name of researcher, date and cell passage number. These were frozen in 'Mr. Frosty' cryovial containers overnight at -80^{o}C, before cryovials were transferred to liquid nitrogen for long term storage.

2.4.2 Monocytes/ Macrophages

Adherent monocytes isolated in the procedure described in 2.3.3 above were washed free of medium. It was replaced with 10ml macrophage medium (see table 1.1) and either 50ng/ml M-CSF or 25ng/ml GM-CSF for differentiation into M2-like and M1-like macrophages, respectively. Cells were differentiated for five days.

On the fifth day medium was removed and cells were provided fresh macrophage medium with 50ng/ml M-CSF or 25ng/ml GM-CSF as above for 24h. The next day medium was removed and cells were washed with PBS, before receiving 5mL cell

dissociation buffer for 20 minutes before being gently scraped from the dish using a bevelled cell scraper. The macrophages, now free in suspension, were pipetted into a 20ml universal and centrifuged at 1800 RPM at room temperature for five minutes. Medium was removed and the pellet resuspended in 1ml macrophage medium. 10µl were mixed with 10µl Trypan blue for cell counting.

2.4.3 T cells

These cells were suspended in T cell medium (see table 2.1) and counted using a haemocytometer. Known concentration of T cells were then maintained in culture using T cell medium supplemented with 10ng/ml IL-2, or seeded in appropriate numbers into plates for experiments.

2.5 Experimental procedure

2.5.1 Repeat dose experiment

Fibroblasts were seeded overnight in 300µl fibroblast medium at 30,000 cells per well of a 24 well plate, unless otherwise stated. The next day, medium was removed and cells received 700µl medium alone, or medium including 10ng/ml TNFα or IL-1α (other concentrations of stimuli are specifically noted in figure legends). After 24h conditioned medium was removed and frozen for future analysis. Cells were washed thoroughly with fresh medium and then rested for 24h in 700µL of growth medium. After 24h the medium was again removed and frozen, before cells were washed and provided with 700µl with or without a second dose of 10ng/ml TNFα or IL-1α for 24h. At the end of this second stimulation, medium was removed for freezing. This experimental design is shown below (Fig 2.2a).

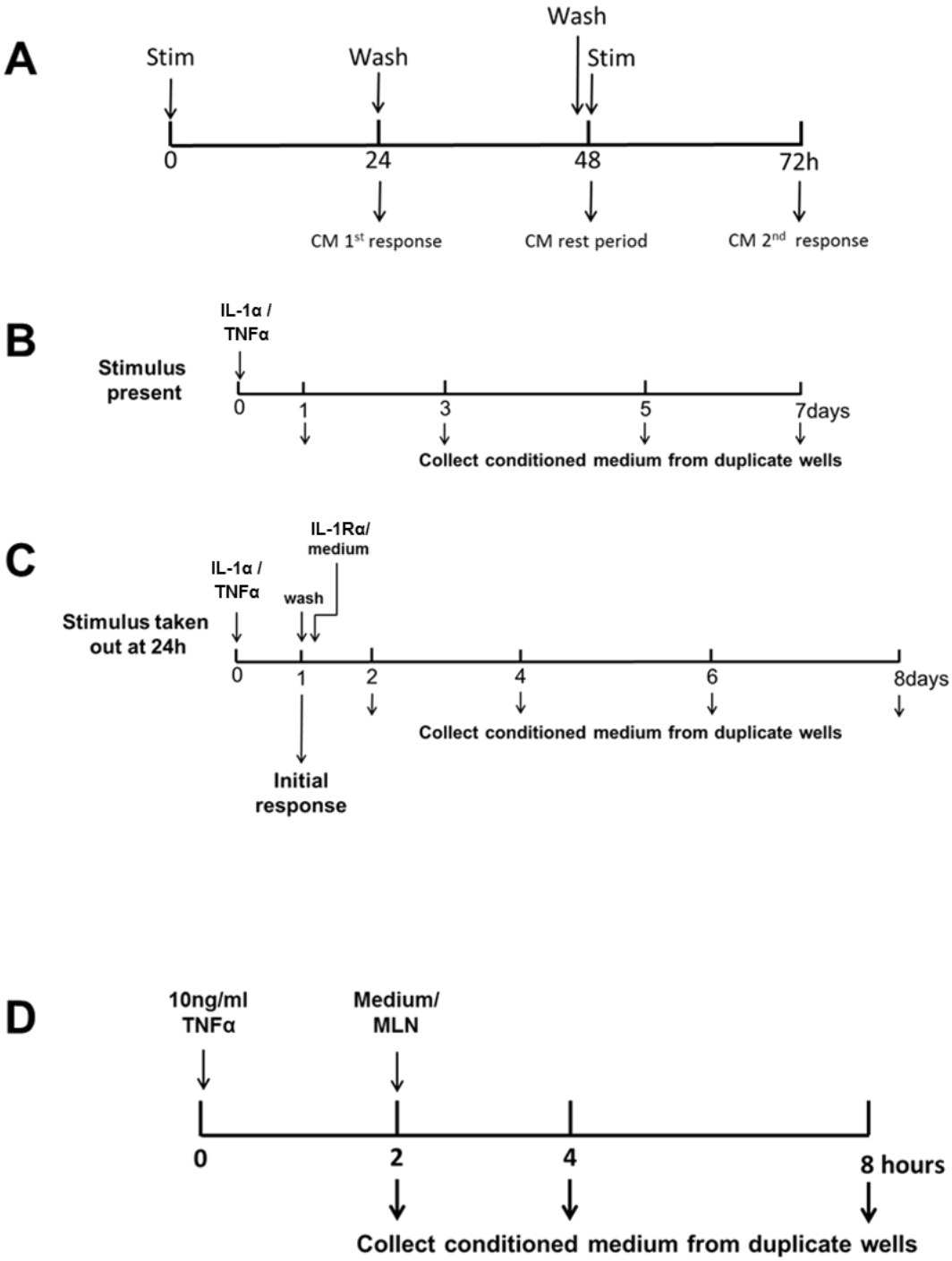

Figure legend overleaf

Figure 2.2: Schematics of experimental designs outlined in 2.5.1-4. A Cells were stimulated for 24h before conditioned medium (CM) was removed. Cells were washed and rested for 24h before medium was again removed. Cells were washed and stimulated again for 24h. CM was once again taken for analysis. **B** Cells were stimulated and CM was removed from duplicate wells at time points indicated. **C** Cells were stimulated for 24h, then CM was removed and cells were washed, then rested in medium or medium containing 1μg/ml IL-1RA for the time points indicated, before CM was removed from duplicate wells. **D** Cells were stimulated for 2h, then medium (control) or medium containing 100nM MLN was added for a further 2 or 6h (up to a total incubation of 4 or 8h). CM was removed from duplicate wells at the time points indicated. Duplicate wells were primed with 10ng/ml TNFα for 24h, then washed and rested in fresh medium before undergoing the same procedure.

Cells were washed with PBS and lysed using 350µl buffer RLT containing β mercaptoethanol. The lysate was frozen for later RNA isolation (see 2.6.4).

The stromal memory in this thesis is based on the repeat dose experiment. Whilst there are permutations on the method described above, these are made clear in the appropriate figure legends, with the main variations as follows:

- **Increased rest period.** In this variant, the rest period is one, three or seven days long.
- **Second dose titre.** Cells received 10ng/ml TNFα or IL-1α for the first dose, but received 10, 1, 0.1, 0.01ng/ml for the second dose.
- **Cross-priming.** Cells were stimulated with either TNFα or IL-1α for the first dose, then received either the same or the other cytokine for the second dose. All stimulations were 10ng/ml.
- **Time courses.** Cells were harvested at time points indicated in figure legends. For time points in the second stimulation, the cells were primed with the standard 10ng/ml TNFα for 24h as a first dose.
- **Macrophage endotoxin tolerance.** Macrophages were seeded at $1x10^6$ cells per well of a 24 well plate in 300µl macrophage medium. Other than this, the design is identical to the fibroblast repeat challenge.

2.5.2 Transient or unremitting response experiment

Cells were seeded overnight at 30,000 cells per well of a 24 well plate in 300µl of medium. The next day this was replaced with 700µL of medium, with or without 10ng/mL TNFα for 24h. After 24h, some wells had conditioned medium removed and were washed then rested in fresh medium for one, three, five, or seven days.

In other wells, TNFα-inclusive medium was left for one, three, five or seven days. Conditioned medium was collected at appropriate time points, and frozen for later analysis. This is shown in figure 2.2b and c above.

2.5.3 NFκB inhibition experiment

BJ fibroblasts were seeded at 30,000 cells per well of a 24 well plate in 300µl overnight before medium was replaced. Cells were stimulated with 10ng/ml TNFα for 2h, then conditioned medium was removed from one well as the '2h time point', whilst duplicate wells received MLN (final concentration 100nM, TNFα concentration remained 10ng/ml). Conditioned medium was removed after 4 or 8h. Other wells received 10ng/mL TNFα for 24h, then were washed and rested before the above treatments were added (primed cells). This is explained in figure 2.2d.

2.5.4 Proliferation test

T150 culture flasks had six crosses drawn on their undersides at equal distance apart. $2x10^5$ FLS were then seeded to adhere to the cross-labelled surface. Every 3.5 days (Monday morning and Thursday afternoon) for three weeks, the flasks were assessed for growth. Using a microscope with inbuilt camera, I focussed on the top right quadrant of a cross in the field of vision as shown in figure 2.3. I then switched to the camera function and took an image without moving the camera. I repeated this process for each cross, on each flask. This ensured that each time images were taken from the same reference point.

Cells were then counted on the Image J software 'cell counter' plug in. Cells per field of view were averaged across the six crosses for the mean number of cells per field of view for each cell line across three weeks. This was plotted as cells per field of view or as percentage increase from the first count as day 3.5.

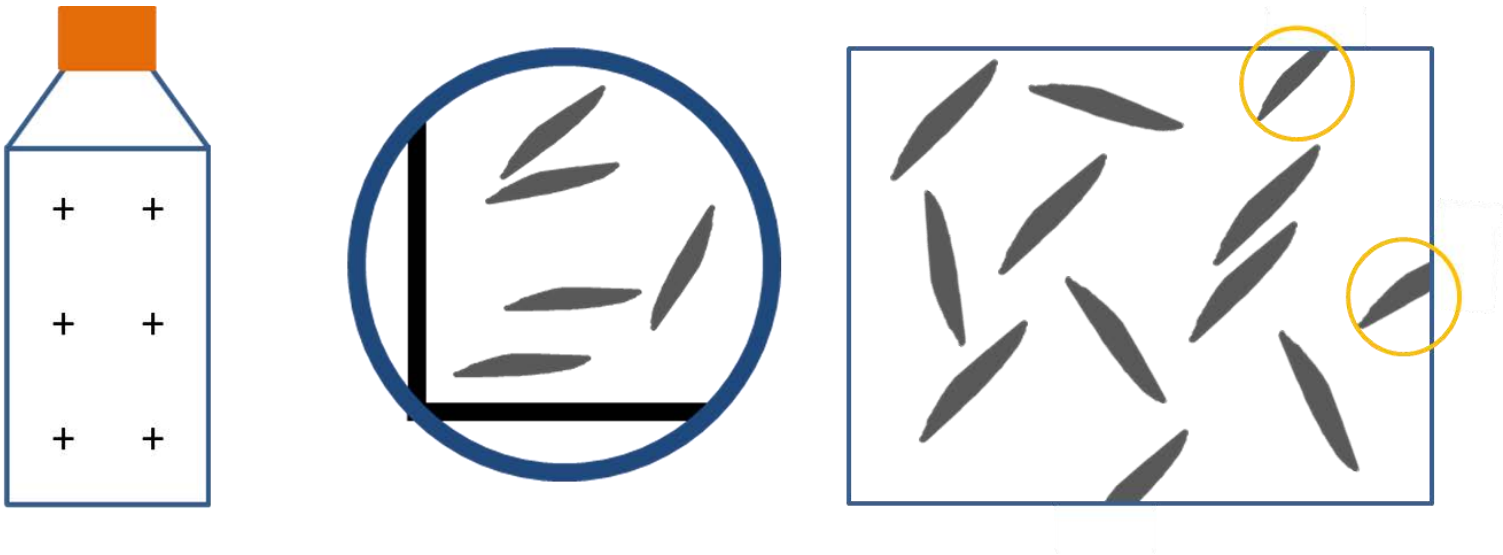

Figure 2.3: Assessment of fibroblast proliferation. **Left** T75 culture flasks were marked with crosses at regular intervals, and had $2x10^5$ cells seeded into them. **Centre** the top right quadrant of each cross was focussed on using a 10x microscope. **Right** camera view was then employed, and cells within the field of view counted. Circled cells would be excluded. Averages of all 6 crosses were taken as number of cells per field of view, per day, per flask.

2.5.5 Macrophage-conditioned medium experiment

Macrophages were seeded at $1x10^6$ cells per well of a 24 well plate in 300µl of macrophage medium, which was replaced the next day with 700µl of macrophage medium with or without 10ng/ml of LPS. After 1h conditioned medium was removed and cells were washed then rested for 23h in fresh monocyte medium. This unstimulated or stimulated conditioned medium was frozen for future use.

Fibroblasts were seeded at 30,000 cells per well in 300µl of complete growth medium overnight. The next day this was replaced with 630µl of fibroblast medium with or without 10ng/ml TNFα. Wells receiving TNFα also received macrophage conditioned medium from the above experiment neat (70µl) 1:10, 1:100, or 1:1000. Control wells received 70µl of macrophage medium. At 24h conditioned medium was removed. Cells were washed with PBS and lysed with 350µl RLT lysis buffer, then frozen for later RNA isolation. This is illustrated in figure 2.4.

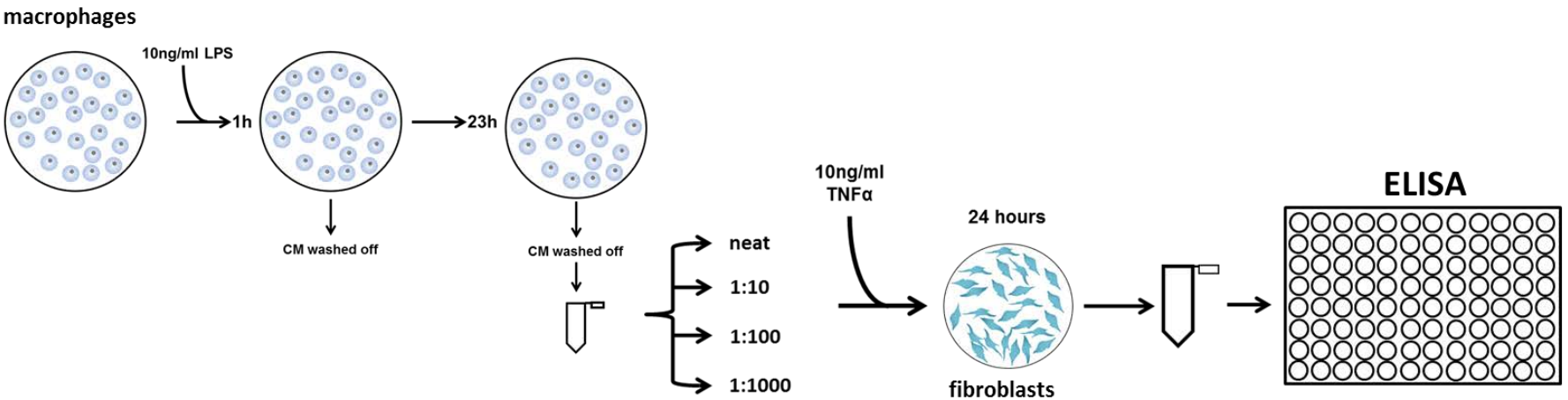

Figure 2.4: Schematic of experimental design for 'effect of macrophages on fibroblasts' experiment from 2.5.4. M1-like or M2-like macrophages were stimulated with 10ng/ml LPS for 1h, before being washed thoroughly to remove residual LPS. The macrophages were then incubated with fresh monocyte medium for 23h, before the conditioned media were removed. The conditioned media were added to fibroblasts either neat, or diluted as shown above. The fibroblasts were concurrently stimulated with 10ng/ml TNFα. Fibroblasts were then left for 24h. Conditioned media were removed and frozen for later ELISA analysis. Fibroblasts were lysed and the lysates frozen for future RNA isolation.

2.5.6 Conditioned medium effect on leukocytes

2.5.6.1 Effect on monocytes

Monocytes isolated as described in 2.3.2 were seeded into 48 well plates at a density of $3x10^5$ cells per well in 200µl macrophage medium. Cells were allowed to adhere for a minimum of one hour, then the medium and non-adherent monocytes were removed. M1-like and M2-like macrophage differentiation was induced using 300µl macrophage medium containing 25ng/ml GM-CSF or 50ng/ml M-CSF respectively. Other monocytes received macrophage medium with no differentiation inducers added. Other wells received 300µl of medium conditioned by fibroblasts responding to first or second TNFα challenge.

Cells were left for six days to allow differentiation, then examined by bright field microscopy as a rough assessment of ongoing viability and differentiation. The medium was removed and cells were washed with fresh macrophage medium. Cells then received 250µl of fresh macrophage medium and 50µl 1mg/ml pHrodo beads for 4h. After 4h exposure to pHrodo beads (which acted not only as a measure of phagocytosis but also as a pro inflammatory stimulus as the beads are derived from *E. coli*) the medium was frozen for future analysis by TNFα ELISA.

The cells were washed and 300µl medium was added. Cells were then examined by bright field and fluorescent microscopy. Most pHrodo beads should have been removed by the wash step following 4h incubation, however they are only capable of fluorescence upon acidification (i.e. inside a phagolysosome). Non-phagocytosed beads are not visible except under bright field conditions. Figure 2.5 shows the experimental procedure and an example image of phagocytosis.

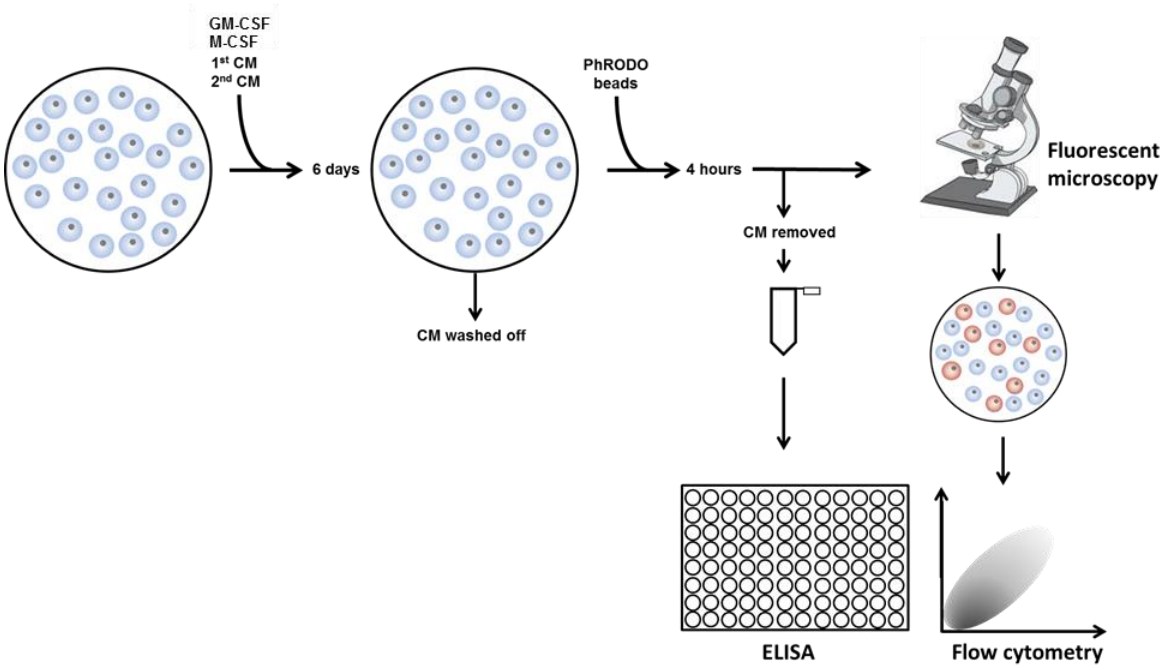

Figure 2.5: Schematic of experimental design for the 'effect on monocytes' experiment of 2:5.5.1. Monocytes were seeded and allowed to adhere before being exposed to differentiation factors or fibroblast conditioned medium for six days. The medium was removed and cells were washed, before receiving pHrodo bead stimulation for 4h. Conditioned medium was removed and frozen for future ELISA analysis. The cells were washed with fresh monocyte medium and viewed using bright field and fluorescent microscopy to produce a merged image of total cells and phagocytic cells. Cells were then removed from wells and incubated with antibodies to assess phagocytosis, differentiation and inflammatory response by flow cytometry. 1st CM= fibroblast conditioned medium from the first response to TNFα. 2nd CM= fibroblast conditioned medium from the he second response to TNFα.

2.5.6.2 Effect on T cells

CD4+ T cells isolated as described in 2.3.4 were seeded at $1x10^6$ cells per well of a 96well flat bottom plate, in 100µl T cell medium. One drop of 'activation beads'- micro beads coated with CD3 and CD28- provided T cells with activation signals. Control wells (designated 'unstimulated') did not receive the activation beads.

Of those wells receiving beads, some received fibroblast conditioned medium from the initial or memory response to TNFα. 100µl of conditioned medium was added per well of T cells, and those cells not receiving conditioned medium received 100µl of T cell medium, meaning all wells had a total volume of 200µl. Cells were incubated for five days, after which they were assessed for intracellular cytokine or transcription factor expression.

2.5.7 Peritonitis experiment

The peritonitis experiment was conducted by Sam Kemble, as I currently lack a home office licence for animal research. Sam was assisted in peritoneal lavage by Jenny Marshall, and both have my sincere thanks.

Conditioned medium was from murine FLS stimulated in the canonical repeat stimulation described in figure 2.2a, using 10ng/ml TNFα for both challenges. Conditioned medium was defrosted and used 1:1 with mouse fibroblast medium (100µl each). Wildtype C57/BL6 male mice at 6-8 weeks old were injected intraperitoneally with 200µl total volume containing mouse medium alone (control), first conditioned medium, or second conditioned medium. In one experiment zymosan was used alone in mouse medium as a positive control for acute inflammation, whilst in another 2µg zymosan was added with mouse medium, or second conditioned medium.

Mice of the same treatment were dispersed amongst cages to avoid cage effect. The housing facility used a 12h/12h light/dark cycle and mice had water *ad libitum* throughout the experiment, which lasted either 6 or 48h, whereupon mice were sacrificed by cervical dislocation.

2.6 Cell product collection and isolation

2.6.1 Secreted protein

Secreted proteins were collected by removing conditioned medium at the appropriate time points. It was transferred to labelled micro centrifuged tubes and frozen at -20^{o}C for future analysis.

2.6.2 Intracellular protein

Total intracellular protein was isolated from cells in Western blot experiments (see 2.7.4). Cells had conditioned medium removed at the appropriate time point and were washed with sterile PBS. 160µl RIPA buffer was added to lyse the cells, before the cell suspension was lifted from the surface with a cell scraper and transferred to a micro centrifuge tube. This was frozen at -20^{o}C for future analysis.

Once defrosted the lysate was centrifuged at top speed for 3 minutes in a QIAshredder tube to further separate the proteins. Samples were then quantified by Bradford assay, wherein densitometry was measured against a gradient of known protein standards.

2.6.3 Nuclear and cytosolic protein

In order to isolate and examine protein in the nuclear and cytosolic fractions of cells from Western blot experiments, the medium was removed, and cells were washed with sterile PBS then lysed with 160µl hypotonic buffer (see 2.1.3.3).

lysed cells were removed with a cell scraper and transferred to a micro centrifuge tube. A large gauge needle was used to further disrupt the lysed cells. The lysate was centrifuged at 16,000 RPM for 3 minutes at room temperature.

The liquid phase was stored as the cytosolic fraction. The nuclear pellet was resuspended in 100µl nuclear extraction buffer (see 2.1.3.3) and placed on a rotating device at 4^{o}C for 20 minutes. This was centrifuged at 15,000 RPM for 10 minutes at 4^{o}C. The liquid phase was removed as the nuclear fraction. The cytosolic and nuclear fractions underwent quantification by Bradford assay.

2.6.4 RNA

Conditioned medium was removed and cells were washed with sterile PBS. The cells were lysed with 350µl QIAgen buffer RLT (with β mercaptoethanol), the lysate was moved to a microcentrifuge tube and frozen for future analysis. RNA isolation was conducted using the QIAgen RNAeasy micro kit as per manufacturer's instructions.

2.7 Cell response analysis

2.7.1 ELISA

Sandwich ELISAs were used throughout and named in section 2.1.4.1. The only deviation from manufacturer's instructions was alteration of the concentrations of the standard curve. Standard concentrations were often low, and were therefore increased to get more treatments onto the same standard curve. Top standards were altered to 2ng/ml, with a 1 in 2 serial dilution throughout, as per manufacturer's instructions.

Briefly, 96 well flat-bottomed plates were coated overnight in coating buffer including capture antibody. This was washed thrice in was buffer (provided) in the morning, then blocked with assay diluent (provided) for 1h before again being washed thrice. Samples were added either neat or diluted in assay diluent, and the standard curve mentioned above was also added. All samples and standards were added in duplicate. After 2h the plates washed thrice, and a detection antibody suspended in asay diluent was added for one hour. After three washes, this was replaced by horseradish peroxidase in assay diluent. After 30mins the plates were washed thrice and the TMB substrate (provided) was added. This substrate cleaves horseradish peroxidase to elicit a colour reaction (clear to blue). The greater the intensity of the blue, the higher the concentration of the target protein. The reaction was stopped after around half the standard ladder had developed a blue hue, using 2M sulphuric acid. This elicited a colour change from blue to yellow.

OD values were analysed via spectrophotometer with an excitation wavelength of 450nm. The values were exported and a standard curve was formed from the known concentrations of the standard. The value of the blank was subtracted from that of the standard controls and the samples, and once dilution factors were accounted for this provided the concentration of the protein of interest.

2.7.2 Luminex

Magnetic bead-based single-plex or multiplex analysis of secreted products was conducted according to manufacturer's instructions in all cases. Briefly, analyte-specific antibodies attached to magnetic beads were coated onto a Luminex black plastic plate, and samples were added at a minimum of 1:2 (diluted in Luminex

diluent) in duplicate. Standards were made by resuspending the lyophilized proteins corresponding to the analytes being examined, and mixing these into one standard, which was serially diluted 1:3 in diluent.

The plate was then sealed with a foil lid and incubated at room temperature for 2h on an orbital shaker at 800RPM. This was then washed on a magnetised plate washer (to avoid washing away the beads) 3x 1minute. Biotinylated antibody cocktail was added and incubated under the same conditions for 1h. The plate was washed as before and Streptavidin HRP was added and the plate incubated under the same conditions for 30 minutes. The plate was washed three times, the samples resuspended in wash buffer, then resuspended again by gentle vortexing again immediately before being ran on the BioRad Luminex reader.

2.7.3 qPCR

RNA was used to form cDNA for qPCR analysis using the reverse transcriptase kit. 1µl reverse transcriptase and 4µl Iscript reaction buffer were added per treatment to RNA amounting to 0.1µg, and made up to 15µl with RNase-free water, giving a final reaction volume of 20µl. This was converted to cDNA in the thermocycler listed in section 1.8.2 with the settings: Lid temperature 105^{o}C, 5 minutes 25^{o}C, 30 minutes 42^{o}C, 5 minutes 80^{o}C. cDNA was then held at 12^{o}C on an infinite hold.

cDNA was made up to 100µl with RNase-free water, making a final cDNA concentration of 0.1µg/ml. 2.4µl cDNA was added to 3µl SYBR green and 0.3µl forward and reverse primers to make a qPCR reaction volume of 6µl per sample per gene. The light cycler conditions were as follows:

Initial denaturation	95 °C	30sec
	95 °C	10sec
40 cycles	59 °C	30sec
	78 °C	20sec
Melt curve analysis	99 °C	

The sequences of primer pairs used is displayed in table 2.2 above.

Once exported from the Light cycler into Microsoft Excel, the expression levels were assessed by the ΔΔCT method:

Technical triplicates averaged = $\bar{x}$

$\bar{x}$- reference gene $\bar{x}$= ΔCT

ΔCT- unstimulated ΔCT for that gene= ΔΔCT

2^- ΔΔCT= fold change from unstimulated expression of that gene

2.7.4 Western blotting

Lysates for Western blotting, whether total protein or isolated intracellular compartments, were subjected to Bradford assay to determine protein concentrations of each sample.

10µl of lysate was added in duplicate to a 96 well plate. Standards provided in the kit and comprising known concentrations of protein were made up to 2000µg/ml to 20µg/ml in serial dilution, and added in duplicates of 10µl/ well to the 96 well plate. ‘Solution A’ and ‘Solution B’ were mixed at a ratio of 50:1, and 200µl was added to

each well. The solution changed over incubation (37^{o}C for 30minutes) from greenish blue to purple, which correlated to the abundance of protein in each well.

OD values were read on a spectrophotometer at wavelength absorbance of 625nm. The blank was subtracted from the standards and samples, and a standard curve was developed, against which samples were compared and quantities of protein determined (averaged between the duplicates).

Following quantification, lysates were loaded to equal abundances of total protein (35µg). Protein volumes were added at a 5:1 ratio to 5x load buffer (see 2.1.4.4) and centrifuged at top speed for 1 minute. Protein samples were then denatured using a heat block at 95^{o}C for 5 minutes before being centrifuged again for 1 minute at top speed.

Samples were loaded into pre cast Western gels with 5µl protein ladder loaded at each side of the sample wells. Currents were run through gels at 120V for 80 minutes before a membrane was placed over the gel and the proteins transferred to the membrane using a trans blotter.

The Western blot was washed with TBS tween then blocked in 5% BSA-TBS tween for 1 hour at room temperature on a plate rocker. After another brief wash with TBS tween, an antibody suspended in either 5% milk-TBS tween or 5% BSA-TBS tween was added to the blot overnight on a plate rocker at 4^{o}C.

The next day the primary antibody was removed and the blot washed in TBS tween 3 times for 5 minutes. A secondary antibody was added and incubated for 1h at room temperature on the plate rocker. The blot was washed three times for 5 minutes before addition of 8ml ECL, made of a 1:1 ratio of the two enhanced

chemi luminescence (ECL) buffers. This was incubated for 1 minute then removed, and the blot was exposed to ultraviolet light using a BioRad chemidoc 'Universal Hood III'. Each protein detected was imaged, and a colorimetric image was also taken to overlay. This allowed quantification of band intensity from the original image, and band size verification using the ladder in the merged image.

Following imaging, the blot was stripped of antibodies by incubation 20ml of a mild stripping buffer on the plate rocker at room temperature for 20 minutes. The blot was then washed with TBS tween and blocked for 1 hour with 5% milk-TBS tween or 5% BSA-TBS tween before a new primary antibody could be added overnight.

Variation in volume loading was counteracted with α Tubulin as a load control. Densitometry of band intensity (explained further in section 1.8.3) was assessed for each protein. Each had its densitometry normalised against αTubulin densitometry for that well, then against the normalised value for the unstimulated well for that protein.

2.7.5 Immunofluorescence

Cells were seeded at 10,000 per chamber of 8 chamber glass slides and stimulated durations indicated in experimental figures. When the time points were reached, conditioned media were removed and cells were washed with PBS. The cells were fixed using 100µl 4% paraformaldehyde at 4^{o}C for 20 minutes, before being washed three times for 5 minutes with PBS.

Cells were then permeabilized with 100µl 0.2% Triton X-100 in PBS at -20^{o}C for 6 minutes, before three 5 minute washes with PBS. The cells were blocked with 100µl 10% horse serum in PBS at 4^{o}C for 1h, before three more five minutes washes with PBS.

The fixed and permeabilized cells were incubated with 200µl primary antibody (anti-NFκB RelA) at 1:200 in 1% horse serum overnight in a wet box at 4^{o}C. Duplicate wells which received identical treatment in the experiment received 200µl isotype control (Rabbit IgG) at 1:200 in 1% horse serum, and after this were treated the same as antibody-treated cells.

After overnight incubation cells were washed three times for five minutes with PBS, then incubated with 200µl secondary antibody (goat anti-rabbit) at 1:200, and nuclear counter stain 4', 6-diamidino-2-phenylindole (DAPI) at 1:100, in 1% horse serum in a wet box at room temperature for one hour. Cells were then washed again three times for five minutes with PBS.

The plastic separating the chambers was removed and each chamber received one drop of 1,4-diazabicyclo [2.2.2] octane (DABCO) before a cover slip was added. The slide was then stored in the dark at 4^{o}C for future analysis.

2.7.6 Flow cytometry

Cells were transferred to a round bottom 96 well plate and centrifuged at 300xg for 5 minutes to pellet. Cells were then permeabilized if necessary using 100µl cytofix/ cytoperm solution for 20 minutes before being washed with 100µl FACs buffer and centrifuged at 400xg for 5 minutes.

Antibodies or isotype controls were made as a master mix or as single colour controls, with 100µl/well. These were added to cells and incubated for 20minutes in the dark at 4^{o}C before being washed with FACs buffer and centrifuged. Cells were then resuspended in 300µl FACs buffer and transferred to flow cytometry tubes. If appropriate, 5µl of absolute count beads were added to samples.

The threshold for auto fluorescence was established using unstained control cells, and compensation of ‘bleed over’ from one fluorophore to another was controlled using single colour controls. In the event of poor cell numbers, especially when low numbers meant spare wells for controls were unobtainable, positive and negative beads were used to provide a positive and negative signal with each single colour control. Compensation of bleed over was enacted internally by the flow cytometer software, which automatically compensates based on signal colour controls. Once the compensation was established each fluorophore was compensated against all the others, and therefore axes were labelled ‘comp’ fluorophore henceforth. Upper thresholds for auto fluorescence were used as starting points for gating the positive samples. Example thresholds are displayed in figure 2.6 below.

The gating strategy for each experiment differed, but after compensation the fragmented cells in the bottom left corner of each graph, and the beads in the top left, were both excluded, leaving the first gate known as ‘all cells’. This gate was applied to all samples and acted as the original parent gate for subsequent gating. The exception to this was the analysis of dead cells via zombie stain, in which case gating on live cells first would be inappropriate.

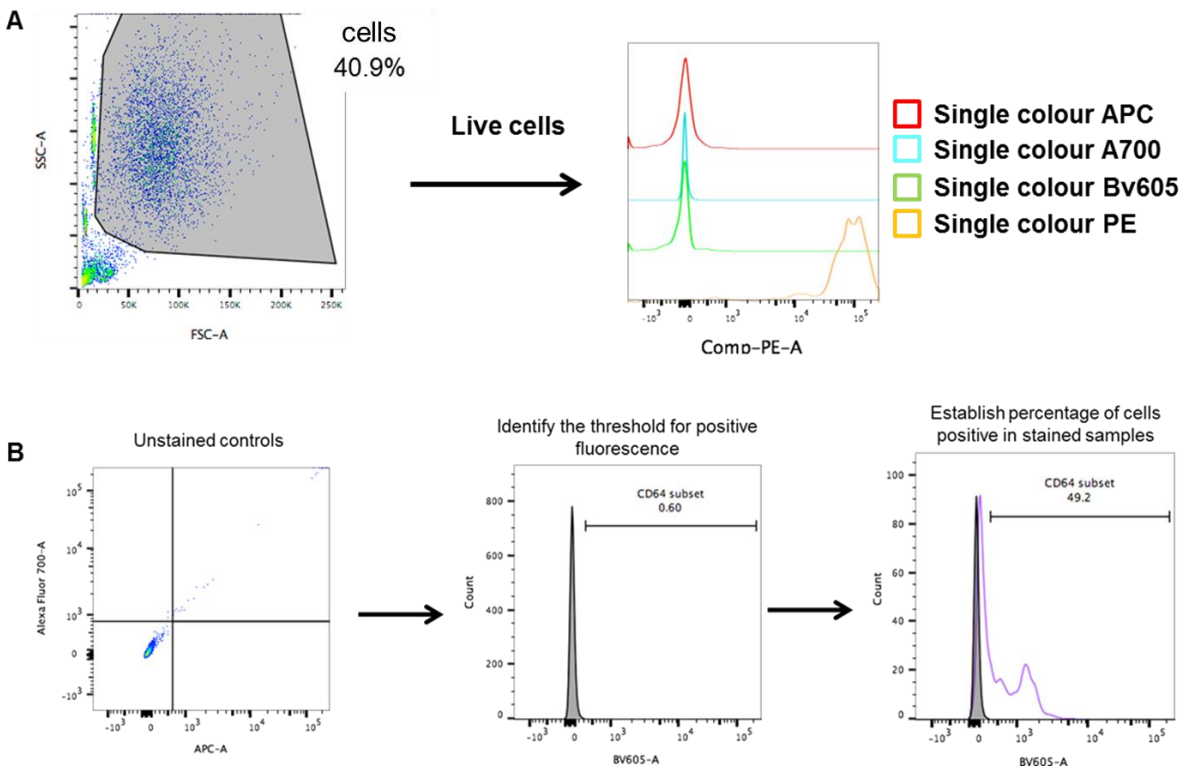

Figure 2.6: Establishment of negative and single colour controls for flow cytometry. A Single colour controls were used, wherein cells incubated with a single antibody were gated for live cells only, and each fluorophore was compared against the others to ensure fluorescence occurred within one channel. **B** unstained controls were again used to illustrate the degree of fluorescence in each channel that was natural, rather than due to use of conjugated antibodies. The negative peak was used as the start point for the positive gate (shown I the middle image), and stained samples were assessed for percentage of cells surpassing this lower limit (right image).

2.7.7 Peritonitis model

The peritoneum of sacrificed mice was flushed with 5ml sterile PBS syringed into the cavity and the abdomen was massaged to encourage cellular infiltrate into solution. The lavage was then removed by syringe. Lavages were transferred to 15ml universals and kept on ice whilst transferred from the animal facility back to the laboratory. The possibility of erythrocyte contamination was addressed by use of 500µl red cell lysis buffer for 2 minutes in each sample. The universals were centrifuged at room temperature, at x400g speed for 5 minutes. After being resuspended in 100µl FACS buffer and transferred to a 96 well round bottom plate, the cells were treated with the same methodology described in section 2.7.6.

The gating strategy for the peritonitis model was formalised separately to the *in vitro* assays. This is summarised in figure 2.7. Briefly, viability was established using the zombie dye, and the negative population was named ‘live cells’. The live cells were then gated on CD3+ vs γδTCR (T cell and γδT cell populations), CD3 vs B220 (T cell vs B cell), Gr1 vs CD11b (double positive neutrophils), and F4/80 vs CD11b (double positive macrophages).

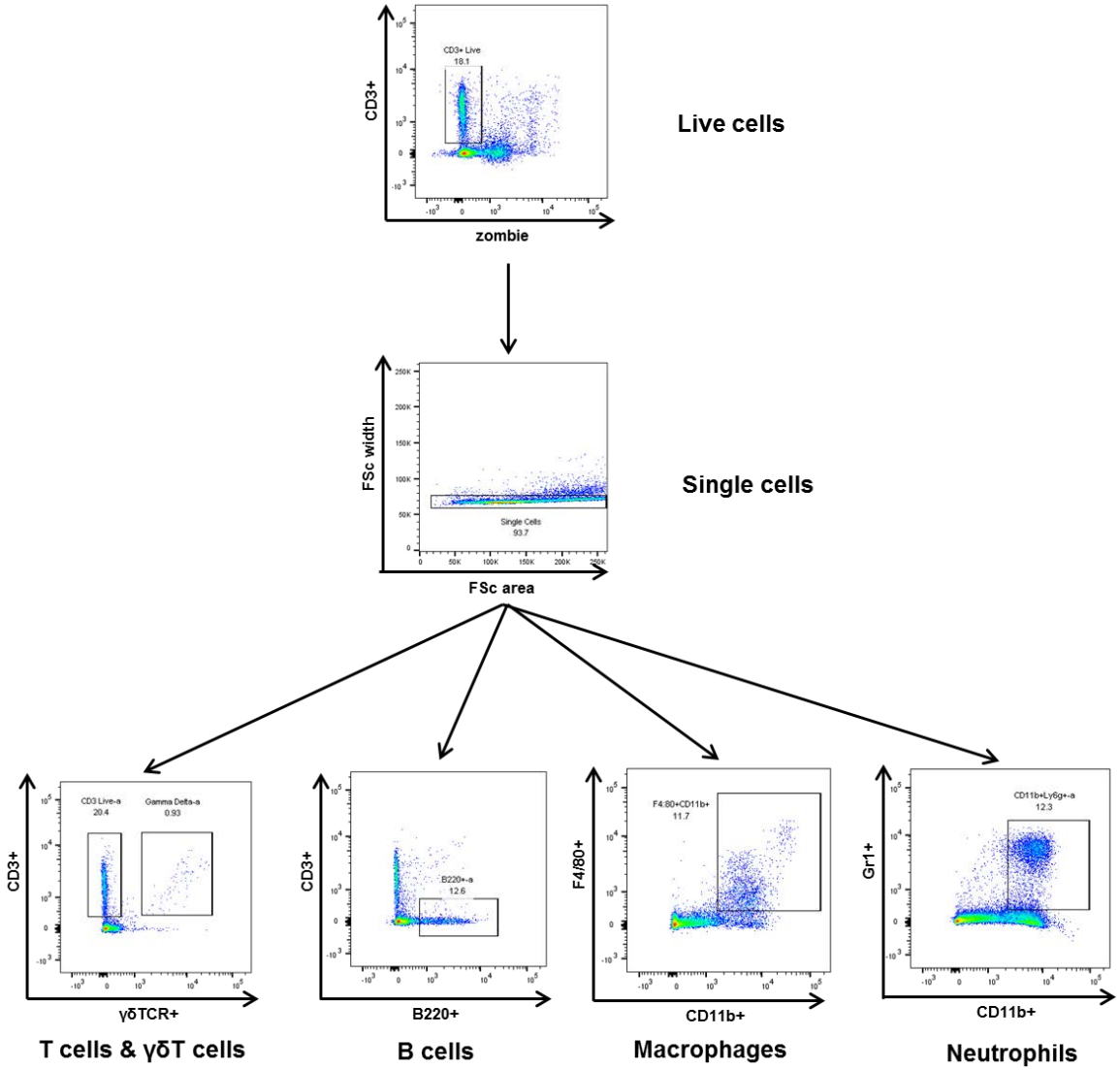

Figure 2.7: Gating strategy for the *in vivo* peritonitis experiments. Isolated and stained cells were sorted based on their uptake of zombie yellow (indicating dead cells), and the negative population was further examined. The doublets were excluded, and then gating strategies isolating populations of T cells, B cells, macrophages and neutrophils were employed. Representative plots.

2.8 Software

2.8.1 ELISA

The BioTek EL808 spectrophotometer used the Gen5 software package for assessment of optical density. The values provided were exported to Microsoft Excel before being transferred for graph production and statistical analysis in GraphPad Prism 5.0.

2.8.2 qPCR

The cDNA synthesis was conducted in a BioRad T100 thermocycler.

The lightcycler 480 hardware used for qPCR experiments used the Light cycler 1.5.1.62SP2 software package for conducting and recording gene expression. Files were saved in the light cycler format, but opened in Microsoft Excel and converted into Excel files for analysis. Values produced in Excel were transferred to GraphPad Prism 5.0 for graph production and statistical tests.

2.8.3 Western blot

The BioRad chemidoc ‘universal hood III’ used ImageLab 5.1 software package. Densitometry values were produced in this software. Images of blots were exported for publication at 600 dots per inch resolution and cropped in Microsoft PowerPoint. Densitometry values were recorded in Microsoft Excel, and then transferred to GraphPad Prism 5.0 for graph production and statistical analysis.

2.8.4 Immunofluorescence

2.8.5 Flow cytometry

Flow cytometry was conducted on the Fortessa flow cytometer, using the Diva software package. Files were exported for examination with FloJo software.

2.8.6 Graph production and statistics

All graphs and statistics were drawn and conducted in GraphPad Prism 5.0.

2.8.7 Schematics and cartoons

Figures produced to illustrate concepts were constructed by myself. This was either in Microsoft PowerPoint or by hand with pens and watercolour paints.

2.9 Statistical analysis

Statistical analysis was conducted in GraphPad Prism 5.0 and was largely split into analysis of raw values and analysis of fold changes.

Tests of significant difference in raw expression or secretion of RNA or protein were conducted using the non-parametric Mann-Whitney *U* test, which assesses significance of difference between data sets without the presumption of normal distribution, which would be inappropriate in small *n* numbers.

Variation in raw values due to inter-patient heterogeneity meant it was also appropriate to assess the difference between first and second response as a fold change. First responses were normalised to one, and second responses expressed as fold changes from this. The fold changes were assessed with the Wilcoxon matched-pairs signed rank test. Comparison between fold change and a

normalised first response (which is always one) meant the Wilcoxon was more appropriate than the Mann-Whitney U test for assessment of fold change.

When comparisons were made between raw values from the BJ line or from mice, inter-patient heterogeneity did not apply. As replicates were genetically identical the parametric comparison of difference is appropriate. This means raw values in these cases were assessed by unpaired t test.

The Kruskal-Wallis statistical test is a non-parametric interrogation of the difference within and between multiple groups (the equivalent of an ANOVA test). This was used to compare the inflammatory responses of fibroblasts from multiple sites of origin against each other.

Error bars represent standard deviation (SD) or standard error of the mean (SEM). In cases such as ELISAs, wherein each sample value represents a mean of experimental replicates, the error bars are SEM. The mean value for each line is taken and combined with those of other lines to provide a ‘mean of means’, and therefore SEM was appropriate. When mean values are not combined, such as in the peritonitis experiment, error bars represent standard deviation.

3 Fibroblast response to chronic and repeat inflammatory challenge

3.1 Introduction

3.1.1 Innate inflammatory memory

Whilst the memory response of the mammalian immune system may usually refer to the cells of the adaptive system, it would be wrong to describe this as the 'traditional' view. For nearly a century findings in plants, invertebrates and mammals have suggested the innate immune system has its own form of memory.

Many genera of plants are capable of mounting stronger defences against infection if they had been previously challenged. This 'systemic acquired response' (SAR) provides the plant with enhanced resistance for months or even years following the primary infection [274, 275]. The response also differs from mammalian adaptive memory in that SAR is not pathogen specific, and can provide protection against a variety of secondary infectious species.

The presence of immune memory of different kinds has been recorded in invertebrates and even lower metazoans (reviewed in [276]). The divergence of these taxa from vertebrates also predates adaptive immunity, although invertebrates have recognized innate immune cells, called hemocytes. These provide a generalized inflammatory response to any infectious agent, in much the same way as the mammalian innate response does in the first 24h of an infection.

One form of innate memory seen in invertebrates is pathogen-specific, and thus provides a similar function to the adaptive immune response of vertebrates.

Drosophila melanogaster, for example, mount an augmented second inflammatory response to *Streptococcus pneumoniae* or the fungus *Beauveria bassiana*, but not three other pathogens [277]. In their review on innate immunity in 2011, Netea and colleagues posited that immune memory of specific-infections (the rarer form) was lost in vertebrates as adaptive immunity evolved; the increased specificity of epitope recognition making the primitive form redundant [278].

The far more common form of invertebrate innate immunity is non-specific. Shrimp, for example, are more resistant to white spot syndrome if previously exposed to viral protein [279]. Similarly, meal worm beetles mount a stronger defence against fungi if previously injected with lipopolysaccharide (LPS) [280].

The mechanisms behind such memory are not fully defined. Although plant SAR researchers investigated the roles of certain gene regulators and signalling molecules (such as reactive oxygen species) [281], the mechanisms allowing memory to be embedded for prolonged timespans are largely still unknown. For years experiments like these were “forgotten”[282], but meanwhile the field of vertebrate innate immune memory was progressing in parallel, seemingly separated from the evidence of these ancient species.

In 1932, Naeslund found that vaccinating children with Bacillus Calmette–Guérin (BCG) reduced mortality rates below that caused by tuberculosis(TB) [283]. The fact that the TB vaccine was saving more lives than were lost to TB suggested an off-target immune defence. Since its discovery, the off-target effects of the BCG vaccine have been shown to provide protection against a host of infections, and even auto-inflammatory reactions such as asthma (reviewed in [284]).

Studies in BCG-vaccinated mice confirmed this improved defence. Crucially, the increased resistance to infections other than *Mycobacterium tuberculosis* was also confirmed in athymic mice [285], proving the off-target defence was at least partially independent of the adaptive immune system.

3.1.2 Monocyte/ macrophage memory

Macrophages are key cells in the innate immune response, and early experiments into mammalian non-specific memory identified macrophages as the repository of innate memory [286-288]. The studies into macrophage memory have spanned decades, showing these cells to be imbued with more than one form of memory.

One form is similar to the prolonged vaccine response-like memory described above. This has been shown with off target vaccine responses like the BCG vaccine protecting mice from candidiasis [289]. Further studies have shown pathogens ranging from *C. albicans* (fungi) to *Staphylococcus aureus* (bacteria) can ‘train’ monocytes and macrophages to mount stronger responses to secondary infections [286-288, 290-292]. The memory response protects non-infected macrophages (in the same tissue as persistent infections) [293] and is transferrable, as splenic dendritic cells are capable of protecting a recipient mouse from lethal bacterial challenge if the donor had been challenged first [291].

Whilst not the life-long protection of adaptive memory, trained immunity has longevity spanning weeks or months [290, 291, 294]. In comparison, the other form of innate memory attributed to macrophages is much shorter-lived. In 1947, Beeson found the pyrogenic effect of bacterial ‘substances’ on rabbits was reduced in successive exposures [295]. In 1965, Hollingsworth and Atkins found that a sub lethal dose of LPS would protect rabbits from a subsequent dose that

was lethal in ‘naïve’ rabbits [296]. This reduction in overt inflammation and mortality rates was referred to as endotoxin tolerance (ET), and some years later it was identified as a characteristic of macrophages (reviewed in [273, 297, 298]).

This form of innate memory is juxtaposed to that previously described; it does not confer a greater resistance to further infection. It does, however, save the host from lethal cytokine storms, a product of over-zealous inflammatory reactions to infectious agents. Interestingly, super-low doses of LPS do not induce tolerance, but maintain a low level of inflammation [299]; supporting the view that tolerance is a tissue-protective mechanism. As early effectors of the inflammatory response, macrophages can be strongly pro-inflammatory. Given continuous stimulation and a pro inflammatory microenvironment (partly of their own making), macrophages can cause death by cytokine in response to a number of immune challenges.

Macrophage ET is not a general refractory state, but a tightly regulated altered response. The initial exposure to danger signals like LPS induces a robust inflammatory response, but upon exposure to further stimuli the cell will release negligible pro inflammatory cytokines (such as IL-6 and TNFα). In contrast, the expression of proteins involved in phagocytosis are increased [300, 301]. In this way, macrophages may respond to an infectious agent, then reduce the risk of causing host tissue damage, whilst still maintaining their phagocytic function. This limits the risk to self but facilitates the removal of inflammation-triggering microbes.

In contrast to the trained immunity discussed above, Macrophage ET is a comparatively transient phenomenon. The refractory state has been found to last from 24-48h [300] to five days [301], before cells revert to a pro inflammatory response to LPS. Endotoxin tolerance is therefore distinct from trained immunity

as it does not prepare the host for future infection. It is a mechanism for managing an ongoing inflammatory episode in such a way as to balance the need to remove pathogens with the much greater need to not kill the host.

3.1.2.1 Endothelial cell memory

Whilst innate memory in leukocytes has been accepted for decades, its existence in stromal cells has taken much longer to come to light. Endothelial cells (EC) have an obvious role to play in the innate response as they form the lining of blood vessels, and therefore facilitate leukocyte and pathogen access to tissue.

Claims of EC memory appear contentious, although this is partly due to differing sites of origin [302-304]. Vein ECs (HUVECs) have been shown to undergo ET similar to that of macrophages (although not necessarily through the same mechanisms) [303, 304], whilst Wang et al found EC lacked endotoxin tolerance [302]. They have, however, also been shown to augment their pro inflammatory roles in response to other agonists, such as TLR3 priming of an augmented response to TLR4 ligands [303].

The field of EC memory is awash with possible roles for the phenomenon. Prior infection with *Schistosoma mansoni* induces EC to a six-fold increase in leukocyte adhesion, presumably resulting in an increased response to subsequent infection [305]. Similarly, chronic exposure of aortic EC to even slightly elevated homocysteine (an endogenous product used as a clinical marker for atherosclerosis) increases leukocyte adhesion in response to subsequent thrombin or LPS exposure. This priming is therefore suggested to play a role in homocysteine's contribution to atherosclerosis [306, 307]. A link between sepsis and atherosclerosis via monocyte inflammatory memory has also been proposed

[308]. Given that sepsis and pneumonia heighten risks of atherosclerosis and increase risk of death months after infection resolves [309, 310], both myeloid and stromal memory may have important clinical ramifications.

In furtherance of this concept, *ex vivo* priming of EC progenitors has been suggested as a therapeutic in regenerative medicine. Priming with CXCL12 improves the functionality of these cells when they are transplanted back into a host and differentiate into mature EC [311].

The examples displayed above suggest a complicated role for EC memory, which appears capable of both negative (tolerance) and positive (priming) facets. This may defend against future infections, but the same mechanisms however, may act as a tipping point contributing to self-induced tissue damage. EC memory therefore acts as a good example of the fine balance the inflammatory response must maintain in order to avoid pathogen and self-induced morbidity and mortality.

3.1.2.2 Fibroblast memory

As with EC, fibroblast memory appears to depend on site and stimulus. Pre-treatment of corneal fibroblasts with LPS causes tolerance in response to *Aspergillus fumigatus* [264]. Similarly, the same group found that zymosan (a TLR2 agonist) caused reduction of IL-8 following *A. fumigatus* infection, and zymosan with LPS induced an even greater tolerance to subsequent fungal infection [312].

In comparison, gingival fibroblasts maintain their IL-6 and IL-8 response to re challenge with LPS. This was explained as a mechanism to maintain inflammatory pressure on oral pathogens whilst macrophages are in a tolerant, refractory state [313]. This lack of memory was confirmed by Zaric et al [314], who also showed

that pre-treatment with a combination of IFNβ and LPS would inhibit an IL-8 response to further stimuli in gingival fibroblasts, similar to a report in corneal fibroblasts [264].

Years earlier, Sakuta et al also showed an effect of IFNα, β, and γ on gingival fibroblast memory [315]. Co stimulation of any of the IFNs and LPS induced IL-8 tolerance, but pre-treatment with IFNs alone followed by LPS would augment IL-8 expression. All of this suggests a role for IFNs in the memory response of gingival fibroblasts, but Zaric and colleagues found no trace of IFNβ in medium conditioned by these cells, suggesting a cell interaction (possibly with infected macrophages) may be necessary to elicit the memory response [314]. Finally, Ara et al showed a dermal fibroblast line, TIG1, to show incomplete tolerance in repeat stimulation with LPS (around a 30% reduction in IL-6) [313].

Whilst displaying different responses, only one theory has been proposed for the evolution of fibroblast memory. Maintenance of inflammatory pressure during oral infection is a reasonable theory, based on the function of macrophages rather than that of fibroblasts. This however would not fit with the roles of IFNs in the above studies. Macrophages release IFNs during infection, but if the infection was in progress fibroblasts would be exposed to LPS at the same time (which induced tolerance rather than maintaining inflammation).

Clearly there is a body of investigation, albeit small, on the innate memory of fibroblasts. The mechanisms behind innate memory (discussed further in chapter 4) are virtually ignored in the above studies, as are the functional consequences.

Fibroblasts have many key roles in inflammation, and many opportunities to contribute to immunopathology in chronic inflammatory disease. It is possible that

fibroblast memory is one such difference between healthy and disease inflammation. With that in mind, I decided to induce fibroblast memory with a challenge-rest-re challenge model. My first hypothesis was that healthy FLS would undergo tolerization in response to a second challenge with TNFα or IL-1α, as a tissue-protective effect like that seen in macrophages. My second hypothesis was that the negative regulation underlying tolerance would be aberrant in RA FLS, leading to maintenance of inflammatory response instead of tolerance.

Given that Lee *et al* had shown an unremitting response to continued stimulation with TNFα [272], I also wanted to examine whether this unremitting response occurred in control FLS. This, then, is how I started my investigations.

3.2 The unremitting inflammatory response of synovial fibroblasts

3.2.1 Chronic and transient stimulation

FLS stimulated with 10ng/ml TNFα or 10ng/ml IL-1α both mounted strong IL-6 responses in the first 24h. The levels of IL-6 in conditioned medium continued to rise throughout the time course. The TNFα-induced concentrations of secreted IL-6 showed a significant difference when assessed by Kruskall-Wallis test (p=0.001). The Dunn's test showed significant differences of the amount secreted after five or seven days compared to unstimulated cells. The increase between days of stimulation did not reach significance, but were nevertheless clear (for example three days 6.745± 2.3ng/ml after three days versus 14.60± 4.5ng/ml at five days).

Incubation with 10ng/ml IL-1α again showed significance by Kruskall-Wallis test (p=0.0018), significance between unstimulated and five and seven days of stimulation, and a non-significant but continuous increase in IL-6 secretion across the time course. Both stimuli therefore induced unremitting inflammatory responses as reported by Lee et al.

Removing stimuli after 24h showed novel results (see figure 3.1b). The levels of IL-6 were still high 24h after removal of TNFα. This did not increase across the next six days however, suggesting IL-6 production in the week post wash occurred in the first 24h. The Kruskall-Wallis test showed a significant difference across the time course, but the Dunn's post-test did not show any significant differences between individual time points.

IL-6 concentrations secreted 24h post-removal of IL-1α were modest. The concentration of IL-6 continued to rise throughout the week, from 13.78± 5.9ng/ml after 24h rest to 100.7 ±23.5ng/ml after seven days of rest. IL-6 levels reached the same concentration as induced by 24h of 10ng/ml IL-1α by day 5. Kruskall-Wallis testing showed significant differences across the time course (p<0.0001), and the Dunn's test found a significant difference between IL-6 concentration secreted after one day of rest and seven days of rest.

The unremitting response therefore appears to occur in control FLS. Removal of TNFα appeared to induce continued IL-6 secretion for a brief period, whilst removal of IL-1α did not stop FLS secreting significant concentrations of IL-6 for a prolonged period.

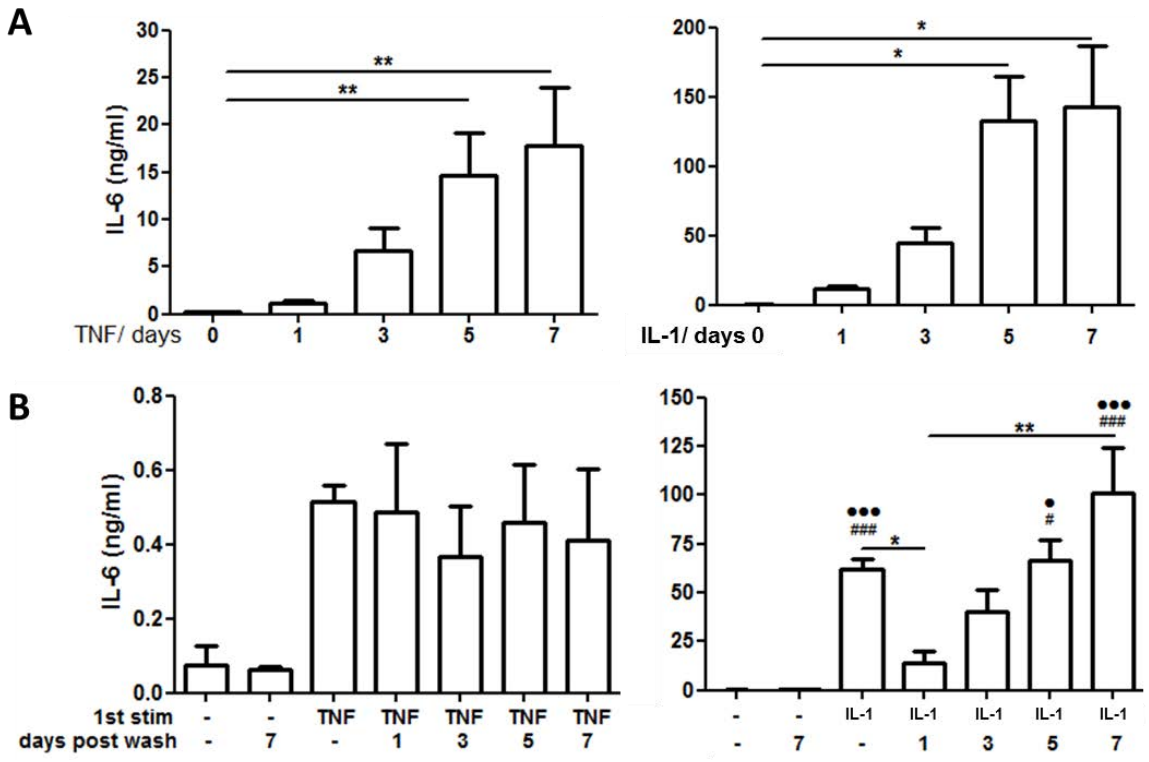

Figure 3.1: The unremitting response of fibroblasts requires ongoing TNFα, but not IL-1α. FLS were stimulated with 10ng/ml TNFα or IL-1α, or received media (control). The treatment was either left on the cells for up to seven days (**panel A**) or removed after 24h. If the latter, cells were washed and fresh medium was added for the indicated durations (**panel B**). Secreted IL-6 was assessed by ELISA. n=4-6. Assessed by Kruskall-Wallis comparison and Dunn's post-test. * p=0.05, ** p=0.01, *** p= 0.001. In **B**, significant difference from one day of medium (- -) signified by #, significant difference from seven days of medium control (- 7) signified by ●, significant difference between cytokine-treated cells signified by *.

3.2.2 The effect of transient stimulation on synovial fibroblast inflammatory secretion

The amount of IL-6 produced in the period post-wash differed according to stimulus. Given the high levels of IL-6 produced in the 24h post-TNF removal, I tested whether this was due to ongoing stimulation, or simply the result of residual mRNA translation from the last few hours of the 24h stimulation period. To this end BJ fibroblasts (which are useful tools for examining fibroblast biology due to their rapid proliferation and an increased number of divisions without undergoing replicative crisis) were stimulated for 24h, before TNFα removal as per previous experiments, before IL-6 secretion over the next 24h was assessed. If the hypothesis were true, IL-6 secretion would be high at early time points in the rest, and negligible in later time points.

As shown in figure 3.2, a robust induction of IL-6 occurred in the 24h of stimulation with 10ng/ml TNFα. The levels of IL-6 secreted post wash however, low (220± 9pg/ml at 2h), and increased slowly throughout the time course. The level at 24h (730± 9pg/ml) was higher than any earlier time point (albeit non-significantly as assessed by multiple comparison), indicating a continued production of IL-6. It was not, however, anywhere near the level secreted in the 24h of stimulation. This contrasts the data from figure 3.1b, which suggested high concentrations being secreted 24h after washing the cells. Figure 3.2 appears to indicate that whilst IL-6 does indeed continue to be secreted, it is not secreted in large quantities.

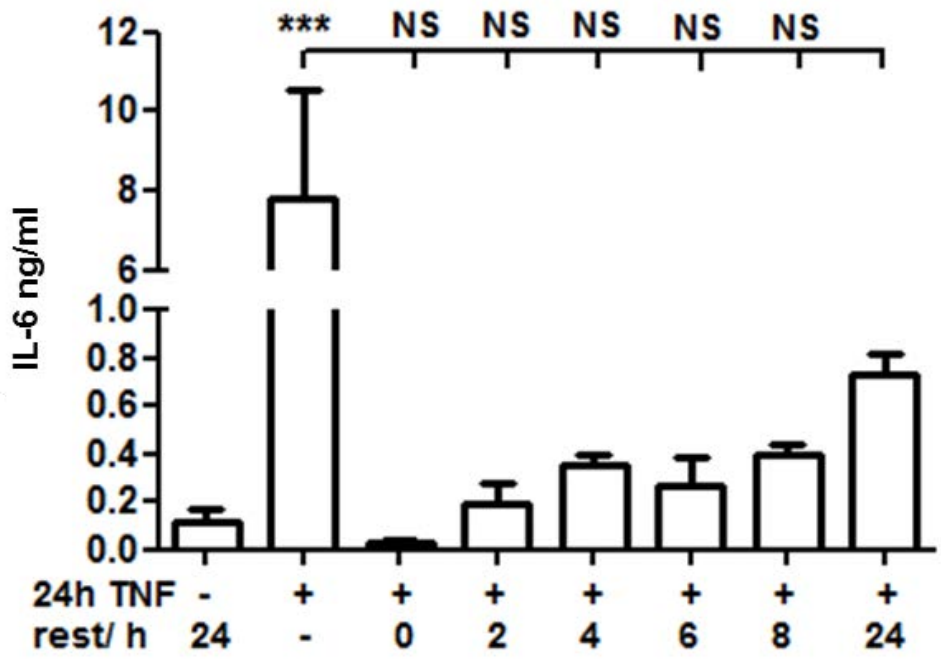

Figure 3.2: IL-6 secretion continues at a low level during the 24 post-removal of TNFα. BJ fibroblasts were stimulated with medium (control) or 10ng/ml TNFα for 24h, before being washed and rested in fresh medium. This was removed at indicated time points and assessed for IL-6 secretion. n=7, one-way ANOVA with Dunnet's post-test against 24h post removal of TNFα (*** $p< 0.001$).

The level of IL-6 following removal of IL-1α were low (see figure 3.1b), but continued to increase throughout the rest period, suggesting continued stimulation of FLS. To test the possibility of residual IL-1α stimulating fibroblasts in the rest period I conducted an IL-1α titration. As shown in figure 3.3, 10ng/ml IL-1α induced the greatest IL-6 response of the concentrations tested (38.11 ±3.54ng/ml). It was not significantly higher than 1ng/ml (35.59 ±2.09ng/ml), suggesting a ceiling effect at 1ng/ml. Lower concentrations induced a significantly reduced IL-6 response, but even 0.1ng/ml (100-fold lower concentration) still significantly increased IL-6 compared to medium alone.

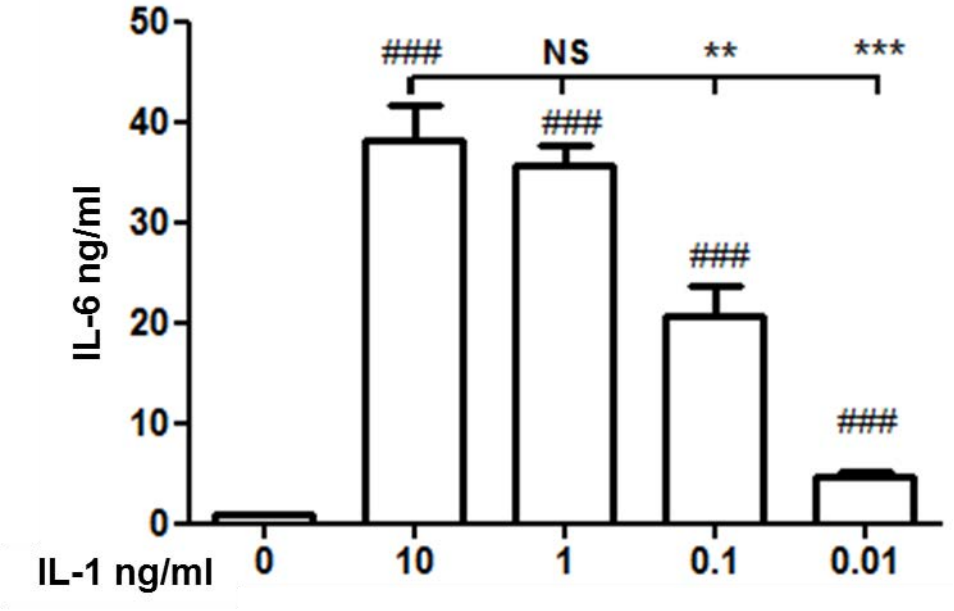

Figure 3.3: Low doses of IL-1α significantly induce IL-6 secretion by fibroblasts. BJ fibroblasts were stimulated for 24h with medium (control) or the concentrations of IL-1α indicated. n=4, one-tailed ANOVA with Dunnet's post-test against unstimulated control (### p<0.001) or against 10ng/ml IL-1α (*** p<0.001).

To test whether residual IL-1α post wash accounted for the IL-6 concentrations seen in the rest period, I used IL-1 receptor antagonist (IL-1RA) as an inhibitor of IL-1 receptor binding. The dose titration shown in figure 3.4a suggests all tested concentrations of IL-1RA (0.1-20μg/ml) were sufficient to reduce 1ng/ml IL-1α-induced IL-6 levels to those of unstimulated cells. 1μg/ml IL-1RA was used in subsequent experiments.

Addition of 1ug/ml IL-1RA at the start of the rest time course did not cause significant reduction of IL-6 secretion following former IL-1 stimulation. There were no significant differences between the IL-6 secreted by cells exposed to medium only and those exposed to medium and IL-1RA in the week following removal of IL-1α. This suggests free IL-1 following the wash was present in negligible amounts. It is worth noting however, that unlike the IL-1-induced IL-6 concentrations displayed in figure 3.1 the IL-6 levels did not continue to rise throughout the time course in figure 3.4. The lack of difference may not therefore prove free IL-1 is not further stimulating fibroblasts, but it certainly does not prove it *is* having an effect.

Based on the above data, I confirmed Lee et al's published findings on the unremitting response of RA FLS to pro inflammatory cytokines. I also showed that control, non-RA FLS also mounted an unremitting response, and that this is dependent on the ongoing presence of TNFα but perhaps not IL-1α.

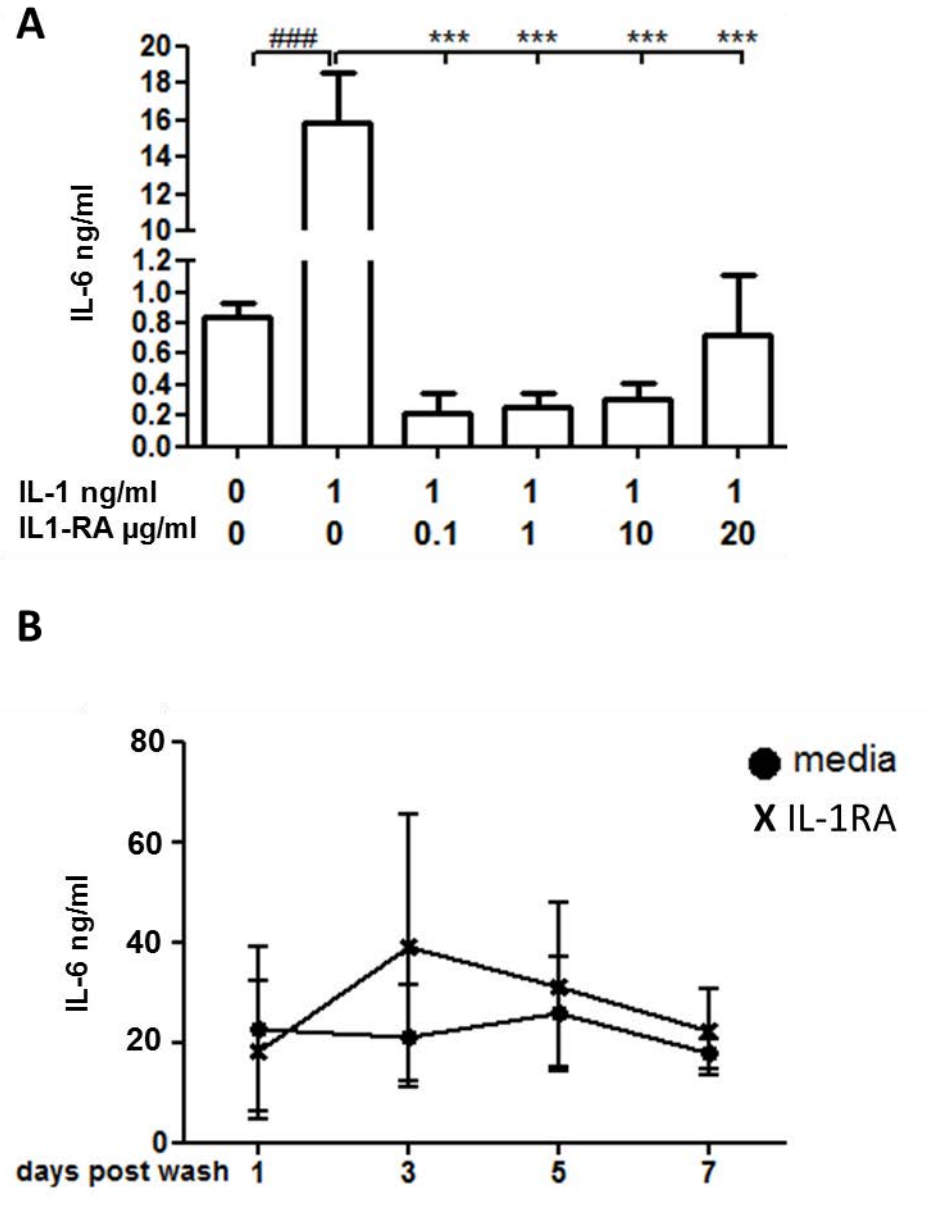

Figure 3.4: IL-1RA does not appear to affect IL-6 secretion following removal of IL-1. A BJ fibroblasts were stimulated with medium or 1ng/ml IL-1α for 24h. Duplicate wells receiving IL-1 also received IL-1RA at the concentrations indicated. n=5, ANOVA with Dunnet's post-test against unstimulated controls (### p<0.0001) and against stimulation with 1ng/ml IL-1α (*** p<0.0001). **B** FLS were stimulated with 1ng/ml IL-1α for 24h then washed free and incubated in medium or medium containing 1µg/ml IL-1RA for the indicated number of days. n=3 per treatment.

3.3 Innate cell memory in repeatedly challenged macrophages

Given that FLS from both the healthy and the RA synovium mount an unremitting response to chronic stimulation, I wanted to assess the effect of stimulating FLS twice. This was inspired by macrophage endotoxin tolerance, wherein second LPS challenge induces a refractory state.

To illustrate the memory response of macrophages, monocytes were differentiated into M1-like or M2-like cells as described in the methods section. Cells were stimulated for 24h with 10ng/ml LPS, before conditioned medium was removed and cells were washed in fresh medium and rested for 24h. Cells were again washed, and then stimulated once again with 10ng/ml LPS for 24h. The conditioned media from the first and second responses were compared for TNFα secretion.

As shown in figure 3.5, both macrophage subsets robustly responded to LPS challenge, with M1-like macrophages secreting the higher quantity of TNFα. In response to the second challenge, however, both M1-like and M2-like macrophages displayed the refractory ‘tolerized’ state, producing significantly less TNFα on re challenge.

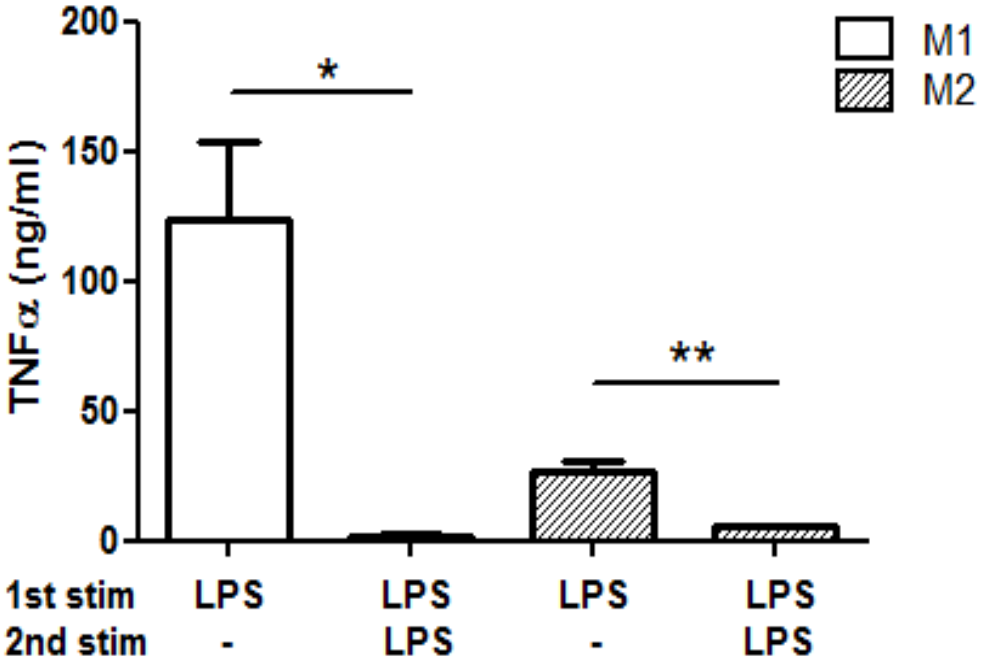

Figure 3.5: Macrophages undergo endotoxin tolerance upon second exposure to LPS. M1-like and M2-like macrophages were stimulated for 24h with 10ng/ml LPS, before conditioned medium was removed and cells were washed in fresh medium, before being rested for 24h. Cells were once again washed, then stimulated again with 10ng/ml LPS for 24h. Conditioned medium was once again removed. First and second responses to LPS challenge were assessed by TNFα ELISA. n=3, * p=0.05, ** p=0.01, unpaired t test.

3.4 Fibroblast response to repeat challenge

3.4.1 Repeat challenge with TNFα

After displaying tolerance in macrophages, I wanted to test it in fibroblasts. My hypothesis was that FLS from a healthy joint would share this refractory state, whilst RA FLS would aberrantly (strongly) respond to stimulation.

In order to test the effect of repeated challenge on fibroblasts and to optimise experimental design, the neonate foreskin dermal line 'BJ' was used. Cells were stimulated as described in the methods section. 10ng/ml TNFα for 24h, then a rest period of 24h before another challenge with 10ng/ml TNFα.

As shown in figure 3.6, BJ fibroblasts secreted negligible IL-6 at rest (57 ±8.3 pg/ml), but showed a robust stimulation by TNFα, secreting 1.2 ±0.25ng/ml IL-6 in the first 24h. Cells that only received TNFα at the 48h point produced comparable quantities of IL-6 (compare columns 2 and 4). This suggests no increase in cell number during the experimental procedure. Cells washed free of TNFα at 24h and receiving only medium henceforth reverted to the secretion of only a small quantity of IL-6 (compare column 5 to columns 1 and 3). In stark contrast, cells which received a second dose of TNFα (column 6) secreted significantly more IL-6 in the second response compared to the first. This suggests that fibroblasts have a form of inflammatory memory. In opposition to my hypothesis however, fibroblast memory takes the form of an augmented, 'primed' response, rather than a negative 'tolerized' response.

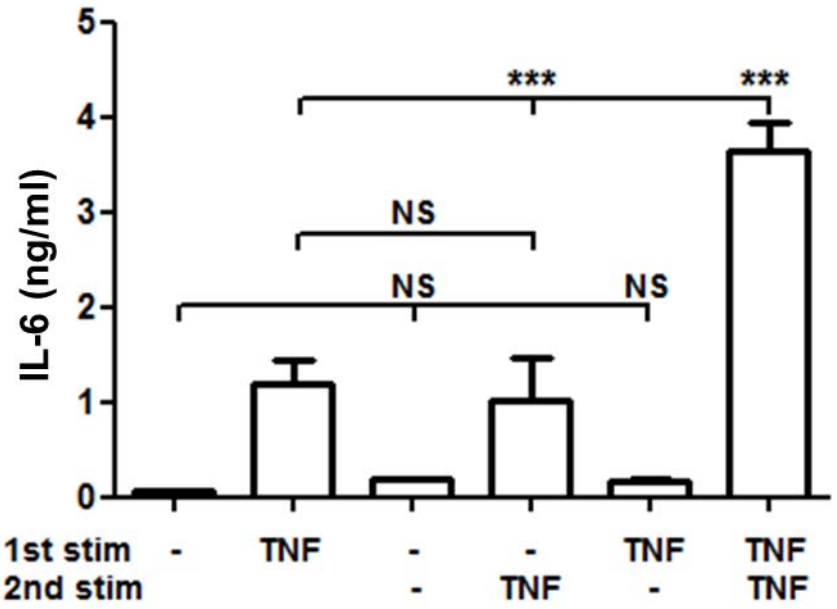

Figure 3.6- Fibroblasts exhibit positive stromal memory. BJ neonate foreskin fibroblasts received vehicle or 10ng/ml TNFα for 24h before conditioned media was removed and cells were washed and rested in fresh media for 24h. Cells then received vehicle or 10ng/ml TNFα for a further 24h, before conditioned media was removed. IL-6 secretion was measured by ELISA. n=3, *** p<0.001 as assessed by one-way ANOVA with Bonferroni's post-test.

As shown in figure 3.7a BJ fibroblasts treated with 10ng/ml TNFα still had around 7ng/ml TNFα left in the medium following 24h incubation. The level of TNFα depletion did not differ between challenges but did suggest a large amount of TNFα was still present and could theoretically continue stimulating fibroblasts during the rest period if not properly washed away.

Columns 4 and 5 of figure 3.6 shown above suggested fibroblast priming by TNFα was not due to continual response. Nevertheless, the anti-TNFα antibody Adalimumab (Ada) was used to ensure that residual TNFα was not maintaining a low level of stimulation during the rest period.

As shown in figure 3.7b, IL-6 secreted by BJ cells in response to 10ng/ml of TNFα could be successfully inhibited by a range of Ada concentrations. The lowest concentration (1μg/ml) was selected for further experiments. Cells were stimulated twice for 24h with 10ng/ml TNFα in the presence or absence of 1μg/ml Ada, or received medium as a control. Cells washed and rested in medium or 1μg/ml Ada for 24h, before being washed and re stimulated with TNFα or medium.

As shown in figure 3.7c, 1μg/ml Ada abrogated IL-6 production following 10ng/ml TNFα (column 2 compared to column 1), in accordance with figure 3.7b. Cells previously primed with TNFα mounted an augmented IL-6 response to second challenge (column 5), and this was not affected by the presence of Ada in the rest period (compare columns 5 and 6). Whilst multiple comparisons suggested a non-significant increase of TNF-challenged from unstimulated, and of second challenge compared to first challenge, the increases are clear, as is the lack of consequence of adding Ada in the rest period. The augmented second response is therefore not due to continued stimulation from residual first TNFα challenge.

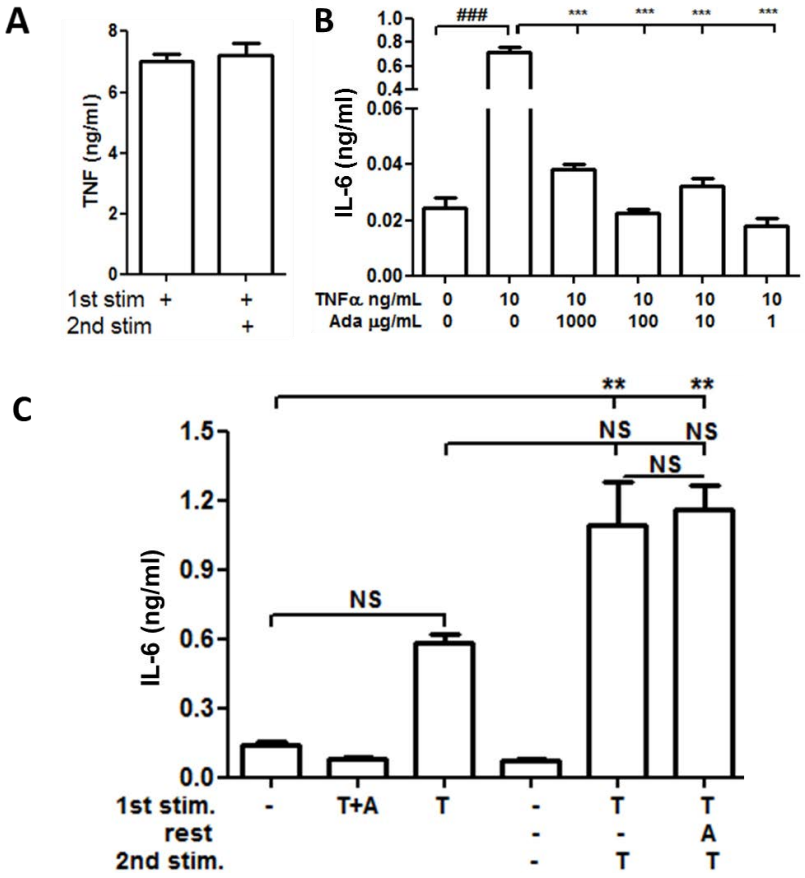

Figure 3.7: The augmented second response is not due to residual TNFα in the rest period. A Residual TNFα after 24h or primed and 24h stimulation with 10ng/ml TNFα. n=4. **B** Titration of Adalimumab (Ada) with BJ fibroblasts stimulated with 10ng/ml TNFα for 24h. n=4 assessed by one-way ANOVA with Dunnett's post-test against stimulation with 10ng/ml TNFα (*** $p< 0.0001$) or unstimulated control (### $p< 0.0001$). **C** BJ fibroblasts were stimulated with 10ng/ml TNFα or medium for 24h in the presence/absence of 1µg/ml Ada. Medium was removed and cells were washed and rested in medium, or medium with 1µg/ml Adalimumab for 24h, then washed again. Cells were then stimulated with 10ng/ml TNFα or medium for 24h. n=3-7, assessed by one-way ANOVA with Bonferroni's post-test (** $p<0.01$).

Having shown that BJ fibroblasts showed inflammatory memory, I returned to FLS to test whether priming also existed in the synovium. Fibroblasts were stimulated as per the experiments above.

As shown in figure 3.8a the quantity of IL-6 secreted by unstimulated FLS in the first and second 'challenges' did not differ (0.13 ±0.07ng/ml and 0.30 ±0.81ng/ml respectively). FLS showed a non-significant increase in IL-6 secretion during the second TNFα challenge (6.82 ± 3.0ng/ml compared to 2.13 ± 0.81ng/ml). To exclude inter-patient variation of absolute secreted concentrations, I also assessed the fold change between doses. As shown in figure 3.8b, the fold change was striking (4.06 ±0.72-fold), and significant when tested statistically (p= 0.004, Wilcoxon matched pairs signed rank test).

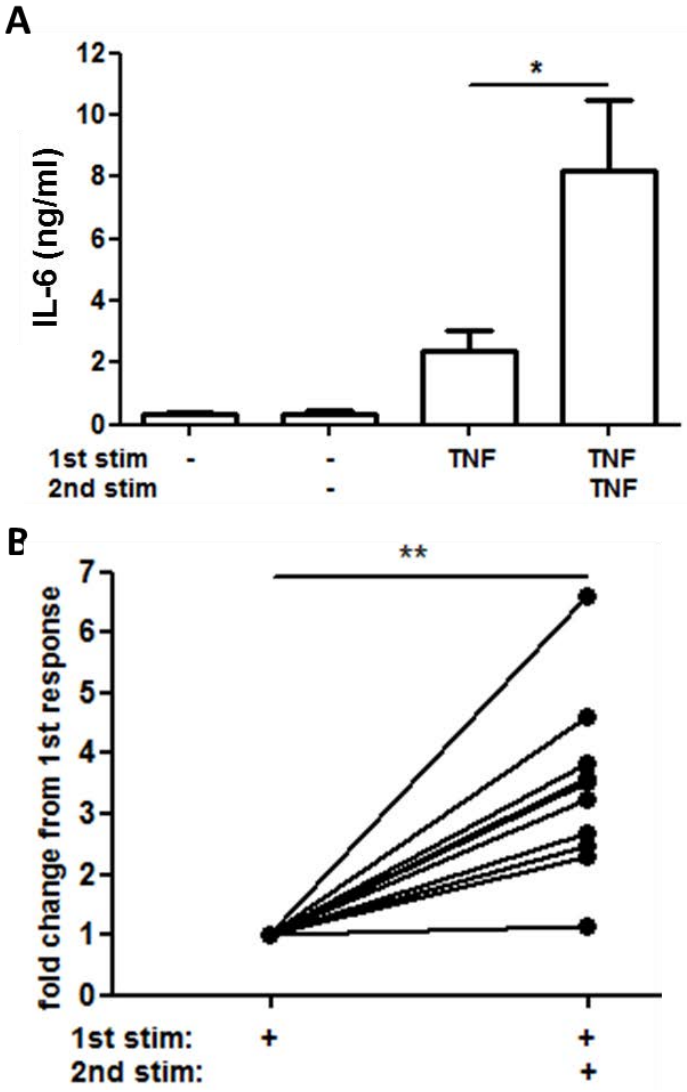

Figure 3.8: FLS mount an augmented second response to second dose of TNFα. FLS were stimulated with 10ng/ml TNFα for 24h. Conditioned medium was removed and cells were washed and rested for 24h. Cells were then washed and treated with 10ng/ml TNFα for a further 24h. Response to first and second dose was measured by IL-6 ELISA. **A** Absolute IL-6 levels secreted in response to first and second doses of TNFα or medium assessed by Mann-Whitney U test. **B** Second response to stimulus represented as a fold change from first response (normalised to one). **, $p<0.01$, Wilcoxon matched pairs signed rank test. n=7.

Having shown FLS to mount an augmented IL-6 response to second challenge, I then assessed whether this was a specific or generic upregulation of protein secretion. Conditioned media from first and second challenge with TNFα were analysed by Luminex or ELISA for a range of secreted proteins (figure 3.9). These were induced by both the first and second TNFα challenge, but the augmented second response was protein-specific.

CXCL10 and CCL5 were both significantly increased, with respective fold changes of 2.58± 0.44 and 5.87± 1.23. Other proteins involved in the inflammatory response showed no priming (CCL2), or even reductions in secretion. The latter was true for IL-8, which had a fold change of 0.64± 0.13-fold. This reduction was non-significant (p=0.06, Wilcoxon matched pairs signed rank test), and has therefore not been considered an example of tolerance.

The memory response displayed by FLS appears to facilitate the augmentation of certain pro inflammatory mediators (namely IL-6, CXCL10 and CCL5). The fact that other proteins heavily involved in the inflammatory response were not augmented suggests a specific role for fibroblast priming.

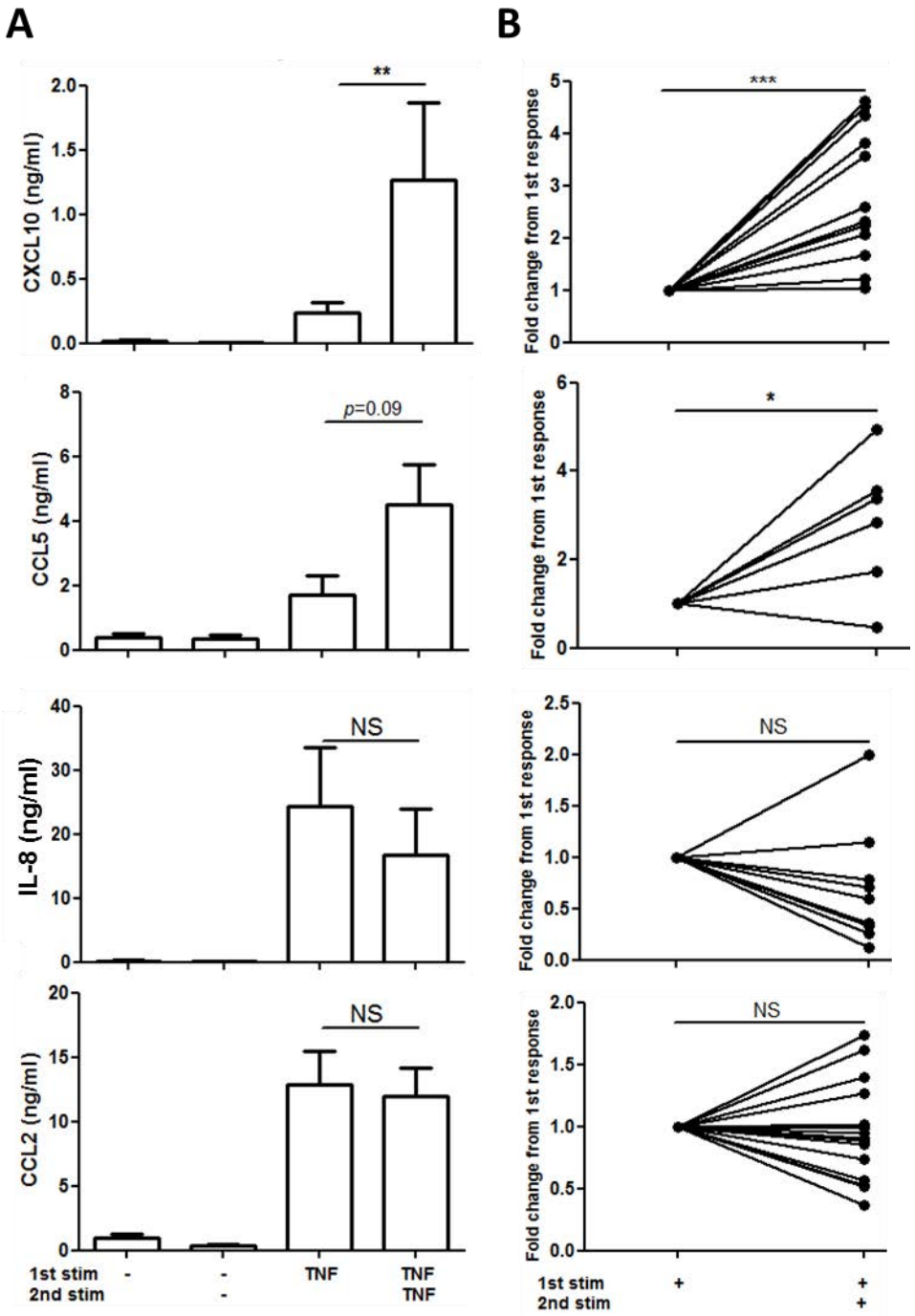

Figure legend overleaf

Figure 3.9: The augmented second response is protein-specific. FLS were stimulated with 10ng/ml TNFα for 24h. Conditioned medium was removed for analysis, and cells were washed and rested for 24h in fresh medium. Cells were once again washed and then stimulated with 10ng/ml TNFα for 24h. Conditioned medium was removed for analysis. Each protein was assessed by ELISA or Luminex. **A** Raw secretion, assessed by Mann-Whitney *U* test**. B** Second response to TNFα represented as a fold change from first response, assessed by Wilcoxon matched pairs signed rank test. * $p<0.05$, ** $p<0.01$. Mean ± SEM. CXCL10 n=10, CCL5 n=9, IL-8 n=6, CCL2 n=9.

Repeat challenge with IL-1α

I conducted the same experimental procedure with IL-1α as with TNFα to assess whether it could also prime FLS to mount an augmented second response. As shown in figure 3.10, FLS mounted a robust response to 10ng/ml IL-1α, and an augmented primed response to the same concentration (32.67± 11.67ng/ml and 74.44± 23.62ng/ml respectively). This augmented response was non-significant due to inter-patient heterogeneity, but the fold increase of second response compared to first (3.22± 0.98) was found significant by Wilcoxon test (p=0.03).

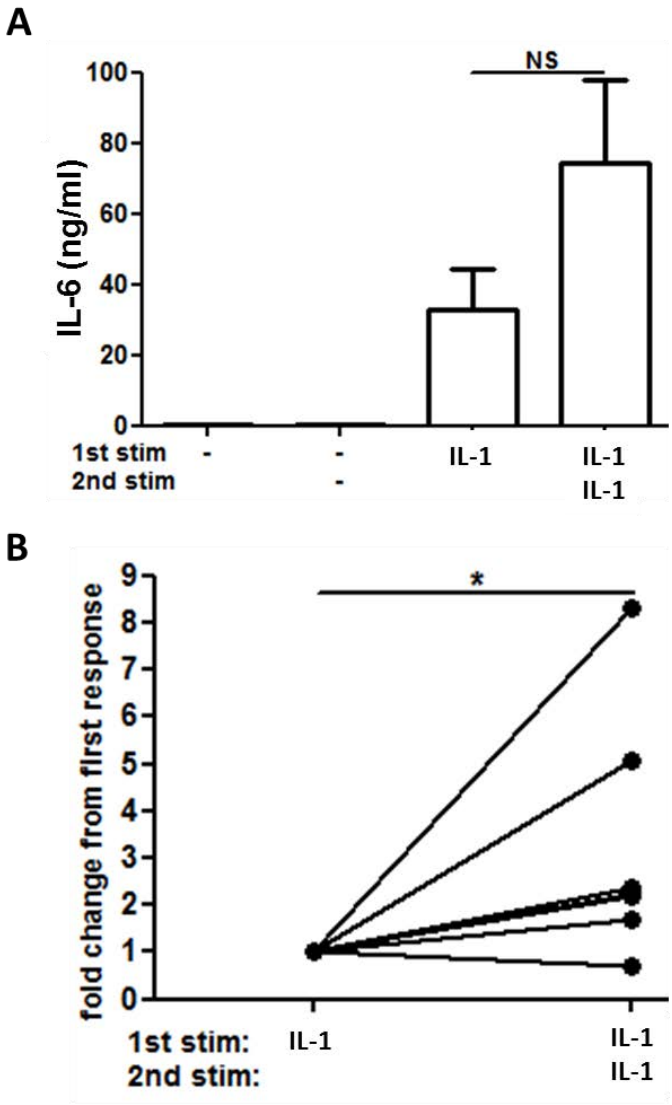

Figure 3.10: IL-1α induces an augmented second response from FLS. FLS were stimulated with 10ng/ml IL-1α for 24h, the conditioned medium was removed and cells were rested in fresh medium for 24h, before being challenged again with for a further 24h. **A** raw secretion in response to first and second challenge with medium or IL-1α. Mann-Whitney U test, NS. **B** Second response to IL-1 represented as a fold change from first response. Wilcoxon matched pairs signed rank test, $p<0.05$. n=7.

3.5 Stromal memory in murine fibroblasts

I wanted to test whether fibroblast memory was conserved in other species. Fibroblasts were isolated from the joints of wildtype C57/BL6 mice and cultured as described in the methods section. Human TNFα and IL-1α have been shown to cross react with mouse cells, so the same reagents, concentrations and protocol were used as with human cells. LPS, a commonly-used example of exogenous stimuli, was also used at 10ng/ml.

Murine FLS mounted robust IL-6 responses to TNFα and LPS (to around the same extent), and mounted a greater response to IL-1α, as seen in human FLS. Upon repeat challenge, TNFα induced a significantly increased secretion of IL-6, and IL-1α induced an augmented response of a lower magnitude (6.5±1.1-fold and 1.61± 0.19-fold respectively). LPS induced a robust response from mouse FLS but did not induce an altered IL-6 response.

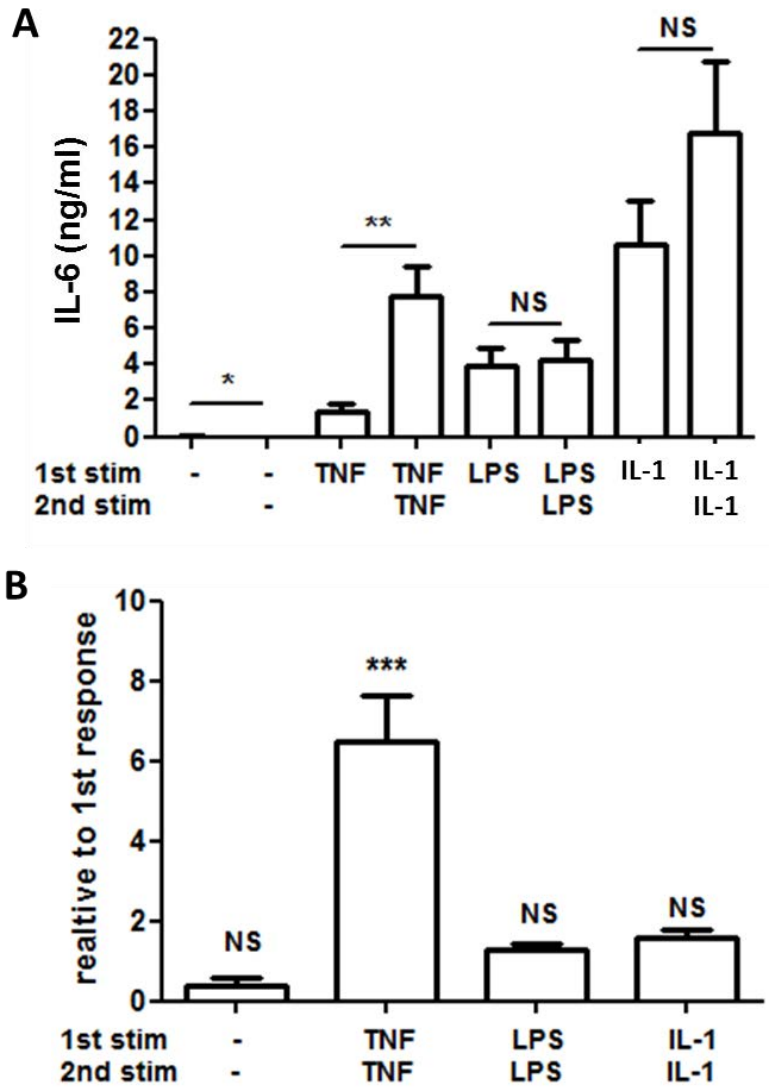

Figure 3.11: mouse FLS augment IL-6 in upon re challenge in a similar manner to human FLS. Mouse FLS were stimulated with 10ng/ml TNFα, 10ng/ml LPS, 1ng/ml IL-1α, or received medium (control) for 24h before conditioned medium was removed. Cells were washed and rested for 24h before being washed and re stimulated with the same stimulus for a further 24h. Conditioned media from first and second response were analysed by IL-6 ELISA. **A** Raw IL-6 secretion. * $p< 0.05$, ** $p<0.01$ by unpaired t test. **B** fold change from first response. *** $p<0.001$ by one-way ANOVA with Dunnet's post-test against the normalised first dose. n=6.

Whilst figure 3.7 showed human fibroblast had a large left-over quantity of TNFα after 24h, I wanted to test this in the mouse conditioned media to see whether residual TNFα would be a confounding variable if I put conditioned medium into an *in vivo* model.

The ELISA failed to show TNFα levels above that of the blank, and therefore fell below the range of detection (the greatest dilution of the standard controls gave a concentration of 78pg/ml). Whilst the possibility remains that some TNFα is present in the conditioned medium from murine FLS, if it is there it is of a very low concentration.

3.6 Discussion

3.6.1 Response following inflammatory stimuli

This investigation started by assessing the unremitting inflammatory response of FLS, and the consequences on control FLS once a stimulus was removed. Following the unremitting response to stimulus originally described in RA FLS by Lee *et al* [272], I confirmed the continued production of IL-6 in response to pro inflammatory cytokines by control FLS.

These cells were isolated from patients experiencing arthralgia, with no discernible inflammation observed during exploratory arthroscopy. Despite this apparent lack of inflammation, the fibroblasts continued to secrete IL-6 for days under the influence of stimuli, suggesting a strong role in the inflammatory response. Lee et al suggesting the underlying mechanism for the unremitting response in RA FLS was a lack of negative feedback regulators [272]. Given my data, such a conclusion should also be drawn when considering control FLS.

I was also interested in prior stimulation of FLS. Given the unremitting response in the presence of stimuli, I tested whether removal of said stimuli would allow FLS to return to basal secretion of IL-6. As shown in figure 3.1, removal of TNFα was followed by FLS secreting a similar amount of IL-6 in the following 24h, but negligible levels thereafter.

The time course of IL-6 secretion in the 24h post wash failed to reproduce this, but the concentration in the original experiment was noticeably lower than most results for 24h of 10ng/ml TNFα. Because of this, I have drawn the conclusion that a residual amount of IL-6 secretion (averaging 0.5-1ng/ml) is produced in the first 24h post TNF-removal, due to transcripts produced in the last hours of stimulation

leading to protein being secreted after TNF-removal. This is confirmed by figure 3.2, in which around a third of the IL-6 secreted in the first day after removal of TNFα was secreted in the first 4h. Combined with the basal level of secretion that fibroblast return to at rest, this could easily account for the levels of IL-6 seen in the 24h post wash. Residual TNF is therefore not necessary for a small level of IL-6 secretion following removal of TNF.

In comparison, removal of IL-1α led to a small amount of IL-6 secretion in the subsequent 24h, which increased through the next week, leading to IL-6 concentrations equivalent to 24h of stimulation. It is possible that this is due to increased transcript stability (IL-1-induced IL-6 mRNA has a greater half-life than TNF-induced IL-6 mRNA [316]) but the first test was whether residual IL-1α was responsible. IL-1α titration showed that the dose dependant IL-6 secretion plateaued at 1ng/ml, but even 0.1ng/ml was capable of inducing IL-6 secretion significantly higher than basal. This suggests even a hundredth of the initial input could continue to stimulate the FLS.

1μg/ml IL-1RA was sufficient to abrogate IL-1α-induced IL-6. Despite this, there was no decrease in IL-6 secretion over the week post-IL-1 removal when IL-1RA was administered (see figure 3.4). This suggests free IL-1 was not present, even at the one hundredth the original input concentration. I cannot however discount the fact that IL-1 bound to receptors may still have been contributing to fibroblast stimulation. IL-1RA would not inhibit bound IL-1, so I can only acknowledge this possibility. Given the sensitivity to low doses of IL-1α, it is difficult to imagine FLS ever reducing their IL-6 secretion once IL-1 was in the milieu. That being said, anti-inflammatory mediators (not least IL-1RA) are present *in vivo*, but may not be

produced by fibroblasts in sufficient quantities *in vitro* to negate the proinflammatory effects of IL-1.

3.6.2 Innate memory in fibroblasts

After describing the effect of transient and chronic exposure of FLS to stimuli I moved on to the main focus of this project: testing the existence of innate memory in fibroblasts. Previous studies on repeat stimulation of fibroblasts were cited in the introduction, wherein a spectrum of memory responses is induced by different stimuli. Repeat exposure to LPS induces a refractory state in macrophages or EC [299, 304], but not gingival fibroblasts. The authors suggested that this was because fibroblasts must maintain inflammatory pressure on pathogens if macrophages are in a refractory state [313].

Exposure to repeat challenge inducing tolerance is an over simplification, as cross priming of TLR ligands exerts differential outcomes. TLR3 agonists prime EC challenged with TLR4 to increase IL-6, whilst TLR2 agonists abrogate IL-6 secretion in response to TLR4 challenge [303]. Similarly the overall outcome at the organism level differs, with increased resistance [317, 318] and susceptibility [319-321] to infection being described.

Whilst the majority of research has focussed on infection studies, others have investigated the role of endogenous mediators of inflammation. Both TNFα and IL-1 induce increased protection against lethal infection [322, 323]. Further, Van der Meer and colleagues found that IL-1's protective effects in increasing survival of infected leukopenic mice was explained by effects on neutrophils, macrophages, or haematopoietic recovery [294]. Increased resistance based on IL-1 priming and

without obvious leukocyte contribution could support the role of fibroblast priming, although this is conjecture only.

During the period of my research, Sohn *et al* published priming of RA FLS with chronic exposure to TNFα [324]. Three days of TNFα primed RA FLS to mount augmented CXCL9, CXCL10, and CXCL11 responses to IFNα,β or γ, whilst non-primed IFN challenge failed to induce these chemokines. In the same vein as Sohn *et al*, I chose to stimulate fibroblasts with TNFα, an endogenous DAMP which fibroblasts would be exposed to during infectious or injurious inflammatory events. My data in FLS and a neonate dermal line (BJ) showed a significantly augmented response to TNFα or IL-1α, which was not due to low level stimulatory effects of residual stimuli during the rest period, thus confirming the findings of Sohn *et al* in non-RA FLS and a dermal line.

The augmented response is opposite to the refractory state of macrophages; a gene-specific upregulation rather than down regulation of pro inflammatory mediators. It is commonly accepted in the field that tolerance of LPS by macrophages defends against tissue damage in that inflammatory episode. Following this rationale, FLS memory must be seen as propagating inflammation. It may be apt to agree with the view of Ara et al, in that FLS may upregulate proinflammatory mediators to compensate for a refractory state of other cells. Alternatively, the joint is ostensibly sterile and therefore repeated DAMPs may warn FLS of a distinctly pathologic occurrence. Testing this theory would require stimulation by exogenous stimuli.

3.6.3 Specific augmentation of IL-6, CXCL10 and CCL5

FLS upregulated IL-6, CCL5 and CXCL10. IL-6 has multiple roles, both pro- and anti-inflammatory (reviewed in [112]). Response to IL-6 requires IL-6R but fibroblasts do not express IL-6R, only gp130 [268], meaning FLS in monoculture cannot use IL-6 as a feedback device. However, *in vivo* increased IL-6 could have pro or anti-inflammatory consequences on other cells. IL-6 increases T cell adhesion to EC, making T cell infiltration more likely. This is an important step in the progression of an inflammatory episode, as the switch from neutrophilic to mononuclear infiltrate is vital to resolution of inflammation [104, 112] [100, 110]. That being said, Komatsu *et al* showed that RA FLS production of IL-6 leads to $CD25^{lo}$ progenitor T cells differentiating into Th17 cells rather than Tregs [200]. IL-17 and IL-6 from Th17 cells and FLS therefore establishes a positive feedback loop.

CXCL10 is an archetypal T cell chemoattractant. It is, however, produced at low concentrations in the data presented above. It will therefore be necessary to assess physiological relevance of these concentrations. Nevertheless, CXCL10 was significantly augmented, confirming the findings of Sohn *et al* who found CXCL9, 10 and 11 were all augmented in primed fibroblasts [324].

CCL5 is augmented from a much higher initial concentration. CCL5 is a mononuclear chemoattractant, again supporting the hypothesis that fibroblast priming is of mediators involved in mononuclear infiltration. Monocytes are recruited after neutrophils in most inflammatory scenarios. They differentiate based on cues provided by cells already in the tissue (such as fibroblasts and neutrophils).

In the light of these data, I propose a model for the influence of the initial and memory response. It is presented in figure 3.12, illustrating that whilst the initial response will recruit a mixture of PMN and MNC, the memory response will shift the balance of chemo attractants, and hence the balance of infiltrating leukocytes.

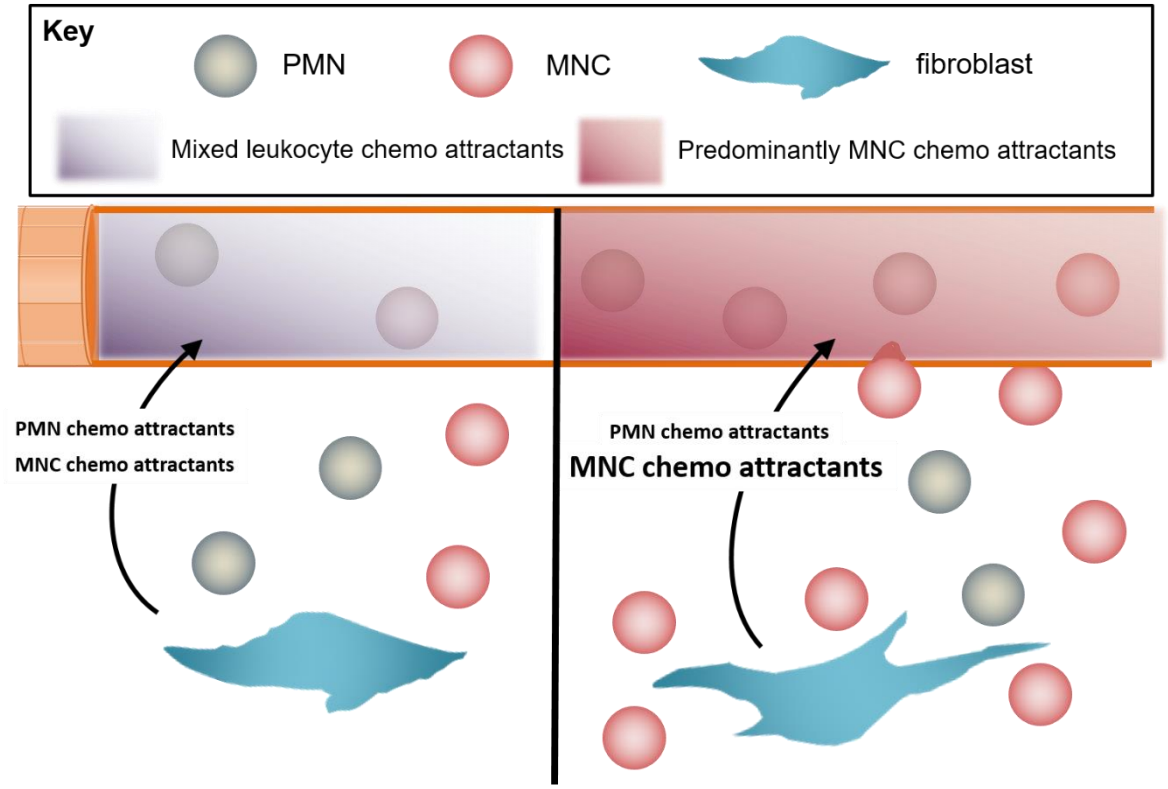

Figure 3.12: Cartoon of 'increased monoculear recruitement' theory. Illustrated theory of how the fibroblast intial and memory responses to stimuli affect leukocyte recruitment. Initial response (left side) would include release of PMN and MNC chemo attractants causing general influx of both sets of leukocytes. Increased MNC chemokine release in the memory response would reusult in increased number of infiltrating MNC, whilst PMN influx remains the same. PMN= polymorphonuclear cells, MNC= mononuclear cells. Key provided.

I propose that fibroblast memory in FLS is inherently inflammatory. The specific mediators shown to be upregulated suggest progression from neutrophilic infiltrate, not necessarily an anti-inflammatory role. Rather it seems to promote a mononuclear infiltrate. As explained earlier, in healthy tissue this is necessary for a healthy peak and subsequent return to (altered [325]) homeostasis. The duration of this response would have to be tightly regulated however, so as not to progress to a chronic state of inflammation.

This theory would match findings from earlier research into trained immunity. Priming of EC by endogenous mediators, for example, may increase long term risks of atherosclerosis and cardiac disease [306], and elevated levels of endogenous mediators remaining high months after resolution of infection increases risk of atherosclerosis and death [310].

Oral pathogens known to contribute to periodontitis have also increase likelihood or severity of a number of extra-oral diseases (reviewed in [326, 327]). It may be that innate memory is an example of the tightrope our immune system walks: a careful balance of both suppressing infection and avoiding self-induced tissue damage.

4 Mechanisms involved in the primed response

4.1 Introduction

4.1.1 Cellular response to stimulation

At rest and during inflammation, cells respond to a plethora of mediators. This may be endogenous or exogenous, secreted or membrane-bound, suppressive or stimulatory, autocrine, juxtracrine, paracrine or systemic.

The way in which those signals are recognised and processed, and the subsequent response from the cell, have profound consequences. As shown in the previous chapter, cellular responses can be dramatically altered, even in response to the same stimulant. This depends on the internal response to stimulants. The cell response is far too broad a subject to be covered by one text, and the following literature is presented merely as examples of the processes relevant to the following data. The simplest representation of a cellular response to pro inflammatory stimulation is represented in figure 4.1.

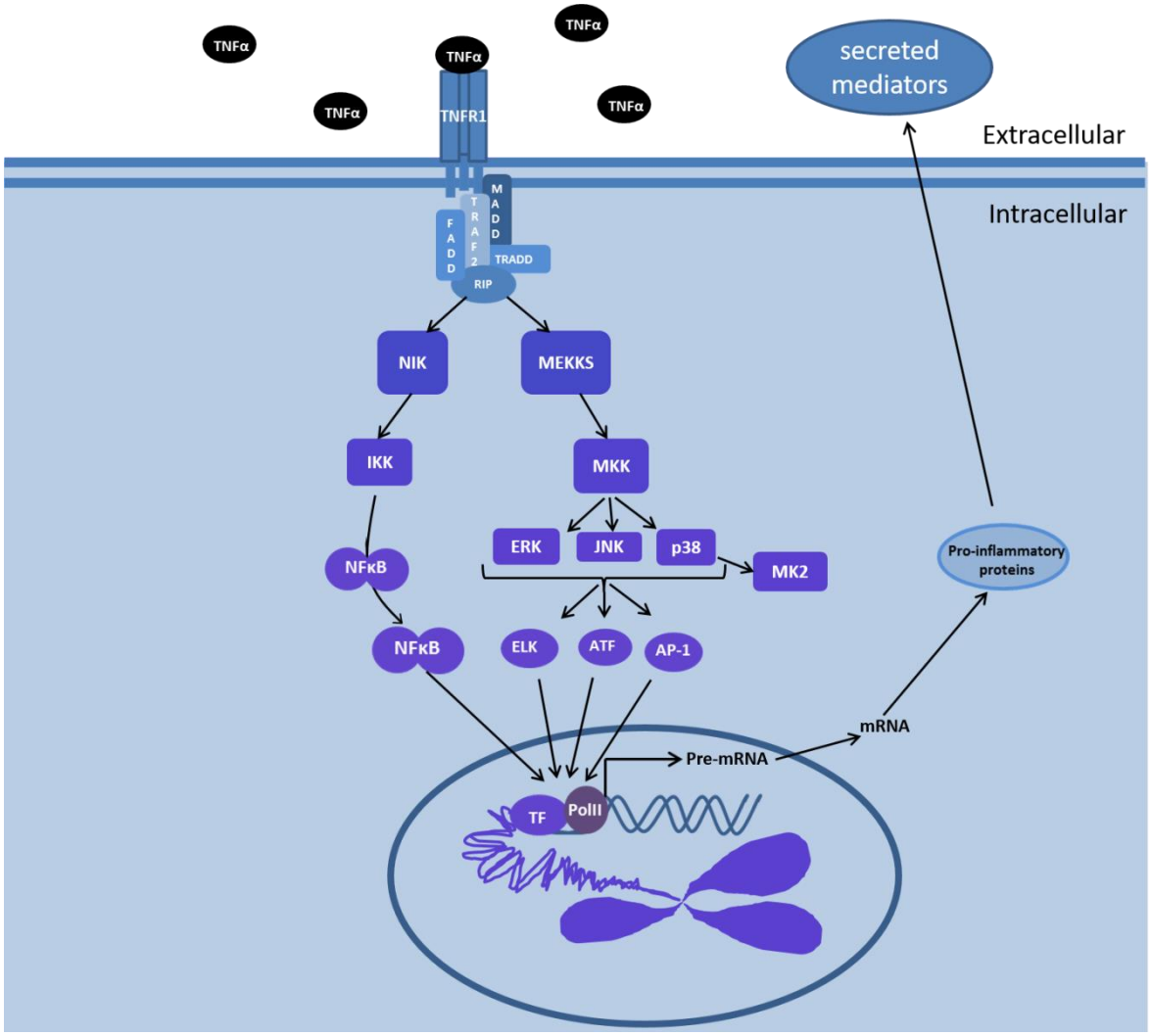

Figure 4.1: Illustration of the cellular response to pro inflammatory stimulation. Cartoon illustrating some of the effects of TNFα on a cell, regarding signal transduction, transcription and release of soluble mediators. For simplicity and relevance to the data in this chapter, only the NFκB and MAPK pathways are shown.

4.1.2 Receptors

Inflammatory mediators act on target cells via cognate receptors. Both TNFα and IL-1 act upon several receptors, however not all are expressed on a cell, and not all result in signal transduction.

TNFα binds both the TNF receptor 1 (TNFR1) and TNFR2. In a similar way to classic versus trans signalling of the IL-6R, it has been suggested that TNFR1 is the more pro inflammatory receptor [328], and is highly expressed at the pannus-cartilage junction [329]. This theory may be a result of murine studies, as transgenic mice overexpressing human TNFα develop RA-like symptoms [330], and TNFα over expressing mice lacking TNFR1 have ameliorated diseases [331] whilst those deficient in TNFR2 develop worse symptoms [332]. TNFR1 is also bound by lymphotoxin alpha (another TNF family member). Lymphotoxin α is found at high concentrations in RA patients, and induces an aggressive phenotype in FLS [333].

TNFR2 is highly expressed on Tregs, and those lacking the receptor cannot inhibit colitis in mouse models [334]. Similarly, haematopoietic cells-specific knock out increases inflammation in mouse models [335], further suggesting a dichotomy for the two receptors. That being said, TNFR2 on RA FLS can receive TNF-like ligand 1A (TL1A) to induce IL-6 secretion [336]. TNFR2 expression has been claimed to be restricted to endothelial cells (EC) and immune cells [328], yet this is disproved by fibroblasts studies [336, 337]. TNFR1 induces the NFκB pathway, whilst TNFR2 does not. TNFR2 did, however, augment the proliferation seen downstream of TNF-TNFR1-NFκB in murine fibroblasts [337].

IL-1 receptors are ubiquitously expressed, and targeted by IL-1α, IL-1β and IL-1RA. IL-1R1 is the main receptor, and IL-1R3 acts as an accessory to transduce the signal. IL-1R2 acts as a decoy receptor [338]. As there is only one signal complex, The ratio of IL-1β to IL-1RA, and of the abundance of soluble receptors (discussed later) dictates the inflammatory nature of this ligand-receptor family.

Receptor abundance limits the extent to which a cell can respond to stimuli. Binding to TNFR1 is permanent, and upon ligand binding the receptor is internalised [328]. Receptor conformation is also important. IL-6-sIL-6R binds two gp130 proteins to induce signal transduction. This means that at higher concentrations of the IL-6 complex the signalling effect is reduced, due to the requirement for twice as much gp130 as IL-6-IL-6R. At the highest concentrations however, the stoichiometry changes, and a tetramer of two IL-6 complexes and two gp130 proteins forms, changing the ratio to 1:1, and so increasing the efficacy of each IL-6 complex [339]. This biphasic response was not seen in the IL-6 family member leukocyte inhibiting factor (LIF), which binds gp130 at a ratio of 1:1 [340], meaning an increase in LIF dose-dependently increases its effect upon the cell.

4.1.3 Signalling pathways

4.1.3.1 Nuclear Factor κB (NFκB)

NFκB is known for driving inflammatory pathways [341], and is crucial to various chronic inflammatory diseases, including RA [342]. It is split into the canonical and non-canonical pathways. The canonical (shown simply in figure 4.1 earlier) is induced by a number of mediators including TNF and IL-1, and begins with NFκB-inducing kinase (NIK) activating the IκB kinases (IKKα and β) and NFκB essential modulator (NEMO, or IKKγ). The IKK complex phosphorylates and marks for

degradation the inhibitor of κB (IκBα) which holds the canonical NFκB p65 and p50 sub units inactive in the cytosol. IκBα is ubiquinated and undergoes proteasomal degradation, after which NFκB may translocate to the nucleus and act as a transcription factor for a range of inflammatory genes.

The non-canonical pathway is activated by a different set of cytokines, although transcription of the non-canonical sub units NFκB2 (p52) and RelB is induced by canonical action [343]. In the alternative pathway, NIK activates IKKα (but not the other IKKs) [344], releasing the active p52 subunit from its inactive p100 precursor. The p52-RelB NFκB translocates to the nucleus to exert its effects.

NFκB's importance in RA has been widely published upon [342, 345-350], with roles in cytokine production, osteoclastogenesis and bone destruction. In fibroblasts, NFκB is necessary for pro inflammatory and degradative mediator production [351-353], and instigates proliferation whilst inhibiting apoptosis [354].

4.1.3.2 Mitogen-activated proein kinase (MAPK)

The MAPK signalling pathways have many roles in homeostasis and inflammation [355]. They are induced through a kinase cascade of mitogen-activated protein kinase kinase kinase (MAP3K), MAP2K, and MAPK, with subsequent induction of transcription factors. These cascades were illustrated in the review by Thalhamer and colleagues [356].

The three MAPK pathways are p38, c-Jun N terminal kinase (JNK) and extracellular signal-regulated kinase (ERK). p38α and β, JNK1/2 and ERK1/2 are the most studied. p38 responds to various stimuli and effects various cellular responses (reviewed in [357]). Some are relevant to RA, such as expression of IL-6 [261, 358-361], other proinflammatory mediators [360-362], and

osteoclastogenesis [363, 364]. Inhibiting p38 has unsurprisingly proved efficacious in animal models [364, 365], but has unfortunately failed in clinical trials for several diseases, including RA [366].

ERKs also have inflammatory roles in arthritis. Proinflammatory cytokines induce robust ERK phosphorylation in FLS [353, 367, 368], which plays important roles in pannus propagation and murine paw pain [369] (both attributed at least in part to ERK activity in fibroblasts). It is key to FLS proliferation, whilst p38 is not [370].

JNK MAPKs play roles in the destructive elements of RA. JNK activity contributes to FLS production of MMPs [370, 371], and JNK-/- mice have decreased FLS production of MMPs. This knockout study showed a modest reduction in inflammation, but completely abrogated radiographic damage in the joints [360].

The complex network of MAPKs and other signalling pathways, such as the NFκB pathway, mean conflicting results and confusing conclusions. Different studies have found, for example, weak [265] or strong [360, 367, 371] JNK activity in FLS. This may be stimulus-specific [372]. TNF was reported as a weak inducer of JNK activation but a stronger inducer of ERK and p38 [368], whilst others reported FLS production of MMPs was IL-1/ERK-dependent, as TNF is a poor inducer of p38 and ERK phosphorylation [353]. The relative contributions of NFκB and MAPKs are often difficult to discern as they interact and mediate each others' functions [353, 361, 362, 372, 373]. They all, however, clearly have roles to play in RA, and are all highly activated in the RA compared to OA synovium [342, 367].

4.1.4 Transcription factors (TFs)

At most basic, TFs are necessary for polymerase II to bind DNA and instigate transcription. General TFs, however, are only part of the story, and other TFs exist

to induce specific cellular responses including differentiation, proliferation, apoptosis, and cytokine production. Ectopic expression of TFs can induce aberrant behaviours in cells, such as the four core TFs of monocyte function inducing myeloid-like traits in dermal fibroblasts [374].

NFκB, as described above, is a major inflammatory TF, and NFκB dimers themselves act as TFs for a huge number of inflammatory genes. The necessity for NFκB in production of pro inflammatory mediators is well known (i.e. IL-6 [351, 352]) and Lee et al showed that the unremitting RA FLS response to TNFα was dependent on ongoing NFκB activity [272].

Canonical NFκB is, however, transient due to its own negative feedback loop of NFκB inhibitor A (NFκBIA) transcription and subsequent IκBα repression of NFκB nuclear translocation. This serves as example of the complexity involving this well-known TF. Whilst IκBα shuttles NFκB p50-p65 out of the nucleus [375], it cannot do the same for non-canonical NFκB dimers. Saccani et al found the prolonged response of DCs to stimulus was due to the switch from canonical p50-p65 to non-canonical p52-p65 NFκB signalling [376].

This also introduces the concept of temporality, as non-canonical NFκB subunits are transcribed by canonical NFκB [343], replenishing the cytoplasmic pool and facilitating the continuance of signalling. Whilst this means the non-canonical pathway may take over, the non-canonical homodimer p50-p50 also inhibits p50-p65 in the nucleus [377], suggesting timing of the two pathways is important.

Interaction between TFs is not restricted to the same family. Activator protein (AP)-1 and NFκB, for example, interact under a range of stimuli [350, 353, 361, 362]. A host of cell, time and stimulus-specific NFκB interactions are also listed by

a review by Vanden-Berghe et al [378]. NFκB also induces transcription of AP-1 subunits by inducing ELK1 (another TF) binding to their promoters [373].

The AP-1 TF itself represents a range of alternatives, with 12 possible subunits forming homo-and hetero-dimers (although the Fos family cannot form heterodimers, and so form heterodimers with Jun members). The range of possible TFs is dependent on cell type, stimulus, abundance of subunits and the dimer formed (reviewed in [379, 380]). This can lead to ‘repressors’, which are weaker trans activating dimers binding the consensus site to the exclusion of stronger transcriptional activators. There are also antagonistic roles, such as fibroblast proliferation induction by JunD-JunB [381] and inhibition by cJun-JunB [382]. This is further complicated by the TF binding of enhancers and silencers sometime kilobases upstream, as evidenced by Qiao et al in priming of macrophage pro inflammatory secretions [383].

An examination of consensus sequences in any inflammatory gene shows a number of TF bindings sites. In inflammatory conditions many TFs are increased, correlating with higher levels of the cytokines that are both the cause and product of their activities [384-387].

4.1.5 Chromatin access

4.1.5.1 Structural access

Chromatin exists as a folded and condensed structure [388], but must be unwound for replication and gene expression. The lowest form of architectural structure in chromatin is the nucleosome: a complex of eight histone proteins (two each of H2A, H2B, H3 and H4), around which 146 base pairs of DNA are wrapped. Whilst these are the most common histones, variants modify

accessibility. For example, the mH2A variant inhibits NFκB and SWItch/Sucrose Non-Fermentable (SWI/SNF, which loosens histone-DNA binding) binding to promoter sites [389]. The H2A.Z variant however, binds certain promoters during repression, but is unusually easy to lose upon stimulation, thus facilitating opening of chromatin [390].

Nucleosomes do not necessarily inhibit transcription complex binding, but must be moved in order for transcriptional machinery to travel along DNA. This is induced by modifications of the histone N terminal domain. Whilst NFκB can immediately bind the NFκBIA gene promoter, it cannot do the same for CCL2, IL8 or IL6, as the consensus sequence is occluded by a histone (a cryptic site). NFκB therefore induces an immediate increase in NFκBIA transcription, followed by inflammatory cytokine transcription, once p38 has induced H3 acetylation, causing it to move and allow NFκB binding [391]. Such structural alterations are transient, and the act of moving a histone via acetylation may induce the loss of this modification, and cause the histone to return to its former site, inhibiting TF binding once more [392].

4.1.5.2 Post-translational modifications

As already touched upon, altering chromatin access is modulated by post-translational modification of proteins or DNA. Epigenetic alteration was initially considered as a semi-permanent, inherited level of control, overlaid over genetic heritability. This has changed in recent years to also include non-genetic amendments dictating gene expression on a shorter time span [393]. The most commonly of such post-translational modifications are phosphorylation (already discussed), methylation and acetylation. That being said, ubiquitination and sumoylation also have important roles [394, 395].

A plethora of histone modifications are listed in Araki 2016, stating their contributions to open and closed chromatin [258]. An oversimplification is that methylation and acetylation respectively induce repressed and open states. Methylation acts on both proteins and DNA. H3K27me3 (tri-methylation of serine 27 on histone 3) is an example of repressive protein methylation [396]. Methylation of DNA is induced by DNA methyl transferase (DNMT) [397], and involves methylating the 5' cytosine in CpG [398], which are often clustered [399]. These CpG islands are found at 70% of human gene promoters [400], but even those distant from promoters influence transcription [401]. A subset of inflammatory cytokines have low level H3K9 methylation, acts as a weak repressor. During the H3K9 acetylation mentioned above, H3K9me is easily lost, facilitating an open chromatin state [402]. Methylation at promoters often negatively correlates with transcription levels [403], and RA patients are globally hypomethylated [404]. Further evidence from both RA and non-RA studies, has shown hypomethylation at the IL6 promoter [405-409], and the promoters of other proinflammatory cytokines [410-412].

Acetylation as a permissive marker is also evidenced by its global increase in RA compared to OA FLS [413], and decreased histone deacetylase (HDAC) activity in chronic obstructive pulmonary disease (COPD) alveolar macrophages [414]. Histone acetyl transferases (HATs) induce acetylation, which is antagonistic to HDACs in the balance of active versus repressive states [415]. The acetylated and therefore transcriptionally available phenotype leads to transcription of many inflammatory mediators [391, 402, 414, 416]. Inhibiting HDAC activity in alveolar macrophages increases IL-8 production [414], and inhibition by the compound FK228 *in vivo* decreases FLS hyperplasia, synovial inflammation and radiographic

damage [417], and induces macrophage apoptosis [418]. Other HDAC inhibitors have also been shown to inhibit proinflammatory mediator expression [418, 419].

Whilst a ‘methylation= decreased inflammation, acetylation= increased inflammation’ paradigm springs to mind, this is far too simplistic. H3K27me3 is repressive, but H3K4me3 is permissive [396]. Further, hypermethylation of the death receptor 3, leads to repression of its expression and thus helps RA FLS to become apoptosis-resistant [420].

4.1.6 Post-transcriptional regulation

Following transcription, mRNA undergoes a number of alterations and interactions before finally being translated. The full-length transcript must be spliced to remove introns, which can vary to induce a huge variety of final mRNAs. It is estimated that around 95% of all human genes are alternatively spliced [421].

A transcript is vulnerable to degradation before ever reaching the ribosome, and this is avoided by various mechanisms. Addition of a 7-methyl guanosine (5’ cap) prevents targeting by 5’-3’ exonucleases, and a string of adenosine residues up to 200 strong (poly-A tail) at the 3’ end of the transcript defends against 3’-5’ exonucleases and other transcript destabilisers. Such mechanisms are helped by additional protein binding, such as the poly-A binding protein, which further protects the 3’ untranslated region (UTR). The secondary structure of the transferrin mRNA are bound by iron-response element binding protein when intracellular concentrations of iron are low. This defends the transcript from degradation whilst iron binding is necessary, and when iron concentrations increase the transcript loses its protective proteins and is susceptible to degradation [422].

Once capped and tailed, the steady state of mRNA is to be stable. Targeting mRNAs for degradation dictate the transcript's longevity and thus the amount of translation (although mRNA contributing to their own translation is also multifactorial, and reviewed in [423]).

Transcript destabilisers (discussed in the next section) are counteracted by transcript stabilisers, which often act by steric hindrance on the transcript or inhibiting transcript destabilisers themselves. Bone marrow tyrosine kinase on chromosome X (BMX) acts in a cytokine-induced manner to stabilise IL-6 mRNA in RA FLS [424]. Human antigen R (HuR)is also capable of stabilising transcripts by outcompeting destabilisers like tristetraprolin (TTP) [425]. During stimulation the p38/MK2 axis shifts the balance towards HuR [426], stabilising inflammatory transcripts. HuR is also capable of increasing mRNA translation [426].

Proinflammatory cytokines can induce also transcript stability, as an inflammatory trigger requires and inflammatory response, one which is carefully repressed under steady state conditions. Several studies have examined TNF or IL-1-induced IL-6 or compared the two, and shown an effect on transcription, but also on mRNA half-life, which differs based on stimulus [316, 424, 426, 427]. The increased stability was often described as being via p38/MK2 [261, 426, 428-430], which played a lesser role in IL6 transcription [316, 427].

The data presented above show the many and complicated mechanisms involved in the inflammatory response (up to translation, which has been omitted for brevity). The 'on' switches, however, are not given free reign. Indeed, there are as many negative mechanisms inhibiting inflammatory response as there are inducing it. A simple illustration of this is shown in figure 4.2.

4.1.7 Negative feedback and mechanisms of repression

Ligand-receptor binding is required to induce many of the signals used by cells during the inflammatory response. To limit the effects of ligands, decoy receptors are used. Both TNFR1 and TNFR2 can be secreted as decoys [431, 432], and another TNF family member (decoy receptor 3) not only binds membrane-bound ligands, but also back-signals through them to inhibit apoptosis and proliferation in RA FLS [433]. IL-1R1 and ILR2 are released as soluble decoys found in RA blood and synovial fluid, and IL-1RA binds IL-1R1 as a decoy ligand [338]. Whilst some decoy receptors and ligands are increased in RA compared to controls (like IL-1R2 [434] and IL-1RA [435]), but the increase is lower than the increase in IL-1, making the ratio of pro-to anti-inflammatory signals smaller in RA than OA [436, 437].

The signal transduction cascades are negatively regulated at each level, often by feedback loops. The NFκB pathway is inhibited at multiple stages (reviewed in [438, 439]). The best example is the IκBα. IκBα holds canonical NFκB inactive in the nucleus until phosphorylated, ubiquitinated and degraded. Its gene, NFκBIA is rapidly transcribed following NFκB translocation [391], and the subsequent *de novo* IκBα shuttles NFκB out of the nucleus and once again holds it inactive [375].

The constitutively high signalling of NFκB in RA FLS induces a depleted IκBα pool. This may contribute to the apoptosis-resistant nature of RA, as NFκB products inhibit Fas-L and TNFα apoptosis signals in RA FLS [440]. Aberrantly low IκBα may also contribute to articular damage, as addition of IκBα expression in MLS and FLS spared anti-inflammatory genes but abrogated MMP and proinflammatory cytokine expression [342].

Repression of chromatin, and of access to it by machinery and activators occurs at several levels. Repressive histones [389], histone modifications [258], and epigenetic marking of the DNA [403] all act to repress gene expression once initiated. Aberrant profiles of such epigenetic traits increase inflammatory mediators [408], and curtail inhibitory aspects [420] of RA and other chronic inflammatory diseases [258, 414].

Transcript stability is a rich source of examples of negative feedback loops. Many inflammation-related transcripts include adenosine-uridine rich elements (ARE) in their 3' UTR. They are not only targeted by stabilisers like HuR, but a plethora of destabilising proteins (reviewed in [441]). TTP was originally described in fibroblasts [442], and is important in multiple immune cell types (reviewed in [443]). TTP destabilises a number of pro inflammatory cytokines, but also IL-10. This latter seems counterintuitive, but is outweighed by the inhibition of pro inflammatory mediators [444]. Destabilisers are induced by proinflammatory stimuli, and so act in a complicated feedback loop to restrict excessive signalling [445]. PP2A de-phosphorylates TTP to activate its anti-inflammatory function. However, p38 also induces MK2 to phosphorylate TTP, thus inactivating it. This process is suspected to be aberrant in RA, as TTP-/- mice spontaneously develop arthritis, whilst genetic modification of TTP to stop its phosphorylation (and therefore its inactivation) is protective. This can also be achieved by artificially inducing PP2A [446].

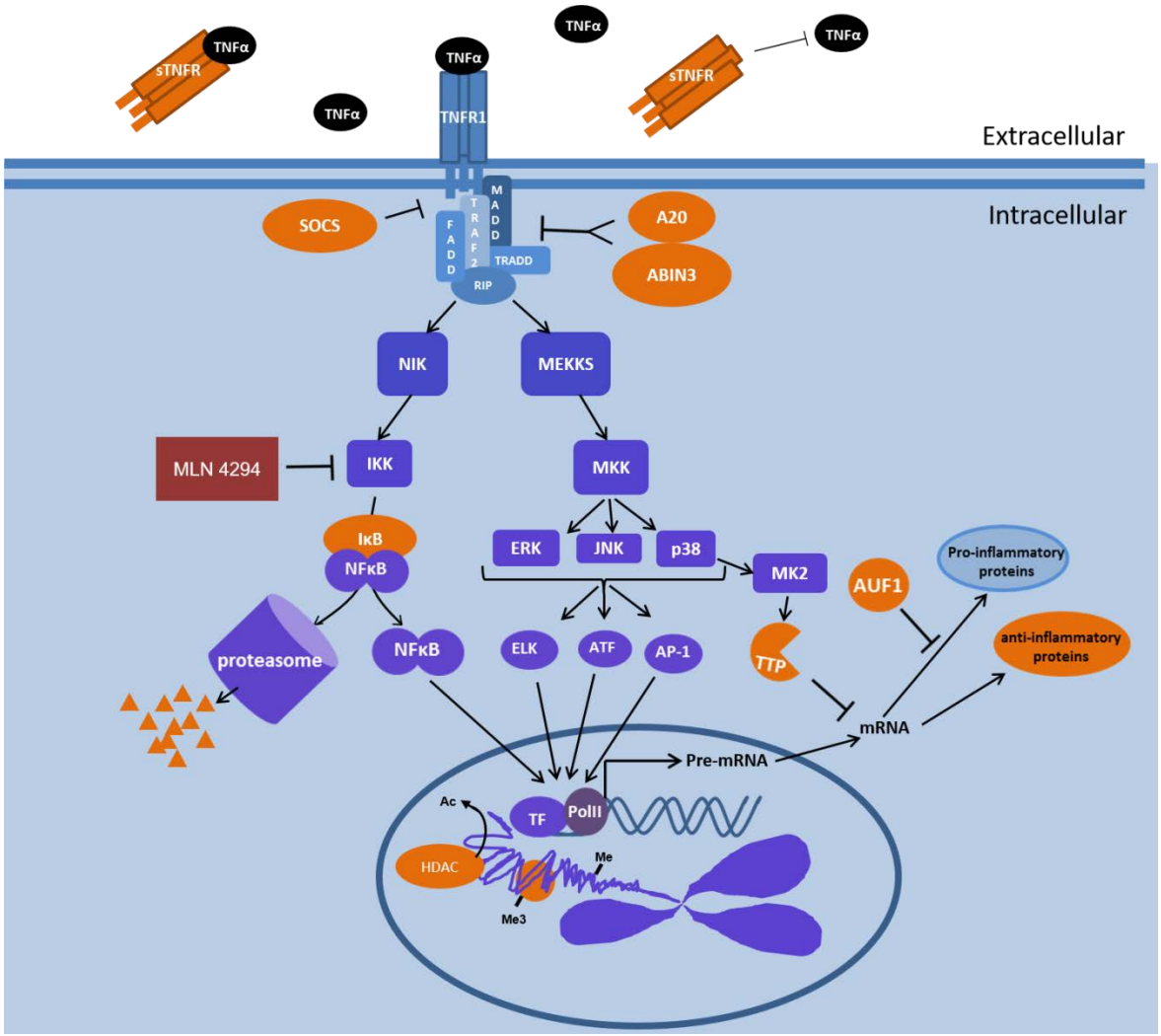

Figure 4.2: Negative regulatory mechanisms in the cellular response to stimulus. Cartoon of the cellular response to stimulus, including example inhibitors of the signal transduction and cellular response. Shades of blue and purple represent the inflammatory response, whilst orange represents inhibitors. Note the red box representing MLN 4294. Whilst not an endogenous inhibitor, it is used in this chapter and therefore included in this figure as illustration of its point of inhibition.

4.1.8 Mechanisms involved in innate memory

As discussed in chapter 3, innate memory has been recognised for decades, and as such there is a body of research regarding its mechanisms. A review of mechanism involved in macrophage endotoxin tolerance discussed various mechanism [273]. These range from depleting TLR4 surface abundance [447] and distal negative regulators (see [273]), through to translational and post-translational regulation of TNFα abundance [448]. The majority of publications now generally agree chromatin access is the key driver of macrophage endotoxin tolerance, as shown by Foster, Hargreaves and Medzhitov [300].

Whilst chromatin access dictates transcription machinery binding to DNA, there is a degree of ‘chicken and egg’ about it as the underlying mechanism. The process of epigenetic tolerance by reduced H4Ac, H3k4me3 [300], H3k10-p [449] or by maintaining H3k9me3 [449] is due to an inducing signal. Whilst the epigenetics of trained immunity is more long term (reviewed in [278] and shown by others to hold specific loci in a permissive state [292, 450, 451]), the modifications in short term innate memory must be more dynamically altered.

TLR4 signals through the Myd88 and TIR-domain-containing adapter-inducing interferon-β (TRIF) pathways. These induce the early cytokine and late/interferon response, respectively. The latter is partly dependent on the former, as MyD88 signalling induces endocytosis of the TLR4 ligand, which can then be used in the TRIF pathway. In macrophage endotoxin tolerance, the TRIF-dependant induction of negative regulators is upregulated, and this feeds back to inhibit MyD88 and TRIF pathways [452-454]. Thus inhibition of distal signalling cascades down to

functional consequences, and chromatin access is presumably increased in the loci responsible for genes coding distal regulators.

Studies of stromal cell memory are few, and those elucidating mechanisms are rarer still. A study of endothelial cell (EC) tolerance to TLR4 stimulation showed that EC endotoxin tolerance was dependent on MyD88, as TLR4 engagement barely activated TRIF. This mechanism was seen to increase IRAKM and decrease MAPK phosphorylation, suggesting a shift in responsible pathway but still a reliance of distal regulators [304].

Koch et al recently conducted a cross-stimulation experiment of TLR2, 3 and 4 on EC [303]. As with macrophage tolerance, repeat TLR4 stimulation brought about tolerance. Challenge with TLR2 or 3 agonists either as the initial or second challenge altered the IL-6 and CXCL10 expression in both tolerant and augmenting fashions, and to varying extents. The mechanism underlying of TLR2 priming of augmented TLR4 responsiveness was claimed to be RIP1 activity in the first response inducing IRF7 protein production which facilitated a greater IFN-response (CXCL10) in the second response. This was independent of the NFκB pathway and MAPKs, except for ERK1/2, were also thought not to be involved. The complexity of the comparison made a mechanistic model difficult, and the lack of NFκB involvement contradicts Wang et all [302], who found repeated TLR4 stimulation induced EC to mount an NFκB-dependant augmented response. It should be noted however that Wang and colleagues did not see tolerance, and so perhaps the role of NFκB is correctly reported by both groups. The repressed state involved reduced NFκB, whilst the augmented response was via increased NFκB.

Fibroblast studies have shown increases, decreases and no effect, all varying dependent on stimulus, read out and site of origin. Zaric et al posited that macrophages cannot tolerize to TLR2 repeat challenge because TLR2 signals through MYD88 and not TRIF (which is necessary for the IFN response). IFNβ is necessary to elicit the tolerant state, and addition of IFNβ along with repeat TLR2 agonism induced tolerance in gingival fibroblasts [314]. Koch et al thought that TLR3 primed EC for a tolerant IL-6 but augmented interferon/CXCL10 response because TLR3 signals through the TRIF/IRF rather than MyD88 pathways. In this sense they both agreed an disagreed with Zaric's work. It seems IFN may be necessary for tolerizing the MyD88-dependant genes, but for augmenting the TRIF-dependant genes. If negative regulators are TRIF-dependent then this may agree with the negative feedback loop cited above [454].

The necessity of IFN in facilitating HGF tolerance was clear, and its reduction of MyD88-dependant cytokines but increase in TRIF-dependent products was shown in EC. It was also shown to act independently in primed FLS. Sohn et al found chronic exposure to TNFα facilitated augmented chemokine responses to IFNβ or IFNγ [324]. The authors found increased Signal transducer and activator of transcription (STAT)1 protein produced by chronic TNFα, facilitating a larger STAT-1-phosophorylation response to IFN challenge. This concept of increased protein abundance agrees with part of the Koch theory, but Sohn et al considered chromatin accessibility to be the principle mechanism of the augmented response.

Much of the work listed above was conducted in myeloid cells, which have numerous important differences in comparison to fibroblasts. Even the studies of EC and formative studies of fibroblast memory provide answers that a far from

clear. The work below is therefore based on our understanding of fibroblast inflammation than that of earlier studies of innate memory.

4.2 Results

4.2.1 Longevity of the mechanism underpinning the primed response

Having confirmed the existence of fibroblast memory, I began examining the mechanisms that may underlie it. The first test was to assess whether it is a permanent or transient phenomenon. As stated earlier, RA FLS are epigenetically imprinted in a fashion distinct from that of non-RA FLS. Elucidating the longevity of memory would give an indication of the mechanism underpinning it.

FLS were stimulated twice, as per previous experiments, but the rest period between stimuli was extended to three or seven days. As shown in figure 4.3, IL-6 secretion did not differ if the cells were only exposed to medium. In comparison, the fold change altered depending on the rest period between challenges with TNFα or IL-1α. In chapter 3, fold changes of response following one day of rest showed significant results. Kruskall-Wallis found the responses to TNFα or IL-1α were significantly different from the normalised first dose (p=0.0006 and p=0.021 respectively). Dunn's post-test following multiple comparisons showed the augmented response to TNFα, but not IL-1α, was significant after one day of rest. The fold changes were similar to those reported in chapter 3 however, suggesting a reproducible and robust increase in IL-6 secretion following stimulation after a 24h rest period.

After three days of rest, the magnitude of the fold change had reduced. TNFα still induced an increase in IL-6 (2.62 ± 0.6-fold, p=0.03), which had declined by seven days of rest (1.81 ±0.55-fold, p=0.38). The IL-6 response to second challenge with

IL-1α also decreased after three or seven days, with the latter. These data suggest that FLS priming of IL-6 is transient and lasts at its peak efficacy for less than three days.

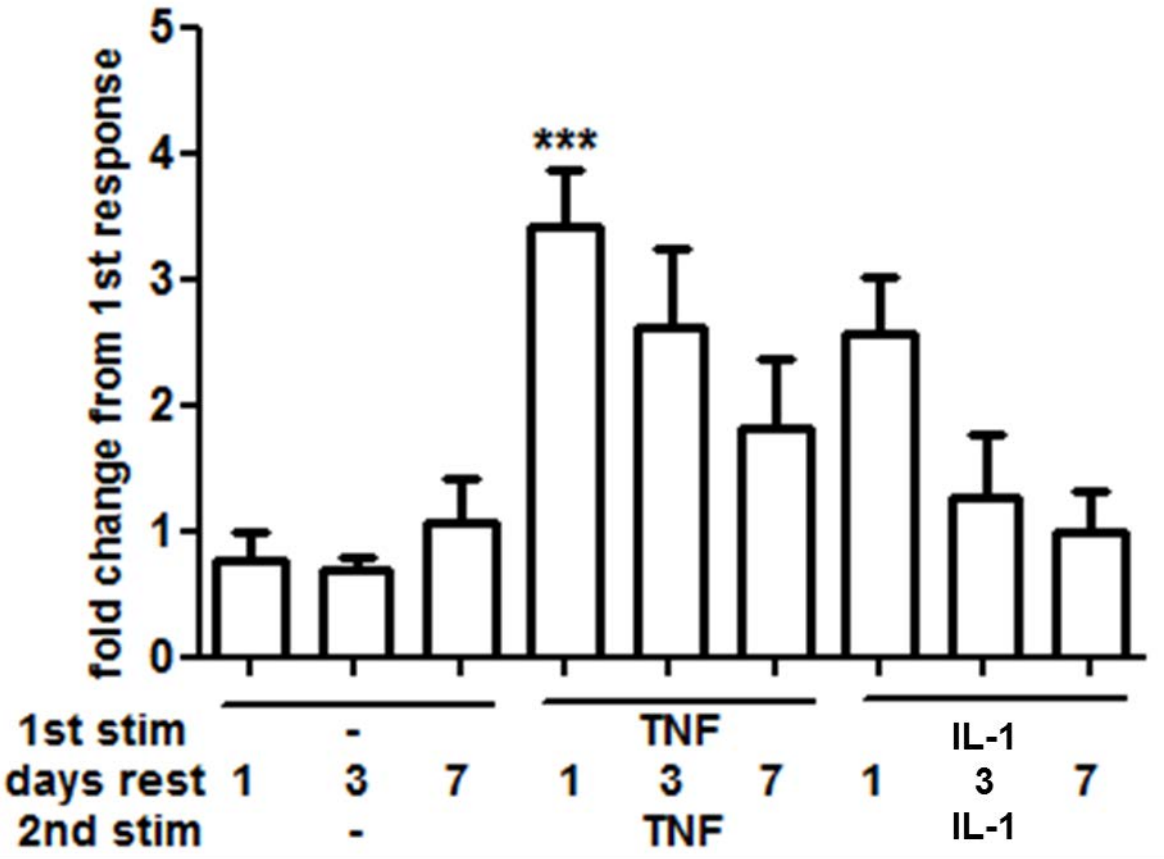

Figure 4.3: Fibroblast memory is temporary. FLS received 10ng/mL TNFα or IL-1α, or medium for 24h. Conditioned medium was removed for analysis and cells were washed and rested in fresh medium for one, three or seven days. Cells were then washed and received a second dose of medium, 10ng/mL TNFα or IL-1α. Conditioned medium was again removed for analysis by IL-6 ELISA. IL-6 secretion in the second response is represented as a fold change of the first dose (which is normalised to one). Mean ± SEM. *p< 0.05, **p< 0.01 (Kruskall-Wallis with Dunn's post-test). n= 5-10. Adapted from Crowley *et al* 2017 [455].

4.2.2 Sensitivity of the primed cell to second challenge

Having displayed the transient nature of fibroblast priming, the next step was to compare the intracellular signalling in the first and memory responses. To test the hypothesis that cells were more sensitive to the second challenge, I primed cells with 10ng/ml TNFα or IL-1α as usual, rested the cells for 24h, then stimulated with a range of concentrations of the same cytokine. An augmented response to reduced concentrations would imply a greater sensitivity, for example by increased receptor abundance.

TNFα was dose-dependent across the range examined. A second dose of 10ng/ml TNFα induced a significant increase in IL-6 secretion (2.99± 0.91-fold, p=0.01), but lower doses failed to induce an increased response (Figure 4.4b). As expected, 1 and 10ng/ml IL-1α both induced an augmented second response (Figure 4.4c); at 5.01± 0.95 fold and 2.81± 0.34 fold increase, respectively. As shown in figure 3.3 IL-1α reaches a ceiling effect on inducing IL-6 secretion at 1ng/ml. It is therefore unsurprising that in figure 4.4 both 1 and 10ng/ml IL-1α induced augmented IL-6 responses following priming with 10ng/ml IL-1α.

Given that concentrations below that which induces maximal response in the first challenge did not augment the primed response, it is reasonable to assume that BJ fibroblasts are not sensitized by the priming dose.

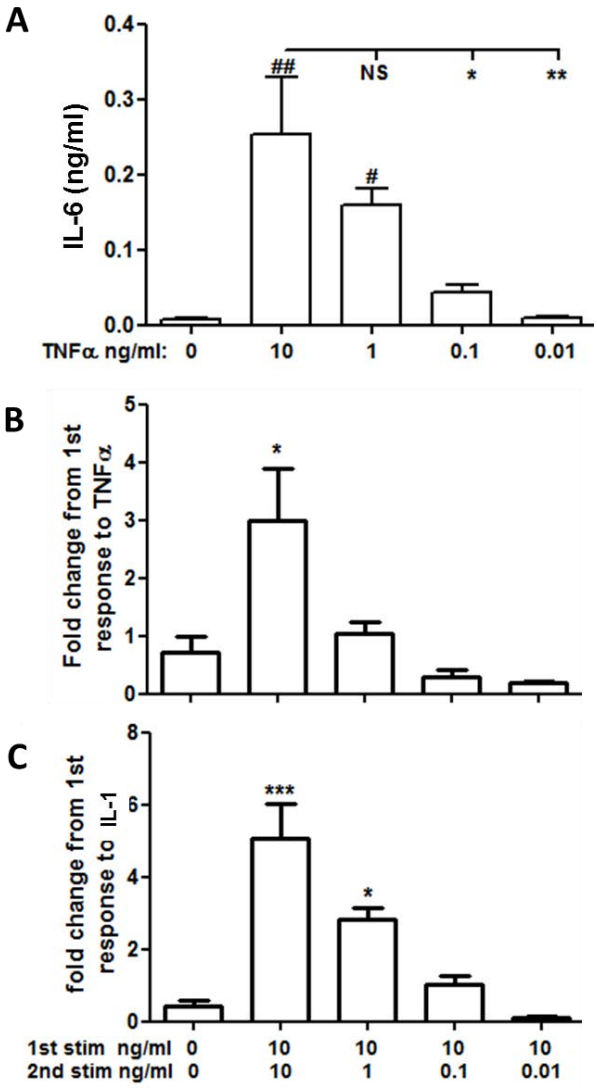

Figure 4.4: Fibroblasts are not sensitized to stimulus by priming. BJ fibroblasts were stimulated with 10ng/ml TNFα or IL-1α for 24h, then washed and rested for 24h. Cells were then stimulated with indicated concentrations of cytokine for 24h. First and second responses to stimuli were assessed by IL-6 ELISA. Second response to stimulus is expressed as fold change from first response. **A** TNFα dose titration, n=3. ANOVA with Dunnet's post-test against unstimulated cells (## p<0.001) or 10ng/ml TNFα (* p<0.05, ** p<0.01). **B** TNFα repeat-stimulated cells, n=7. **C** IL-1α repeat-stimulated cells, n=6. ANOVA with Dunnet's post-test against normalised first dose (* p<0.05, ** p<0.001).

4.2.3 Receptor specificity of the primed response

Whilst still examining the concept of receptor involvement I considered whether the augmented response must be induced through the same receptor as the initial response. To this end I primed fibroblasts with TNFα or IL-1α then used either the same, or the other cytokine for the second challenge.

As shown in figure 4.5, challenging with the same cytokine twice induced an augmented IL-6 response, as previously shown. The use of different cytokines to prime and re challenge also induced an augmented response. The amount of IL-6 secreted in response to IL-1α following priming by TNFα, for example, was over three-fold greater than the IL-6 released in response to IL-1α without prior stimulation. Multiple comparisons showed significant increases in fold change, but Dunnet's post-test showed only TNF-TNF and TNF-IL-1 reached significance increases from the normalised first response.

This nonetheless confirms work in other cell types, that innate memory is not stimulus-specific, and once primed cells will mount augmented responses to stimuli of different pathways. These data, in combination with those of figure 4.4, suggests the priming mechanism is not stimulus specific, nor due to sensitization. The mechanism is therefore more likely to manifest further down the signalling cascade.

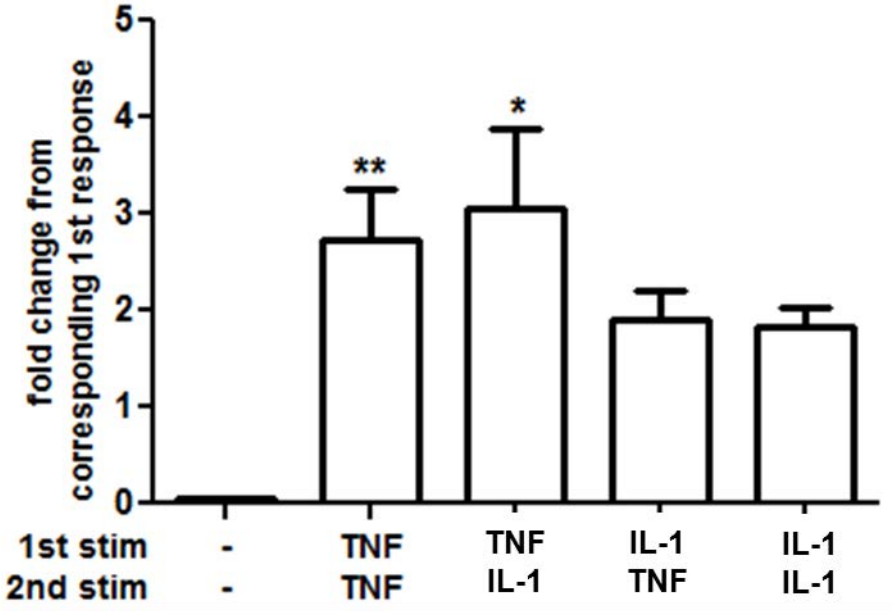

Figure 4.5: Priming an augmented second response does not require the same receptor in both doses. BJ fibroblasts were stimulated with 10ng/mL TNFα or IL-1α, or medium, for 24h. Conditioned medium was then removed, and cells were washed and rested for 24h. Cells were then washed and stimulated again with either the same or opposite cytokine, again at 10ng/mL for 24h. Conditioned medium was again removed, and the first and second responses to stimuli were assessed by IL-6 ELISA. Second dose responses are expressed as fold changes from first responses to the same stimulus. Mean ± SEM. *p=0.05 **p=0.01 as determined by ANOVA with Dunnet's post-test against the normalised first dose. n=6. Adapted from Crowley *et al* 2017 [455].

4.2.4 Intracellular signalling in response to the second challenge

The next step was to assess signalling pathways following receptor binding. Whilst previous data rules out first dose residue stimulating cells in the second challenge, I couldn't rule out a role for receptor-bound cytokines. If true, the signalling pathways may be maintained in a higher state of readiness during the rest period. This would facilitate a stronger response to second challenge.

To test whether activation of signalling pathways continued through the rest period, I used Western blots to assess NFκB and MAPK activity. Phosphorylation (a marker for activation) of NFκB p65, ERK1/2, JNK1/2 ad p38 MAPK was induced by proinflammatory stimulation, but 24h after the removal of TNFα, signalling of all four pathways had returned to basal levels (Figure 4.6).

Figure 4.3 showed that the primed response is high after one day of rest but decreases as the days without second challenge progress. I therefore posited that signalling would still be high 24h post-wash and would then decrease. In refutation of this the levels of phosphorylation did not differ between the first and third day of rest.

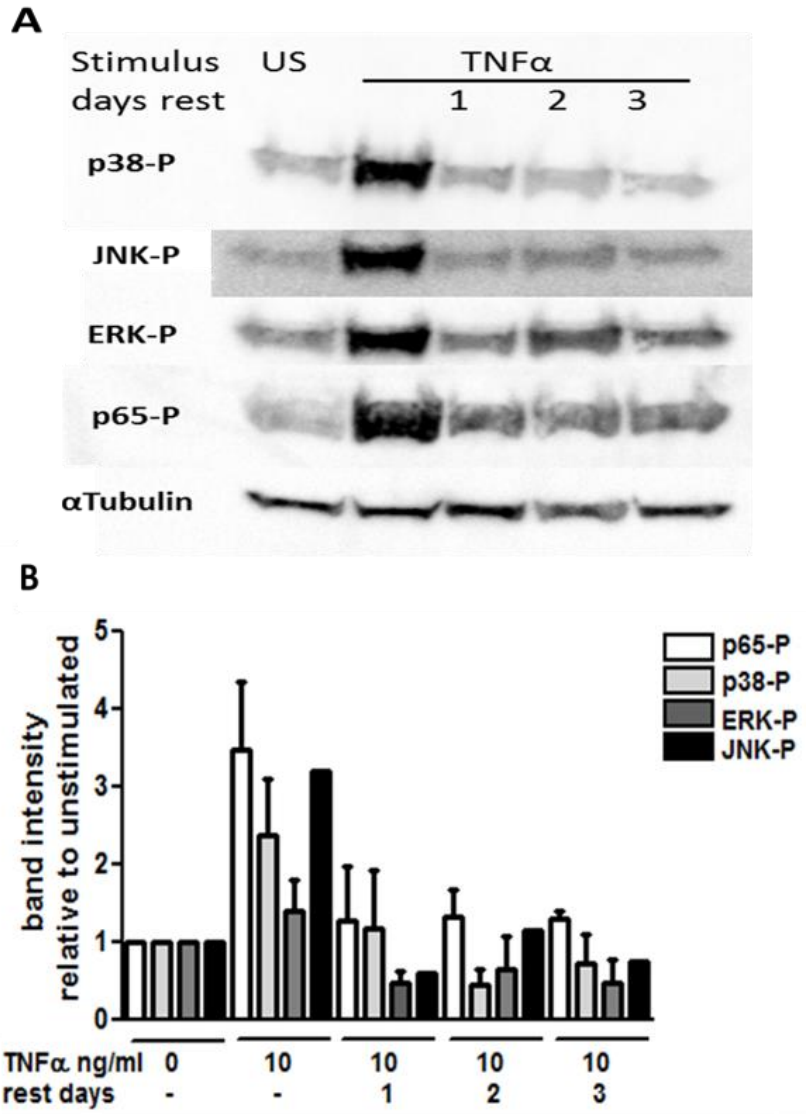

Figure 4.6: signalling activity returns to basal following removal of stimulus. BJ fibroblasts were stimulated for 24h with 10ng/ml of TNFα. Cells were either lysed, or washed with medium and rested in fresh medium for one, two or three days before lysis and protein isolation. **A** Western blots of the one dose and subsequent rest. Representative of three blots. **B** Densitometry plot of treatments relative to unstimulated cells. All protein densitometry was normalised against respective treatments' αTubulin levels. Representative of three blots (JNK representative of one blot). Mean ± SD, n=3. From Crowley *et al* 2017 [455].

Signalling in the rest period did not suggest an altered basal state of activity. I therefore tested whether signalling was altered between the two responses to stimuli.

In figure 4.7, the signalling in response to 10ng/ml TNFα was robust from 30 minutes. The duration differed between blots, but largely phosphorylation was transient, returning to basal levels within 2-4h. As with figure 4.6 above, all proteins' phosphorylation levels returned to basal by 24h after TNFα was removed.

In contrast to the initial response, the MAPK pathways, particularly p38 and JNK, showed abrogated phosphorylation in the second challenge, and ERK1/2 remained phosphorylated for a shorter period than in the initial response. The phosphorylation of Ser536 on NFκB p65, however, appeared to be prolonged in the second response. Whilst the duration differed from blot to blot, all experiments displayed a prolonged phosphorylation of NFκB p65 in the second challenge. Phosphorylation at this site represents a useful marker of NFκB activation.

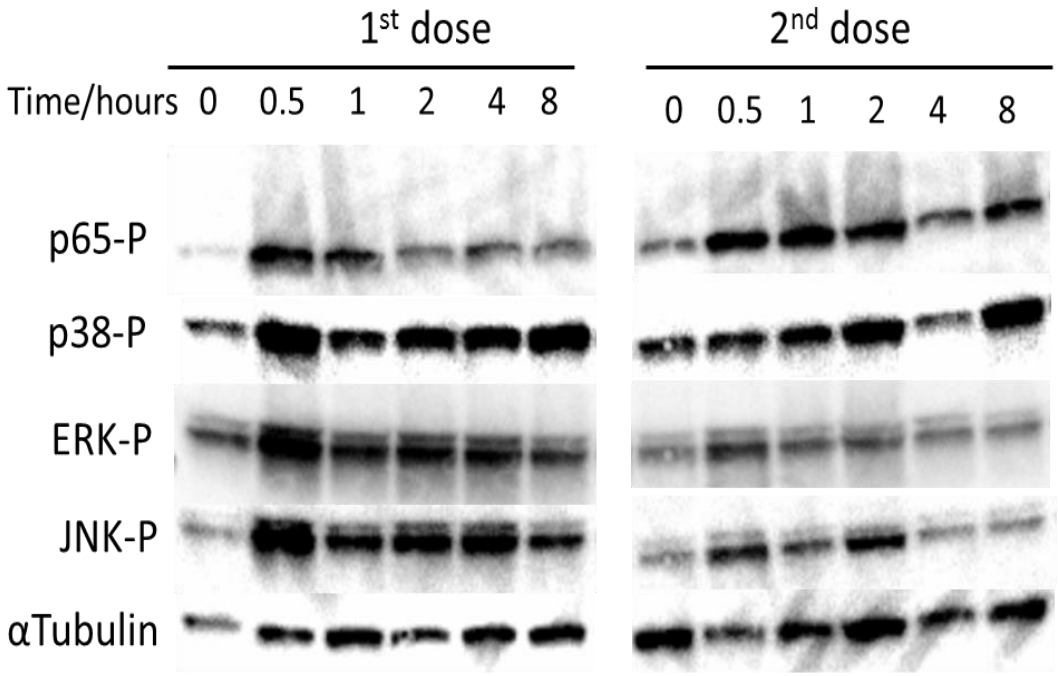

Figure 4.7: NFκB p65 remains phosphorylated for longer in the second response to stimulation. BJ fibroblasts were stimulated with 10ng/mL TNFα for the times indicated, with or without a priming exposure to 10ng/mL TNFα for 24h. Phosphorylation state of NFκB, p38, ERK1/2 and JNK1/2 were assessed by Western blot. Representative of five blots. From Crowley *et al* 2017 [455].

I sought to confirm the prolonged activity of NFκB by other methods. To this end I repeated the experiment using isolated cytosolic and nuclear fractions. This allowed me to assess the length of time NFκB p65 remained in the nucleus (its site of activity) in initial and memory responses to TNFα.

As shown in figure 4.8, the amount of NFκB p65 present in the BJ fibroblast nucleus after 2h of exposure to TNFα was significantly higher in the primed response than the initial response. The loading of the Western blot was unequal, but it is still easy to see that relative to the control protein in each well, there is increased nuclear NFkB time points in the memory response. This is made clearer by a densitometry plot (figure 4.8b). This suggests that NFκB p65 activity, not just phosphorylation, is prolonged in response to challenge if the fibroblast is primed.

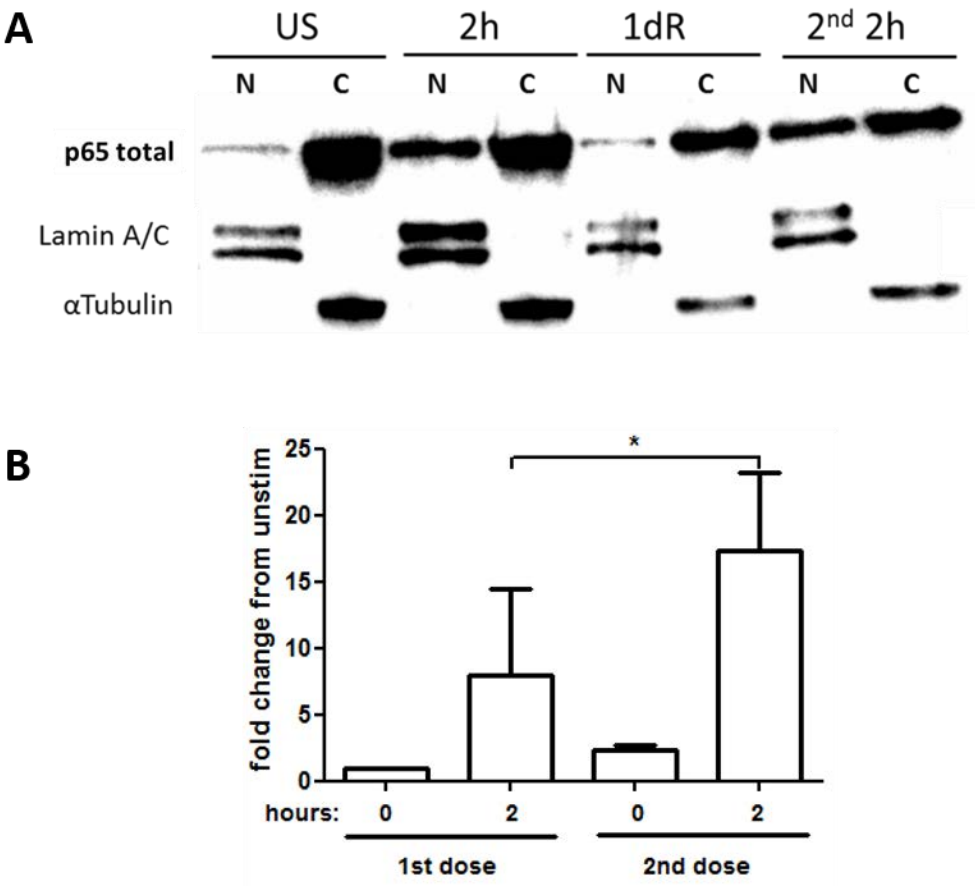

Figure 4.8: NFκB p65 remains localized to the nucleus for longer in primed fibroblasts under challenge than those receiving their first challenge. BJ fibroblasts were stimulated with 10ng/ml TNFα for the times indicated, with or without priming. The proteins isolated from each treatment were split into nuclear and cytosolic fractions and Western blots were used to assess NFκB p65 abundance in the nuclear fraction. **A** Western blot. **B** densitometry of NFκB p65 intensity relative to unstimulated nuclear levels, all normalised against Lamin C. Representative of three blots. Taken from Crowley *et al* 2017 [455].

As further evidence of this difference, BJ fibroblasts were also examined by immunofluorescent test of nuclear localization. Cells were stimulated with 10ng/ml TNFα for 30 minutes, 2h or 4h (figure 4.9a), or primed for 24h and rested for 24h, before undergoing the same time course (figure 4.9b).

BJ fibroblasts at rest (figure 4.9a, top image) had a clear absence of NFκB p65 (designated by green fluorescence) in the nucleus, as expected under basal conditions. Nuclear localization is evident at 30 minutes, in that the nucleus is no longer dark. The transient nature of NFκB p65 nuclear localization is evident, however, as the nucleus appears darker at 2h, and darker still at 4h, at which point nuclear sparing is similar to that of unstimulated cells.

As with the Western blot data, primed fibroblasts had relatively little evidence for NFκB p65 in the nucleus before re challenge (figure 4.9b, top image), although some cells still showed nuclear localization. After 30 minutes of TNFα stimulation, cells displayed greater nuclear localization in the second response compared to the first, and this robust nuclear fraction remained high at 2 and 4h.

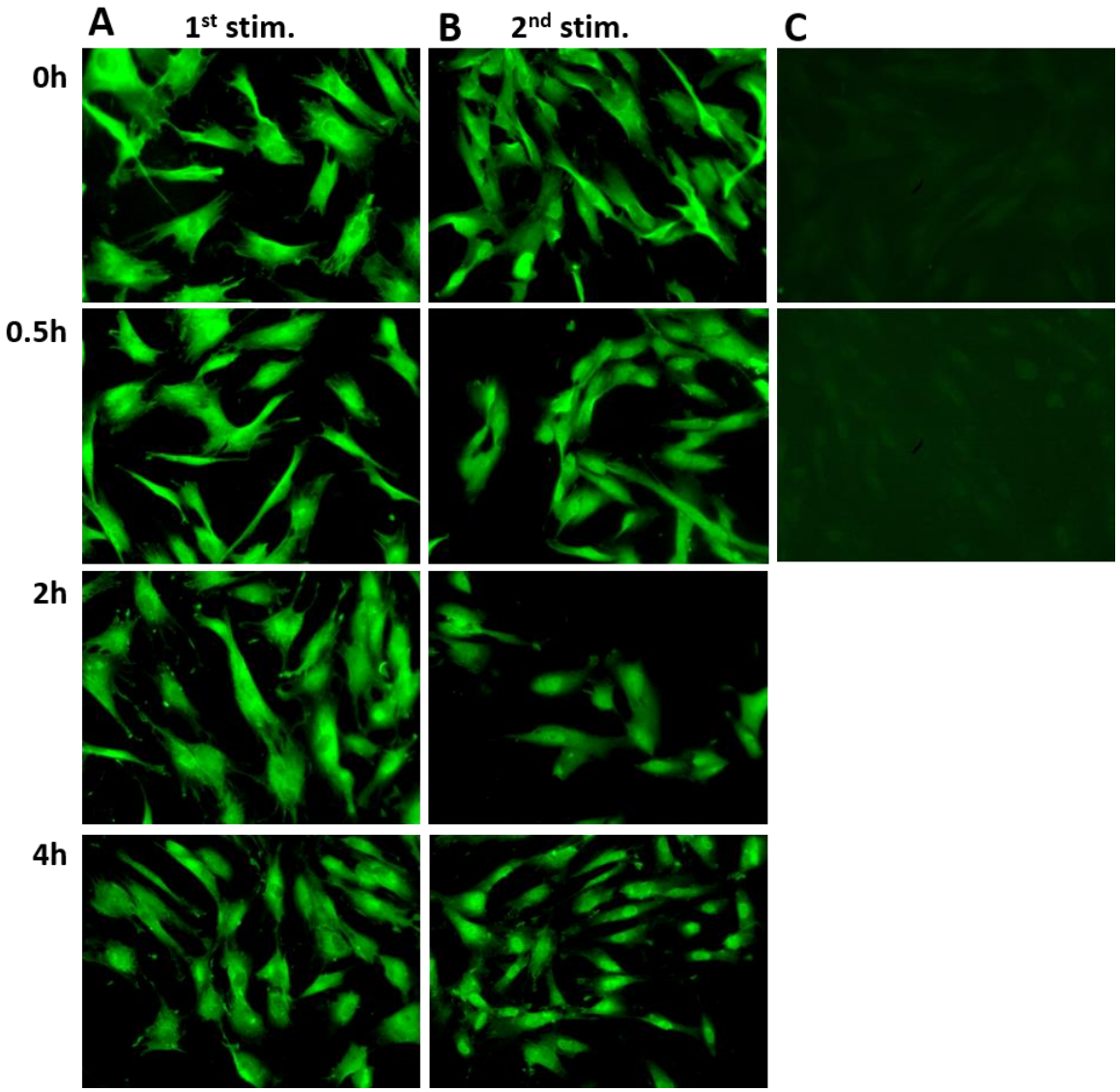

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Figure 4.9: NFκB p65 nuclear localization is prolonged in the second response to TNFα. BJ fibroblasts were stimulated with 10ng/mL TNFα for 0.5, 2 or 4h, or for 24h before being washed, rested for 24h, and then stimulated again for 0.5, 2 or 4h. Cells were fixed and permeabilized, before being incubated with anti-NFκB p65 antibody overnight, and an appropriate secondary antibody the next day. Control chambers underwent the same experimental conditions, but did not receive a primary antibody incubation. p65 localization was examined by fluorescence microscopy at x20 magnification. **A** Localization of NFκB p65 in the first response to TNFα. **B** Localization of NFκB p65 in the second response to TNFα. **C** Example control wells from the first and second responses. Representative of three independent experiments. Taken from Crowley *et al* 2017 [455].

The evidence for prolonged NFκB activity, both in terms of phosphorylation state and intra cellular localization, has been shown by three different techniques in independent experiments. Confirmation of this phenomenon in FLS would prove difficult by Western blotting, as FLS grow at a slower rate and are therefore difficult to conduct large scale experiments on.

It was, however, possible to confirm the prolonged nuclear localization by immunofluorescence. As shown in figure 4.10, the basal levels of NFκB p65 in the nucleus was slightly higher than in BJ fibroblasts at rest, but this is not surprising given the higher basal production of pro inflammatory cytokine like IL-6.

Irrespective of the basal nuclear localization seen in RA FLS, the same pattern of a robust yet transient increase was observed in the first response to TNFα (figure 4.10a), whilst the nuclear localization in response to second challenge was much more prolonged. This evidence adds to that of the BJ cells in demonstrating prolonged NFκB activity in the memory response compared to the initial response.

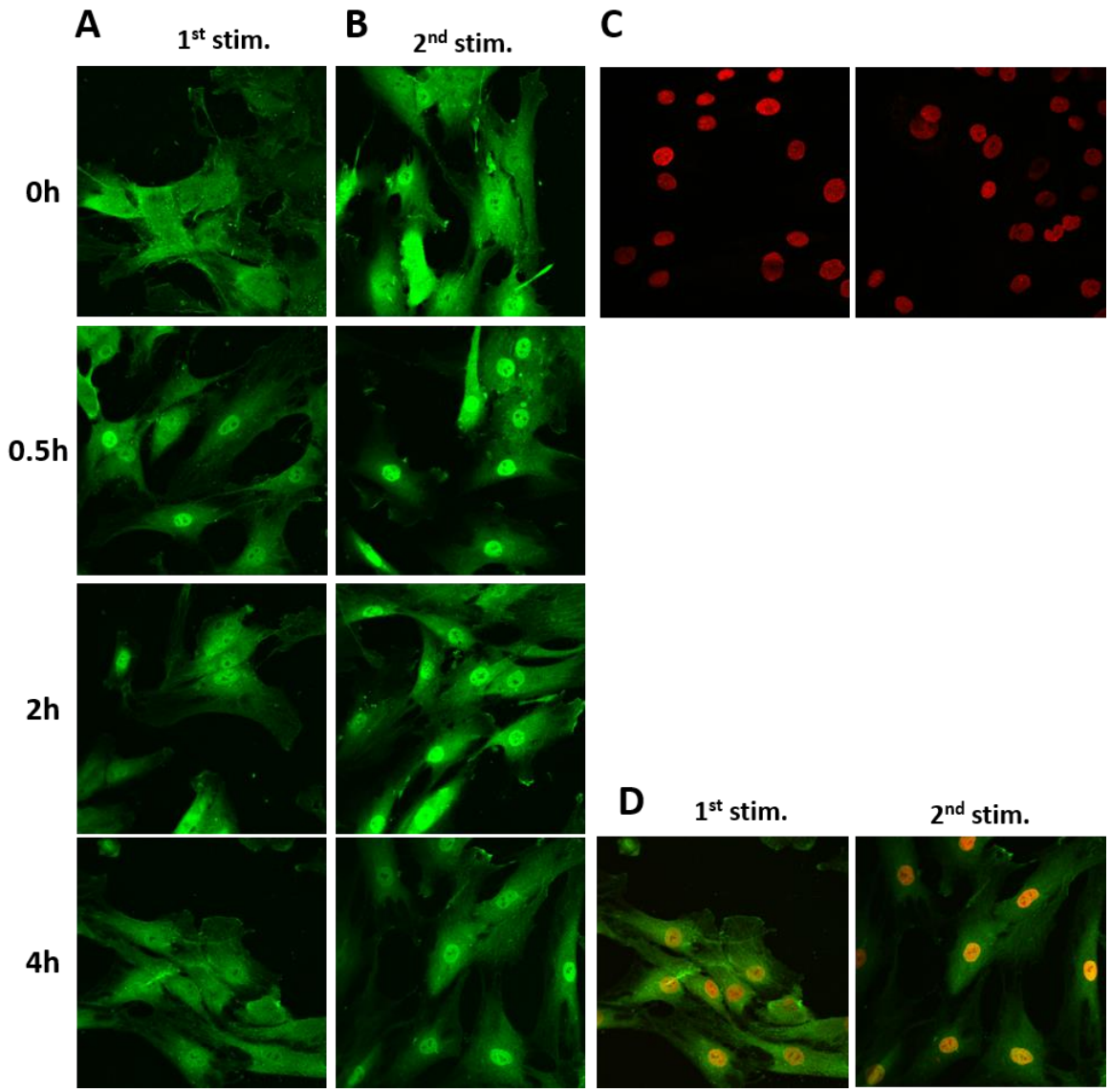

Figure 4.10: FLS display prolonged NFκB p65 nuclear localization during second TNFα challenge. FLS were stimulated with 10ng/ml TNFα for the time points indicated either with (column **B**) or without (column **A**) priming with 10ng/ml TNFα for 24h. NFκB p65 is shown in green. **C**) Example isotype controls with DAPI nuclear counterstain in red. **D**) 4h time points in primed (right) and un primed (left) time course, with nuclear counterstain in red. Representative of three independent experiments. Taken from Crowley *et al* 2017 [455].

Once the prolonged NFκB response to second challenge had been ascertained and confirmed in FLS, I tested whether it was causative or simply correlated. To this end I used the NFκB pathway inhibitor MLN4924. An MLN titration was used to find a dose sufficient to inhibit the IL-6 secreted by BJ cells in response to 10ng/ml TNFα (Figure 4.11a).

As the prolonged NFκB activity separated the initial and primed responses at 2 or 4h, I stimulated cells with 10ng/ml TNFα for 2, 4, or 8h, with or without priming. In duplicate wells, 100nM MLN was added at the 2h point of the 4 and 8h samples.

The 8h time course showed the usual augmented response in primed fibroblasts (Figure 4.11b open squares). The addition of 100nM MLN after 2h had a negligible effect on IL-6 secretion during the first dose, indicating a similarly negligible role for the NFκB pathway after this point (Figure 4.11b closed squares, left graph). In stark contrast, addition of MLN at 2h of the memory response caused a significant reduction in IL-6 secretion at 4 and 8h (Figure 4.11b closed squares, right graph), reducing the IL-6 secretion to levels similar to those of the first response to TNFα. This differential effect of MLN displays an increased importance of the NFκB pathway after 2h in this primed response.

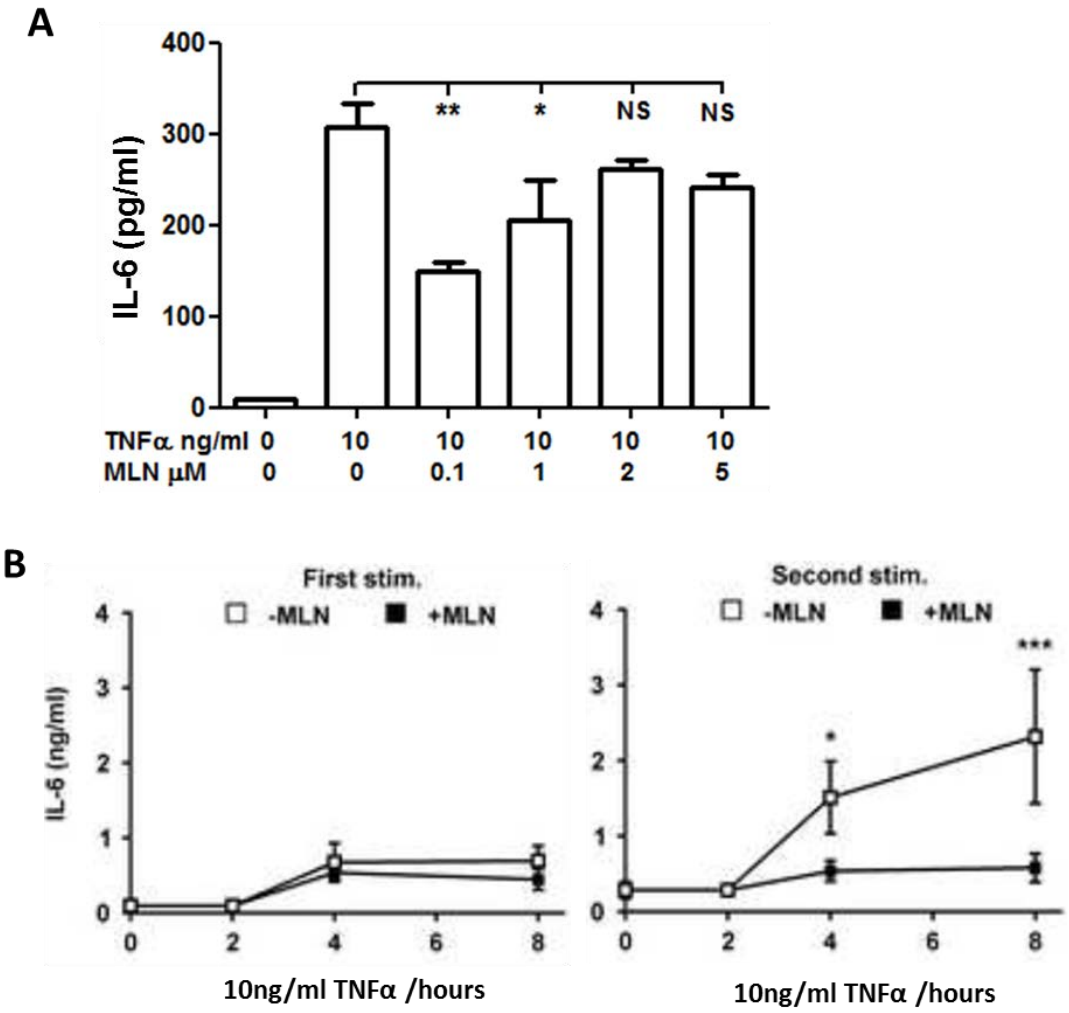

Figure 4.11: The augmented secretion of IL-6 in primed fibroblasts requires prolonged NFκB activity. A BJ fibroblasts were stimulated with 10ng/ml TNFα for 24h with or without the indicated concentrations of MLN. n=4, $p< 0.05$, ** $p< 0.01$, as assessed by ANOVA with Dunnet's post-test against 10ng/ml TNFα. **B** BJ fibroblasts were stimulated with 10ng/ml TNFα for the times indicated, with (right graph) or without (left graph) priming. 100nM MLN was added at the 2h point. IL-6 secretion at each time point was assessed by ELISA. n=6. * $p< 0.05$, *** $p< 0.001$ (Student t test). Section B taken from Crowley *et al* 2017 [455].

4.2.5 Transcriptional response to the second challenge

The IL-6 production at different time points in the first and second response indicated altered kinetics of the second response. As shown in figure 4.12, the amount of IL-6 produced at four, eight and 24h of stimulation with 10ng/ml TNFα was significantly higher in the second response.

The augmented second response at later time points was unsurprising after demonstrating the necessity of prolonged NFκB activity in the second response (Fig. 4.11). Earlier time points, however, also showed an augmented response. The second two hour point displayed an average IL-6 secretion 68% higher than the first response at the same time point, which represented a non-significant increase (p=0.12, unpaired t test).

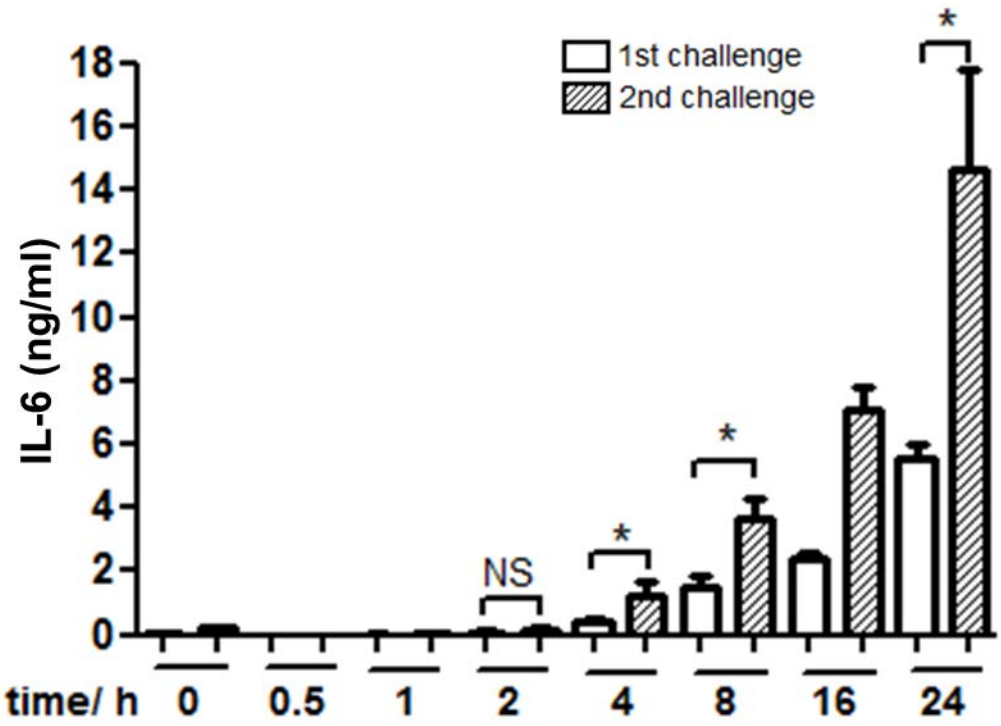

Figure 4.12: Priming by TNFα induces greater IL-6 secretion at early time points. BJ fibroblasts were stimulated with 10ng/ml TNFα for the time points indicated, with or without priming. IL-6 secretion in the first and second response were assessed by ELISA. n=5, * $p < 0.05$, unpaired t test.

Given the previously published description of increased chromatin access after initial stimulation, the increased IL-6 secretion at earlier time points led me to hypothesize a widened transcriptional window. In this model, primed genes would undergo earlier *and* later transcription compared to the initial stimulation.

In order to test this hypothesis, cells were stimulated with 10ng/ml TNFα for the time points indicated in figure 4.13, and transcription was assessed by measuring primary transcript abundance. Primary transcripts are rapidly converted into mature transcripts and are therefore a useful proxy measure of transcription.

Technical difficulties resulted in data too variable to trust, but it is included as it's pattern matches the western blot and immunofluorescence data but disputes the 'widened window' hypothesis. The initial IL-6 transcriptional response had a strong but temporary peak, whilst the memory response lacked a dramatic peak but maintained its expression level for longer. This contrasted the IL-8 transcription data, in which the memory response expression level was never higher than that of the initial response.

These preliminary data may support the prolonged response model. The mature transcript levels of IL-6 also confirmed this result, the memory response being held at a higher level than the initial response at 6h. Also, the difference between initial and memory response levels at 4h was much closer, implying a possible role for increased transcript stability. The IL-8 data did not match those of its primary transcript, and actually showed increased mature transcript in the memory response at three of the four time points measured.

As stated, these data are not trustworthy enough to facilitate conclusions, and are included only to help illustrate the hypotheses being explored.

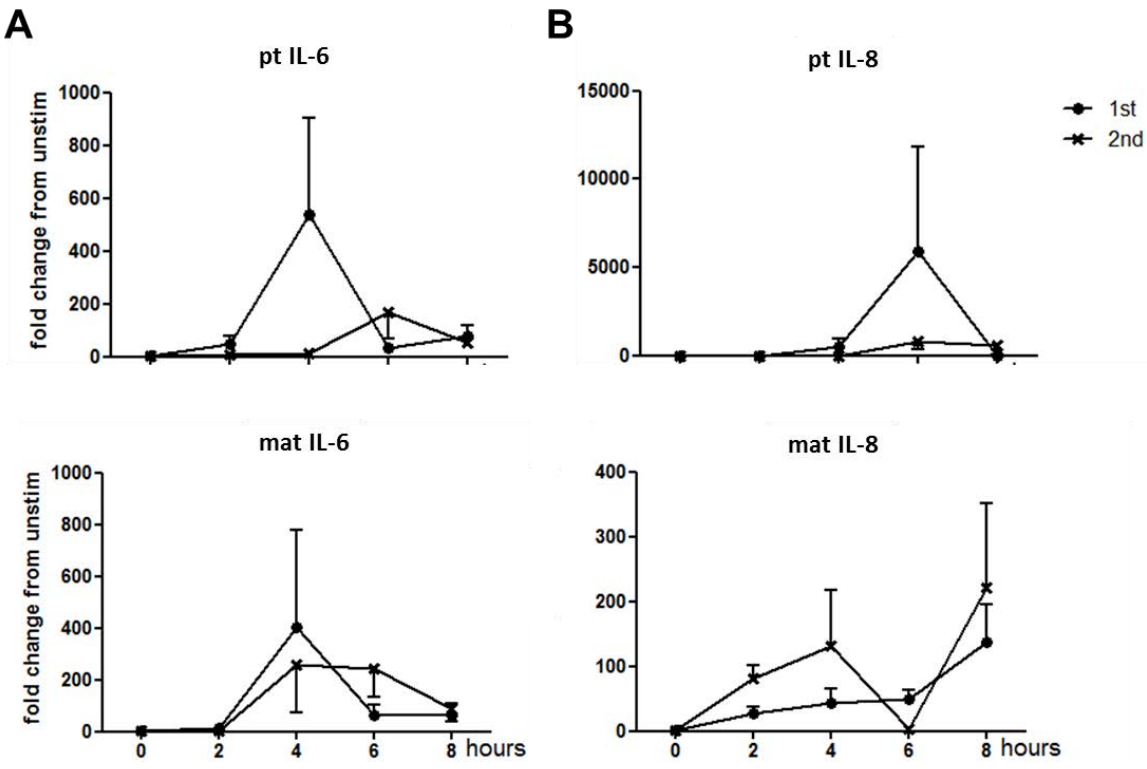

Figure 4.13: Gene expression kinetics following first and second TNFα challenge. BJ fibroblasts were stimulated with 10ng/ml TNFα for the time points indicated, either without priming or following 24h 10ng/ml TNFα and 24h rest in fresh medium. IL-6 (column A) and IL-8 (column B) primary (top row) and mature (bottom row) transcripts were assessed by qPCR. n=3 BJ replicates, mean± SEM.

4.3 Discussion

As discussed in chapter three, myeloid cells have received a great deal of the attention paid to innate immune memory, particularly with regard to mechanistic studies. Some comparisons show conserved mechanisms and shared findings, such as TLR4 repeat challenges inducing tolerance in macrophages and EC, or TLR2 repetition not inducing the refractory state [303, 314]. Others do not match as well, as two challenges with TLR2 or TLR4 induce tolerance in macrophages [456], whilst the EC IL-6 and CXCL10 are augmented and reduced respectively under the same conditions [303].

These differences raise the salient point that cell types are phenotypically and functionally distinct. Even comparisons between EC of different sites have shown differences in repeat stimulation experiments [302, 303]. However, given the paucity of mechanistic studies in fibroblast memory it is worth comparing memory in different cell types.

4.3.1 Transiency of innate memory in fibroblasts

Myeloid memory may be short term tolerance or long-term training, and therefore has two levels of transiency. The duration of the rest between TNFα challenges negatively correlated with the magnitude of memory response, implying a length of memory much more reminiscent of endotoxin tolerance than trained immunity.

Sohn et al's paper on fibroblast priming stated that "Since the priming effect of chronic exposure to TNFα decays slowly over a period of a few days after washing and blocking TNFα" [324]. Whilst they did not publish this data, it confirms that the mechanism behind fibroblast memory is transient. This paper found chromatin accessibility was involved in priming, but the transiency suggests a mechanism

distinct from the epigenetically-altered endothelial cells [305] and fibroblasts [259] which transfer inflammatory characteristics through passages *in vitro*.

4.3.2 Receptor abundance and sensitivity

Increasing the abundance of receptors on the cell surface facilitates cell sensitivity. Whilst complicated by the oligomeric nature of some receptors (such as trimeric or tetrameric formation of gp130-IL-6R complexes [339]), in many cases the increase in receptors correlates with increased responsiveness.

Fibroblast IL-6 showed dose dependency to both TNFα and IL-1α, which was mirrored upon re stimulation with the same cytokine. Such a result suggests that the cells were not sensitised to a second challenge, and the implication is that augmented IL-6 levels were not due to an increased level of receptors.

This would have been confirmed by flow cytometry had more time been available, as preliminary (excluded) data did not show that cytokines significantly increased transcription of their cognate receptors. This is supported by the findings that TNFα induced and augmented response to IL-1α, and vice versa. If the augmented response was due to increased receptor abundance, it may only be present upon re challenge with the same cytokine.

This appears to disprove increased receptor abundance as the underlying mechanism for memory. Other studies however, viewed this theory more favourably. Epithelial studies have varied, showing viral infection increases TLR3 expression and subsequent response to double stranded RNA [457], whilst TLR2 levels were not found to play a role in the augmented response to pneumoniae following influenza, despite its induction of increased TLR2 expression [458].

Cross-priming of TLRs has shown mixed results in macrophages, EC and fibroblasts [303, 312, 456]. TLR2, TLR3 and TLR4 have been challenged repeatedly, or in cross-stimulation experiments. These showed both augmented and abrogated responses upon second exposure, and this was often theorised as due to signalling below the receptors [303, 314]. Given the lack of evidence from my proxy experiments, and the number of publications which have failed to draw a consensus on receptor abundance being causative, I believe the predominant mechanisms for memory are downstream of the receptor-ligand interaction.

4.3.3 NFκB and MAPK pathways

I next examined the roles of two classic inflammatory signalling pathways: NFκB and the MAPKs. Signal transduction pathways often feed into one another but, the studies conducted revealed some clear answers. Assessing whether signalling continued after removing TNFα achieved two aims. First, to see if cells started off their second response from a higher signalling baseline and second, if heightened signalling decreased over days. It therefore tested whether continued signalling and its ‘wearing off’ accounted for the transiency seen in figure 4.3.

Koch et al recently theorised that the initial EC response induced increased interferon response factor (IRF) 7, allowing a greater interferon response to the second challenge [303]. Unfortunately, the authors did not detect IRF7 phosphorylation, so couldn’t ratify their theory. My own examination revealed that signalling had returned to basal after one day of rest, thus disproving both my hypotheses. As I did not test total protein levels I cannot draw parallels with the EC study. I therefore cannot exclude increased protein abundance, but can rule out a higher baseline of activity.

The signalling in repeated challenges was interesting, clearly demonstrating the changes involved in a memory response compared to the initial challenge. My main finding was of prolonged NFκB p65 phosphorylation. In contrast, p38 phosphorylation varied between repeats, JNK phosphorylation was poor, and ERK1/2 phosphorylation was refractory.

MAPKs contribute to a variety of TFs, which can modulate the effects of NFκB [350, 361, 391, 402, 459, 460]. The limited changes in JNK and p38 activity match EC memory responses [303, 382]. ERK plays roles in fibroblast proliferation [370, 381, 382] and inflammatory secretions [382], and increases in activity during EC endotoxin tolerance [303, 304]. If ERK phosphorylation increases in tolerance, but decreases in my augmented response model, it implies a negative regulatory role for ERK in memory responses.

The NFκB response gave a clear result and a mechanism to examine further. A group in New York have shown that continued NFκB is necessary for the unremitting response of RA FLS to TNFα [272, 324], and the augmented response to IFN [324]. During a primed response to IFN, FLS mounted a stronger and more prolonged inflammatory response. My research did not show a stronger NFκB response upon re challenge, but it did show prolonged phosphorylation.

This result was confirmed by nuclear versus cytosolic fraction Western blot and immunofluorescence. The subcellular fraction Western blot suffered technical difficulties with assessing total protein abundance, making equal protein loading difficult. Nevertheless (especially with the aid of densitometry) it is clear that NFκB p65 is in the nucleus for longer in the memory response. The immunofluorescent test of BJ cells confirmed this. When we used this figure in a manuscript the

reviewers suggested we include isotype controls and nuclear counterstains. Repeating the experiment in primary FLS with relevant controls confirmed the results. Nuclear counter-staining actually made it harder to examine nuclear localisation, so I presented my data without it in the paper [455] and this thesis.

Finally, an inhibitor study tested whether prolonged NFκB activity was causative or coincidental. MLN is a commonly employed NFκB pathway inhibitor. It inhibits the post-translational modification of IκBα by the IKK complex, thus holding canonical NFκB inactive state in the cytoplasm.

Whilst there are both canonical and non-canonical NFκB dimers, I focussed my investigation on the p65 subunit. It is commonly in the canonical dimer (p50-p65) but can also exist in non-canonical dimers like the p52-p65 dimer used by DCs to maintain inflammatory secretions (it is resistant to removal from the nucleus by IκBα) [376]. This subunit is widely implemented to study canonical NFκB activity. Whilst p65 is inflammatory, p50 also forms a homodimer, which inhibits the canonical dimer [377], and is increased in macrophage endotoxin tolerance [461]. Memory studies in fibroblasts once examined NFκB dimer usage. The authors found inhibiting NFκB abolished the augmented memory response, and that p50 and p65 were the only subunits which could be manipulated to alter the augmented inflammatory response [315].

This suggests that inhibition via MLN is a reasonable method of assessing the role of the canonical pathway. Its addition after 2h clearly displayed an inhibitory effect on the augmented IL-6 response to TNFα. It is worth considering off target effects (such as on other NFκB dimers), but it seems reasonable to claim that the NFκB pathway does indeed mediate the augmented response. These data and the

publications cited above support my initial finding of prolonged NFκB activity. Whether decreased [303, 304, 452, 453] or increased [302, 324] in the memory response (of various innate cells), NFκB activity correlates with cytokine secretion.

4.3.4 Altered kinetics and the transcriptional window

Reports suggest that innate memory occurs at the chromatin access [300, 324, 450, 461] and transcriptional level [264, 314, 315]. Whilst distinct steps, chromatin opening is a major step in initiating the transcriptional response. My own attempts at ChIP examination were not worthy of inclusion in this thesis, but Sohn et al, have shown a role for chromatin accessibility in fibroblast memory [324].

My research suggested prolonged activity of NFκB was responsible for priming. Accessible chromatin would also suggest a faster response, as the chromatin of primed cells should be in a permissive state. One theory I did not have time to test was that the p38-induced removal of a histone from the cryptic NFκB binding site in the IL6 promoter would not be necessary in primed cells, leading to faster initiation of response. I considered models of a prolonged transcriptional response (based on my results) and a ‘widened transcriptional window’ (allowing for data suggesting chromatin was readily accessible).

In a time course of primed or un-primed cells, the difference in IL-6 secretion widened in a time-dependent fashion. The difference at 2h (before the divergence in NFκB activity) was non-significant. This suggests that if chromatin was indeed more readily accessible upon re exposure, it did not necessarily mean a quicker response. Another fibroblast repeat challenge experiment (after LPS or IFNγ-primed LPS response) reported differences in magnitude, but not kinetics [315]. This confirms that primed cells react with the same kinetics.

My qPCR evidence, as stated in the results section, is poor. I therefore make no claims based on them, and given time would develop trustworthy RNA data. The IL-6 primary and mature transcript data point towards a correlation with NFκB activity (a high, transient peak in the first response and a more stable memory response), whilst IL-8 primary and mature transcripts lacked a consistent pattern. I cannot be sure that this experiment worked technically, as I do not have ELISA data showing an augmented secretion of IL-6 in the second challenge.

Publications extolling transcriptional control have shown fibroblast IL-6 and IL-8 mRNA to be altered in the memory response [264, 315, 462], but few publications have used the same methodology. This means determining the molecular basis underpinning my augmentation of IL-6 but not IL-8 protein is difficult. One publication showed an IL-8-specific reduction of transcription and protein abundance in primed cells [462], confirming my gene-specificity data, as do other studies in fibroblasts, macrophages and ECs [300, 303, 314]. Whatever the mechanism underpinning priming is, it is fine-tuned to only affect certain genes.

4.3.5 Conclusions

In this chapter, I sought to elucidate a mechanism for the augmented protein response in fibroblasts repeatedly challenged with TNFα. I examined sensitivity to lower doses and other stimuli, signalling pathways and TF contributions. I also made preliminary investigations into altered kinetics.

There are many questions still to be answered. One of these is the fine-tuning of priming to only certain genes, which could be at a number of levels. Interlinking pathways like MyD88 and TRIF-dependent mechanisms are more interactive than first thought [303], and an array of TFs downstream of the MAPKs and JAK/STAT

pathways interact with NFκB to modulate its effects. Chromatin access can be permissive on a gene-by gene basis, and transcript stabilisers and destabilisers have differing affinities to different gene transcripts [430, 444].

Chromatin access and epigenetic markers have not been assessed, and have only been assessed in one fibroblast-memory publication [324]. This publication did not compare primed and un primed inflammatory genes, and only used RA FLS. This ignores the altered chromatin access and epigenetics in RA [258, 404, 409-413], therefore may be confounded by its lack of comparison to healthy equivalents.

Whilst I did not include my data regarding NFκBIA (the IκBα gene) expression due to inconsistency, it is worth noting its role here. IκBα is more stable in endotoxin tolerant cells, and replenishes its cytoplasmic pool faster in tolerant cells [453, 461]. Given that IκBα holds NFκB inactive in the cytosol, and nascent IκBα shuttles NFκB out of the nucleus, it would be interesting to test the hypotheses that in the augmented memory response there is less IκBα at later time points, and that this is due to decreased transcription of NFκBIA.

The conclusion of my mechanistic examinations is that NFκB activity is not increased in magnitude, but prolonged, and the extra time when NFκB is present in the nucleus results in the augmented IL-6 response seen in the second stimulation. This is simply illustrated in figure 4.13.

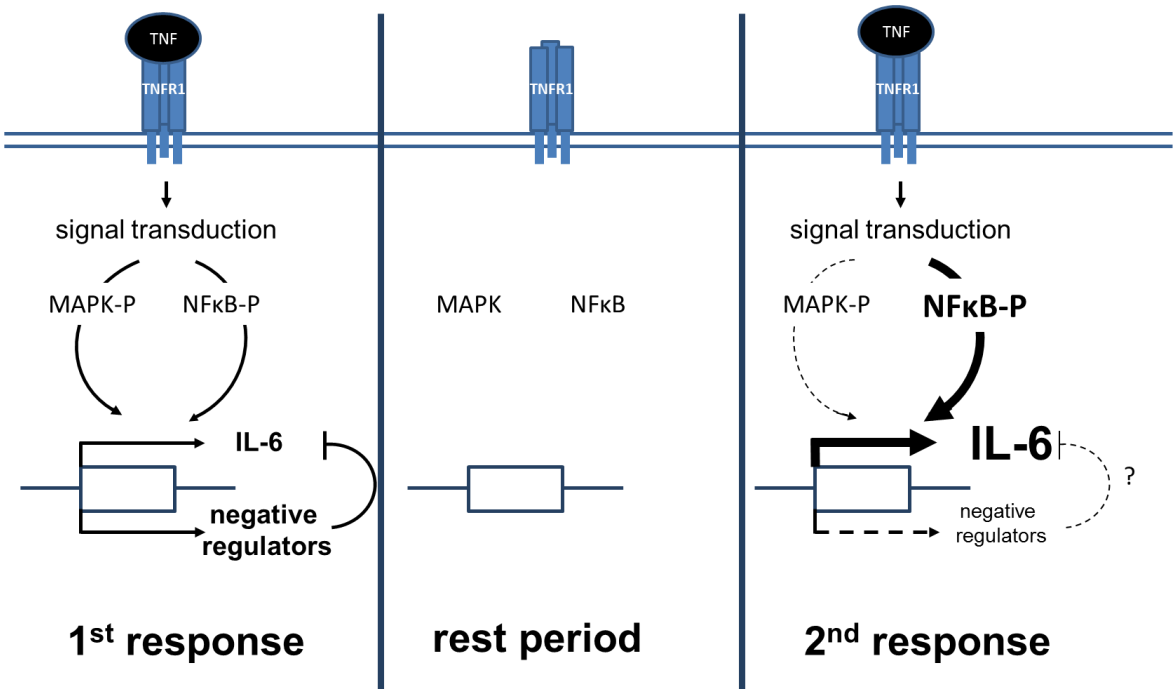

Figure 4.14: Schematic of the mechanisms underpinning fibroblast memory. Cartoon illustration of the signalling pathways downstream of the TNF receptor as evidenced by my research in this chapter. On the left is the response to an initial TNFα challenge. In the middle is the signalling occurring during the rest period, whilst on the right is the prolonged NFκB response (signified by thicker arrows and bold print) leading to an augmented IL-6 response. The negative regulators aspect is based on the balance inferred by an augmented proinflammatory response, rather than data presented in this chapter.

5 The site- and disease- specificity of fibroblast memory

5.1 Introduction

5.1.1 Site-specificity of chronic inflammatory disease

Chronic inflammatory disease may occur due to improper resolution of an inflammatory episode. Many mediators of the inflammatory response are systemic. Blood concentration of C reactive protein is regularly measured as a marker of inflammation, as it correlates well with the severity of several diseases. Circulating leukocytes are vital to pathological inflammation. Cells and their secreted products can all be considered systemically available contributors to inflammation.

That being said, the majority of chronic inflammatory diseases are tissue-specific. Whilst co-morbidities are common, each disease 'prefers' a particular tissue. Exceptions exist, as conditions like Behcet's syndrome, familial Mediterranean fever and even to an extent systemic lupus erythromatous cause systemic or multi-tissue inflammatory disorders. Excluding exceptions however, the majority of chronic inflammatory diseases have a target organ, suggesting tropism allows systemically available circulating mediators to only affect certain tissues. Below are some examples diseases, with particular focus on their leukocytic infiltrate.

5.1.1.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) has been discussed earlier, so only a brief introduction is warranted here. RA affects over 1% of the UK population [118], causing chronic inflammation and irrevocable damage in synovial joints.

Relatively few susceptibility alleles for RA are joint-specific. ACPA antibodies, for instance, target many proteins found in sites other than the joint. Retarded CTLA4

migration to the immunological synapse has been reported, but again this is not restricted to a particular tissue. Both examples implicate lymphocytes, as do animal models (CIA is based on immunity against collagen in joints). Macrophages are also implicated, with drastically increased numbers in the RA synovial lining layer [152]. Monocytes can also differentiate into osteoclasts. The RA cellular infiltrate is heterogenous, with no 'dominant' leukocyte. Neutrophils [173], T cells, B cells and monocytes [172] are all increased in RA synovial tissue or fluid. T cells appear to be resting [192, 193] but nevertheless induce FLS IL-6 and IL-8 [193]. The most prominent subsets are Th1 cells [190], with lesser fractions of Th17 cells and Treg also present [203, 207].

Muller-Ladner et al showed that RA FLS removed and passaged then transplanted into SCID mice could still degrade human cartilage in the absence of leukocytes [259], suggesting inflammatory autonomy. Far from maintaining joint lubrication and ECM homeostasis, RA FLS become proliferative, secrete a range of inflammatory and degradative mediators, and provide RANKL for osteoclast differentiation. RA is arguably the disease in which the most evidence exists for altered fibroblast behaviour (recently reviewed in [463]).

5.1.1.2 Psoriasis

Psoriasis is another common disease which affects 2-3% [464] of the world population. It is specific to the skin, with three distinct forms. Guttate and pustular [465] plaque are highly inflammatory, but acute- not chronic- diseases. Plaque plaque however, is a chronic inflammatory condition with epidermal thickening and keratinocyte hyperplasia, leading to characteristic 'scaling' of the skin (see figure 5.1) [465]. The canonical inflammatory sequence of neutrophilic infiltrate, followed by monocyte and lymphocyte influx is not followed in psoriasis, which has an initial

monocyte infiltrate [466] followed by lymphocytes [466, 467]. Neutrophils act to exacerbate inflammation, and counts correlate with lesion progression and disease severity. There is a greater neutrophil burden in active vs. inactive lesions, with an even higher burden in pustular and guttate psoriasis [465].

Psoriasis is considered an epidermal disease, and psoriasis epidermis and scales express increased concentrations of neutrophil chemo attractants [468, 469] and adhesion molecules [470], leading to neutrophilic infiltrate of the epidermis. Psoriasis lesions include large amounts of CCL5 [471] and CCL2 [472], but monocytes and macrophages are restricted to the dermis [466], suggesting a role for this compartment in the disease.

Whilst it is agreed that T cells dominate the infiltrate in psoriasis [465], which subset is most abundant is still divisive. CD8+ [473] and CD4+ [474-477] T cells have been proposed, and Th17 cells have also been linked to psoriasis, along with several other autoimmune diseases [208]. Th17 cells are known for their signature cytokine IL-17, which is found at high levels in psoriatic skin [478]. The differentiation of naïve T cells into Th17 cells can be induced by DC release of IL-23 [479], which establishes a feedback loop of IL-17 and IL-23 known to play an important role in psoriasis [480]. Inhibition of IL-23 leads to a subsequent decrease in a range of proinflammatory cytokines [481]. Th17 cells are not the only IL-17-producing cells that may play a role in psoriasis pathology, gamma delta T (γδT) cells are also major producers of the cytokine, and play a role in psoriasis [212].

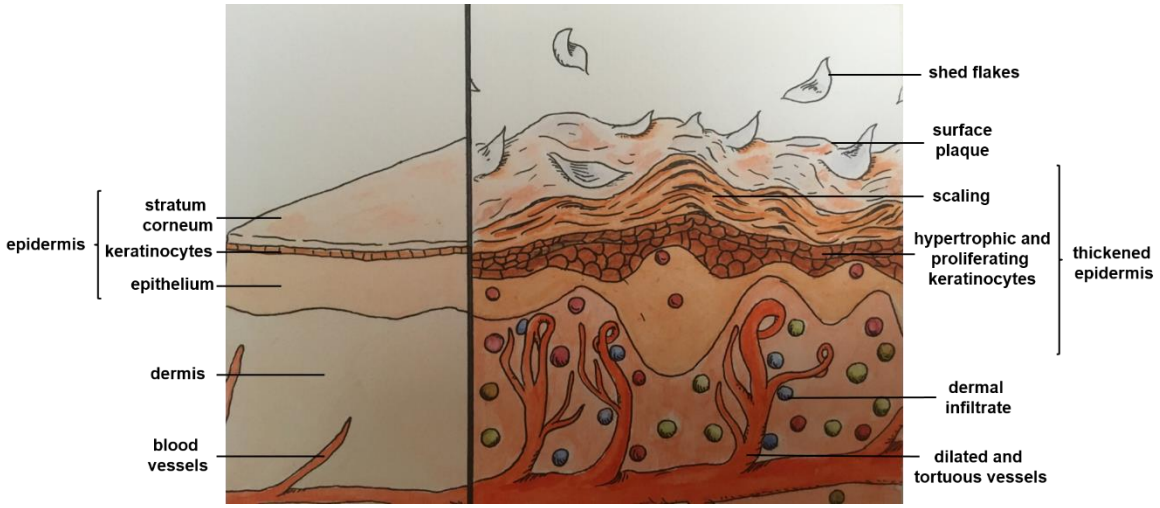

Figure 5.1: Comparison of healthy and plaque psoriasis skin. This illustration shows the major layers of the skin on the left, and the common pathological changes see in plaque psoriasis on the right. Particular attention should be paid to the increased size and number of keratinocytes. Also, to the relative abundance of leukocytes in epidermis and dermis. Leukocytes are distinguished by colour, with neutrophils (purple), mainly perivascular monocytes (blue) and predominant T cells (green).

5.1.1.3 Periodontitis

Periodontitis (PD) may be the most common chronic inflammatory disease, thought to affect around 20% of the population [482]. In contrast to RA and psoriasis, which may have a microbial trigger only at their outset, PD is not an auto-inflammatory disease. It is initiated and perpetuated by a constant misbalance of microbe-immune cell interactions. Both damage host tissue, providing a niche for pathogens and exacerbating inflammation [327].

Whilst the most obvious manifestation of PD is tooth loss, teeth are not targeted in the disease. Initial host-microbe damage to the gingiva induces a chronic state of tissue damage which deepens degraded pockets into the soft tissue, periodontal ligament and alveolar bone. This results in a loss of support for the tooth, which falls out without being damaged. This non-dental tissue loss is shown in figure 5.2.

As in the gut, a shift in predominant microbial species (largely towards gram negative anaerobes [483]) is associated with disease progression. *Porphyromonas gingivalis* is an accepted PD-associated pathogen, and is scarce in healthy but bountiful in PD mouths [484]. *P. gingivalis* uses proteases to degrade host tissue, and can resist complement, which damages tissue, providing niches for the pathogen to expand into [485]. Thus host and microbe conspire against the oral tissue.

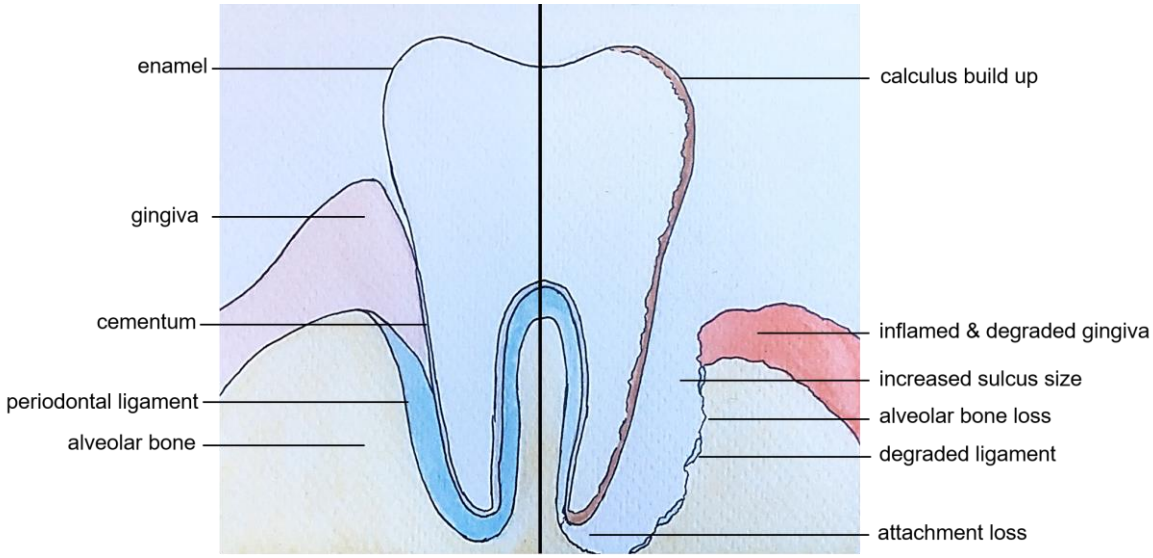

Figure 5.2: Illustration of healthy and periodontitis periodontal region. The major anatomical structures of the tooth and alveolar area are labelled on the left, in a representation of healthy tissue. On the right, the pathological features of periodontitis are shown and labelled.

Reports on the predominant leukocytes in PD vary, partly due to classification of the disease (gingivitis may or may not be early stage PD [486]), or temporality [487]. Leukocytes exist in healthy gingiva and in mild PD, suggesting an immunological presence before overt inflammation is observed [488]. Neutrophils may protect against PD, with numbers inversely correlating with disease progression and bone loss [489, 490]. Contrary to this, neutrophils exist in greater numbers in severe PD, particularly in inflammatory abscesses [491]. Neutrophil products kill oral bacteria but also degrade host tissue, thereby both helping and hindering resolution (reviewed in [492]).

Mononuclear cells are commonly described as predominant in severe PD [493], particularly B cells and plasma cells [491, 493-496]. B cells produce auto-antibodies in the lesions [497], suggesting a pathologic role, but conflicting studies found increased bone loss upon B cell donation [498], and depletion [499].

Others describe T cells as predominant [487, 500]. Autoreactive cells have been detected in PD lesions [501, 502], and Th17 cells, or their signature proteins contribute to the PD microenvironment [487, 489, 500, 503]. IL-21 is high in PD [487, 504, 505], and correlates with Th17 cell numbers . Ebersole et al's temporal study of PD showed IL-21 to rise through disease progression and then plateaus, implying maintenance of the Th17 response [487]. Addition or removal of T cells gives varied results on disease outcome and bone loss [506-509]. In fact the only subset found not to exacerbate PD is Th2 cells [507]. Differences may be due to timescale, as Ebersole et al showed different T cell repertoires in different stages of disease progression [487]. It may be possible that neutrophils and lymphocytes have positive and negative effects based on temporality, periodontal area, and microbe burden.

The best examples are Tregs, which have both positive and negative effects in PD. Ebersole *et al* found Tregs to be equally dominant with Th17s in initiation [487], and mouse studies have shown that Tregs, IL-10 and TGFβ dampening of the immune response results in persistence of pathogens in gingiva [500, 510, 511].

5.1.1.4 Chronic pulmonary disorders

There are several chronic inflammatory diseases of the airways and lung. Chronic obstructive pulmonary disease (COPD) is set to become the fourth biggest worldwide killer by 2020 [512]. The increase in female smokers [513] (smoking being the major risk factor for COPD) and increased smoking in the developing world [514] are thought to contribute to this increased burden.

Airway inflammation and obstruction characterize both COPD and asthma, although the obstruction in asthma is usually reversible, whilst only partially reversible in COPD. Although distinct diseases, asthma and COPD may be difficult to differentiate in severe cases [515] or older asthma patients, in whom airway obstruction often resembles that of COPD [516].

Structural alterations to airways occur in all chronic pulmonary diseases. Asthma involves the muscular layer, with smooth muscle cells proliferating and becoming hypertrophic, narrowing the airway [517]. COPD does little to alter this layer [518], instead causing damage to the epithelium and lamina propria, again causing airway narrowing [517]. This difference is illustrated in figure 5.3.

Airway remodelling in COPD is associated with chronic inflammation, and leads to progressive bronchitis [519], hypersecretion from mucous glands, and lesions in the epithelium [518] (which lead to a loss of barrier function). This latter may

further perturb homeostasis, as the lung is not a sterile organ, and the microbiome of COPD lungs is different to that of a healthy counterpart [520].

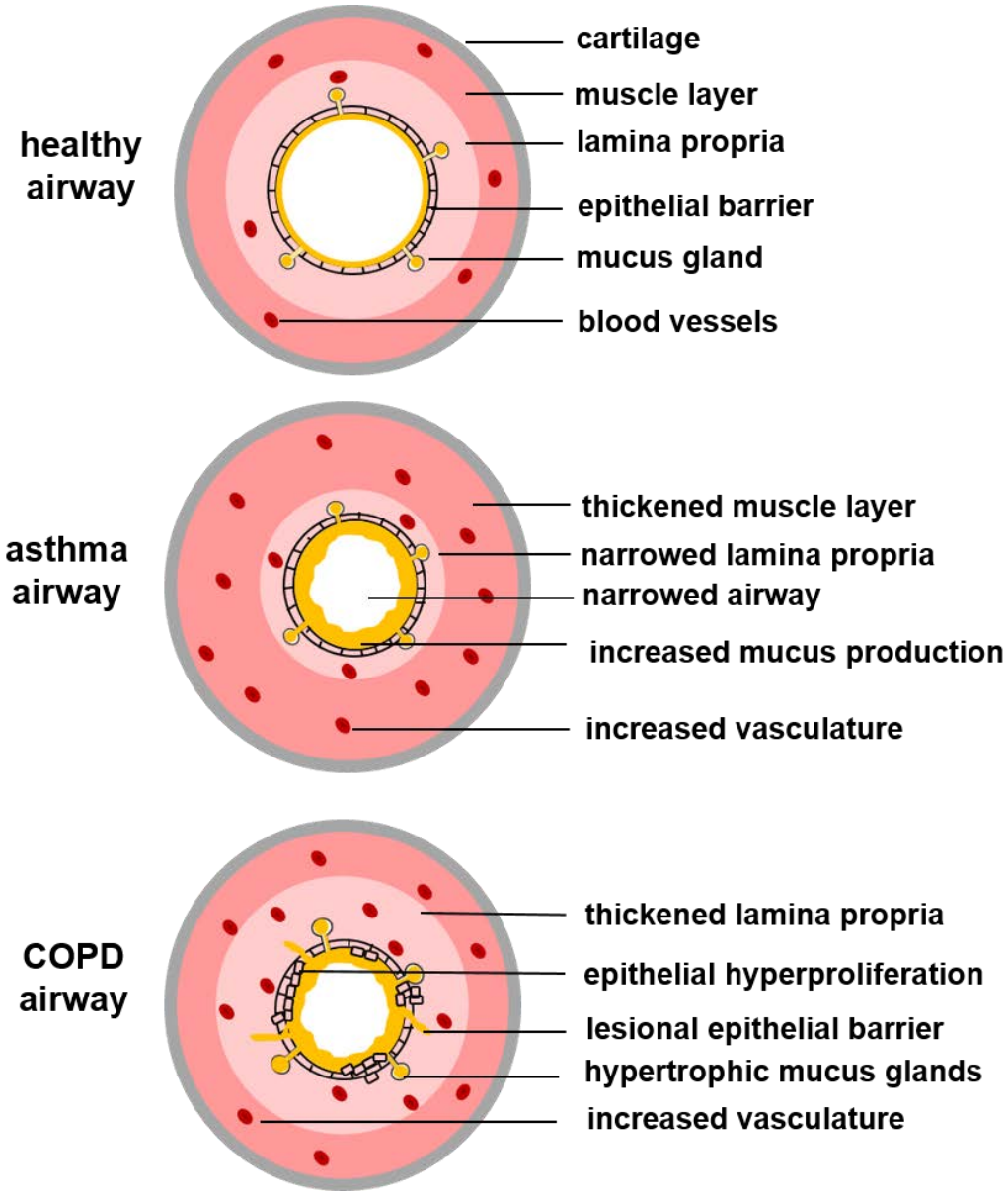

Figure 5.3: Cross-sectional illustrations of healthy, asthmatic and COPD airways. The general features of the healthy airway are shown in the top illustration. The pathological changes to airways, with particular attention to obstruction, are shown in the lower two images. These represent an asthmatic airway (middle) and a COPD airway (bottom).

The inflammatory infiltrate in chronic pulmonary diseases shows heterogeneity. It is generally accepted that asthma is driven by Th2 and eosinophils [521], however severe asthma may be propagated by neutrophils too [522]. COPD appears to be dominated by B cells, with degranulated mast cells also present, but CD4+ T cells less common [518]. Bronchitis is a symptom of COPD and is correlated with macrophage numbers (but not with neutrophils) [523]. Circulating eosinophilia has been used as a predictor of acute exacerbation of COPD [524]. Eosinophils are involved in asthma, but tissue-infiltrating and circulating eosinophils are different may be valid ways of differentiating the two diseases.

Fibroblasts play an important role in chronic pulmonary diseases, particularly their role in matrix deposition. As explained above, fibroblasts act as structural cells, and also provide the ECM proteins which act as the non-cellular scaffold upon which tissue is built. In COPD, therapeutic intervention with bronchodilators reduced the inflammatory infiltrate and allow epithelial regeneration, but did not reduce the numbers of myofibroblasts in the deep lamina propria [518], suggesting a retained inflammatory signature.

Idiopathic pulmonary fibrosis (IPF) is another progressive pulmonary disease with pathologic fibrosis resulting in loss of function and mortality. It is heterogenous and involves both slow and rapid forms, along with complicating pulmonary comorbidities [525, 526]. In both COPD and IPF the balance of ECM proteins is perturbed, whether by fibroblasts production or by targeted degradation. MMPs are heightened in COPD [527], and degrade the flexible ECM protein, elastin [527, 528]. Both COPD and IPF involve stiffening the lung ECM [528, 529], and thus altered fibroblast function [530]. Fibroblasts proliferate faster on stiffer surfaces [531], and assume a pro fibrotic phenotype. Addition of healthy fibroblasts to the

IPF lung induces their differentiation towards pro fibrotic cells [532], and similarly taking IPF fibroblasts and transferring them to a healthy lung result in their loss of this phenotype [533].

This may start with an acute inflammatory episode (like gingivitis turning into PD), as survivors of adult respiratory distress syndrome (an acute lung pathology) undergo chronic pulmonary fibrosis [534]. A feedback loop may be established, wherein the initial wound healing response of pulmonary fibroblasts induces stiffer ECM, which in turn differentiates fibroblasts towards a pro fibrotic phenotype in a wound response that leads to loss of lung function and thus a decline in health.

A host of secreted mediators are implicated in chronic pulmonary diseases, and some are reviewed in Clifford et al [535]. The asthma airways secrete a panoply of mediators, with noticeable ‘hyper secreted’ mediators including IL-8 in severe asthma [536] and bronchitis [518]. Interestingly CXCL10 is upregulated in asthma [537] but down regulated in IPF [538], acting as an example of how different chronic inflammatory diseases of the same organ may differ. As already stated, MMPs play key effector roles in COPD and IPF [527, 528].

5.1.2 Site-specificity of stromal cells in chronic inflammation

Fibroblasts, as already described, are a ubiquitous cell existing in all organs, and certain roles are shared between fibroblasts in all tissues. These include the ‘traditional’ roles, namely structural and mechanical support and ECM production. Along with the fibrotic process of wound healing, these are crucial roles of fibroblasts, and are key to the maintenance of homeostasis in resting conditions.

Fibroblasts from different tissues also have shared inflammatory roles. Fibroblasts may act with tissue-resident macrophages as immune sentinels, responding to

DAMPS and PAMPs with proinflammatory mediator release. Further, whilst by no means professional phagocytes or APCs, fibroblasts have at least a small capacity for both roles [249, 539]. During inflammation, fibroblasts modulate EC chemokine and integrin expression, and regulate extravasation [228, 253, 540]. In this way they can dictate the magnitude, 'flavour', and duration of the inflammatory influx.

In chapter one I introduced some data regarding tropism in fibroblasts. Despite shared characteristics, fibroblasts from different sites differ in many ways, from HOX gene [541] expression to stromal postcode [542]. This shows fibroblasts from different tissues have tailored functions to better suit their site. And yet in inflammatory episodes, these differences may break down. For instance, peripheral tissue fibroblasts take on many characteristics of lymphoid stroma, a trait that is aberrantly continued in chronic inflammatory disease [254, 543].

A number of chronic inflammatory diseases have been described above, all with a specific tissue as the focus of inflammation and aberration. What causes such specificity? What allows such specificity when so many contributing factors are found in the circulation? The data described above show that fibroblast behaviour differs according to site and disease state. It may be therefore, that these tissue-resident cells could provide a tissue-specific pathology that drives the inflammation and perturbation in one particular organ.

This thesis have so far relied on data from FLS and the dermal line, BJ. In this chapter, there are two main hypotheses under scrutiny: Fibroblast memory is site-specific, and memory will differ in fibroblasts from the same tissue of origin, according to that organ's status as healthy or chronically inflamed.

5.2 Results

5.2.1 Fibroblast priming exists in multiple anatomical locales

In this chapter I wanted to assess fibroblast priming in a wider setting, examining fibroblasts from other sites and those from control or chronically-inflamed tissue. The aim of this research was to determine how far reaching fibroblast priming is. To assess the question, I cultured fibroblasts from a number of anatomical locales. As with earlier experiments, the cells were stimulated twice with 10ng/ml TNFα for 24h with a 24h rest period between challenges. Control cells received medium in place of TNFα. The responses were initially assessed by IL-6 ELISA.

5.2.1.1 IL-6 protein secretion

The resting secretion of IL-6 varied between sites but given the heterogeneity between patients this did not differ significantly based on site of origin (see Fig5.4a). The mean responses to one challenge with TNFα (Fig.5.4b) varied depending on site; with the lowest mean secretion of IL-6 being dermal fibroblasts (HDF) and the highest being gingival fibroblasts (HGF) (1.27±0.36 and 6.82± 3.85ng/ml respectively). Given this range it is not surprising that Kruskal-Wallis analysis showed fibroblasts from different sites vary significantly in their IL-6 response to TNFα (** p= 0.003).

The response to second challenge showed even greater variation. HDF showed no evidence of memory, and fibroblasts of the bone marrow (BMDF) did not consistently increase IL-6 upon re challenge. HGF showed an intermediate degree of augmented second response, which did not reach statistical significance. Fibroblasts of the synovium (FLS), tonsil (HTF) and lung (HLF) however showed a much more dramatic increase in IL-6 secretion. Each showed significant

upregulation compared to first response. The Kruskal-Wallis test showed variation between second IL-6 responses to TNFα was even more significant than the difference between initial responses (p<0.0001).

Inter-patient heterogeneity was mitigated by normalising each sample's second response to its first and expressing the data as a fold change. Individual samples' fold changes are displayed in figure 5.4c to show both inter- and intra- site variation. When the fold change was assessed statistically, fibroblasts from the synovium, tonsil and lung all showed significantly augmented IL-6 responses to re challenge (3.61-fold ±0.47, 5.17-fold ±1.28, and 3.47-fold ±0.62, respectively). HGF showed a large fold increase (3.17-fold ±1.10) which failed to reach signifcance. As expected, neither HDFs nor BMDFs showed significant average fold changes at 1.32 ±0.2 and 1.76 ±0.38 fold respectively. Kruskal-Wallis examination found a significant difference in fold change between fibroblasts of different sites. Dunn's post-test comparing sites showed significant differences between skin and synovium, lung or tonsil, and multiple comparisons against the normalised first response showed significant increases in the synovium, tonsil and lung.

These data illustrate that fibroblast response to inflammatory challenge differs according to site. Whilst this is not a novel result, site-specificity becoming more pronounced upon re challenge is. Fibroblast priming was shown to be site-specific, and present in multiple sites.

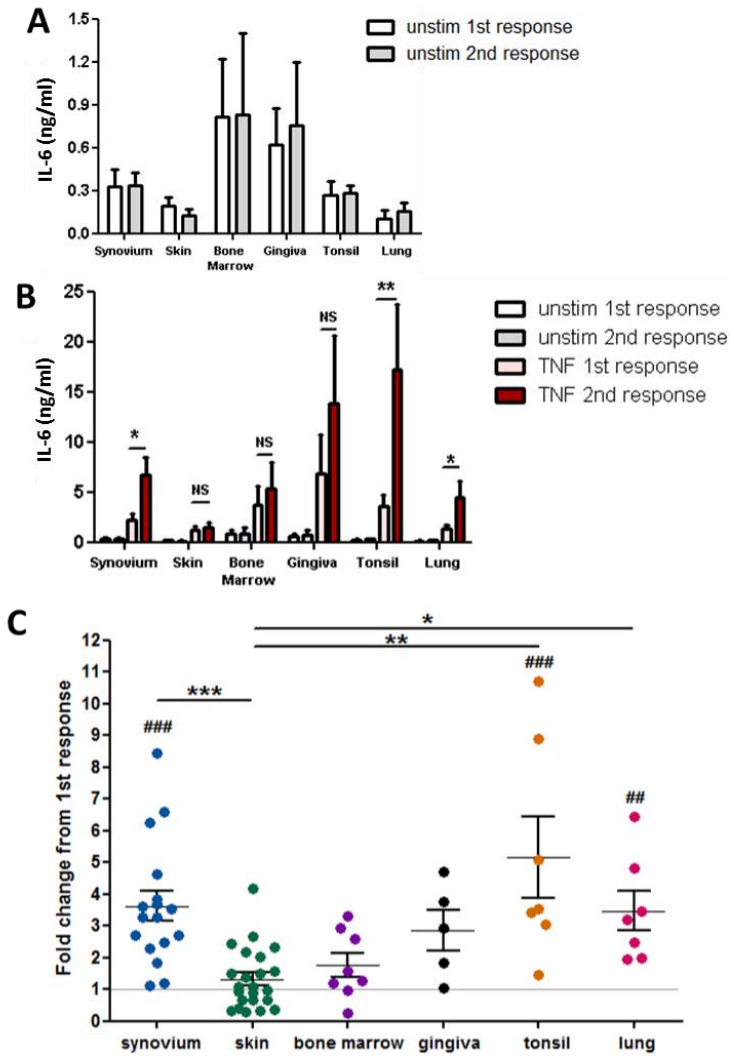

Figure 5.4: Fibroblast priming of IL-6 is site-specific. Fibroblasts were stimulated with 10ng/ml TNFα or medium for 24h, with or without prior challenge with TNFα or medium. IL-6 response to first and second challenges were assessed by ELISA. Raw IL-6 concentrations produced in first and second responses to **A** medium or **B** TNFα. *p<0.05, **p<0.01 first versus second response (Mann-Whitney U test). **C** Fold change of second relative to first response, assessed by Kruskal-Wallis with Dunn's post-test against normalised first dose (## p<0.01, ### p<0.001), or between sites (*p<0.05, **p<0.01, ***p<0.001). Individual samples with mean ±SEM for each site. Synovium n=17, skin n=23, bone marrow n=8, gingiva n=5, tonsil n=7, lung n=7.

5.2.1.2 Other secreted proteins

I also assessed neutrophil and monocular cell chemo attractants (IL-8 and CCL2), to examine whether gene-specificity seen in FLS (see fig 3.9) were upregulated by fibroblasts of other tissues.

Comparison of fibroblasts of the joint, the skin, the lung and the tonsil revealed site-specific upregulation of IL-8 and CCL2. IL-8 was induced to robust levels in fibroblast of all sites by TNFα challenge. The re-challenge separated skin and synovium from tonsil and lung, with the former pair showing no real evidence for an altered response (FLS 0.77 ±0.2-fold, HDF 1.21 ±0.4-fold). HLF and tonsil HTF showed increased secretion of IL-8 which did not reach significance. When fold changes were assessed by multiple comparisons, a significant difference was revealed between sites, and the memory response of HTFs was significantly increased from first response.

Synovial and dermal fibroblasts secreted similar amounts of CCL2 in response to TNF, whereas lung fibroblasts secreted less and tonsil fibroblasts more. The CCL2 response upon re challenge was noticeably increased in both HTF and HLF (2.58 ±0.4-fold and 2.96 ±0.4-fold respectively). Multiple comparisons again showed significant differences between sites and showed the memory response of HLF was significantly increased compared to the normalised first response.

These data (presented below in figure 5.5) showed that not only is innate memory in fibroblasts dependant on site of origin, it is also tailored to each site, with fibroblasts of different tissues upregulating different pro inflammatory mediators.

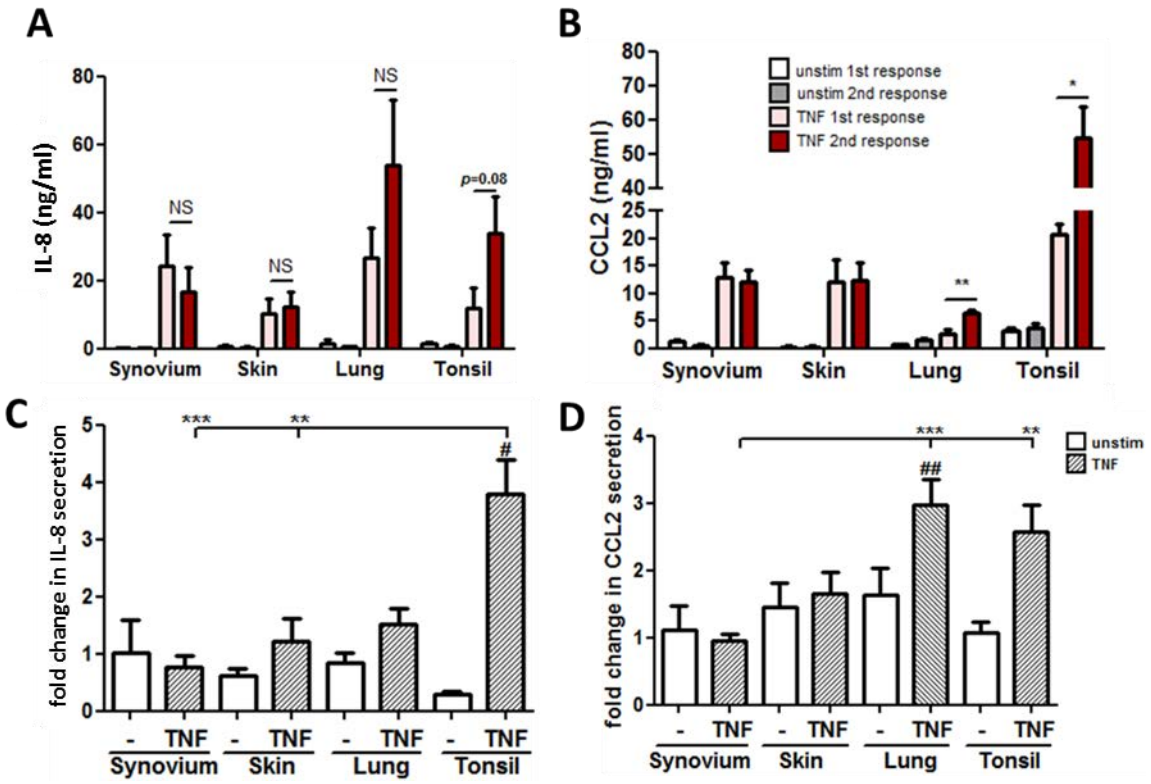

Figure 5.5: Proteins augmented in the second response vary dependent on site. Fibroblasts were stimulated twice for 24h with 10ng/ml TNFα, with 24h rest without stimulus between the two challenges. Conditioned medium from the two challenges were assessed for secreted proteins by ELISA. Top graphs: raw concentration of **A** IL-8 and **B** CCL2 secretion. First and second response to TNFα compared by Mann-Whitney *U* test, * $p<0.05$. ** $p<0.01$. Bottom graphs: fold change of second response against normalised first response (first response= 1) assessed by Kruskal-Wallis test with Dunn's post-test. Significant differences were assessed between memory responses and normalised first responses (# $p<0.05$, ## $p<0.01$) or between sites (** $p< 0.01$, *** $p< 0.001$). Synovium n=15, skin n=13, lung n=9, tonsil n=8.

5.2.2 Comparison of fibroblasts from control and chronically-inflamed tissue

After displaying site-specific variation in the memory response, I wanted to assess the effect of chronic inflammatory disease on fibroblast memory. Whilst not practicable in all sites, the synovium, skin and gingiva could be divided into non-chronically inflamed controls and samples from patients suffering RA, psoriasis, and PD respectively.

18 control and 20 disease samples were plotted based on disease state rather than site of origin (Figure 5.6a). The mean fold changes were control 2.1 ±0.46 and disease 2.55 ±0.29. This difference between the two groups approached, but did not exceed the level of significance (p=0.06, Mann-Whitney U test). When FLS were excluded from the analysis (figure 5.6b), the two groups had statistically different re challenge responses (control 0.74 ±0.11-fold, and disease 2.25 ±0.32-fold, p=0.0004, ***, Mann-Whitney U test). This suggested that the IL-6 priming of certain sites (the skin and gingiva) could be separated based on disease state, whilst others (the synovium) could not.

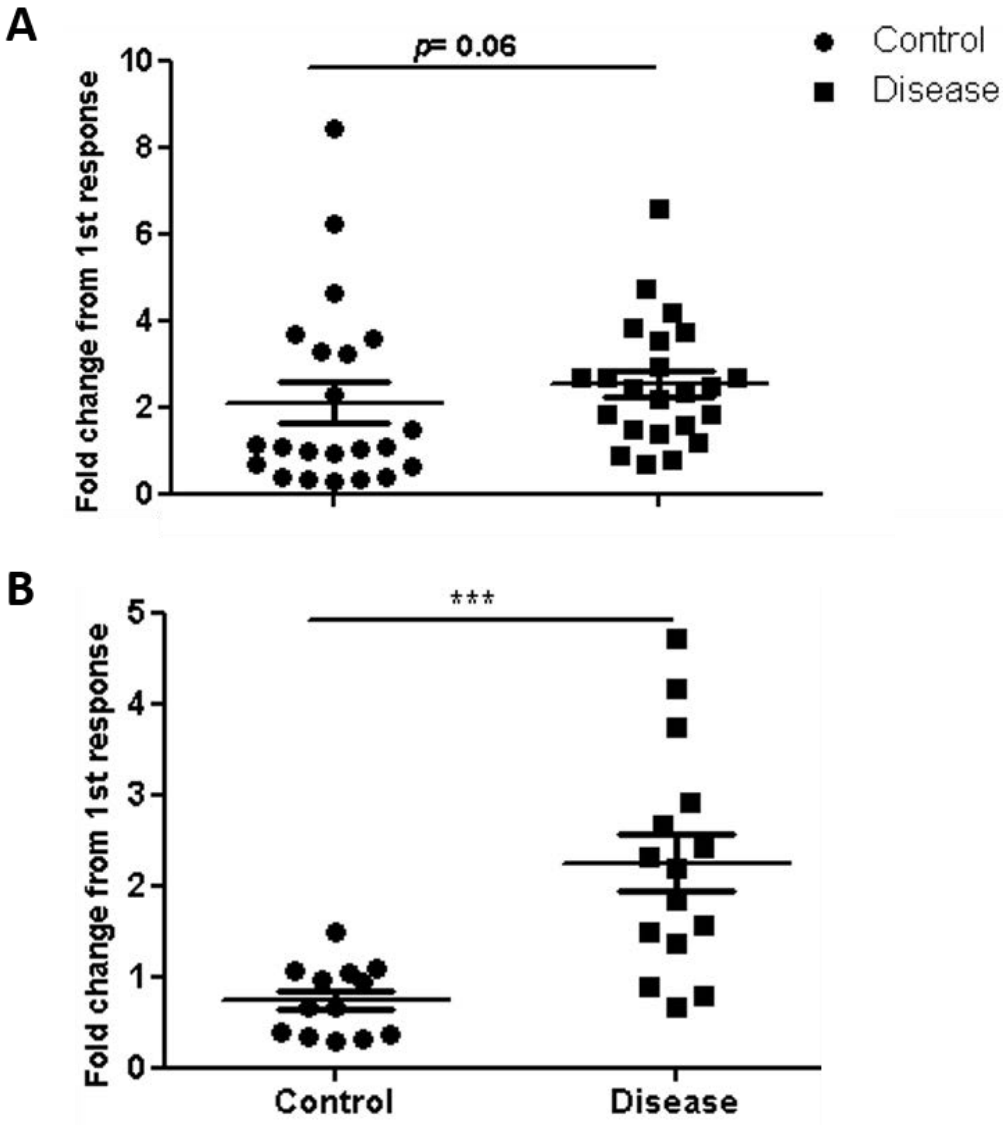

Figure 5.6: IL-6 priming cannot separate based on disease state unless FLS are removed from the analysis. Fibroblasts from the synovium, skin and gingiva were stimulated twice with 10ng/ml TNFα for 24h with a 24h rest between, and the fold change in IL-6 secretion of second compared to first response was calculated. **A** Samples from synovium, skin and gingiva were separated based on disease state and irrespective of site. Control n=22, disease n=23. **B** Fibroblasts from skin and gingiva were grouped according to disease state and irrespective of site. Healthy n=13, disease n=15. *** p<0.001, Mann-Whitney U test.

Given this finding, I then compared control and chronically inflamed samples by site. As shown in figure 5.7, both control and RA FLS mounted a significantly augmented IL-6 response to second challenge (4.06 ±0.72 and 3.11 ±0.58 fold), but there was no significant difference based on disease state. In contrast, healthy HDF failed to augment IL-6 in the second challenge (0.72 ±0.11 fold), whilst psoriasis HDF showed a significant increase (1.98 ±0.29 fold) and a significant difference from control HDF (p=0.001, *** Mann-Whitney U test).

The difference between control and PD gingival responses was striking. The control sample had a fold change of 1.04-fold, and this was validated in several repetitions (data not shown). The four PD samples exhibited an IL-6 fold change of 3.31 ±0.61-fold increase. This difference could not be tested statistically due to the difficulty of obtaining control gingival samples.

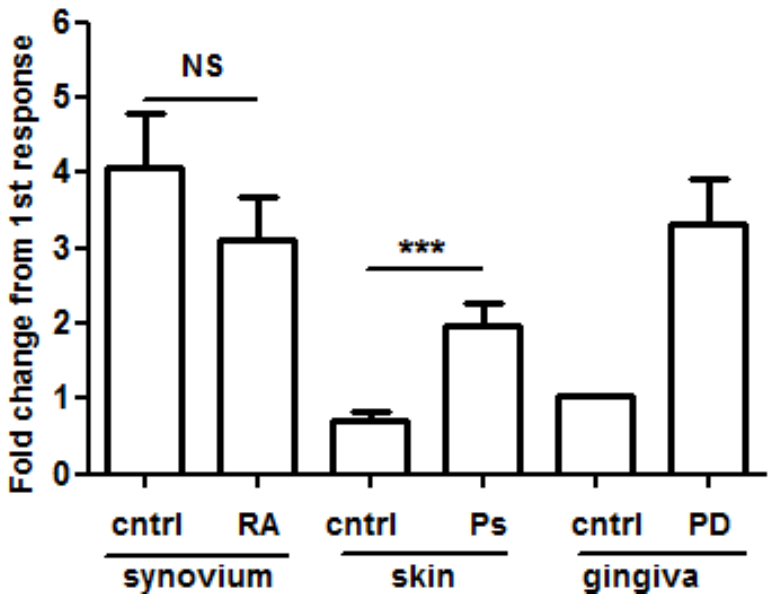

Figure 5.7: Disease state separates IL-6 priming of skin but not synovial fibroblasts. Fibroblasts from control or chronically inflamed synovium, skin, and gingiva were stimulated twice with 10ng/ml TNFα for 24h, with a 24h rest between challenges. Conditioned medium was removed after each 24h period. Response to first and second challenge was assessed by IL-6 ELISA, and the second response was expressed as a fold change from the first response. Fold change of memory response was compared between control and disease fibroblasts in each site (Mann-Whitney *U* test, ***$p<0.001$). Synovial control n=9 disease n=8, skin control n=12 disease n=11, gingiva control n=1 disease n=4.

In order to further compare the inflammatory secretions of healthy and disease fibroblasts, I conducted a custom Luminex on the first and second response of control and disease FLS and HDF. Figure 5.8 displays the HDF data.

CCL21 secretion was undetectable in many samples, and so not presented. GM-CSF secretion was low, but showed a 2.5-fold increase in the second response of psoriasis HDFs, whilst the control HDF only increased it by 40%. CXCL10 expression was low (potentially make the data untrustworthy), but psoriasis HDFs consistently expressed higher concentrations than the control cells. The average fold change for controls was 2.4-fold, whilst psoriasis HDFs increased CXCL10 secretion 4.9-fold in the primed response. Despite these robust increases, and perhaps due to the low number of replicates and low concentrations, neither control nor psoriasis HDFs were found to significantly upregulate CXCL10.

CCL2 was expressed at high concentrations by both control and psoriasis HDFS in the first response. Interestingly this was subsequently lowered by control and raised by psoriasis HDFs in the primed response. The fold change of psoriasis HDF CCL2 secretion (2.14± 0.34) was significant, but only brought the raw CCL2 concentrations up to comparable levels with healthy secretion. This may be due to one outlier in the control samples; its exclusion would have resulted in the psoriasis HDFs secreting a higher average concentration of CCL2 than the controls.

Secretion of CCL5 was not altered by priming control fibroblasts (1.41± 0.54 fold increase) but was significantly augmented by psoriasis HDFs (2.74± 0.47 fold increase). The absolute concentrations were also much higher in psoriasis HDF responses to TNFα, and the primed CCL5 response of psoriasis HDFs was

significantly higher than that of the control primed response when raw concentrations were compared (0.16± 0.02ng/ml and 0.97± 0.39ng/ml, p= 0.04, Mann-Whitney U test).

CXCL12, a T cell chemoattractant and retention factor, was highly expressed by unstimulated fibroblasts and did not appear to be altered by TNFα, with or without priming. The psoriasis HDF did significantly augment CXCL12 (2.74± 0.47, p=0.04, Wilcoxon matched pair signs rank test). This should be treated with suspicion, given the apparent similarity between CXCL12 secretions under all conditions.

None of the fold changes in secretion differed significantly between groups, although this may be a power problem rather than a lack of biological difference.

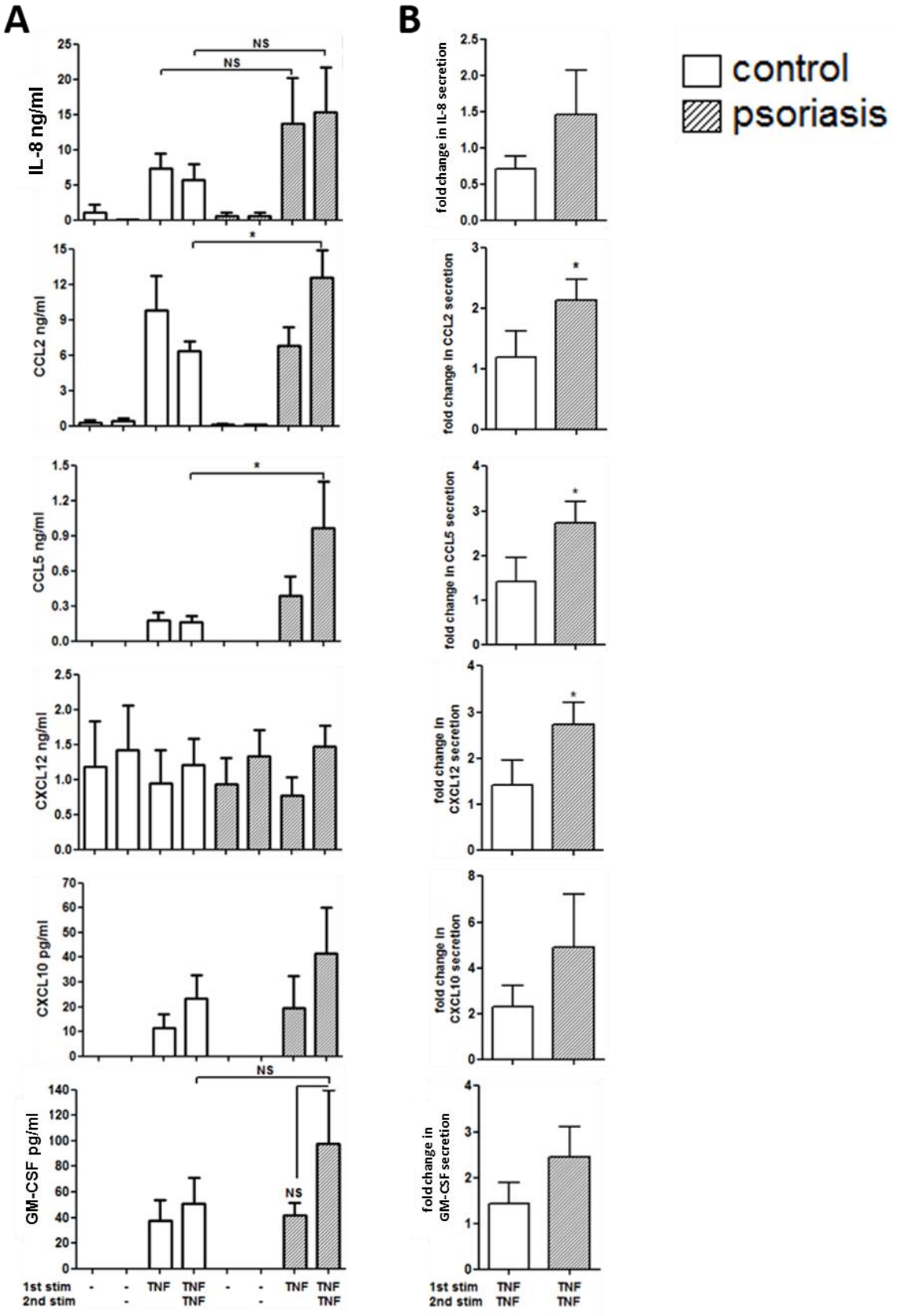

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Figure 5.8: A range of mediators are augmented in the memory response of psoriatic, but not healthy, skin fibroblasts. Conditioned medium from the first and second response to medium (control) or 10ng/ml TNFα was assessed by multiplex analysis for inflammatory mediators. **A** Raw concentrations secreted in response to medium or TNFα, by control (open bars) or Psoriasis (cross hatched bars) HDFs. Difference between first and second response to TNFα is assessed by Mann-Whitney U test, * $p<0.05$. **B** Healthy (open bars) and Psoriasis (cross hatched bars) memory response represented as fold changes from the initial response. Fold change of second response compared to first response to TNFα is assessed by Wilcoxon matched pairs signed rank test, * $p<0.05$. Mean ±SEM, control n=7, Psoriasis n=8.

The same Luminex panel was conducted using control and RA FLS-conditioned medium. Unlike the HDF, CCL21 secretion by FLS was present even in unstimulated samples. The secreted levels secreted were similar between control and RA FLS, and both showed significant increases upon re challenge when raw concentrations were assessed. The fold change increase of control FLS was just below significance (p=.006), whilst the RA FLS did reach significance (p=0.008).

The IL-8 data presented here includes the control FLS data shown in chapter 3, but here there is a comparison of control and RA FLS. FLS did not significantly alter their IL-8 response upon re challenge. The fold change of RA FLS was higher than that of control FLS (1.46 ±0.29-fold and 1.09 ±0.16 respectively) but responses did not reach significance over initial responses, nor between control and disease groups.

CCL5 was secreted at comparable levels by control and RA FLS, and both showed significant increases in secretion upon re challenge (both in raw concentrations and fold change). The difference between disease states was not significant in initial or memory responses, nor in fold change.

This Luminex confirmed my earlier ELISA showing CXCL10 was significantly augmented (see control FLS data in figure 3.9). The RA FLS produced higher concentrations of CXCL10 in response to both first and second TNFα challenge, but this did not significantly differ from control samples in either case. Due to inter patient heterogeneity, increase in CXCL10 secretion was not significant for either group. However, the RA FLS fold change showed a significant increase (4.12± 0.64-fold, p= 0.016). The lack of significance in control FLS apparently comes from the high variation in fold change, as the mean increase was 8.78 ±2.57-fold).

CXCL12 induction was limited, with the stimulated levels not greatly increased above basal levels. Nevertheless, both groups responded to TNFα by upregulating CXCL12. This was significantly increased in the response to second challenge by FLS of both groups. The fold change of control FLS was significant (p=0.03). Whilst the RA FLS failed to reach significance, the *p* value was 0.055.

GM-CSF levels were low and similar in both groups, with a slightly lower initial response and slightly higher secondary response by the control group. This led to a significant increase by the control but not the RA group. Despite this, both groups showed significant fold change increases in GM-CSF.

Figures 3.9 and 5.5 showed that FLS do not augment CCL2. In this Luminex however, the robust initial CCL2 response was noticeably increased upon re challenge. This was significant for control but not RA FLS, with the former starting with a lower initial secretion (7.46 ±1.12ng/ml compared to 12.59 ±3.92ng/ml), and a roughly equivalent second response by both. Both groups showed significant fold changes in CCL2. Naturally this disparity between Luminex and ELISA is concerning, and will be discussed later in this chapter. Therefore, no conclusions are being made about CCL2 response.

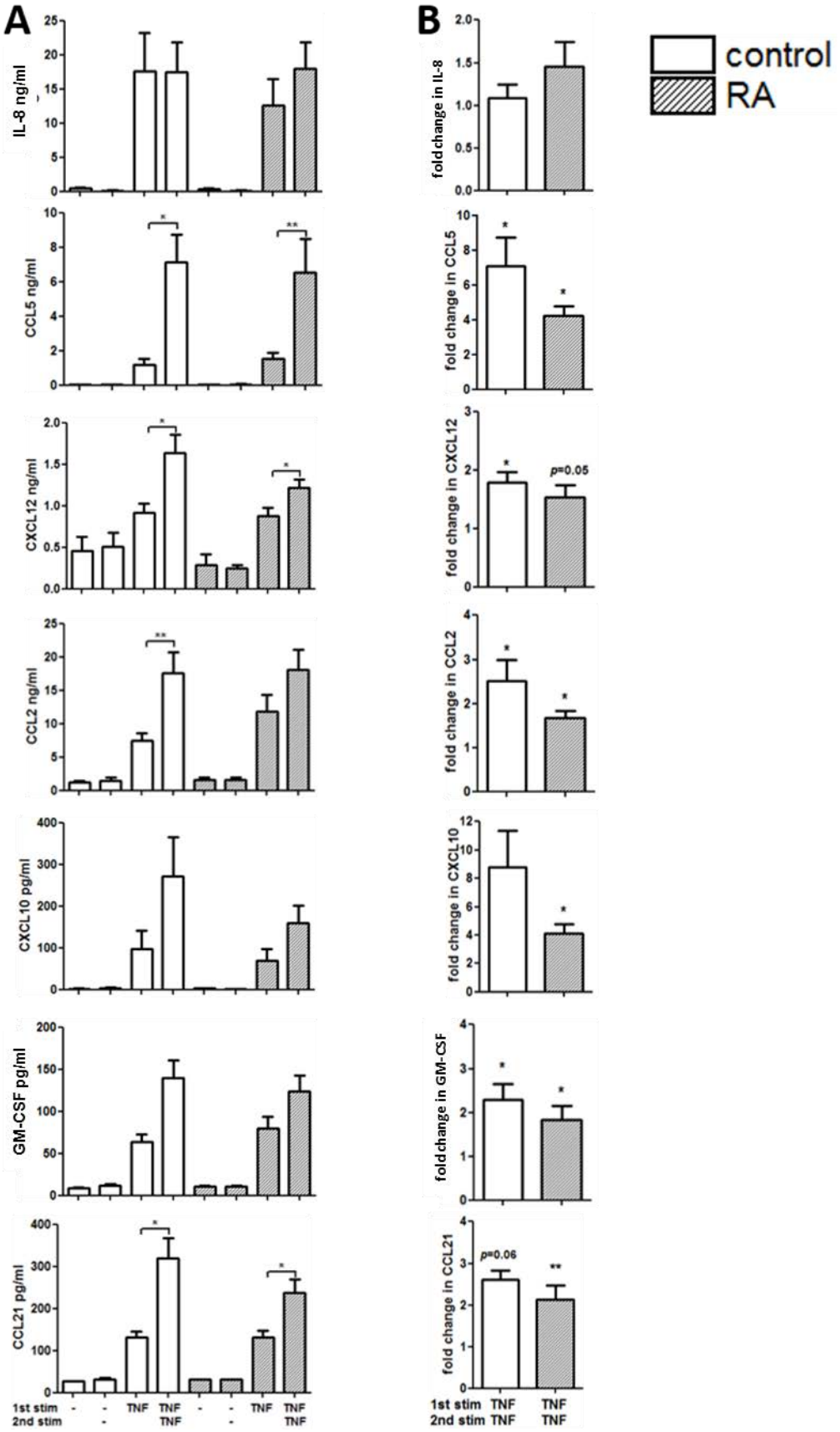

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Figure 5.9: The gene-specific augmented memory response is very similar between control and RA FLS. Conditioned medium from control (open bars) and RA (cross hatched bars) FLS were analysed by multiplex. **A** Raw concentrations of mediators secreted in response to medium or 10ng/ml TNFα. * $p<0.05$, ** $p<0.01$, Mann-Whitney U test. **B** Memory response represented as a fold change from initial response to TNFα. * $p<0.05$, ** $p<0.01$, Wilcoxon matched pairs signed rank test. Control n=6, RA n=8.

As the results above display no difference in concentration of secreted mediators between disease states, I considered the biology of the healthy synovium and RA pannus. The pannus includes more fibroblasts than the healthy synovium, so it is possible that non-significant differences are due to using the same number of cells for both control and RA samples *in vitro*.

To this end I conducted a simple proliferation test to compare the growth rate of FLS. As shown in figure 5.10, RA FLS proliferated significantly faster than control FLS. This rate of proliferation did not, however, differ across the space of one week, suggesting that during my experiments the control and RA FLS were of approximately equal number. This illustrates the artificial nature of using the same number of cells *in vitro* when studying control and RA FLS but rules out any confounding feature of different cell densities. It therefore allows greater belief in the fact that FLS from control and RA joints do indeed share an inherent, gene-specific innate memory.

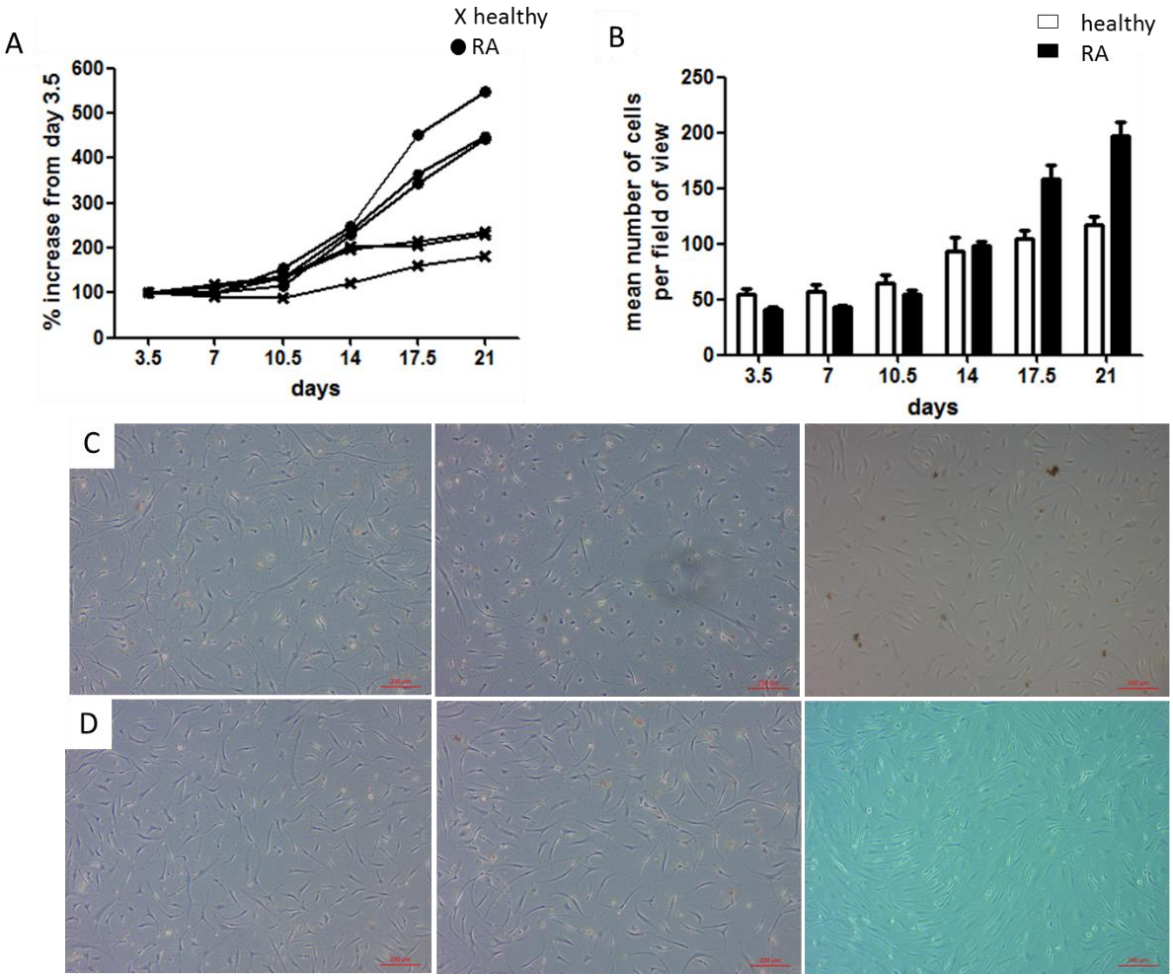


Figure 5.10: RA FLS proliferate faster than control FLS. FLS from healthy or RA individuals were seeded at $2x10^5$ per T75 flask. Crosses were marked at regular intervals on the flask, and used as reference points to return to the same area every 3.5 days. Each area (four per flask) was photographed and cells in the field of view counted, before the numbers were averaged as mean number per flask per day. **A** cell numbers represented as percentage increase from the first count at day 3.5. **B** mean number of cells for healthy or disease lines per day. **C** healthy cells at day 21. **D** RA cells at day 21. Plotted values are mean ± SEM error bars. n=3 healthy and n=3 RA.

In chapter 4 I showed evidence for prolonged NFκB activity being a contributing mechanism in fibroblast priming. The experiment in figure 4.10 was conducted on FLS, which mount an augmented second response irrespective of disease state. A test of this mechanism's role in priming was to examine cells that do, and do not show the augmented second response. I repeated the experiment in control and psoriasis HDF, to assess their subcellular localization of NFκB. Given the lack of innate memory in healthy cells I hypothesized that the second challenge would not induce the prolonged NFkB localization seen in figures 4.9 and 10, whilst, psoriasis HDF would match the FLS with prolonged nuclear localization.

As shown in figure 5.11 the healthy and psoriasis HDFs were similar in their first response. Both exhibited minimal nuclear localization at rest, followed by clearly increased nuclear NFkB levels. This decreased throughout the time course irrespective of disease state and in agreement with both BJ and FLS. The initial response was therefore similar between control and psoriasis HDFs.

The nuclear localization of cells rested for 24h was noticeably higher in the control than psoriasis HDF example, suggesting continued signalling. The response to second challenge of control HDFs once again increased the intensity of the nuclear staining, but not strongly, and this once again decreases through the time course. Psoriasis HDF, on the other hand, maintained their nuclear localization throughout the time course. The intensity of total NFkB p65 staining in control HDFs appears higher than that of psoriasis HDFs, but the latter have a much clearer nuclear localization, implying a prolonged localization, in comparison to a lack of clear nuclear localization in healthy HDFs. Whilst only qualitative, this concurs with the difference between healthy and psoriasis HDF cytokine and chemokine secretion.

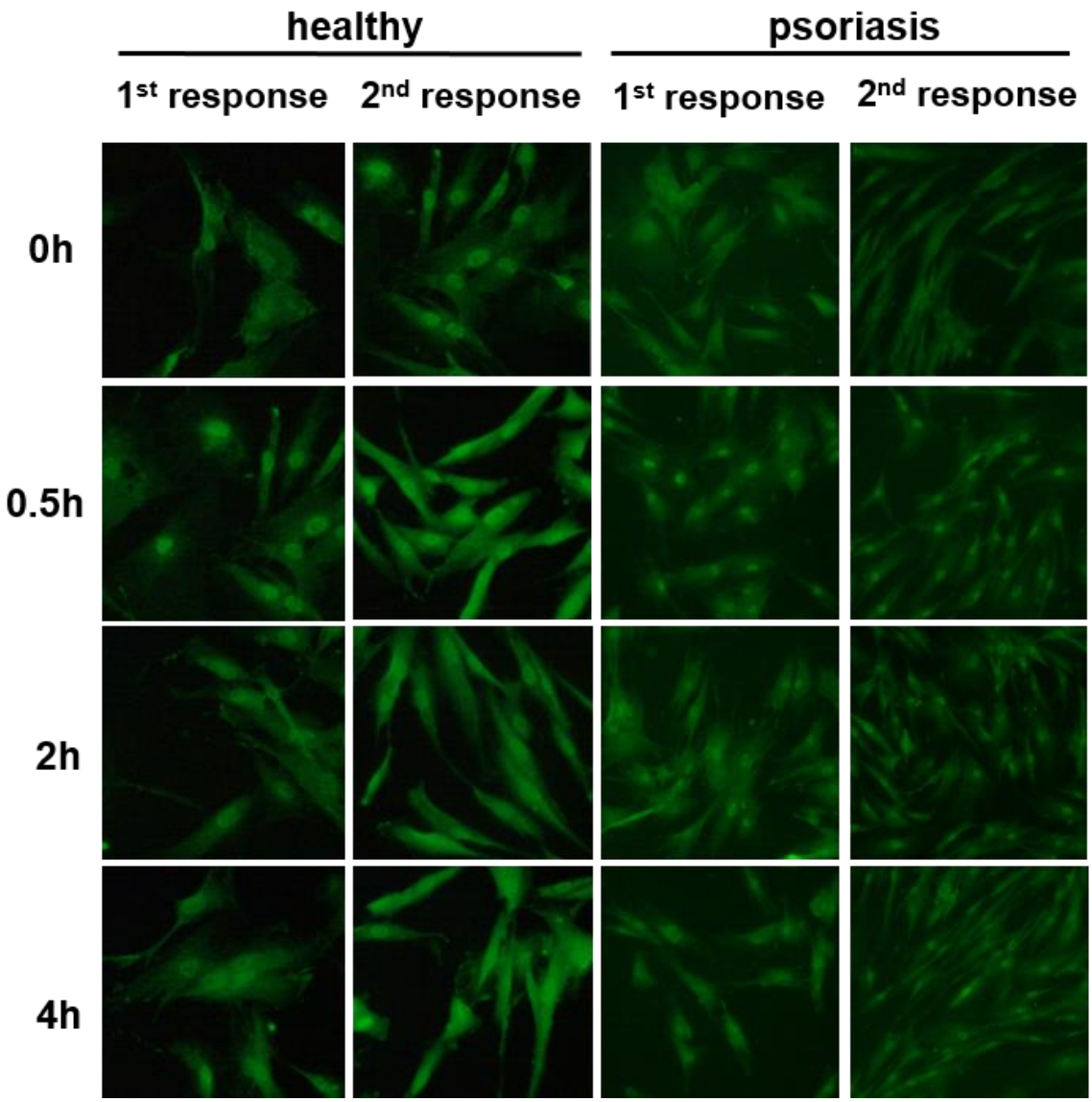


Figure 5.11: NFκB Nuclear localization is prolonged in the memory response of Psoriasis but not healthy HDF. Dermal fibroblasts were stimulated with 10ng/ml TNFα for the time points indicated, or primed and rested, then stimulated for the time points indicated. Cells were then fixed, permeabilized and exposed to anti-NFκB p65 antibody. The fluorescence microscopy was conducted at x40 magnification. Representative of n=3 healthy and n=4 psoriasis dermal fibroblast samples.

5.2.3 The consequences of fibroblast priming in different sites

The above data show that fibroblast innate memory varies between sites, both in terms of the magnitude of memory response, and in terms of which genes are upregulated. It also suggests that fibroblast innate memory may vary according to disease state. Analysis of individual mediators doesn't account for the myriad redundancies and antagonisms seen between said mediators. Neutrophil chemo attraction, for example, is often attributed to IL-8, but IL-17, CXCL1 (Groα), CXCL2 (Groβ) and leukotriene B4 (LTB4) all have neutrophil attractive properties. It is therefore dangerous to assume (in this example) that innate memory does not increase chemo attraction of neutrophils based on data on IL-8 secretion.

In the following section, I attempted to assess the consequences of fibroblast memory from injecting conditioned medium into mice, or by culturing leukocytes with conditioned medium.

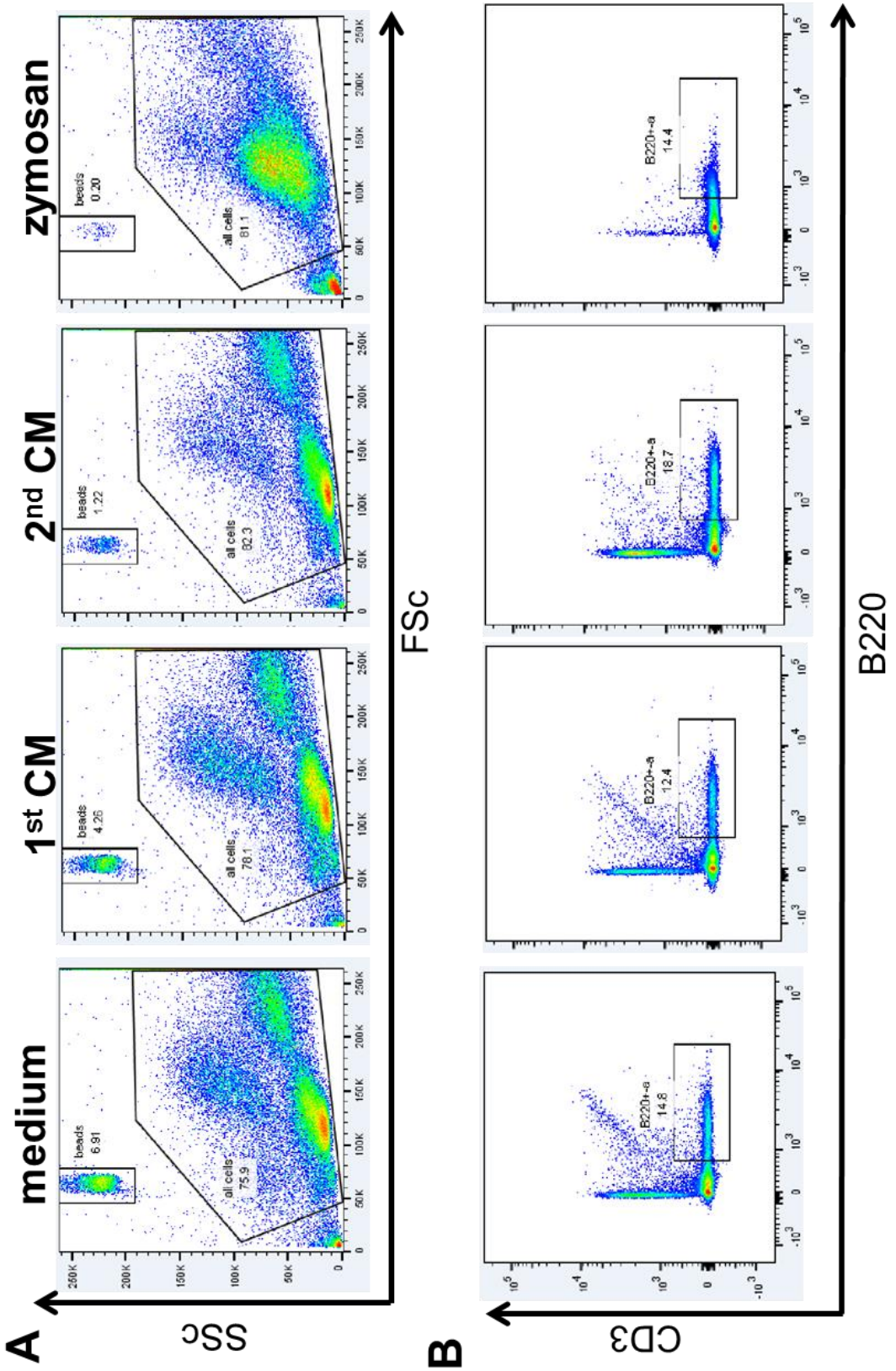
5.2.3.1 *Effect of first and primed response* in vivo

I wanted to test the effects of the fibroblast memory response *in vivo*, but using human conditioned medium (CM) ran the risk of allotype reactions and whilst many human mediators function in mouse systems I could not guarantee all could. With these considerations, I chose to use CM from murine FLS which, as shown in chapter 3, mounted an augmented second response similar to that of human FLS. The CM from murine FLS first and second response to TNFα was injected into the peritoneum of wild type C57/Bl6 mice for six or 48h, before mice were sacrificed and their peritoneal infiltrate was lavaged for analysis.

The six-hour incubation is shown in figure 5.12. As shown in section A the zymosan control induced a huge infiltrate compared to CM, which failed to induce

more infiltration than plain mouse medium. This suggested that CM did not induce cellular infiltrate, which seemed unlikely. Analysis beyond total number showed the zymosan-induced infiltrate had a greater proportion of viable infiltrating cells (91.4%) whilst the control medium, first CM and second CM had similar levels of viable cells (78.9 ±2.35%, 80.0 ±1.59%, and 82.6 ±3.31% respectively).

Examination of the constituents showed a large percentage of the infiltrate was neutrophils and macrophages following zymosan. The control medium, first and second CM induced similar percentages of each leukocyte subset.



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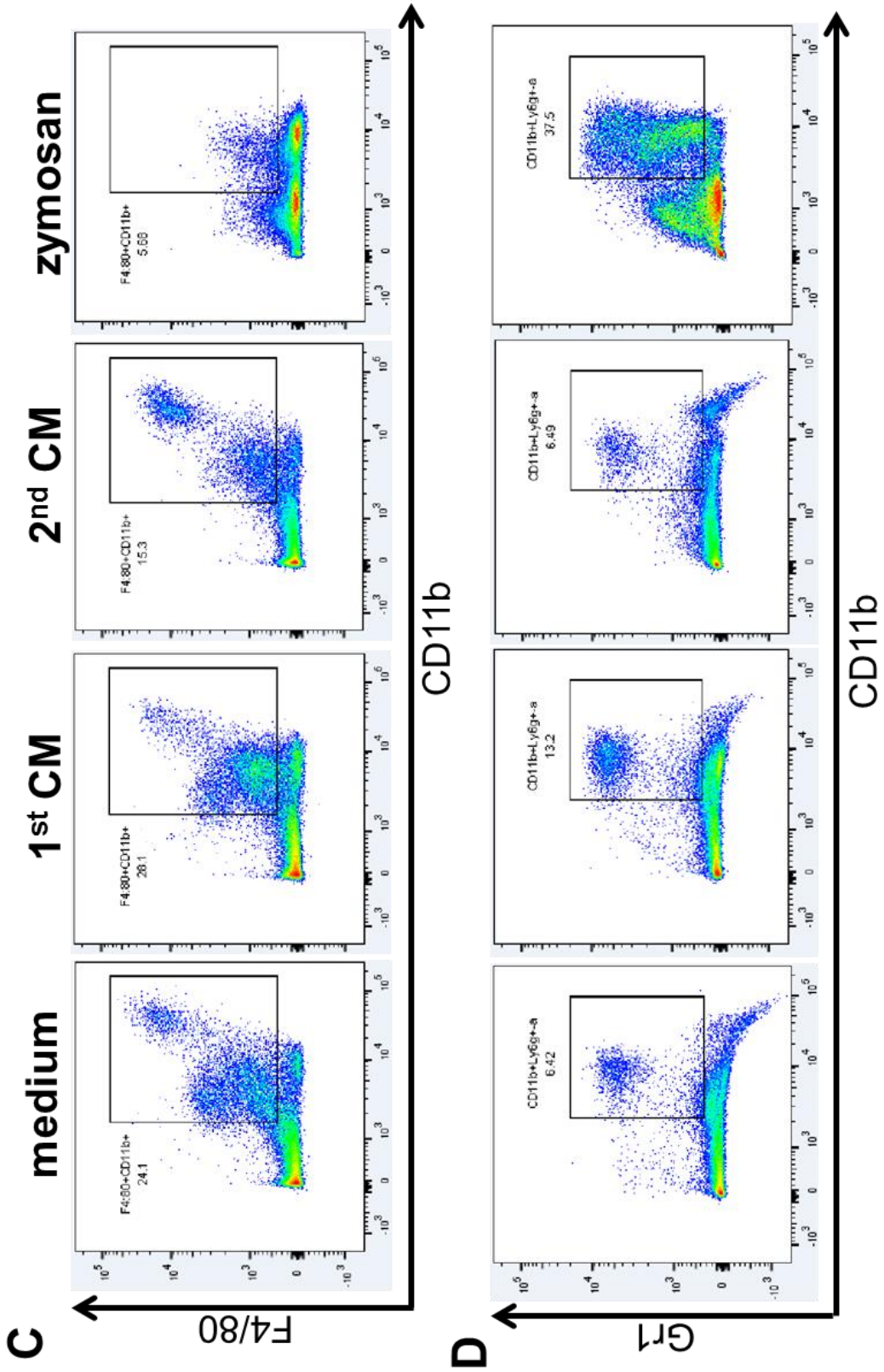

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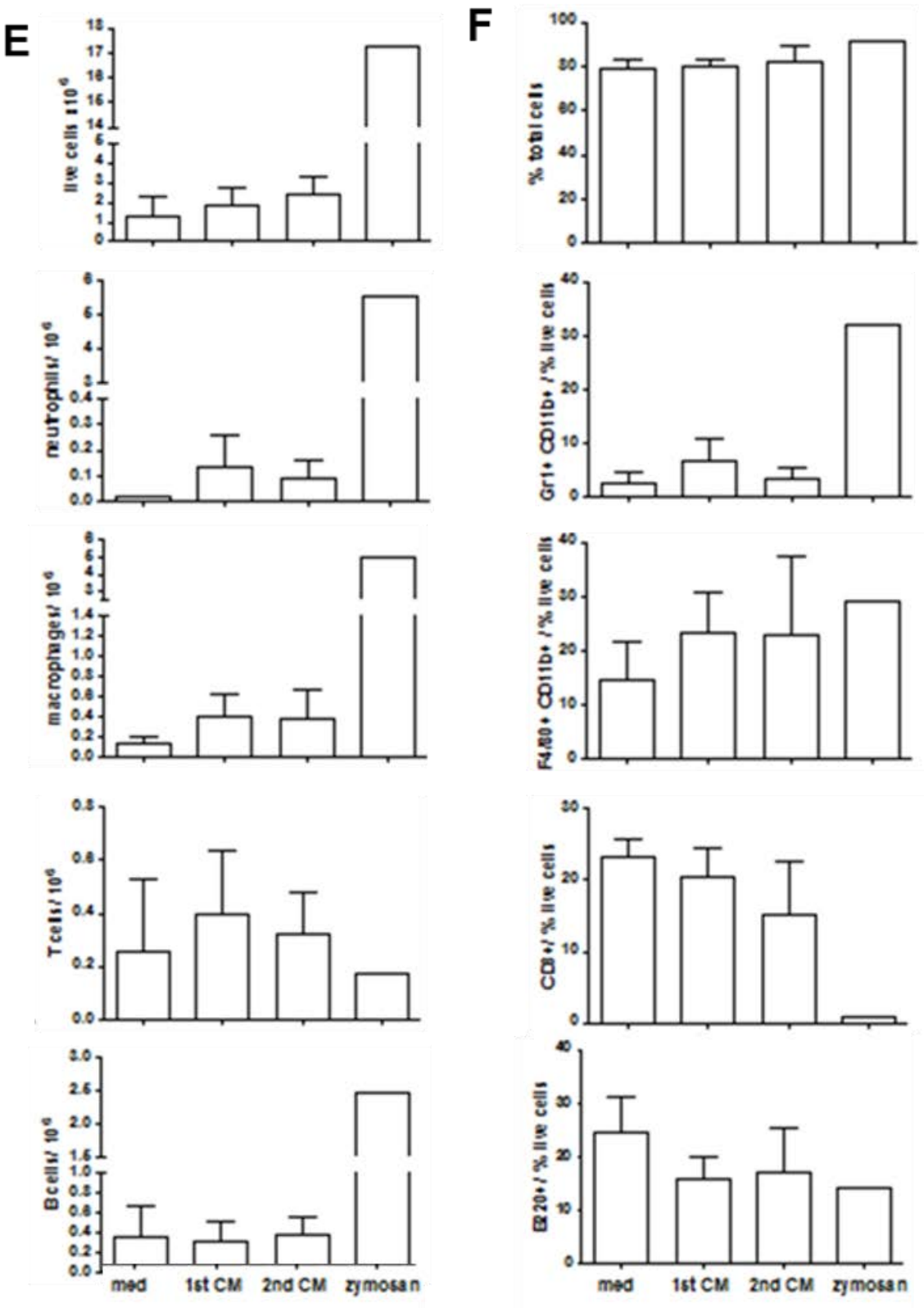

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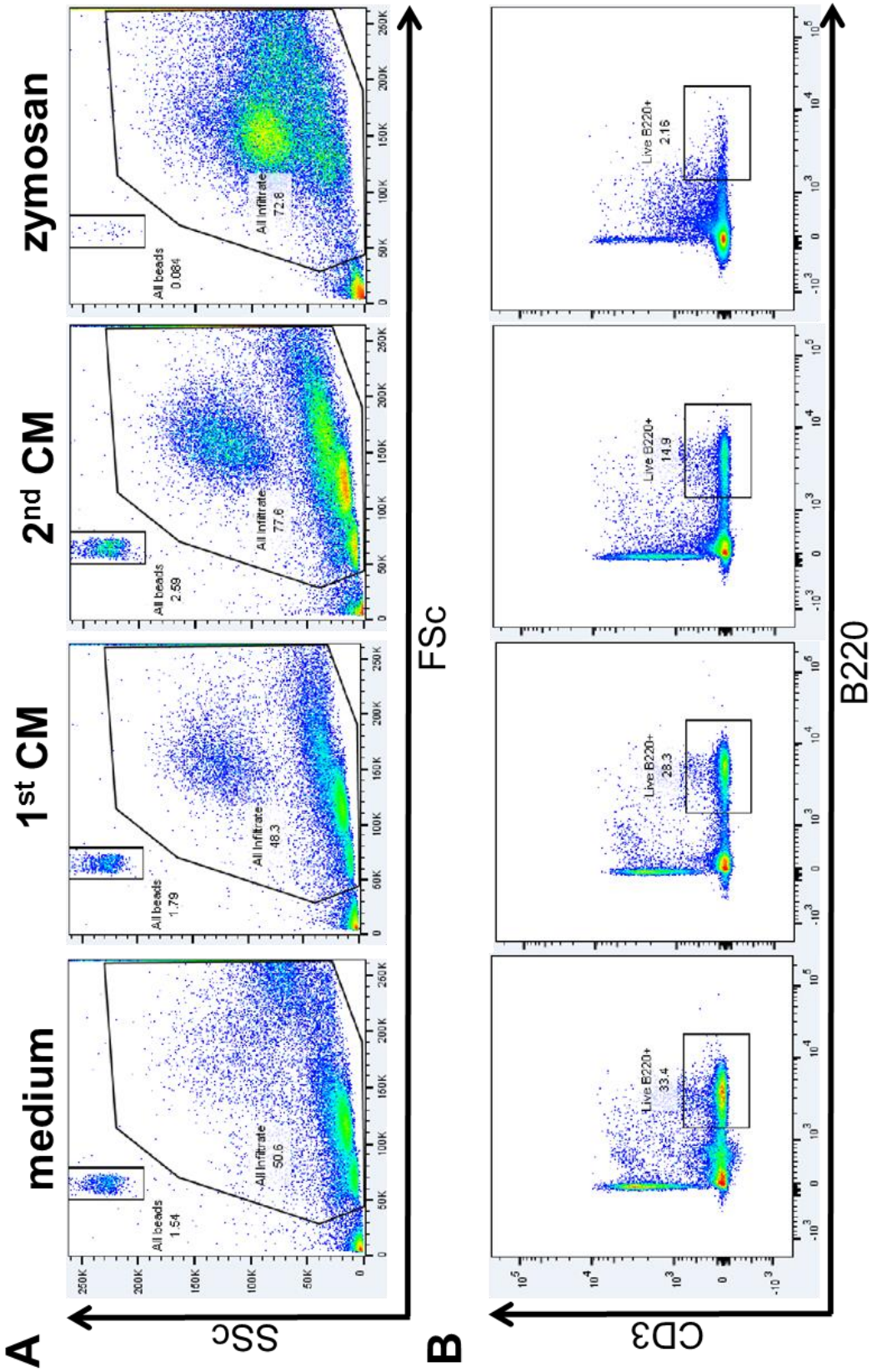
Figure 5.12: Inflammatory infiltrate 6h-post intra-peritoneal injection of fibroblast CM. Medium, zymosan, or CM from fibroblast initial or memory response to 10ng/ml TNFα were intraperitoneally injected into wildtype C57BL6 mice and the infiltrate was removed by lavage after 6h for flow cytometry. **A** Representative blots of total infiltrate following each injected stimulant. **B** CD3+ vs B220+ (T vs B cells). **C** F4/80+ vs CD11b+ (macrophages). **D** Gr1+ vs CD11b+ (neutrophils). **E** bar graphs of each subset by absolute count. **F** bar graphs representing % live cells of total events, and % of cells per subset, mean± SD. Medium n=4, 1st CM n=5, 2nd CM n=5, zymosan n=1. med= medium, CM= conditioned medium.

The lack of CM effect at 6h was surprising, and I next analysed the results from the 48h incubation (see figure 5.13) to test whether delayed kinetics may be the explanation. Once again, the zymosan positive control induced a huge inflammatory infiltrate, whilst the CM failed to raise infiltrate numbers above those of control medium. The percentage of cells still viable (i.e. zombie dye negative) was still higher in the zymosan control.

As with the 6h incubation, the zymosan-induced infiltrate was high in neutrophils, although interestingly the proportion of macrophages had decreased. Percentage influx of the latter was much more akin to those of medium or CM at 48h, and the memory response CM induced the greatest percentage macrophage influx (26.9 ±2.47% compared to less than 20% in the other treatments. B cell infiltrate was once again negligible in the zymosan control, and highest in the 1st response CM.



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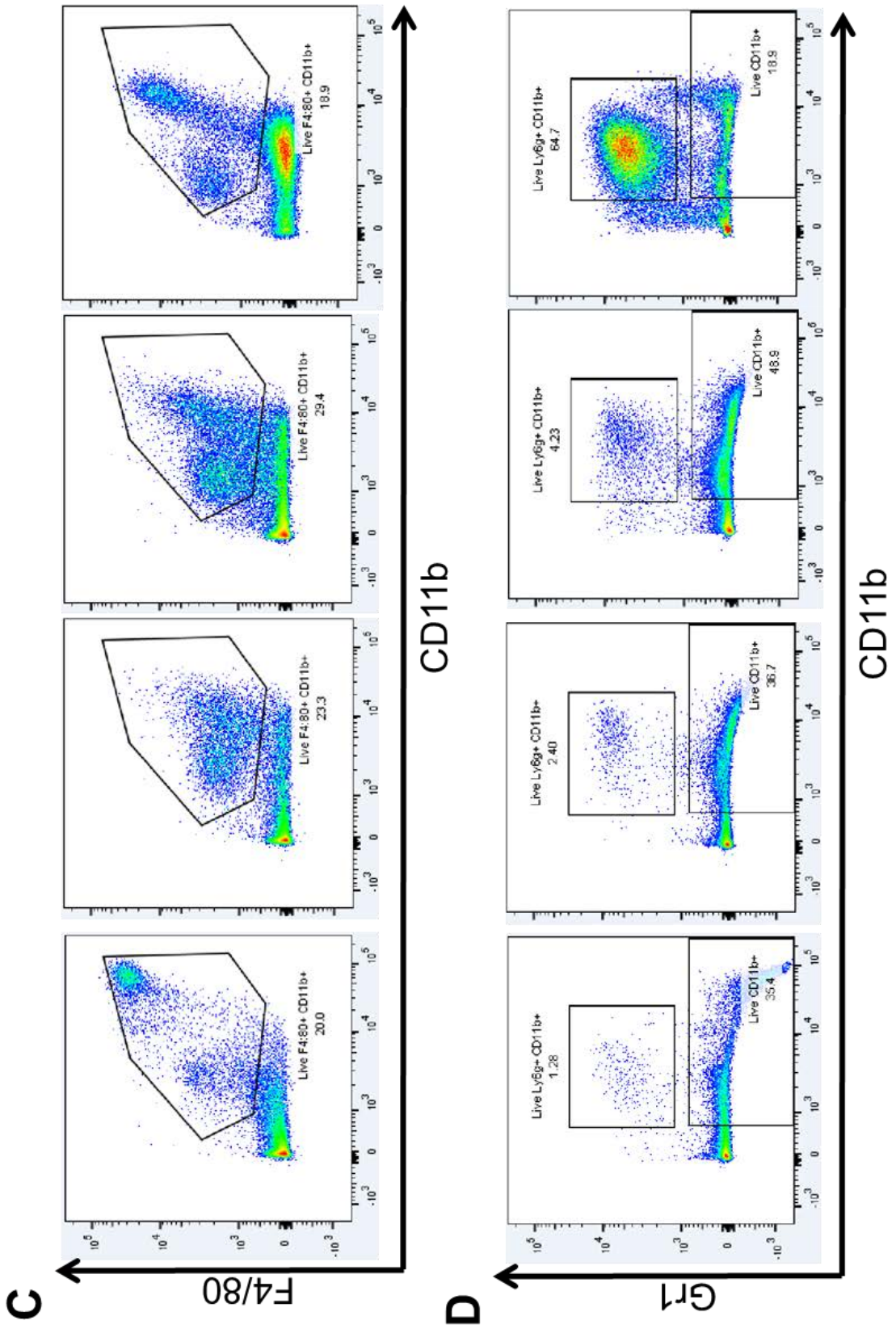

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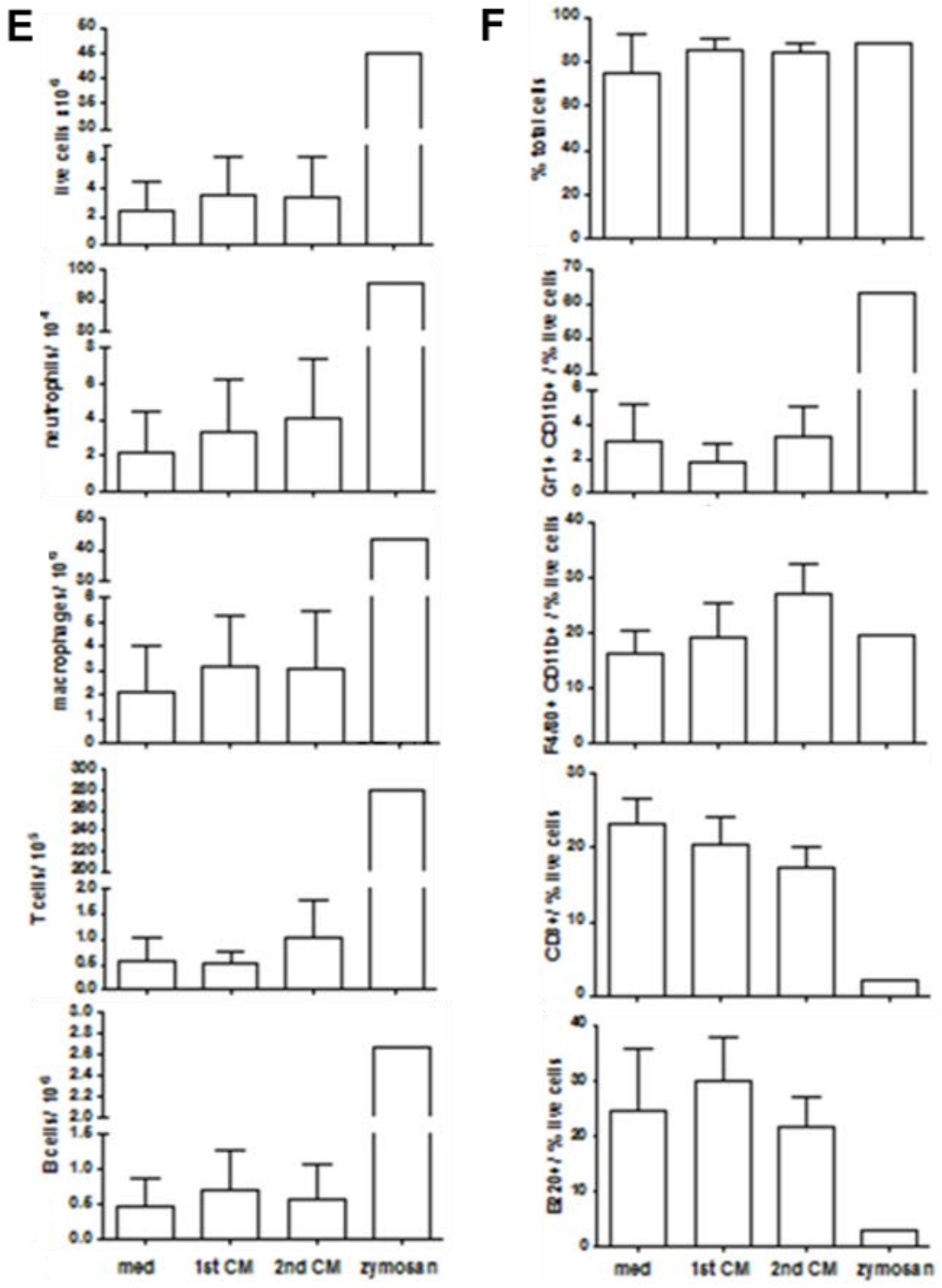

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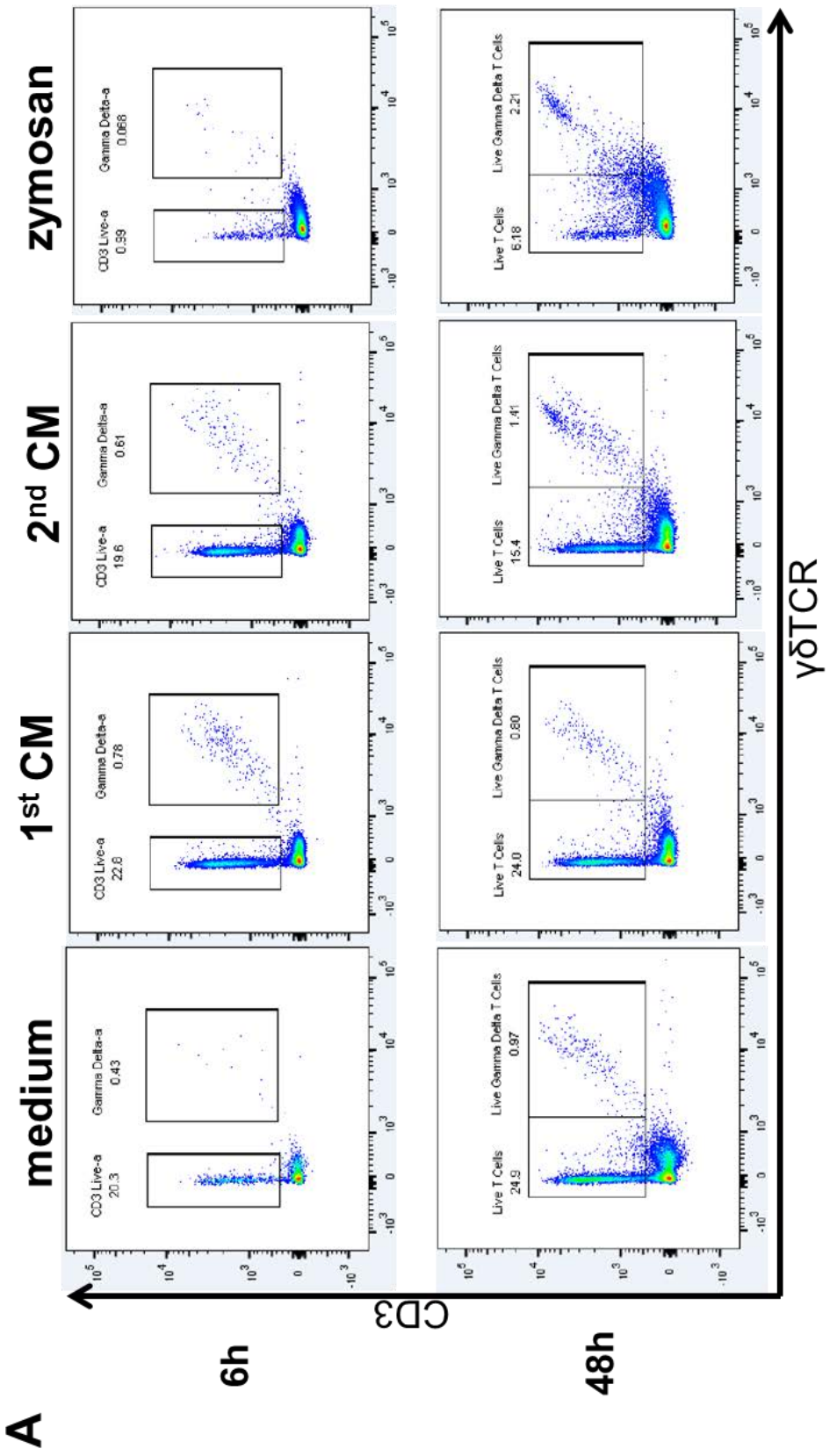
Figure 5.13: Inflammatory infiltrate 48h-post intra-peritoneal injection of fibroblast CM. Medium, zymosan, or CM from fibroblast initial or memory response to 10ng/ml TNFα were intraperitoneally injected into wildtype C57BL6 mice. Peritoneal infiltrate was removed at 48h and analysed by flow cytometry. **A** Representative blots of total infiltrate following injected stimuli. **B** CD3+ vs B220+ (T vs B). **C** F4/80 vs CD11b+ (macrophages). **D** Gr1+ vs CD11b+ (neutrophils). **E** bar graphs representing live cells and leukocyte subsets. **F** bar graphs representing % live cells of total events, and % of cells per subset, mean± SD. Medium n=5, 1st CM n=5, 2nd CM n=5, zymosan n=1. med= medium, CM= conditioned medium.

A rapidly expanding area of research in the field of both infectious and sterile inflammatory episodes is the role of non-canonical T cells. We often consider αβT cells, which are the classic antigen-responding T lymphocytes, however the roles for NKT cells, other ILCs and γδT cells are increasingly appreciated. Based on this, I also included an antibody against pan-γδTCR to see if this subset of T cells was differentially recruited. The data for T cell and γδT cell influx in both 6h and 48h incubations are shown in figure 5.14 below.

The pan-T cell infiltrate formed a negligible percentage of the zymosan-induced influx at 6h, and this was only marginally raised at 48h. Comparison of medium, first CM and second CM showed similar percentages of T cells in the infiltrate at 6h, and this was almost exactly maintained after 48h, implying no change in influx over the time course.

The percentage of γδT cells (of the total live T cell infiltrate) was increased over five-fold in the zymosan induced infiltrate between 6h (6.85%) and 48h (35.7%). The γδTCR+ percentage of total T cells at 6h was comparable between both CM and zymosan, all of which were slightly less than double that of medium alone, but all still as a low percentage of total T cells (within the 3-7% range). By 48h, the proportion had dramatically increased in the zymosan mouse (as already described) and had risen slightly in in the 2nd CM mice. The 1st CM mice however, decreased their γδT cell proportion to that of the control medium. This divergence of at 48h of first versus second CM (4.58 ±0.5% and 7.41 ±1.0%) was statistically significant (p=0.04), implying γδT represent a higher proportion of the infiltrate following memory response than initial response.



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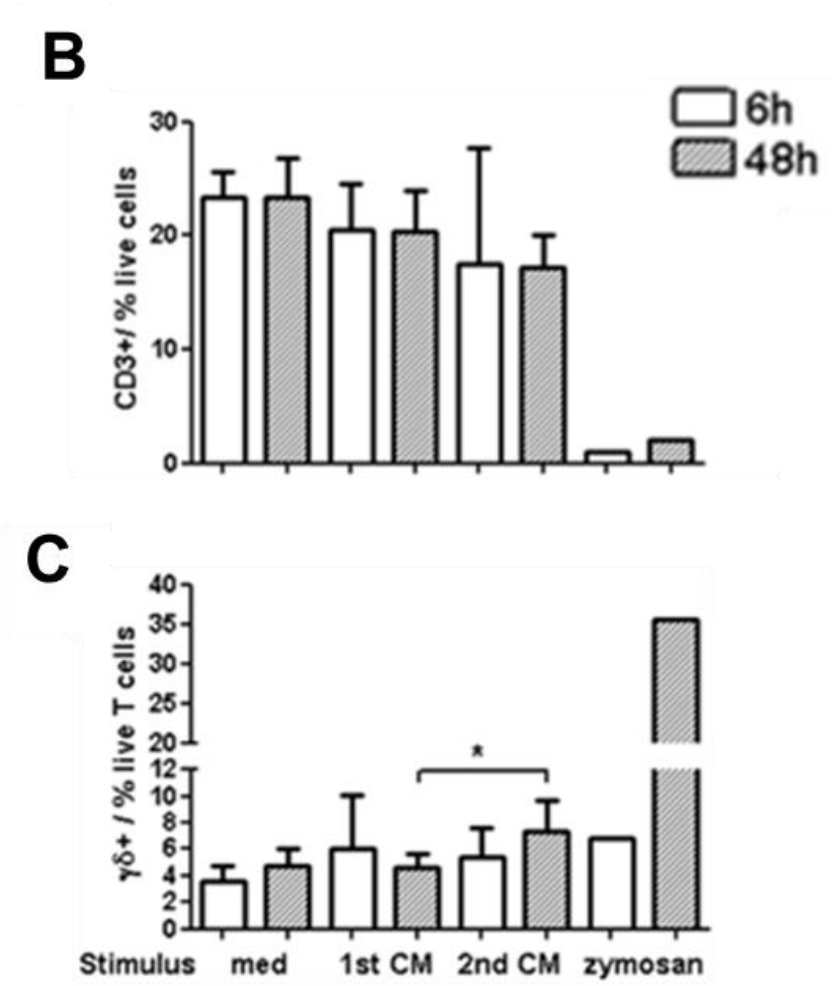
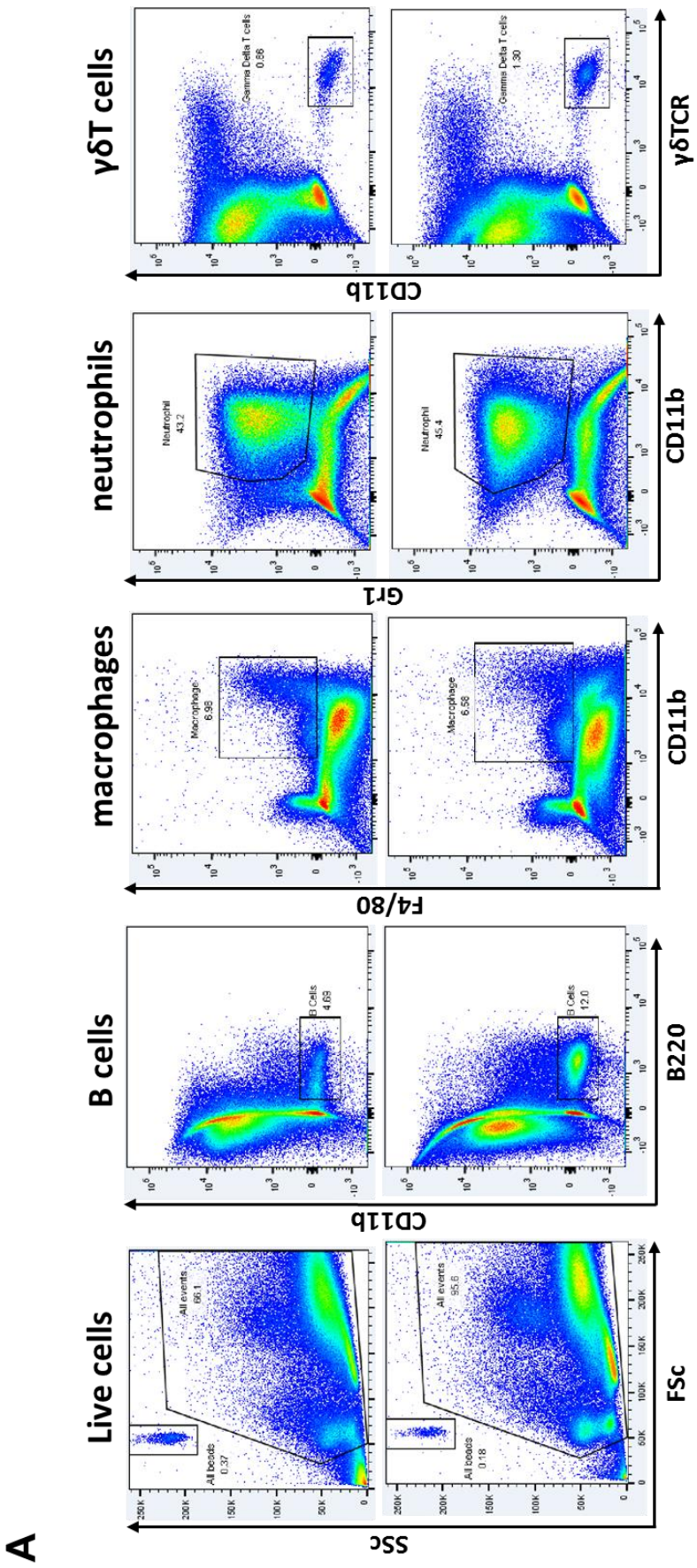


Figure 5.14: Mouse FLS memory response alters the γδT cell constituent portion of T cell influx during 48h peritoneal inflammation. Medium (control), zymosan, or 1st or 2nd conditioned medium from murine FLS TNFα challenge were injected into mouse peritoneum. After 6h (top row of **A** and open bars of **B** and **C**) or 48h (bottom row of **A** and cross hatched bars of **B** and **C**) the mice were sacrificed and peritoneum lavaged to harvest cellular infiltrate. **A** representative plots of T cell and γδT cell influx in response to each treatment at 6 and 48h. **B** Percentage of live infiltrate represented by CD3+ T cells. **C** Percentage of T cell infiltrate represented by γδT cells. Mean ±SD, zymosan n=1, medium n=5, 1st response CM n=5, 2nd response CM n=5. * p <0.05, Mann-Whitney U test.

The last three figures did not show FLS inflammatory responses capable of recruiting leukocytes above the level of growth medium. Given the large quantities of chemokine release shown by human FLS this seemed hard to believe. There is also abundant evidence for the role of FLS as leukocyte attractants in both human and mouse studies. I therefore decided to use zymosan in conjunction with CM. I originally avoided this, as I was concerned zymosan may skew the proportions of leukocyte subsets being recruited to the peritoneum. Zymosan is a TLR2 agonist, and acts as a fungal PAMP. If the secreted products of FLS initial or memory response were not fungus-response skewed, zymosan may override the FLS effect and cause any other 'skew' to be lost.

Given the results of figures 5.12, 13 and 14, however, I conducted a smaller test experiment using zymosan with mouse medium (control), or murine FLS second response CM. This experiment was conducted over 48h, which had promised the greatest differences between initial and memory response effects in the earlier experiment. The experiments presented above show zymosan to induce much greater infiltration than CM, and using both induced only marginally more cells to infiltrate than zymosan alone (see figure 5.15). This limited differences again suggests limited ability of the CM to recruit leukocytes.

Due to technical difficulties, the CD3+ compartment could not be analysed, but the γδTCR positive cells were analysed. These showed no difference between percentage of the infiltrate responding to zymosan or zymosan and CM. This was also true for neutrophils (the predominant infiltrating cell type), with over 40% in both treatments. The macrophage and B cell compartments both showed increased percentages with the addition of CM, although this was from low percentages (less than 10% in each case).



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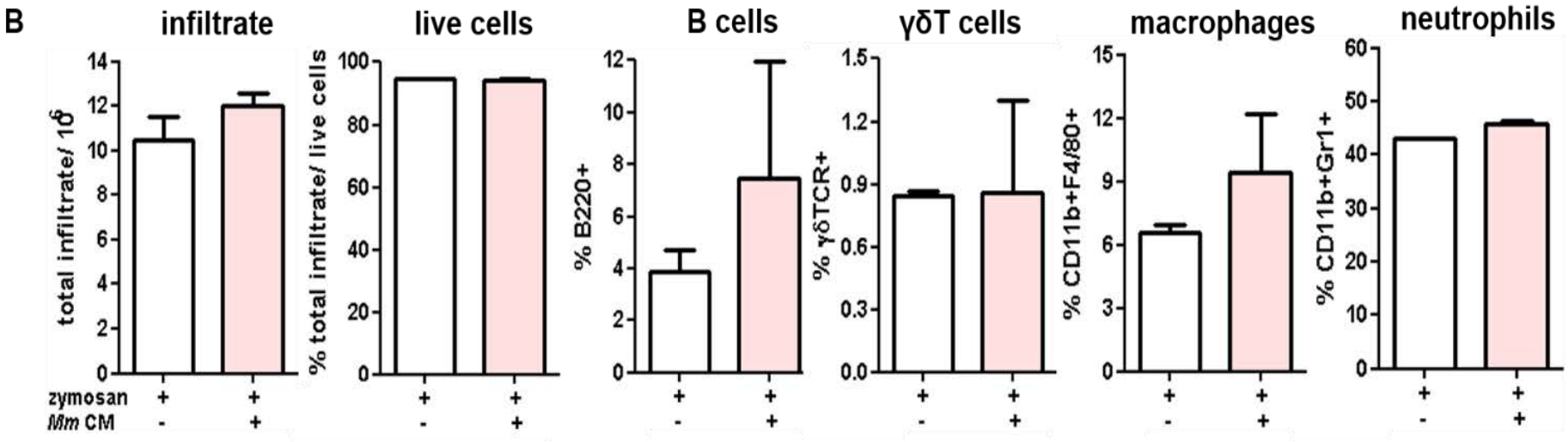

Figure 5.15: fibroblast CM increases peritoneal infiltration following injection of zymosan. Zymosan or zymosan with mouse fibroblast conditioned medium were injected into the peritoneum of mice. After 48h the mice were sacrificed and the peritoneum lavaged to harvest the inflammatory infiltrate, which was analysed by flow cytometry. **A** flow cytometry plots of total, live and leukocyte subset influx in response to zymosan (top row) or zymosan and conditioned medium (bottom row). **B** bar graphs of total, live, and leukocyte subset influx. Mean ±SD, n=2.

5.2.3.2 Effect on monocyte differentiation and macrophage function

Monocyte infiltration is a key aspect of both healthy and pathologic inflammatory episodes. Monocytes modulate the milieu through secretion of mediators, in response to the microenvironment they find themselves in. This environment dictates how monocytes differentiate in the tissue, therefore dictating their role. As fibroblasts are a key stromal cell, and important mediators of inflammation in many tissues, I was keen to assess how the memory response of fibroblasts affected monocyte differentiation and the subsequent macrophages' effector functions.

To this end I conducted the experiment outlined in methods section 2.5.6.1, wherein fibroblast CM from the first or memory response to TNFα was added to monocytes and incubated for six days, before the cells were challenged with pHrodo beads, to assess their phagocytic ability and capacity to secrete TNFα in response to PAMPs.

First, however, I had to assess the same read outs in M-CSF- and GMSCF-differentiated macrophages. This allowed me to examine the functions of these polar opposite macrophages. I could then assess where on the spectrum monocytes would fit after responding to fibroblast inflammatory cues.

M-CSF and GM-CSF-differentiated macrophages had distinct phagocytic capabilities. The difference following pHrodo challenge was clear from the percentage of cells PE+ cells (indicative of phagolysosomes acidifying the internalised pHrodo beads). This was shown by flow cytometry, and merged bright field and fluorescent microscopy images in figure 5.16. No PE signal was found by microscopy or flow cytometry in the absence of pHrodo beads.

The M2 macrophage marker CD206 (mannose receptor) was also shown to be expressed at a higher level in the M-CSF-differentiated macrophages (M2-like cells), offering confirmation for the functional study. Whilst clear segregation could be seen based on CD206 expression, intracellular TNFα expression appeared similar in M1-like and M2-like. This was contradicted by a TNFα ELISA, which showed a striking difference in TNFα secretion in response to PAMP challenge (184 ±8.68ng/ml in the M1-like compared to 53.88 ±7.33ng/ml in the M2-like cells).

With the exception of intracellular TNF staining, these data indicate a clear separation in functional effects based on M1-like and M2-like differentiation. This matches data from our group and the wider literature.

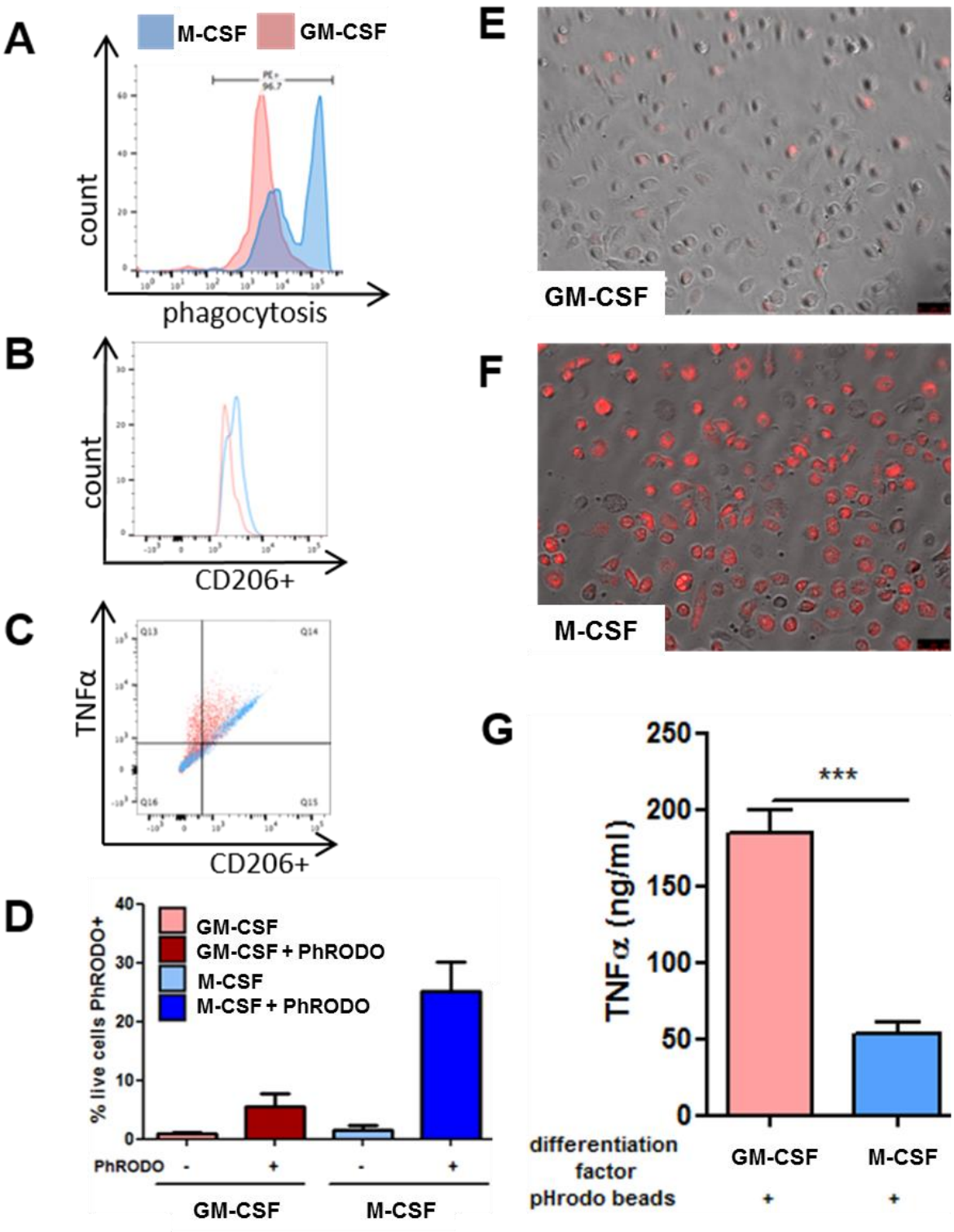

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Figure 5.16: Effector function of GM-CSF and M-CSF-differentiated macrophages. Monocytes were cultured with 25ng/ml GM-CSF or 50ng/ml M-CSF for 6 days, then cells were washed and exposed to pHrodo beads for 4h. Cells were once again washed then assessed by microscopy and flow cytometry. **A** Comparison of phagocytic ability. **B** Comparison of expression of M2 macrophage marker CD206. **C** Assessment of levels of intracellular TNFα and expression of CD206. **D** Separate experiment assessing fluorescence in the 'phagocytosis' channel in GM-CSF and M-CSF differentiated macrophages with or without pHrodo challenge. n=2. **E** and F bright field and fluorescent merge images of GM-CSF and M-CSF differentiated macrophages undertaking phagocytosis. Scale bar= 50μm. **G** TNFα ELISA of GM-CSF and M-CSF macrophages following pHrodo stimulation for 4h. n=5 GM-CSF, n=5 M-CSF, assessed by unpaired t test *** $p<0.0001$.

When CM was added to monocytes, it was added in place of M-CSF or GM-CSF. CM from HTF was added in parallel to the differentiation factors. As shown in figure 5.17a, the use of any differentiation factors or CM appeared to increase cell survival beyond that of monocytes receiving growth medium, although this was non-significant as assessed by multiple comparisons. The number of phagocytosis-capable cells was significantly higher following incubation with memory than initial response CM. This is shown in both graphical and photographic form, although perhaps more clearly in the graph shown in 5.17b.

As with the number of cells capable of phagocytosis, the concentration of TNFα secreted in response to pHrodo challenge was also significantly higher in cells cultured with memory response CM (see figure 5.17f). As shown in figure 5.17g, the memory response induced over 3-fold more TNFα release over 4h in the presence of pHrodo beads (45.38 ±5.27ng/ml versus 14.61 ±1.23ng/ml). These data indicate a stronger pro inflammatory, and a stronger anti-microbial response mounted by macrophages differentiated by the memory response compared to the initial response.

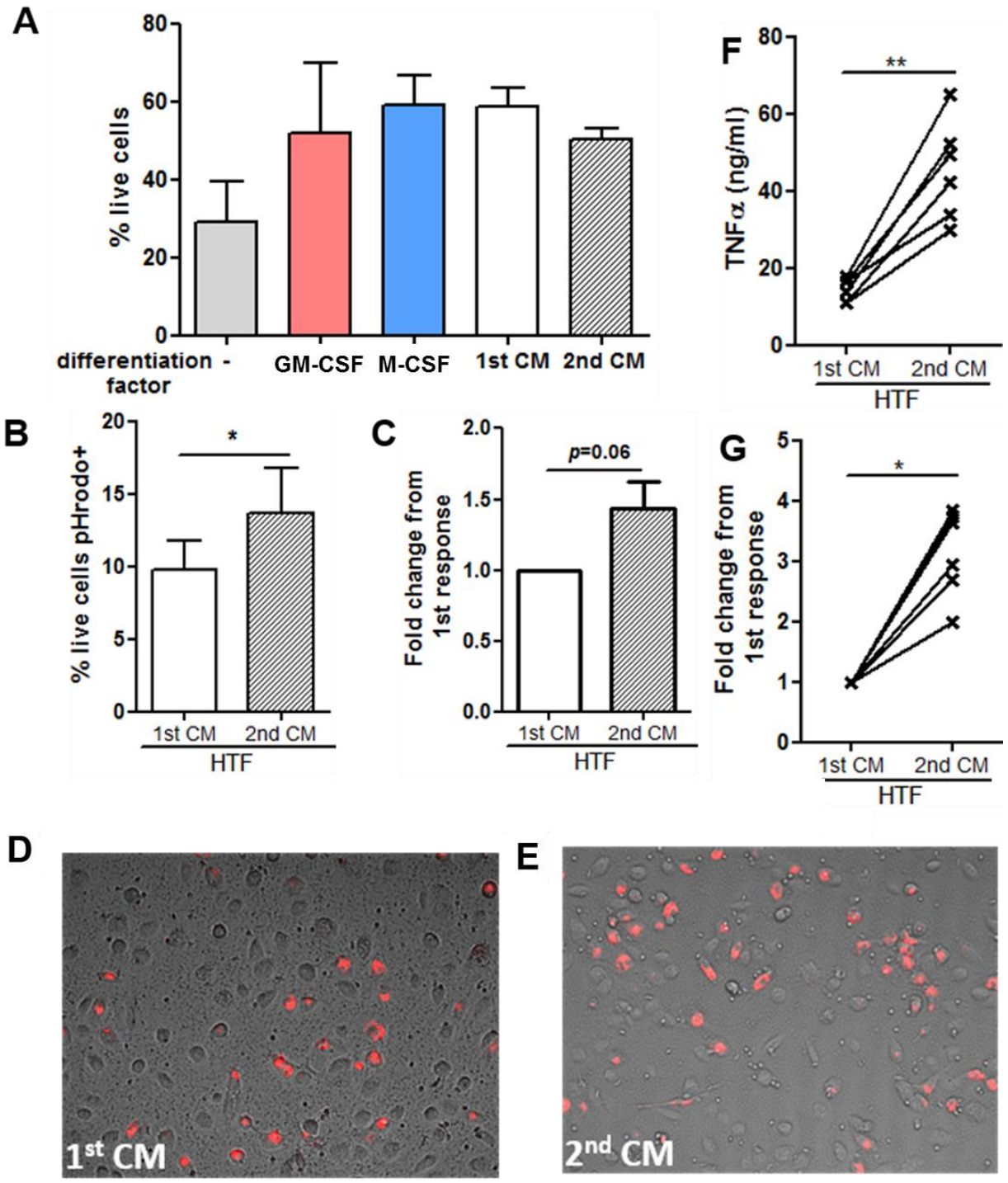

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Figure 5.17: Tonsil fibroblast memory makes macrophages more phagocytic and pro inflammatory. Monocytes were exposed to differentiation factors, or first or second response CM of HTFs for six days. **A** Percentage of live cells after six day incubation by flow cytometry. **B** Percentage of cells exhibiting phagocytosis (Mann-Whitney U test **) and **C** fold change of second dose CM represented vs. first dose CM effect on phagocytosis (Wilcoxon matched pairs signed rank test). **D** and **E** merged bright field and fluorescent microscopy images of pHrodo-challenged cells exposed to first and second CM respectively. TNFα secretion by pHrodo-challenged monocytes exposed to first or second CM expressed as **F** raw concentrations (Mann-Whitney U test **), and **G** fold change (Wilcoxon matched pairs signed rank test *). * $p<0.05$, ** $p<0.001$. n=6 HTF donors using same monocyte donor. CM= conditioned medium, HTF= human tonsil fibroblast.

Given the site-specificity of fibroblast memory, I wanted to compare the result in HTFs with fibroblasts from another tissue. To this end, I repeated the experiment using initial and memory CM from control and psoriasis HDFs (see figure 5.18).

As shown earlier in figure 5.16, the intracellular TNFα stain appeared to show comparable cytokine levels in M1-like and M2-like cells. When the effect of HDF was examined it again showed little difference, with perhaps a decrease in intracellular TNFα in cells exposed to memory response CM from psoriasis HDFs (see figure 5.18a and b). The relative expression of CD206 in figure 5.18b appeared to subtly decrease following memory response CM of either control or psoriasis HDFs, suggesting a shift towards a more M1-like phenotype.

As shown in figures 5.18c and d, the extent of phagocytic ability was increased compared to monocytes receiving no differentiation factors (greater than 67% functionality in the CM treatments, compared to 59.47 ±4% cells phagocytic capable following control medium) but was not significantly altered between CM treatments. The memory response of psoriasis HDFs, for example, induced only a 14% increase in phagocytic ability.

The lack of difference was mirrored by the TNFα ELISA (see figure 5.18e), which was demonstrated by multiple comparisons finding no significant difference across the treatments. The memory response of control HDFs induced a noticeable increase from challenged macrophages (30.98 ±7.49ng/ml compared to first CM 20.39 ±3.71ng/ml), but this was non-significant. Another important observation is that the control M-CSF and GM-CSF-differentiated cells also failed to separate based on TNFα secretion, suggesting a technical issue, rather than a lack of differential effect of the CM.

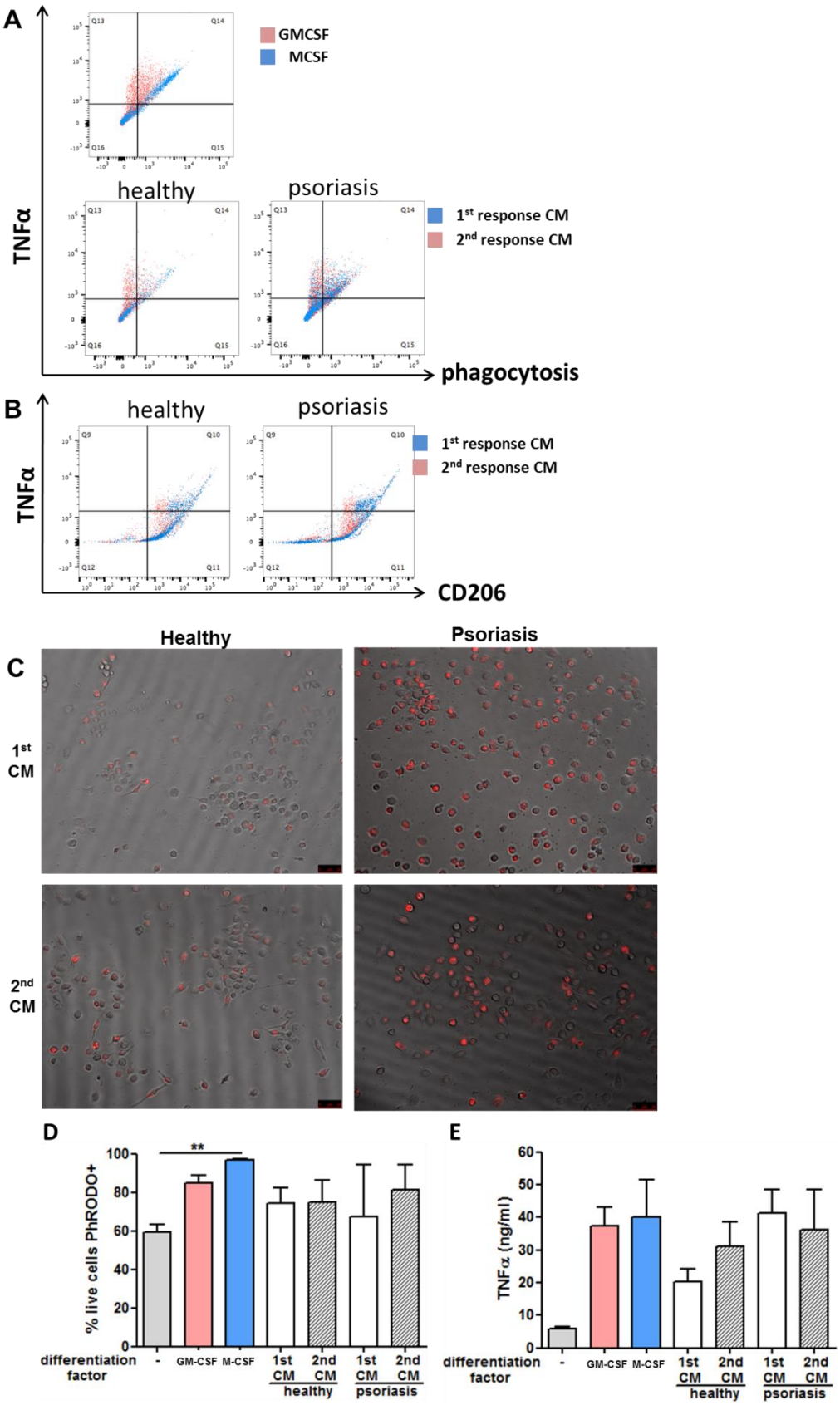

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Figure 5.18: Dermal fibroblast memory does not alter macrophage function. Monocytes were differentiated for six days in the presence of M-CSF, GM-CSF, or HDF first or second response CM. Macrophages were challenged with pHrodo beads for 4h. **A** M-CSF, GM-CSF, and HDF CM effect on macrophage intracellular TNFα and phagocytic ability. **B** HDF CM's effect on macrophage intracellular TNFα and expression of the M2 marker CD206. **C** Bright field and fluorescent merged images of cells exposed to healthy or psoriasis HDF first or second CM. Scale bar= 50μm. **D** Bar graphs representing percentage of live cells showing evidence of phagocytosis, with bars representing mean ± SD Kruskal-Wallis test with Dunn's post-test (** $p< 0.01$). **E** TNFα ELISA of macrophages following 4h exposure to pHrodo beads. Bar graphs represent mean ± SEM. Monocyte (medium alone), M-CSF-differentiated, GM-CSF-differentiated, all n=3 with same monocyte donor. Fibroblast conditioned medium n=4 per treatment. CM= conditioned medium.

5.2.3.3 Effect on T cell activation and function

The CM incubation with monocytes provided some evidence for the consequences of fibroblast memory. I therefore attempted to continue this line of investigation by examining the effect of CM on T cells. The T cells used in this study were from blood cones and provided by Kalvin Sahota, another PhD student in our department. As Kalvin was specifically isolating CD4+ T cells, these were cells used in my studies. The details of the experiment are available in section 2.5.6. CM from lung fibroblasts (HLF) was used in the experiment shown in figure 5.19. Unlike circulating monocytes, circulating T cells may have already differentiated, as we did not remove the CD4+ memory population. This bias in T helper subset can be seen by the high proportion of IL-10+ cells, even in control wells. IL-4+ and IL-17A+ populations were too low to be worth displaying in this figure.

The percentage of cells positive for IL-10 was high (77 ±17.7%), and this increased only slightly after five days in the presence of activating beads (80.77 ±7.56%). In comparison, HLF CM appeared to reduce the number of IL-10+ cells, with 70 ±15.4% of cells IL-10+ in the presence of first CM, and 52.78 ±15.99% IL-10+ in the presence of memory CM. When expressed as a fold change, the difference between initial and memory responses was an average of 33% reduction but did not reach significance.

The other major population in this experiment were IFNγ+ cells. Negligible in control wells (less than 1%), they expanded to account for 17.8 ±2.6% of the population in the presence of activating beads. The percentage of IFNγ+ cells was increased by 1st response CM and decreased by memory response CM when compared to activating beads alone. Multiple comparisons showed only 1st CM significantly differed from medium in ability to significantly increase IFNγ+ cells,

and a pairwise comparison of 1st versus 2nd CM displayed a non-significant difference (not shown). When expressed as a fold change however, the memory response showed a significant reduction in the percentage IFNγ+ cells (an average decrease of 47%) compared to the percentage of IFNγ+ cells induced by the initial response.

These data once again demonstrate that fibroblast memory can alter the behaviour of leukocytes known to play key roles in inflammatory situations.

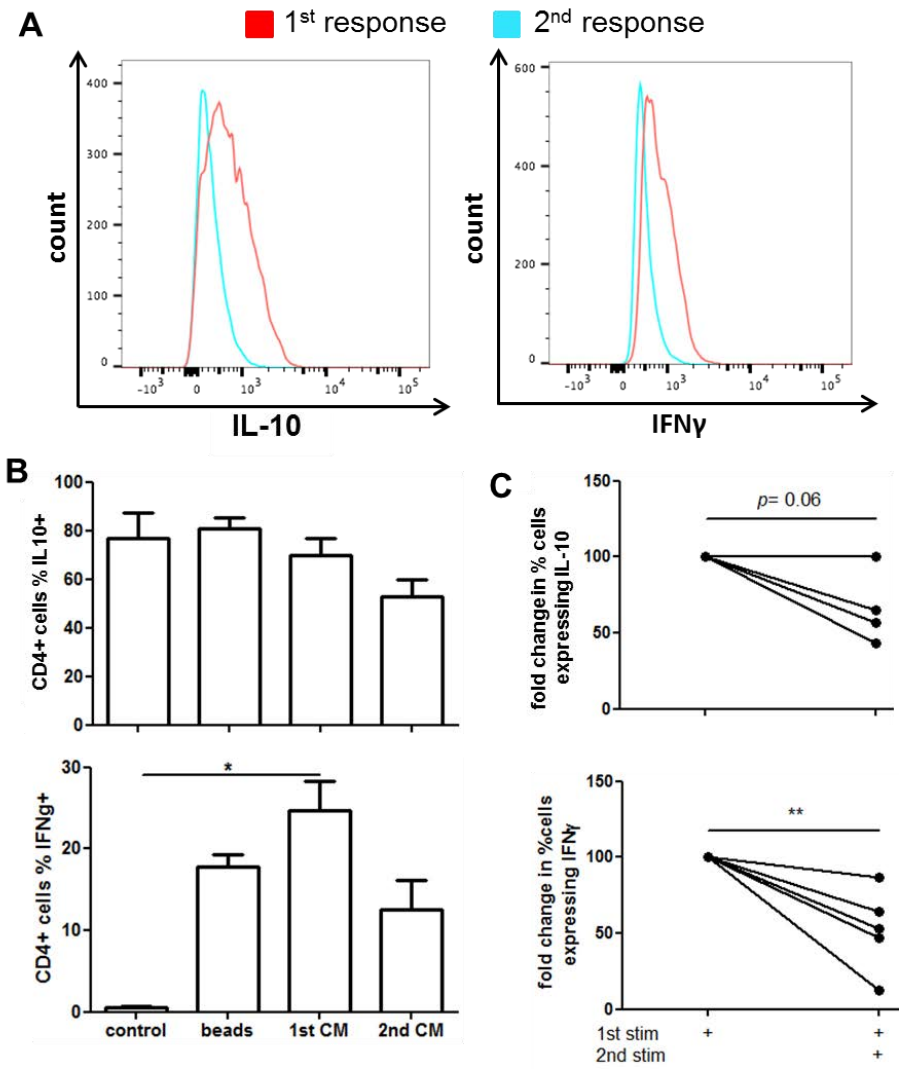

Figure 5.19: Lung fibroblast memory response reduces intracellular cytokine levels in CD4+ T cells. First or second response CM from HLF was added to CD4+ T cells for five days with activation beads. Intracellular cytokines were then assessed. **A** Representative histograms for IL-10 expression (left) and IFNγ expression (right) following each treatment. **B** Percentage of cells expressing IL-10 (top) or IFNγ (bottom). Mean ±SD, Kruskal-Wallis with Dunn's post-test (*p<0.05). **C** fold change of percentage expressing IL-10 (top) or IFNγ (bottom) following 2nd response CM relative to 1st response CM. Wilcoxon matched pairs signed rank test, **p<0.01. n=3 control, n=3 beads, n=6 HLF 1st CM, n=6 HLF 2nd CM.

In another experiment, I used control and RA FLS CM to assess effects on T cells. The effects on three subsets were negligible, and for simplicity only the effect of on IFNγ expression is shown (see figure 5.20). CM from first and memory responses of control FLS did not differ in their induction of IFNγ expressing cells. The memory response of RA FLS however, induced a small increase in the percentage of IFNγ+ cells when compared to the first response CM. When expressed as a fold change, the increase was not significant.

These data opposed their equivalent test using HLF CM, which significantly reduced the percentage of cells expressing IFNγ. This suggests, as with the effect on macrophage function, that the effects of fibroblast memory on leukocytes are dependent on the tissue from which the fibroblasts were removed.

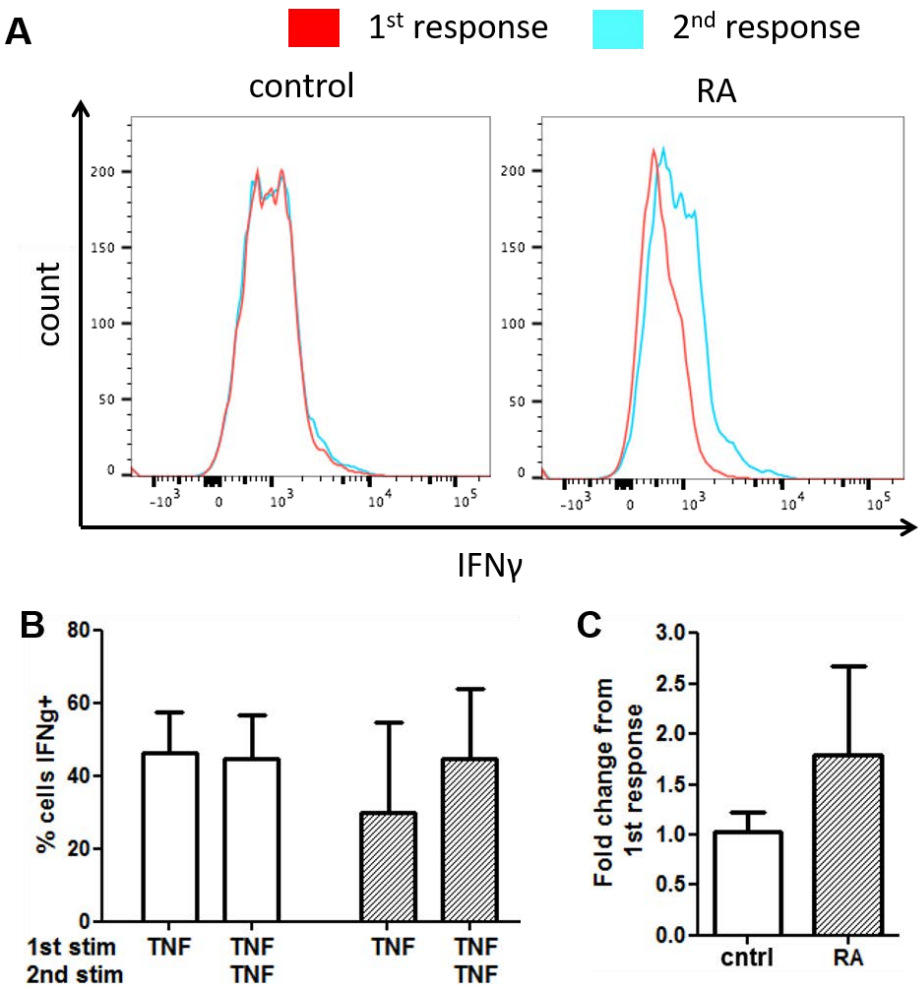

Figure 5.20: The memory response of RA- but not control- FLS increases the percentage of IFNγ+ CD4+ T cells. CD4+ T cells were incubated for five days with activation beads and control or RA FLS first or second response CM. Intracellular cytokine expression was then assessed by flow cytometry. **A** Representative histograms of control and RA FLS effect on T cell IFNγ expression. **B** Percentage of cells expressing IFNγ following each treatment. **C** Fold change of effect of second versus first response CM. Mean ±SD, control n=4, RA n=4.

5.2.3.4 Effect of soluble mediators on fibroblast inflammatory secretion

Fibroblasts do not exist in isolation. They interact with multiple cell types, subsets, and membrane bound or secreted mediators. Whilst *in vivo* methods are holistic, they are difficult to tease apart. Using a limited number of mediators *in vitro* gives a simpler opportunity to see how mediators affect fibroblasts.

The research on this subject so far has been limited to TNFα and IL-1α, but infiltrating cells provide pro resolution and anti-inflammatory mediators too. As there has been little evidence thus far to show fibroblasts capable of turning off their own inflammatory program, I wanted to test which mediators could turn off fibroblast inflammatory secretions.

As explained in chapter 1, fibroblasts are incapable of responding to IL-6, as they lack the IL-6R required to induce signal transduction by the ubiquitous gp130. IL-6R is provided by shedding from other cells, particular neutrophils. In the dogma of acute inflammation, neutrophils are the first infiltrate damaged or infected tissue. Upon passage through tissue and during apoptosis, neutrophils shed IL-6R, which may then bind IL-6 and the dimer can induce gp130 signal transduction.

Given the propensity of fibroblasts to release IL-6 upon inflammatory challenge, I considered the possibility that neutrophil influx may act as the first step in switching off fibroblast inflammatory secretions. In the model illustrated in figure 5.21, neutrophil-derived sIL-6R would bind fibroblast-derived IL-6, feedback into the fibroblast population and negatively regulate IL-6 production. This would help the switch from acute to resolving phenotypes described in many tissues.

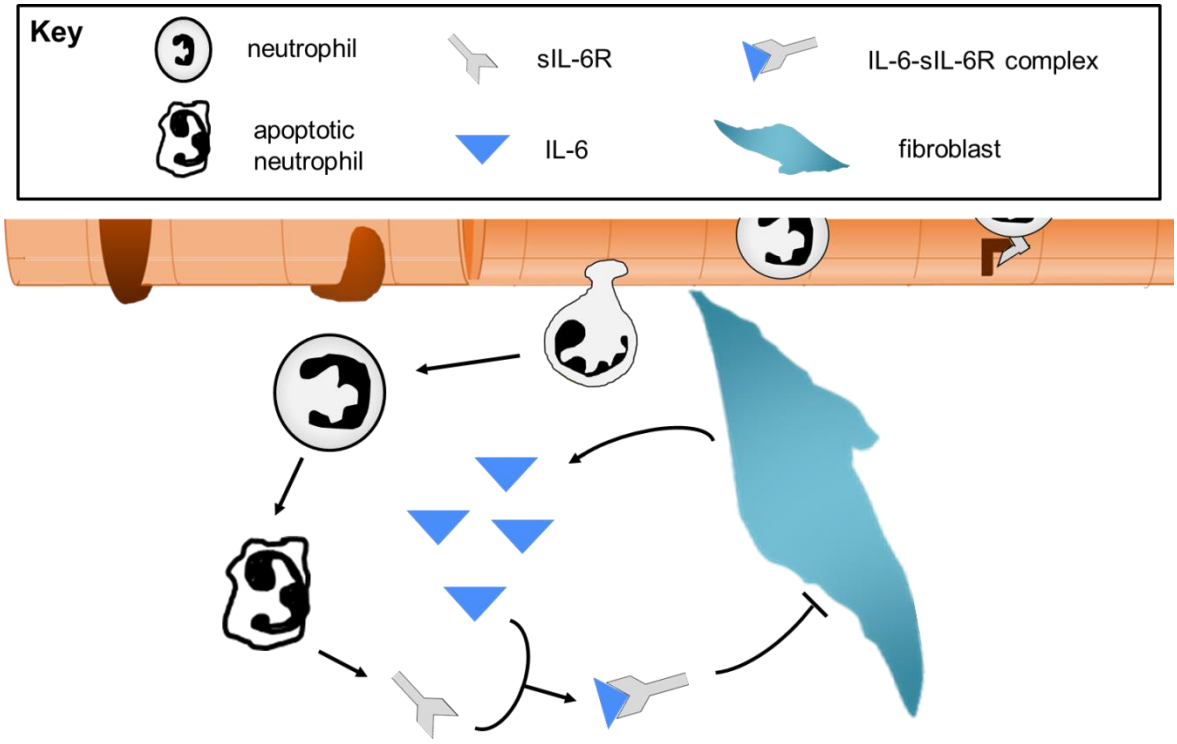

Figure 5.21: Illustration of a proposed negative feedback loop between neutrophil sIL-6R and fibroblast IL-6. Apoptotic neutrophils shed IL-6R (grey shape), which binds IL-6 (blue triangle) to form a complex capable of binding gp130 on fibroblasts and inducing signal transduction in said cell. In this model, that feedback loop would inhibit fibroblast production of IL-6.

BJ fibroblasts were challenged with TNFα for 24h in the presence or absence of sIL-6R. As shown in figure 5.22, qPCR evidence showed that at the 4h point, higher doses (10 and 100ng/ml) of sIL-6R appeared to augment expression of IL-6, IL-8 and CCL2 mRNA. The fold increase in CCL2 mRNA relative to TNFα did not alter much by 8h, and whilst the fold increase in IL-8 mRNA did increase, variation between replicates appeared to make this untrustworthy. The fold increase in IL-6, however, rose dramatically from 4h to 8h, with a dose dependant increase that was over 500 times greater than TNFα alone at the strongest sIL-6R concentration. Despite the large increase in IL-6 mRNA at 8h, there was no significant difference as assessed by multiple comparisons. The other genes analysed also failed to show significant differences from TNFα alone when sIL-6R was supplied.

The large increase in IL-6 expression at the transcript level was contradicted by the ELISA data shown in figures 5.22a. The IL-8 ELISA data also showed no effect of sIL-6R on TNFα-induced IL-8 secretion, which matched the qPCR data for mRNA expression. The effect of sIL-6R and by extension, the model I proposed in figure 5.21 appears to be disproved by the data presented in figure 5.22.

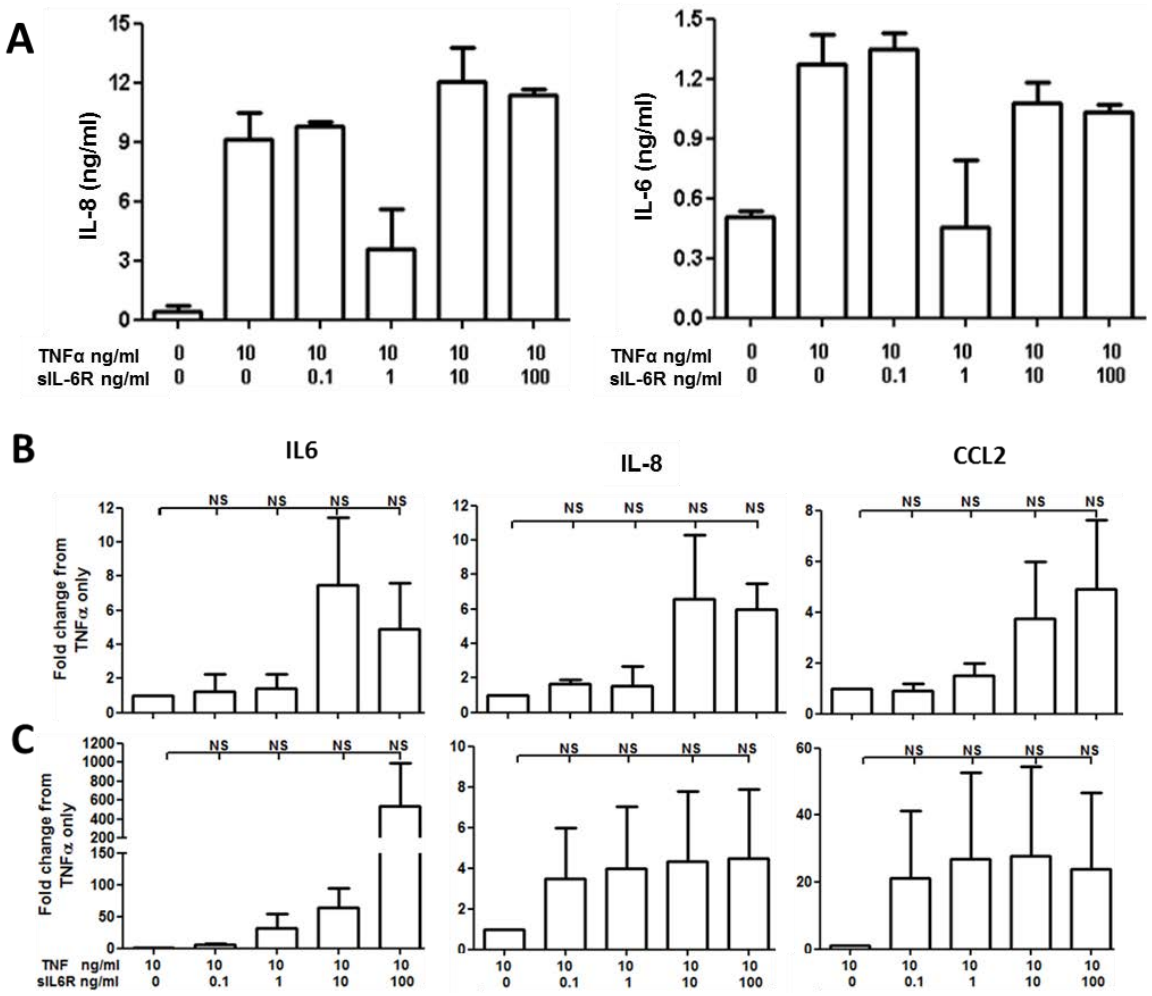

Figure 5.22: sIL-6R may dose-dependently affect fibroblast IL-6 mRNA, but not protein levels, and does not affect other genes. BJ fibroblasts were challenged with medium (control), 10ng/ml TNFα, or TNFα and sIL-6R (at concentrations indicated). **A** IL-8 and IL-6 secreted concentrations were assessed after 24h by ELISA. Mean ±SEM, IL-8 n=3, IL-6 n=6. Gene expression of IL-6, IL and CCL2 were assessed by qPCR after 4h **(B)** and 8h **(C)** incubation. Assessed by one-way ANOVA and Dunnet's post-test against 10ng/ml TNFα alone. Mean± SEM, n=3.

I have already presented evidence for an effect of the fibroblast memory response on monocytes, and so looked for a reciprocal effect of macrophage CM on fibroblasts inflammatory mediators. The effects of M1 macrophages are strongly proinflammatory, and so I focussed instead on the effect of M2-like macrophages. For the sake of simplicity, M2 macrophages are generally thought of as anti-inflammatory, particularly because of the levels of IL-10 they release. This is perhaps an over simplification, however, and M2 cells are 'less pro inflammatory' than M1 cells and have a pro-resolution role. It is not immediately clear, therefore, whether M2 macrophages would inhibit the inflammatory secretions of fibroblasts.

As explained in methods section 2.5.5, I stimulated M2-like macrophages for 1h then washed them to remove LPS and collected the CM 23h later. This was added to BJ fibroblasts challenged with TNFα. As shown in figure 5.23a, there was a slight decrease in fibroblast IL-6 secretion after 24h of incubation. When neat M2 CM was used alongside TNFα, the average fold change was 0.69 ±0.14 fold that of TNFα alone (a 31% decrease in IL-6 secretion).

The limited effect on IL-6 secretion suggested that whilst the net effect of M2-like macrophage secretions is IL-6-reducing, multiple mediators exert different effects on fibroblasts. To test whether fibroblasts can be 'shut off'; I also used a classic anti-inflammatory cytokine: IL-10.

As shown in figure 5.23b, the strong TNFα-induced IL-6 response from fibroblasts was inhibited by IL-10. This inhibition was incomplete, but nonetheless displayed a significant reduction in IL-6 secretion in all but one concentration. Interestingly, there was no observable effect of IL-10 on the TNFα-induced release of IL-8.

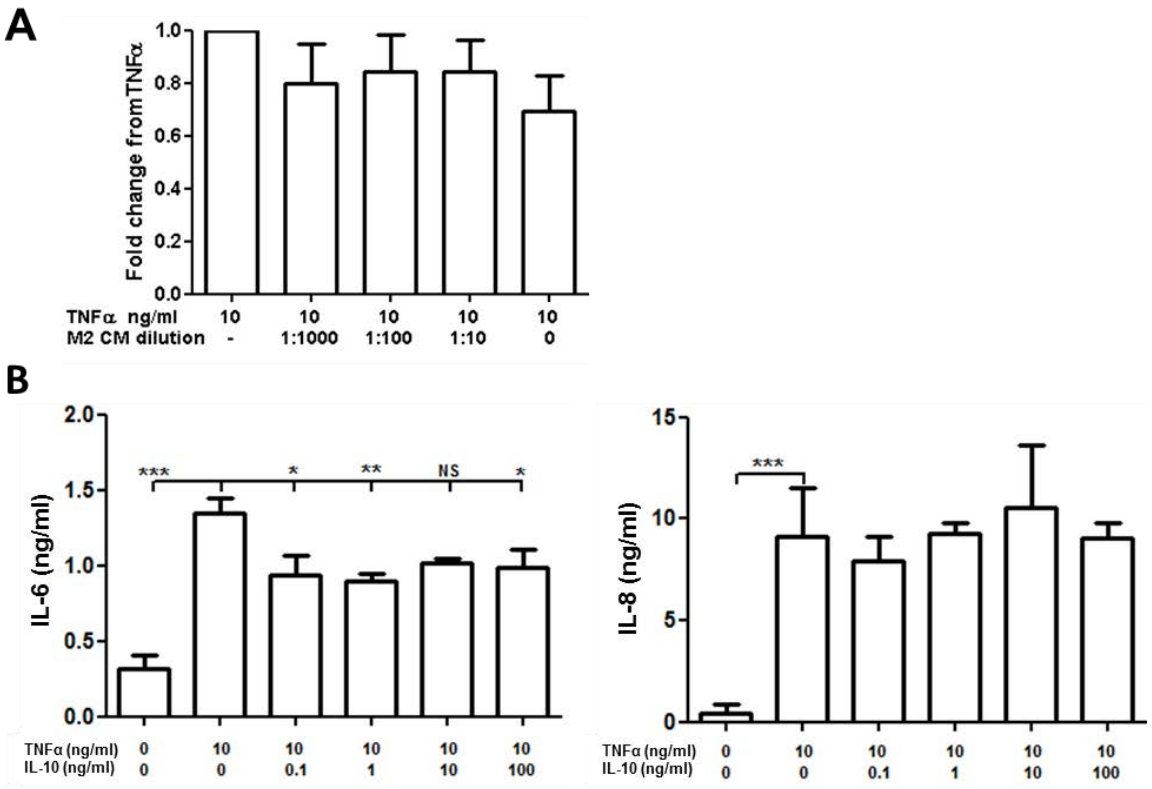

Figure 5.23: Effect of M2 macrophage CM or recombinant IL-10 on fibroblast inflammatory mediators. M2 macrophages challenged with 10ng/ml LPS for 1h were then washed and rested to condition fresh medium for 23h. This CM was added various dilutions to BJ fibroblasts which were simultaneously challenged with 10ng/ml TNFα for 24h. n=4 M2 donors, each on 3 BJ biological replicates. **A** IL-6 secretion represented as fold change from TNFα-induced IL-6 secretion. **B** Effect of IL-10 dose titration on TNFα-induced IL-6 and IL-8 over a 24h period. Mean ±SEM, *p<0.05, **p<0.01,*** p<0.0001, as assesses by One-way ANOVA with Dunnet's post-test against 10ng/ml TNFα. IL-8 n=3, IL-6 n=6.

5.3 Discussion

5.3.1 Site-specificity of innate memory in fibroblasts

My publication showed that FLS mounted an augmented memory response to second challenge with endogenous cytokines. It also showed that HDF from RA and OA patients did not display memory [455]. This, in tandem with data showing no memory in HGF but slight decreases in HDF [313], gave clear evidence for tropism in fibroblasts inflammatory memory.

The data displayed early in this chapter confirmed this hypothesis. The level of IL-6 secreted at rest varied depending on site, suggesting different extents to IL-6's role in homeostasis of different tissues. Comparable basal levels of IL-6 and IL-8 in FLS and HDF conforms to previous publications from our department [176, 544].

Statistical analysis of IL-6 response to initial TNFα challenge revealed significant differences based on site, matching previous literature [176, 544]. It was triggered by TNFα, suggesting IL-6 was part of an inflammatory response. The significant, or near significant, increase upon re challenge in many sites suggested a heightened pro inflammatory response. The extent of the memory response differing according to site may suggest more aggressive fibroblasts in those tissues.

The induced IL-8 and CCL2 responses also exhibited site-specificity. Interestingly, the tonsil and lung fibroblasts varied in the magnitude of their memory responses and share a robust fold induction of all three mediators examined. It is worth considering how a large magnitude of fold change in all mediators may contribute to the biology of these sites.

Tonsil are inherently inflammatory lymphoid structures. Lymphoid organs support the development of lymphocytes, along with other leukocyte populations like DCs which emigrate from the circulation. Neutrophils play roles during inflammatory responses in secondary lymphoid organs like the tonsil [545]. It may be therefore that multiple challenges induce the lymphoid structure to inflammatory secretions in response to persistent danger signalling.

Neutrophils are immediate responders in acute inflammation, and aberrantly prolonged in chronic inflammation. The augmented IL-8 memory response warrants further study therefore, especially functional assays. Prolonged neutrophil influx would perturb the resolution pathway, as it is acknowledged that cessation of neutrophil activity is necessary for resolution to begin [100, 104, 110].

In figures 5.5 and 5.9, I described variable data regarding CCL2 augmentation. This unfortunate disparity makes a conclusion difficult. The secreted concentration of CCL2 in each figure was robust, suggesting a key role for this mononuclear chemokine, but without consistent findings it is impossible to state whether it is augmented or not. A role for increased CCL2 would certainly fit with the theory proposed in chapter three, wherein I suggested the memory response acts as a progression mechanism; driving the inflammatory reaction towards a greater proportion of mononuclear influx and thus the resolution phase. Given time, the disparity in CCL2 ELISA and Luminex results for HDF and FLS will be resolved by reanalysing Luminex samples by ELISA to form stronger evidence for conclusions.

Skin, synovial and bone marrow-derived fibroblasts have been compared regularly in our department, particularly by Filer and Parsonage. They repeatedly showed basal secretions, particularly those of synovial and bone marrow fibroblasts, to be

similar, but stimulation with a range of mediators induced distinctly different concentrations of IL-6, IL-8, GM-CSF, and mononuclear chemokines [176, 544, 546]. Many other groups have shown different fibroblast inflammatory responses based on tropism too. Fibroblasts from tissues including joint, mouth, lung, tonsil, lymph node, skin, and lung have been shown to vary in response to TNF, IL-1, Galectin3, IL-4, IFN, wound induction and *S. aureus* infection [176, 542, 544, 546-549].

As illustrated by the glut of publications in this chapter's introduction and discussion, the concept of fibroblast tropism is by no means novel. What is novel however, is that fibroblast memory (itself a field in its infancy) is not only site-specific in its presence, but the mediators augmented differ according to site.

5.3.2 Innate memory in control and chronic inflamed fibroblasts

Publications have reported differences between control and RA inflammatory mediators, whilst my own data showed little to no difference in absolute quantities secreted by control and RA FLS. This leads me to suggest that "higher in the RA synovium", as is so often reported, is due to the increased number of FLS in the pannus. This idea is supported by figure 5.10, which showed a clear difference in the proliferation rate of control and RA FLS. The combination of my proliferation test and secreted product analysis furthers the theory that "higher in the RA" represents the sum of secreted products of many more cells in the pannus than the healthy synovium.

Nevertheless, reports have specifically shown differences between control and disease fibroblast secretions, such as the base level of IL-6 secreted by FLS [261, 550]. The difference between control and disease fibroblast memory has only

been studied in one paper (my own [455]), and a comparison based on site and disease state has not been published. It is interesting that only by excluding FLS from the data can a difference based on disease-state be shown to be significant. This suggests that FLS are inherently inflammatory, displaying memory even when isolated from joints with no obvious inflammation.

The augmented response in dermal and gingival fibroblasts from chronically inflamed tissues suggests acquired memory. The comparison of control and disease FLS and HDF reinforced my theory ‘inherent to the joint, acquired in the skin’. IL-8 did not alter in the second response, nor did it differ in concentration between control and RA FLS. It did increase (albeit non-significantly) in both concentration and fold change in the psoriasis HDF. As stated earlier, neutrophils are correlated with lesion-expansion in plaque psoriasis [465], and skin flakes from psoriasis patients show high levels of neutrophil chemo attractants [468, 469].

This result suggests fibroblasts contribute to psoriasis (a fact that is often overlooked in a disease considered to be driven by epithelial cells and keratinocytes). Analysis of other mediators, like CCL2 and CCL5, are both high in psoriasis keratinocytes [471, 472], and both significantly augmented in the memory response of psoriasis HDFs. Further to this, the initial response of psoriasis HDFs was not significantly higher than control HDFs, but the concentrations secreted in the memory response were significantly higher than those of control HDFs under re challenge. This suggests that repeated stimulation induces psoriasis HDFs to increase monocyte chemo attraction; a fact which may have therapeutic possibilities, and which reinforces my theory on memory.

Both CCL2 and CCL5 were significantly augmented in FLS in the Luminex study (the disparity between this and earlier data with regard to CCL2 has already been discussed). Neither differed between control and RA FLS, although the initial response to TNF induced noticeably more CCL2 in RA FLS, perhaps representing a greater propensity for monocyte recruitment (which would match data on the huge number of monocytes in the RA synovium [46, 172]).

Neither the increase in GM-CSF nor CXCL10 reached statistical significance in psoriasis HDFs, but both were clearly augmented. Both mediators could play crucial roles in psoriasis, as the former facilitates monocyte differentiation into M1-like macrophages and prolongs neutrophil life *in vitro* [177], whilst the latter recruits T cells, with obvious ramifications given the T cell-dominated infiltrate in plaque psoriasis [465, 473-475, 477, 478].

Both were similarly increased in the control and RA FLS re challenge, once again showing an inherent trait in the synovium. As with the skin, significantly increasing either could be important in the joint, as GM-CSF is higher in the RA synovium than that of controls [551], and GM-CSF-/- mice are immune to CIA [552]. CXCL10 was shown to be superinduced in the innate memory paper of Sohn et al [324], and confirmed in both control and RA FLS in my own [455]. Whilst both mediators were expressed at low concentrations, this does not invalidate biological relevance. Indeed, GMSCF was shown to keep neutrophils alive longer at 10pg/ml [176].

CCL21 was either below, or only just above, the range of detection in HDFs, but was detectable even in unstimulated FLS. This chemokine is usually considered in lymphoid structures (reviewed in [542]), and has also been shown to recruit CD8+

γδT cells [553]. CCL21 was augmented by both control and RA FLS. The lack of significance in the former appears a mathematical rather than a biological one, as the average fold change was actually higher than that of RA FLS.

The fact that tertiary lymphoid structures form in the chronically inflamed synovium may point towards this augmented secretion being a pathological step towards chronicity. Repeated challenge, or chronic exposure to stimuli, is thought to induce chronicity in a number of diseases. The increase in CCL21 may represent a propensity towards ectopic lymphoid structure development under recurrent inflammatory pressure, although this obviously requires further testing.

At the start of this chapter I suggested that innate memory may be inherent to the joint but aberrantly acquired in other tissues. The similarity between control and RA FLS across a range of mediators, and of psoriasis HDFs to FLS certainly appears to confirm this. The link between inflamed skin and synovium is a cogent one, as around one third of psoriasis patients develop arthritis, and are re diagnosed as psoriatic arthritis (PsA) patients [554].

This disease represents a breakdown of the tissue specificity of chronic inflammatory diseases (outlined earlier in this chapter). PsA is distinct from RA (reviewed in [555]) and yet the joints of patients suffering either condition have similar radiographic damage and show upregulation of IL-6, TNFα and IL-1β [556]. Indeed, van Kuijk and Tak stated that the conditions “showed more similarities than differences” [555]. This is particularly interesting in that the synovium of PsA patients recruits skin-specific γδT cells, suggesting an erroneous stromal address code contributing to pathology in the ‘wrong’ tissue.

Alongside the aberrant recruitment of leukocytes, research has proven FLS from inflamed joints may migrate to uninvolved joints to instigate polyarthritis [260]. If migration may instigate inflammation at a secondary site, it may be possible for psoriasis HDFs to migrate into underlying joints. There is already a precedent for this, as not only can FLS migrate between joints to spread disease, but a review by Fries et al from 1994 suggested a pathologic subset of fibroblasts migrate to the skin to trigger skin inflammation in scleroderma [235]. Given the inherent inflammatory traits of FLS, this may provide a novel trigger for arthritis in psoriasis patients.

The attempts to confirm the underlying mechanism of memory in psoriasis HDFs showed evidence for prolonged NFkB activity in psoriasis HDFs compared to control HDFs in the second challenge. The immunofluorescent test showed the transient localization of NFkB p65 in the nucleus following initial TNFα. The similarity between control and psoriasis HDFs correlated well with IL-6 response to initial challenge. Similarly, the limited evidence for strong nuclear localization in control HDFs following second challenge matched the slight reduction in IL-6 compared to initial response, whilst the prolonged response of psoriasis HDFs matched the augmented secretion of various pro inflammatory mediators in the second response. Confirming this result with an MLN experiment (as in chapter 4) would be a useful validation.

5.3.3 In vivo use of conditioned medium from memory responses

Given the abundance of data regarding increased cytokine and chemokine secretion in the memory response, a natural progression was to test the effect of increasing chemokine release on leukocyte recruitment. The theory I suggested in

the discussion of chapter three could be supported by evidence of a shift in infiltrate bias towards increasing mononuclear recruitment. My attempts to confirm this with Boyden chambers did not progress as hoped, and I had no usable data for this thesis. Instead, following the finding that murine FLS also augmented IL-6 in a way reminiscent of their human counterparts, I performed an intraperitoneal injection of murine FLS CM to look for differences in infiltrate composition.

The effects of zymosan alone were in keeping with previous reports on its use in C57/BL6 mice peritoneum for the same duration (total infiltrate, neutrophil, macrophage and lymphocyte counts all match previous reports [325, 557]). This suggests that the experimental procedure and execution were valid.

A flaw of using the murine FLS rather than the human was that their secretions had not been well characterized (only IL-6 was analysed), and so it is possible that chemokines were not altered in the memory response as seen in their human counterparts. This would however, only account for the lack of difference between initial and memory response CM, not the lack of increased induction compared to control injections. The implication is that murine FLS CM has no chemotactic power whatsoever. It is unlikely that these cells produce no chemokines (and work from other in our department show abundant chemokine secretion in mouse FLS). Even working solely with my murine FLS data, I would expect increased recruitment of leukocytes. IL-6 (secreted at nanogram quantities by my murine FLS) acts as a chemo attractant of monocytes [558] and T cells [559] *in vitro* at concentrations relevant to this study. Further, the recruitment of leukocytes was shown to be dose-dependent, and therefore the six-fold increase in IL-6 in the memory response CM should have resulted in discernible differences.

The only significant difference was in γδT cell infiltrate at 48h. Whilst all the aforementioned reasons suggest this result is not trustworthy, it is worth noting the importance of γδT cells in inflammation. These cells are associated with a number of chronic inflammatory diseases (reviewed in [208]). Their role is, however, confusing. Inhibited prophylactically, they significantly delay the onset of CIA, and yet inhibition during the time course immediately induces an increase in arthritis severity [209]. Clearly, they have both pro and anti-inflammatory roles [210, 211], but the tenuous nature of the evidence linking them to my research means drawing a conclusion for their role in fibroblast memory is impossible.

5.3.4 Consequences of fibroblast memory on leukocytes

The effect of fibroblasts on recruitment, differentiation and effector function of leukocytes has been studied to some extent. Studies of chemotaxis are the most common, and have shown fibroblasts from a range of tissues and diseases are capable of recruiting a range of leukocyte subsets [190, 228, 250, 266, 560-565]. Some of these studies compared healthy and disease cell recruitment, such as comparison of infiltrate in different arthropathies (the role of fibroblasts is heavily implied, although the data is clinical rather than co culture in nature) [564, 565]. Other studies can be compared to draw conclusions about disease state, such as one showing scleroderma HDF increase the infiltration of T cells through endothelium [562], whilst another showing non-inflamed skin fibroblasts inhibiting leukocyte adhesion and transmigration through endothelium [228].

Direct comparisons have shown site and disease differences. Research in our department has shown control FLS and HDFs inhibit EC adhesion of leukocytes, and migration of the latter through the former. RA FLS however, increase

leukocyte adhesion to and migration through EC. This difference was due to the relationship of IL-6 with TGFβ to suppress EC function, and IL-6 combination with other factors to increase it [228]. This effect on EC was also reported by another group studying myofibroblasts in chronic liver disease [560]. In systems lacking EC intermediaries, the same pattern of RA FLS increasing recruitment (specifically of T cells) was due to their increased secretion of CXCL12 [250]. RA FLS stimulated with Cyr61 (an ECM protein increased in the RA synovium and known to induce FLS proliferation) increase IL-8 and thus recruit neutrophils [266].

Fibroblasts effect leukocytes in other ways than simply recruitment. Related studies from our department showed that resting FLS, BMDF and HDF had similar efficacies in keeping neutrophils alive, which was improved (particularly in FLS) by TNF-induced GMSCF release [176], and that RA FLS release GM-CSF, thus keeping neutrophils alive twice their usual span [177]. Komatsu et al also showed RA FLS to secrete high levels of IL-6, which induce $CD25^{lo}$ T cell progenitors to differentiate into Th17 cells, which released IL-17 to induce RA FLS to release more IL-6 [200]. RA FLS have also been shown to directly affect one third of macrophage inflammatory genes, and the authors posited the effects on genes relating to differentiation into alternatively-activated macrophages may play a role in RA pathogenesis [271]. This statement should be treated with caution however, as the same paper showed similar effects from OA FLS and lung fibroblasts.

5.3.4.1 Effect on monocyte/macrophages

Attempts at Boyden chamber experiments to assess chemoattraction never progressed beyond technical attempts, and there was not time to conduct fibroblast-EC co cultures. Nevertheless, some of the results regarding fibroblast memory altering leukocyte behaviour appear compelling.

The functional studies of effector function in M-CSF- and GM-CSF- differentiated macrophages match published data regarding their phagocytic function and TNFα secretion (reviewed in [566, 567]). The polar opposite forms of macrophage established a framework within which the effect of fibroblasts could be studied.

To my knowledge there are limited publications regarding fibroblast effects on macrophages (other than chemoattraction tests). This niche led to the PhD project of Jason Turner, who is studying the effects of FLS on monocytes in co culture. He has shown alterations in macrophage gene expression (unpublished data), which is concordant with that of Donlin et al [271]. Given the range of inflammatory mediators shown to be secreted by both tonsil and dermal fibroblasts in earlier figures, it is not surprising that they should keep monocytes alive roughly as well as the differentiation mediators, or induce robust TNFα release upon challenge.

What is surprising is that the memory response CM of HTF should induce such an increase in TNFα secretion from pHrodo-challenged macrophages. A caveat of this experiment is that it was not possible to exclude TNFα from the fibroblast CM, and figure 3.7a shows that TNFα was more than likely present in the CM used to differentiate monocytes. That being said, the levels of TNFα in the first and second response CM did not differ, and so cannot account for the significant increase in TNFα present in the macrophage CM. Further, the fibroblast CM was washed clear of the macrophages before pHrodo challenge, and so should not have contributed to the levels seen in figure 5.17.

Previous publications have suggested M1-like cells are TNF^{hi} poor phagocytes, whilst M2-like cells secrete less TNF and are good phagocytes [566, 567]. This was confirmed by figure 5.16. The number of phagocytosis-capable cells also

increased in the memory response-differentiated macrophages suggests that the same cells were not responsible for phagocytosis and hi TNFα secretions. This implies that different populations in the same well were responsible for increased phagocytosis and extracellular TNFα.

This heterogenous population makes sense given that the HTFs will secrete a mixture of differentiation factors, rather than only M-CSF or GM-CSF (as in the control wells). Taken together, these data appear to show the memory response of HTFs induces an increase in functionality of both ‘M1-skewed’ and ‘M2-skewed’ macrophages, but with a bias towards the TNFα-producing M1-like cells. This would match *in vivo* data from the Gilroy lab. They showed macrophages from a resolving episode (‘rM’) would be converted towards an M1 phenotype when donated into a chronic wound [557]. rM are not M2-like cells, again showing the ‘mixed phenotype’ seen in vivo, or when CM is used to differentiate monocytes.

The limitations of the equivalent study with dermal fibroblasts were mentioned in the results section. The similarity between M1-like and M2-like macrophages in their functional readouts meant the experiment was unlikely to be trustworthy. It may be that the low concentrations of secreted mediators, or the significant but not huge fold changes in the memory responses, could not provide the monocytes with strong enough signals to provide the compelling results seen of the HTF CM. Given the importance of macrophages in psoriasis [466] however, provides enough incentive to repeat this experiment if the opportunity arises.

5.3.4.2 Effect on T cells

The effects of fibroblasts on T cells have been presented, and vary according to both site and disease state. Importantly for this study, the role of fibroblasts in T cell behaviour is at least partly via secreted mediators [190, 200, 228, 250].

Frequently inhaled aerosols mean that the response in the lung must needs be suppressive. Given that it is not a sterile organ even in healthy individuals this is not surprising (consider the tight control over immune reactions in the gut for example). With this in mind I was interested to see that HLF CM very slightly reduced the number of IL-10+ (most likely Treg) CD4+ cells, and yet increased the number for IFNγ+ (Th1) cells compared to activation beads alone. In comparison, the memory response noticeably decreased the IL-10+ cells, and severely decreased the IFNγ+ cells. Whilst speculation only, this may suggest an initial response to stimulus, but an immunosuppressive effect of fibroblasts following continued challenges. Certainly, reducing the IFNγ+ population makes sense in this context, but decreasing the IL-10+ population does not.

The literature on the effect of HLF on T cells is inconclusive. Two studies using co culture of lung fibroblasts with CD4+ T cells were diametrically opposed; one finding a pro inflammatory pairing of increased Th17 differentiation and increased fibroblast proinflammatory cytokines, whilst the other found a decrease in T cell activation markers and intracellular TNFα and IL-10 [568, 569]. The former used asthma patients' cells whilst the latter used tumour-adjacent fibroblasts. Whilst this could explain the difference in their results, I used a mix of tumour-adjacent and COPD samples, which would presumably have opposing results in my system.

In comparison to the apparently suppressive effect of memory in HLF, the effect of RA FLS was a small increase in IFNγ+ cells. IFNγ is known to be high in the RA synovium and the main Th subset responsible for its production (Th1) is considered a major T cell population in the synovium [190].

Whilst this was not a chemo attraction experiment, the increase in this subset is interesting, as RA FLS preferentially recruit Th1 over Th2, and predominantly use CCL5 to effect this (elucidated by inhibition of CCL5, CCL2, CXCL12 and CXCL10) [190]. CCL5 is highly expressed by RA FLS and increased over four-fold upon second challenge. The attractive theory is that the memory response of RA FLS increases Th1 polarization, thus contributing to the infiltrate seen in the synovium. It is important to note however, that the concentrations and fold change of CCL5 by RA FLS was matched by the non-inflamed counterparts, and that the increase in IFNγ+ cells did not reach significance.

Attempts to examine T cell TFs and thus polarization were abandoned due to poor staining and in favour of cytokines, which display a clearer picture of the effector response of cells. T cells may be plastic [206, 207, 570] (distinct from the progenitor differentiation described earlier [200]) and therefore cytokines give more useful information than TFs.

With hindsight, it may have been more appropriate to conduct this study with naïve T cells only, as even using cytokines as a readout was biased by Th subset (i.e. the overwhelming IL-10+ population shown in figure 5.19). Memory T cells may have entered this experiment with designated TFs and therefore Th subset delineations. Further, Th cells have been shown to obtain cytokine memory on a cell by cell basis [571], meaning the modest changes I saw in the FLS CM test

may have been the result of naïve T cell changes in cytokine response only. Whilst reported plasticity may induce change (such as IL-17+ Th1 [572]) I did not have the time and expertise to properly elucidate this transition in my experiment. On a more prosaic note, this study was conducted as and when spare CD4+ T cells became available, and such limiting it to only naïve cells may have reduced the available cells to unpracticable numbers.

5.3.5 Other mediators' effects on fibroblast inflammation

The final set of results was intended to display the effects of leukocytes on fibroblasts, as interactions are never one way. Given the large amount of data in this thesis and in the literature suggesting fibroblasts lack off switches, I wondered which cell type or mediators they rely on to perform this role for them.

The concept of a negative feedback loop of neutrophil sIL-6R and fibroblast IL-6 was a solution to the question of how the balance is tipped towards resolution. Data has shown the apoptosis of neutrophils to be a 'tipping point' in an inflammatory episode, facilitating the progression towards mononuclear infiltrate [110-112, 268, 573]. My data showed IL-8 was not affected, matching previous data on IL-8 and other neutrophil chemo attractants [573, 574]. Also, my data regarding increased CCL2 secretion match published literature [268, 573]. Whilst this all fits with the hypothesis that neutrophil apoptosis and sIL-6R shedding provides a step to resolution, my data on IL-6 disproved the negative feedback loop hypothesis.

This may have been predicted, as soluble gp130 inhibits IL-6 production induced by trans-signalling [187]. Numerous papers and reviews have claimed *trans* signalling to be the pro inflammatory arm of IL-6 signalling [100, 110, 112, 268,

573], and the fact that its antagonist has such therapeutic promise [187, 575] appears to support this. In this case, the feedback loop of IL-6 must presumably have a positive role in healthy inflammation. There are pro-resolution and regenerative roles for IL-6, but they may be overridden or perverted towards a perpetually inflamed state in the wrong microenvironment.

The effects of M2-like macrophages were limited, but caused a decrease in IL-6 secretion by fibroblasts. As briefly discussed in the results section, M2 macrophages are not strictly anti-inflammatory, and release strong concentrations of TNFα. The M2 CM displayed the ‘less pro inflammatory’ nature of these cells. Direct co culture may confirm the resolving interaction. Fernando et al showed that IL-6 makes M2 macrophages behave ‘more M2-like’; increasing IL-10 secretion and inhibiting CD4+ T cell polarization [576]. Given that IL-6 is secreted by fibroblasts, and augmented upon re challenge, this may actually drive the M2 macrophages to strongly inhibit fibroblast pro inflammatory secretions a feedback loop.

The increased IL-10 secretion may be vital in this regard, as it significantly but incompletely reduced fibroblast IL-6 secretion. Publications have shown various concentrations of IL-10 secretion by fibroblasts [577, 578] and have shown it to be present in the RA synovium [578]. I found it to have no effect on IL-8, agreeing with Seitz [579] but not Katsikis [578]. The limited effect IL-10 had on IL-6 suggests a higher concentration is required, or other mediators are involved in reducing IL-6 production by fibroblasts.

5.4 Conclusion

This chapter (I am sure the reader is aware) was significantly longer than the first two. This was due in part to the similar background literature and goals of the investigations herein, which meant two draft chapters were combined.

The first half of the chapter was a comparison of fibroblast innate memory in cells of different tissues and disease states. The evidence for variation in magnitude and gene-specificity of the augmented response is strong, and as such provides a novel insight into the nuances of tissue-specific stroma.

The differences between control and chronically-inflamed tissue were no less stark when the right site was chosen, and in sufficient numbers to establish robust statistical analysis. The working theory of ‘inherent in the joint, acquired in the skin’ is- as I say- just a theory. It is however, adapting all the time, as the evidence of RA- but not control- FLS increasing IFNγ+ cells has shown.

The *in vivo* data had a clear remit which it failed to deliver upon, and future work would do well to improve upon my attempts at both *in vitro* and *in vivo* examination of the leukocyte recruitment. Nevertheless, examining the effect of memory on monocyte/macrophages and T cells produced significant results. Both have displayed that the fibroblast response to second challenge is not only significantly altered but also has a significant effect on leukocytes.

Finally, the small study into the effect of other cell types and their mediators on fibroblasts displayed the complex nature of the interactions in the inflamed microenvironment. Given time, a more complex investigation would be interesting. In place of this, an illustration of the possible interactions, based on the data presented above, will be provided in the brief discussion chapter hereafter.

6 Discussion

6.1 Addressing the objectives

In the introduction chapter, I set out a project aim, with objectives for each chapter of results. Whilst the data have been discussed in their respective chapters, in this section I will summarise the degree to which each was achieved.

6.1.1 Do FLS mount unremitting responses to- or following-inflammatory challenge?

The work of Angela Lee strongly influenced this thesis, by illustrating an unremitting response and lack of negative feedback mechanisms in RA FLS [272]. I sought to test whether control FLS would also display this phenotype.

The experiments conducted confirmed that control FLS mount unremitting responses to both TNFα and IL-1α.

It also suggests that prolonged responsiveness to TNFα is dependent on continued TNFα presence, once more in agreement with Lee et al [272]. The prolonged response to IL-1α, even after its removal, was not due to residual free IL-1α, as experiments using IL-1RA had no effect on IL-6 secretion. This suggests that IL-1α exerts a profound effect on control FLS. There is an argument for an uncoupling of inflammation and erosion based upon TNFα and IL-1 [580]. This difference in unremitting response after removal of one but not the other cytokine could therefore have interesting repercussions. Unfortunately, this was not examined further, and so remains conjecture only.

6.1.2 Do Fibroblasts alter their response upon second challenge?

This question has been asked more than once in the past, although a series of papers in the 1990s appear to have been forgotten in the intervening years (discussed in a review by myself, Chris Buckley and Andy Clark, manuscript in preparation). Our inspiration for studying memory was actually the concept of endotoxin tolerance in macrophages [273]. I hypothesized that FLS would become refractory to proinflammatory stimuli, as seen in macrophage endotoxin tolerance.

The finding that FLS and a foreskin line actually augmented their second response to stimuli was the central focus of this thesis. From this initial observation, I attempted to study a range of aspects, from intracellular mechanisms to conservation between anatomical locales. The details of these different aspects were assessed in other objectives, but the simple answer to this objective is that fibroblasts display innate memory, which manifests as an augmented response to second challenge.

6.1.3 What is the underlying mechanism responsible for the memory response?

As shown in the introduction for chapter 4, there are myriad pathways and mediators involved in the cellular response to stimulation. These differ based on the stimulus (and its concentration), cell type, microenvironment, and a number of other factors. Figure 4.1 gave a simplified version of the signal transduction from agonist to secreted product, and with more time I would have liked to assess each step in more detail.

Proxy experiments suggested altered receptor abundance was not responsible, although this was not confirmed with analysis of surface expression as would have

been proper. I would also have liked to assess the mechanisms occurring in the nucleus in greater detail, as this has been shown recently to be important in FLS memory [324], in RA [258], and the inflammatory response in general [581]. Similarly, an antibody array to assess the roles of non-canonical NFκB and other TFs may yield valuable information, particularly regarding the gene-specificity of memory.

As discussed by Serhan et al, negative feedback loops are necessary for resolution of an inflammatory episode [582]. These loops would be particularly interesting to examine in fibroblast innate memory, as gingival [313] and synovial [272] fibroblasts appear to lack them, suggesting an inability to switch off inflammatory secretions. As it appeared to be reliant on external factors, I tested medium conditioned by other cells or mediators to assess what *could* turn off fibroblast inflammation. Greater assessment of both the autocrine and paracrine negative regulators would have been interesting, particularly in regard to whether prolonged NFκB was due to decreased expression or function of negative regulators.

Finally, the Achilles heel of my project was studying RNA levels. Without clear results I could not fully answer the question of whether the augmented response induced an increase in transcription, an earlier/ longer transcriptional window (the models proposed in chapter 4), or whether transcript stability was responsible for the augmented response. This latter aspect would have suited our laboratory, and with extra time I would continue this line of enquiry.

Naturally not all pathways could be examined, but nevertheless I found a mechanism that could be illustrated by various techniques (see chapter 4 and

Crowley *et al* 2017 [455]), namely prolonged NFκB activity. The interesting kinetics of MAPK signalling shown in the same experiments were unfortunately not followed up on, although this would have complimented other studies of stromal innate memory [303].

No attempt to test the effects of metabolism were made, despite its role in inflammatory responses [583]. An in-house collaborative project is underway however, and the preliminary data look promising. Whilst too formative to be referenced herein, I hope a publication of the metabolic changes seen in fibroblast memory responses will be available in the near future.

6.1.4 Can this mechanism be manipulated?

Whilst understanding a mechanism is important, the ability to manipulate it is key to translational studies. To progress to that stage, I would have to discern the consequences of fibroblast memory *in vivo*. This was unfortunately not successful in my thesis investigations.

Nevertheless, this objective was achieved, in that inhibition of NFκB after the point at which it had fulfilled its nuclear role in the first response was sufficient to abrogate the augmented response to the second challenge. This means the underlying mechanism of fibroblast innate memory can indeed be manipulated.

6.1.5 Does fibroblast site of origin influence innate memory?

Site tropism was discussed in greatest detail in chapter 5, but examples of site-specific responses were cited in each chapter. Myriad examples display the diversity of fibroblasts [233, 235]. The comparison of fibroblasts from different sites was therefore one of the most interesting aspects of this thesis.

The results were fully discussed in chapter 5, but the answer for this objective is that site does indeed play a role in fibroblast innate memory responses. Perhaps unsurprisingly, the pleiotropic cytokine IL-6 was augmented in most sites. The variation in magnitude of this augmented response ranged from a five-fold increase to a non-significant decrease, which may indicate different requirements for IL-6 in the inflammatory responses of different tissues.

Not only did the augmented response differ from site to site, it also differed according to which inflammatory mediator was assessed. Variation in CCL2 and IL-8 was strong between sites. Different cytokines were shown to be augmented in different sites, and the literature provides evidence for fine-tuning of inflammatory episodes and recruitment of leukocytes, by alterations in chemokine production [190, 251, 267, 325]. Again, the roles of these cells in different tissues/ inflammatory diseases were discussed earlier, but it is worth reminding the reader that neutrophil and mononuclear cell infiltrates often represent distinct stages of the inflammatory response [100, 111, 325]. If IL-8 is augmented by fibroblasts of some sites and not others, it suggests a stronger role for different leukocyte subsets in different sites.

6.1.6 Does fibroblast disease state influence innate memory?

The examination of site variation in fibroblast memory showed differences in magnitude, but also commonalities, such as the increase of IL-6, IL-8 and CCL2 in both tonsil and lung fibroblasts. The role of chronic inflammation in altering cell behaviour is widely acknowledged. Given the samples taken from many sites were from chronically inflamed tissue, the question that arose was whether their healthy, non-inflamed counterparts would behave in the same way.

This was addressed by comparisons of healthy versus psoriatic skin, and RA versus non-inflamed synovial, fibroblasts. The synovial samples mounted similar responses irrespective of disease state. This suggests an inherent proinflammatory phenomenon in the joint, although caution should be exercised when considering the control fibroblasts 'healthy'. The cells may be healthy, in which case fibroblast priming is inherent to the joint. Alternatively, they may be pre-symptomatic arthritis samples (RA-specific auto antibodies and arthralgia may exist years before the onset of synovitis [584-586]). This leads to two intriguing possibilities. If the former is true then FLS are inherently inflammatory, which may help explain why arthridites (including but not solely RA) are so common. If the latter is true, priming may be a useful predictive tool for treating RA patients before their disease becomes irrevocable [178].

The comparison between *bona fide* healthy and psoriasis skin fibroblasts showed a clear difference between health and disease. This unveiled a disease-specific phenomenon, which was maintained across a number of inflammation-related mediators. This comparison would ideally have been made in gingival samples too, but limited healthy samples made this impossible. As posited in chapter 5, the fact that a healthy gingival sample reproducibly didn't show memory, but the PD samples did, would imply a pattern similar to the skin samples.

No conclusion can be made based on the gingival samples, but a pair of testable theories based on the skin samples are proposed. The first is that the chronically-inflamed state of psoriatic skin induces an acquired pathology in the form of fibroblast memory. The other is that people with skin fibroblasts capable of mounting memory responses are predisposed to psoriasis. Given more time, this

latter could be tested, as my collaborator has samples not only from active lesions, but from uninvolved skin from psoriasis patients.

6.1.7 Does fibroblast innate memory alter leukocyte behaviour?

This final objective required the most learning of new concepts and techniques. In chapter 3, I expounded the theory that the increase in mononuclear chemokines but not IL-8 would result in a shift to a mononuclear infiltrate. In chapter 5 I sought to confirm this hypothesis. My attempts to perform trans-well assays did not make it into the thesis, and the *in vivo* tests did not match the hypothesis. The three most likely explanations are: a) the conditioned medium did not include a successful augmented second response, b) the conditioned medium was removed from the peritoneum before the larger amounts of mediators in the memory response could manifest an effect, or c) that the fibroblast augmented response does not alter *in vivo* movement of leukocytes.

This is properly discussed in chapter 5, but the wide array of literature concerning the strong chemotactic abilities of a number of the mediators examined, suggests a technical issue, rather than a biological result. I cannot, of course, confirm this. Given the opportunity, I would repeat this experiment in the air pouch model, which was specifically developed to assess chemotactic effects *in vivo* [325].

The more successful arm of this objective was to assess leukocyte behaviour. Data are provided illustrating the difference in functional abilities of both monocytes and lymphocytes after incubation with fibroblast initial or memory responses. Particular interest should be paid to the effects on monocytes. The tonsil fibroblast conditioned medium from the memory response appeared to

increase both TNFα secretion and phagocytosis. Further examination could reveal whether distinct populations of macrophages were induced [46, 53].

This not only confirms that fibroblast memory does play a role in leukocyte behaviour, but also suggests that this can be contact-independent. Fibroblast effects on other cells can be exerted by both contact dependent and independent mechanisms [176, 177, 228, 253]; therefore future exploration would involve direct co-culture. This objective was achieved, in that evidence shows the fibroblast innate memory response alters leukocyte behaviour.

6.2 Future directions

6.2.1 Mechanistic investigation

As discussed, the mechanisms underpinning fibroblast memory have only been examined in a very few publications. There are still many unanswered questions, and a better understanding of the molecular mechanism would help to develop therapeutics if fibroblast memory was proven to be pathological.

Particular attention in such research should be paid to negative regulators and epigenetic markers. The former could be under represented during the memory response [272, 313], whilst the latter may be responsible for fine-tuning the memory response [324]. There is also a lack of molecular studies with regard to TLR agonists in fibroblast memory, and given the strength of literature around this in monocytes (and ECs to some extent), research into this field would have the advantage of inter-cell comparison.

6.2.2 Memory contributing to chronic inflammatory disease

The theory suggested above of fibroblast memory being pathologically acquired could be a novel therapeutic opportunity. If fibroblasts do acquire memory in chronically-inflamed sites then inhibition would be of an inflammation-propagating mechanism. For example, CCL21 was raised in disease but not healthy dermal fibroblasts, and is used in lymphoid structure formation [543]. Its aberrant regulation in psoriatic skin could increase recruitment and polarization of lymphocytes.

This field could be progressed by better healthy-disease comparisons. It is difficult to acquire healthy lung samples, for example. Use of recently post-mortem samples may be a suitable method of acquiring ‘healthy’ tissue for proper comparison. If multiple sites displayed the acquired memory phenotype, this could represent a target in multiple diseases. As shown in figure 6.1, every tissue has its accompanying inflammatory disease, and all tissues have fibroblast populations. It is possible that distinct subsets of fibroblasts in a tissue drive pathology [242]. This concept could be (and indeed is being) applied to innate memory in fibroblasts. Inhibition of fibroblast memory could therefore be used to treat multiple diseases, and would only affect tissue-resident cells. This would also have the advantage of leaving the patient immunocompetent.

Data from the cross-over of clinical and *in vitro* settings has already shown stromal cell memory may increase the risk of atherosclerosis [306, 307], and thus the concept of fibroblast memory contributing to inflammatory disease is not revolutionary. Naturally, therapeutic targeting is a long way off, but a manuscript

based on the results shown in chapter 5 is in preparation, and may provide evidence for progression of this field.

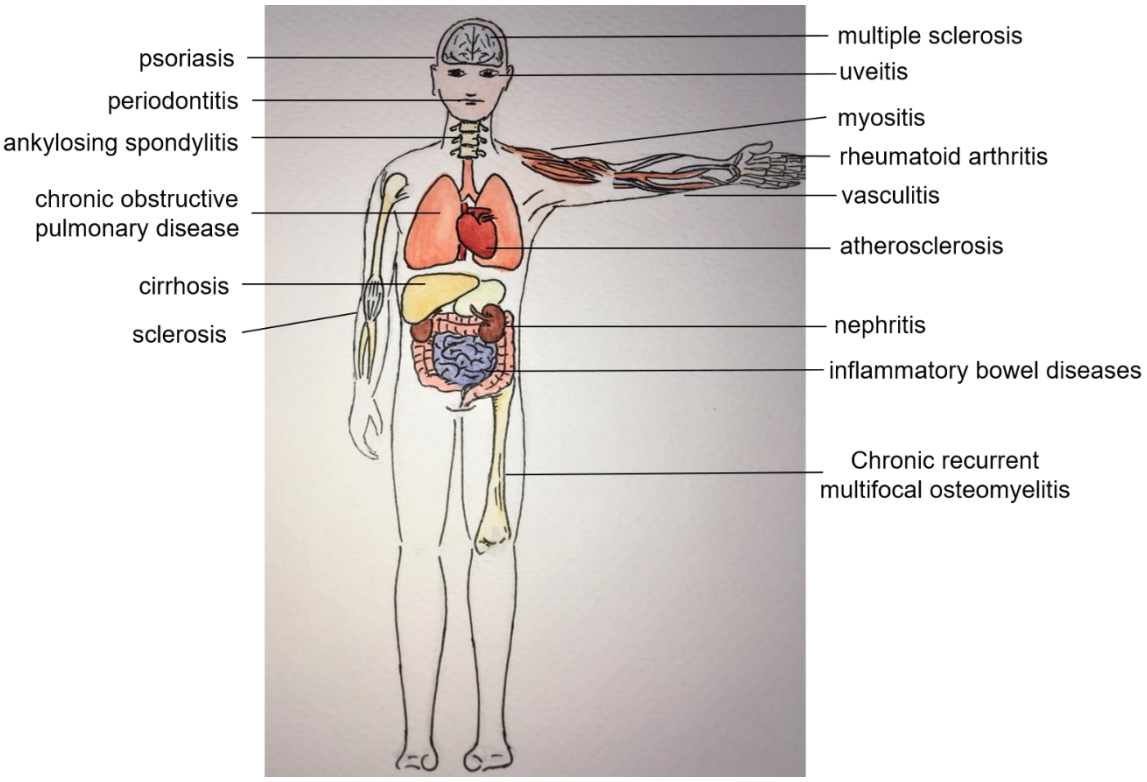

Figure 6.1: Illustration of chronic inflammatory diseases. Cartoon showing the tissues affected by different chronic inflammatory diseases.

Memory as a tissue-wide phenomenon

Another future direction for this field is a more holistic approach. Myeloid lineage memory has been studied *in vivo* for decades, albeit in the context of infectious disease. My personal interest would be to assess the inflammatory memory of whole tissues. Lymphocytes were originally thought to be the sole repository of memory in mammals, but the distinct roles of innate and adaptive immunity are being reconsidered. Lymphocytes are now ascribed innate functions (i.e. those of γδT cells [208, 212]), whilst innate cell memory is receiving increasing attention. Monocytes, macrophages, natural killer cells, epithelial cells, ECs and fibroblasts have all been shown to exhibit memory [303, 455, 587]).

The natural history of infection in the lung has been posited as a signature which dictates the outcome of future inflammatory events [588]. Combining this concept with the study of each tissue-resident cell type that displays memory would offer a new approach to understanding the natural history of our inflammatory episodes and outcomes.

6.3 Summary model of fibroblast innate memory

In this thesis, I have explored the under studied concept of fibroblast innate memory. In chapter 3 I aimed to characterize the phenomenon, in chapter 4 I sought to elucidate an underlying mechanism, and in chapter 5 I examined both its anatomical extent and its wider consequences.

Combining the results herein with those in the fields of stromal memory, fibroblast biology, and rheumatology allows me to present a model of fibroblast innate memory. This is of course speculative, and given more time I would have liked to have confirmed some of the predictions with direct experiments.

The unknown quality that has the greatest bearing on whether my model is realistic is whether fibroblast innate memory is a long-term or short-term protective mechanism. These two concepts are distinct in monocytes (trained immunity and endotoxin tolerance, respectively [282]), and occur in different inflammatory scenarios. Stromal memory in non-infectious contexts has been suggested as a long-term priming towards greater inflammatory reactions [307], whilst the short-term nature of repeat challenges is more reminiscent of adaptations to continuing inflammatory events seen in haematopoietic and stromal cells [302-304, 324, 587].

To my knowledge, no long-term studies of the kind favoured by Mihai Netea (Kleinnijenhuis 2012, Arts 2016) have been examined to ascertain the presence of trained immunity in fibroblasts. Also, my results showed a transiency to fibroblast memory, albeit *in vitro*. This means I have no support for a model of fibroblast memory protecting against subsequent infections (although Dakin et al [589] hypothesized this for sterile injury). Instead, I favour the adaptation idea, that fibroblast memory may be a mechanism of altering the tissue response as an inflammatory insult occurs. This is already accepted in the field of macrophage endotoxin tolerance, and is supported by data illustrating the plasticity of differentiated macrophages and T cells as microenvironmental cues alter [43-45, 207].

Below is set forth a model of the consequences of fibroblast innate memory (based on the synovial fibroblast, as appropriate for a project conducted in a rheumatology department). I have also accounted for the proinflammatory conditions of the RA synovium, in order to posit the effect of fibroblast innate memory in the setting of a chronic inflammatory disease.

In an instance of infection or injury, fibroblasts recognize DAMPS and PAMPs and respond by releasing a range of mediators including IL-6 and chemokines. The chemokines recruit leukocytes, with neutrophils arriving first, followed by monocytes and lymphocytes [325]. Whilst inflammatory stimuli induce ECs to upregulate adhesion molecules, FLS IL-6 and TGFβ inhibit EC adhesion of leukocytes and forward migration once the leukocytes have extravasated [253]. Nevertheless, leukocytes infiltrate tissue, and neutrophils release inflammatory factors [69, 173, 180] which act the second challenge for FLS primed by the injurious stimulus. These FLS will therefore upregulate mononuclear chemokines and IL-6, but not IL-8 [455].

The monocular leukocytes recruited by the initial FLS response will reach an inflammatory microenvironment, but the timely apoptosis of neutrophils would result in a reduction in proinflammatory mediators, would induce macrophage differentiation into pro-resolution cells [70], and would provide sIL-6R for trans-signalling [112]. Trans-signalling would increase FLS release of IL-6 and CCL2 but not IL-8 [100, 109-111]. Augmented IL-6 release would further limit the infiltration allowed by EC, whilst the increase in mononuclear cell chemokines would result in a higher proportion of infiltrating cells being mononuclear. The increasingly pro-resolution milieu would support a progression to the altered homeostasis seen following acute inflammation [590]. All of this is illustrated in figure 6.2.

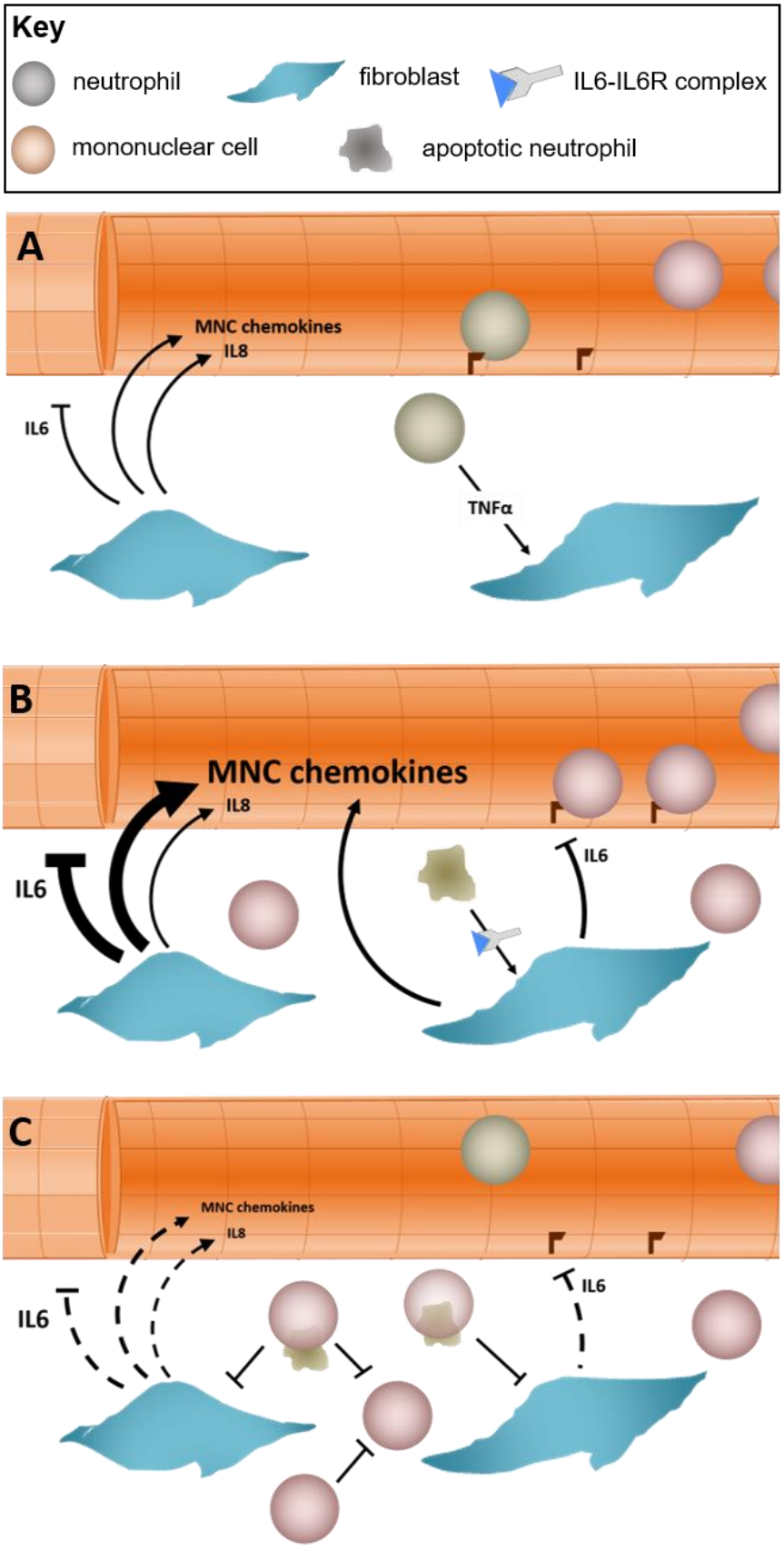

Figure legend overleaf

Figure 6.2: Model of fibroblast memory contributing to the inflammatory response. A An initial insult leads to fibroblast release of mediators which induce recruitment of leukocytes, and modulate adhesion to an extravasation through endothelial cells. Neutrophils supply the second stimulation to fibroblasts. **B** fibroblasts augment IL-6 and MNC chemokines to increase MNC recruitment whilst restricting total infiltration. Apoptotic neutrophils facilitate trans-signalling to increase IL-6 and MNC chemokine release. **C** the MNC infiltrate removes apoptotic neutrophils and provide anti-inflammatory and pro-resolution mediators to end the fibroblast inflammatory response. Key provided. Thickness of arrows represents magnitude of mediators. MNC= mononuclear cells.

In the chronically inflamed setting of RA, numbers of FLS increase [160, 163]. The increased number means a greater concentration of chemokines and IL-6. RA FLS soluble mediators act on ECs to increase their permissive state with regard to leukocyte infiltration [228]. Neutrophils would again provide the second challenge, but the large quantities of survival factors in the RA synovium (released by FLS) would keep neutrophils alive [177, 178]. This would maintain the proinflammatory milieu [175] and not provide the ‘off switch’ for macrophages normally induced by efferocytosis of neutrophils [591].

The large quantities of survival and proinflammatory factors would maintain an inflammatory milieu [37, 46, 101, 109, 165], which induces proinflammatory polarization of myeloid and lymphoid cells [44, 46, 49, 106, 195, 200, 207]. This would combine with RA-associated phenomenon such as deficient Tregs [205], auto antibodies [592] and spontaneous NETosis of neutrophils [182] which are already primed for inflammatory responses [181]. All of this would make reduction of the inflammatory milieu and removal of neutrophils very difficult, thus propagating the inflammatory response. This is summarized in figure 6.3.

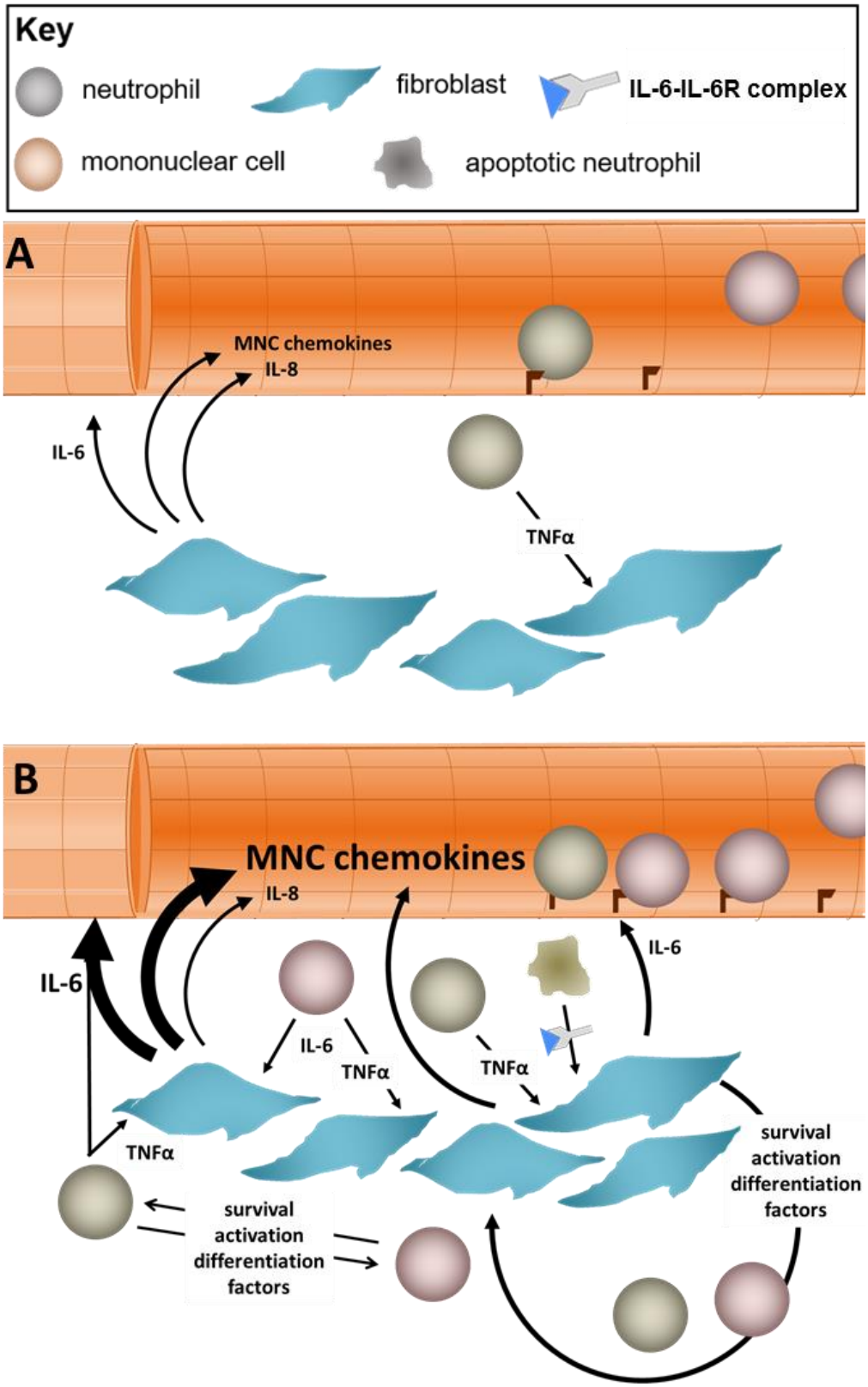

Figure legend overleaf

Figure 6.3: Model of fibroblast memory contributing to chronic inflammation. **A** An initial insult induces fibroblasts to release mediators to recruit leukocytes and encourage endothelial cell-leukocyte adhesion and extravasation. Neutrophils provide a second challenge for fibroblasts primed by the initial insult. **B** Fibroblasts upregulate mediators and MNC chemokines to encourage further extravasation. Some neutrophils become apoptotic and facilitate trans-signalling to increase MNC infiltrate, whilst others continue to release proinflammatory stimuli. Prolonged neutrophil action leads MNC differentiate into proinflammatory subsets. Regulatory cells are dysfunctional and unable to dampen inflammation. Chronic inflammation is propagated. Key provided. Thickness of arrows represents magnitude of mediators. MNC= mononuclear cells.

As previously stated this model is far from proven but I do not believe that it is based too heavily on suppositions. Whilst it does not of course account for every detail of the complex interactive process of inflammation, I offer it as a possible role for fibroblast memory. Perhaps future research will go on to refute or corroborate the ideas set forth in it.

6.4 Final remarks

This project, like all projects, has adapted and evolved across the four years of its duration. I have addressed many questions, but have ended with far more than I started with. They say a good project is never finished, and I sincerely hope that I and others can continue to build upon the results presented herein. I have shown that fibroblasts have innate memory, at least in part due to prolonged NFκB activity. It is gene, site, and disease-specific, with consequences on leukocyte behaviour. The fact that fibroblasts have memory was not widely publicised before I started, and the other findings were novel at the time of their discovery. I hope the effort invested, and the standard and novelty of these findings convinces the reader of their value.

The field of stromal innate memory is not new, in fact it spans at least 27 years. It is however, sparse, and an informed consensus on any aspect is lacking simply by lack of publications. I cannot therefore fit my research into a well-formed niche in an established field. Instead, I add it to the knowledge of this community in the hope that it provides one more small piece in the puzzle of inflammation biology.

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