THE PRESENCE OF C-REACTIVE PROTEIN (CRP) ISOFORMS DURING MURINE WOUND HEALING

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Declaration

With the exception of any statements to the contrary, all the data presented in this report are the results of my own efforts and have not previously been submitted in candidature for any other degree or diploma. In addition, no parts of this report have been copied from other sources. I understand that any evidence of plagiarism and/or the use of unacknowledged third party data will be dealt with as a very serious matter.

Julia Hynes 16 May 2013

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AP-1	Activator protein 1	23
CHD	Coronary Heart Disease	22
CRP	C-Reactive protein	13
CVI	Chronic venous insufficiency	21
D14	Day 14	13
D21	Day 21	13
D3	Day 3	13
D7	Day 7	13
ECM	Extracellular Matrix	16
EDTA	Ethylenediaminetetraacetic acid	26
EGF	Epidermal Growth Factor	17
EOC	Endothelial Outgrowth Cells	24
ERK	Extracellular signal-related kinase	62
FcγRI	Fc gamma receptor 1	23
FcγRIIA	Fc gamma receptor 2A	23
FGF	Fibroblast growth factor	17
FGF2	Basic fibroblast growth factor	16
GMCSF	Granulocyte macrophage-colony stimulating factor	61
HUVEC	Human Umbilical Vein Endothelial Cells	24
ICAM	Intracellular Adhesion Molecule	15
IFNγ	Interferon γ	15
IL-1	Interleukin 1	20

Abbreviation	**Meaning**	**Page**
IL-1β	Interleukin 1β	24
Il-2	Interleukin 2	61
IL-6	Interleukin 6	20
IL-8	Interleukin 8	15
KGF	Keratinocyte growth factor	16
LPC	Lysophosphatidylcholine	22
MCP-1	Monocyte chemoattractant protein 1	15
mCRP	Monomeric/modified C Reactive protein	13
MMP	Matrix metalloproteinases	16
nCRP	Native C Reactive protein	13
NFκβ	Nuclear factor kappa beta	23
OVX	Ovariectomised	52
PAF	Platelet activating factor	63
PAI	Plasminogen activator inhibitor	16
PDGF	Platelet derived growth factor	15
RBC	Red blood cell	63
ROS	Reactive oxygen species	15
TBS	Tris-buffered saline	27
TGFα	Transforming growth factor α	17
TGFβ	Transforming growth factor β	15
TIMP	Tissue inhibitor of metalloproteinases	17
TNFα	Tumour necrosis factor-α	15

VEGF	Vascular endothelial growth factor	16
α1-PI	α1-proteinase inhibitor	20
α2-M	α2-macroglobulin	20

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Abstract

Native C reactive protein (nCRP) is a pentameric oligoprotein composed of 5 identical protomers. Levels of this acute phase protein increase up to 1000 fold with the onset of inflammation; dissociation into single subunits creates a new isoform known as modified/monomeric CRP (mCRP). To date there has been no investigation of the presence and role of CRP in cutaneous wound healing. Our work presents a temporal and spatial profile for the presence of both CRP isoforms from 6 different time points post-wounding spanning 3 major phases of the wound healing process. Wax embedded murine (BALB/c) wound samples were sectioned and stained by immunohistochemistry with monoclonal antibodies (mCRP; 8C10 and nCRP; 2C10) specific to each isoform. CRP-positive cells were quantified by microscopy and expressed as a ratio of the total cell count to allow comparison between samples. During the acute wound healing process significant differences between expression of each isoform were found at day 3 post-wounding (D3) in inflammatory cells ($p=<0.0001$), and at both D7 and D14 in blood vessels($p=<0.0001$). Moreover, expression of each CRP isoform was dependent on cell type and time point post-wounding. Expression of both isoforms decreased significantly in inflammatory cells between early and late inflammation (D3 and D7) ($p=<0.0001$). A similar pattern was seen in fibroblasts between D14 and D21, indicative of the late proliferative and early tissue-remodeling phases. However, levels of nCRP increased significantly in blood vessels between D7 and D14 ($p=<0.0001$), compared to no significant difference in mCRP expression.

We also investigated CRP expression in the early inflammatory phase (D3) of an acute (intact) and delayed (ovariectomised) model of murine wound healing and found mCRP to be expressed

significantly higher ($p=0.013$) than nCRP in the delayed (impaired) model of wound healing. However, there was no significant difference in mCRP expression between the acute and impaired healing models but rather a significantly decreased expression of nCRP in the delayed model of healing. To conclude the varying levels of expression in a cell type and time-specific manner suggests each isoform may exert differing biological activities important to the wound healing process that warrants further investigation. Moreover, differences in expression between acute and delayed wound healing models suggest CRP isoforms may mediate age-related impaired healing and chronic wounds. Further studies are required to determine the differential effects of CRP isoforms on cutaneous healing processes and the precise roles they may play during wound repair.

1. Introduction

1.1 Pathophysiology of acute cutaneous wound healing

Acute wound healing is a dynamic interactive process involving soluble mediators, blood cells, extracellular matrix and parenchymal cells. It consists of three overlapping phases: inflammation; tissue formation (proliferation) and tissue remodelling (Singer and Clark, 1999).

1.1.1 Haemostasis

The healing cascade begins when platelets adhere to exposed collagen in the disrupted tissue, degranulate and release clotting factors and cytokines. Clotting factors facilitate the formation of the haemostatic plug. Cytokines such as platelet derived growth factor (PDGF) and transforming growth factor beta (TGF-β) initiate chemotaxis of neutrophils, monocytes and fibroblasts, enhance proliferation of fibroblasts and production of extracellular matrix (Werner and Grose, 2003; Diegelmann and Evans, 2004).

1.1.2 Inflammation

Neutrophils are the first predominant cell to reach the wound via diapedesis whereby the endothelial cell wall is activated by proinflammatory cytokines such as interleukin 1-beta (IL1-β), tumour necrosis factor-α (TNF-α) and interferon gamma (IFN-γ.) The endothelial cells are induced to express adhesion molecules crucial for neutrophil infiltration such as P- and E- selectin and intracellular adhesion molecule-1 and -2 (ICAM-1,-2) Adhesion molecules interact with integrins on the neutrophil cell surface helping them to move out of circulation into the extravascular space. Cells are then directed towards the wound via chemokines such as: Interleukin 8 (IL-8), growth related oncogene-α, monocyte chemoattractant protein 1 (MCP-1) and the accumulation of bacterial products such as lipolysaccharides and formyl methionyl peptides (Eming *et al.,* 2007). The function of the neutrophil is to remove foreign material, bacteria, non-functional host cells and damaged matrix components that may be present at the wound site. Neutrophils achieve this by phagocytosis, release of anti-microbial peptides, generation of reactive oxygen intermediates and trapping microbes in extruded nets composed of histones and DNA

(Brinkmann *et al.,* 2004; Theilgaard-Monch *et al.,* 2004). Neutrophil infiltration peaks by day 3 (D3) post-wounding and is followed by an influx of monocytes which mature into larger phagocytic macrophages.

Monocyte infiltration peaks at D7 post-wounding and gradually declines during subsequent phases (Martin, 1997). During inflammation the main function of the macrophage is the removal of dead or dying cells including apoptotic neutrophils via phagocytosis and also the debridement of damaged tissue to pave the way for fibroplasia and angiogenesis (Trowbridge and Emling, 1997). Macrophages are also important producers of a battery of growth factors necessary for subsequent events in the wound healing process such as PDGF, TGFβ, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) which are important for stimulating the proliferation of fibroblasts and endothelial cells, the formation of granulation tissue and the induction of angiogenesis (Li *et al,*. 2007). The importance of the macrophage in the proliferative stage of the wound response has also been reported *in vivo* as depletion results in severe haemorrhage and failure to progress into the subsequent stage of tissue maturation due to impaired wound closure (Lucas *et al.,* 2010).

1.1.3 Proliferation

In this phase of healing cellular activity predominates, with major events including re-epithelialisation, angiogenesis and fibroplasia. Re-epithelialisation is the process of restoring an intact epidermis characterised by epidermal keratinocyte proliferation and migration. To enable migration, around 12 hours after wounding keratinocytes flatten and elongate, develop pseudopod like projections of lemellipodia, lose cell-cell and cell-matrix contacts and retract intracellular tonofilaments at the edge of their cytoplasm. Extracellular matrix (ECM), matrix metalloproteinases (MMPs) and growth factors are implicated in the migration of keratinocytes by providing direction and degradation allowing cells to leave the basement membrane and migrate into the wound. Proliferation occurs near the cells of the migrating tongue ensuring an adequate supply of cells to migrate and cover the wound. When migration ceases keratinocytes re-attach themselves to the underlying substratum, restore the basement membrane

and terminally differentiate to create a stratified epidermis. Epidermal growth factor (EGF), keratinocyte growth factor (KGF) and transforming growth factor-α (TGF-α) are thought to be among the important stimuli behind this phenomenon (Li *et al.,* 2007). Fibroplasia describes fibroblast proliferation and migration into the wound fibrin clot (Li *et al,*. 2007). Fibroblasts deposit fibronectin, interstitial collagens and proteoglycans to create granulation tissue (Trowbridge and Emling, 1997). Some evidence suggests that fibroblasts may differentiate into myofibroblasts that have actin microfilament bundles similar to smooth muscle cells which contract, thus aiding wound closure (Tomasek *et al.,* 2002). TGF-β provides critical signals during this phase, by increasing transcription of most matrix proteins such as fibronectin, collagen and proteoglycans. In addition to this TGF-β can also interfere with proteolytic degradation of matrix proteins by reducing synthesis of specific proteases including a serine , a thiol and a metalloprotease, and increase the production of protease inhibitors such as plasminogen activator inhibitor (PAI) and tissue inhibitor of metalloproteinases (TIMP) (Roberts *et al.*, 1992) (Sporn *et al.,* 1987). TGF-β can also upregulate the expression of αβ1 integrin receptors and stimulate fibroblast migration (Gailit *et al.,* 1996) (Heino *et al.,* 1989). EGF and fibroblast growth factor (FGF) have also been shown to stimulate fibroblast proliferation and migration (Li *et al.,* 2007). Due to a high metabolic rate within the wound site there is an increasing demand for oxygen and nutrients. Reduced pH, reduced oxygen tension and increased lactate within the wound site induce the release of factors needed initiate the formation of new blood vessels in a process named angiogenesis (Diegelmann and Evans, 2004). Angiogenic cytokines include FGF, VEGF and TGF-β produced by epidermal cells, macrophages, fibroblasts and vascular endothelial cells. These cytokines induce endothelial cells to assume the correct phenotype for capillary tube formation and stimulate their proliferation which provides a continuous supply for capillary extension. Angiogenic capillary sprouts invade the wound clot and organise into a micro vascular network throughout the granulation tissue restoring blood flow. The newly forming blood vessels deposit matrix including fibronectin and proteoglycans but ultimately form mature vascular basement membrane (Tonnesen *et al.,* 2000).

1.1.4 Tissue Remodelling

The final stage of wound repair is tissue remodelling which begins 2-3 weeks after wounding and can last for up to a year or more. The remaining endothelial cells, macrophages and myofibroblasts either undergo apoptosis or leave the wound site leaving mostly collagen and a few extracellular matrix proteins. The repaired tissue is continually strengthened by the active remodelling of collagen from type I to type III a process carried out by MMPs. (Gurtner *et al*., 2008).

1.2 Chronic wound healing

1.2.1 Definition of a chronic wound

A chronic wound is defined as one which has failed to proceed through the phases of healing in an orderly and timely process to produce anatomic and functional integrity; timeliness is relative and determined by the nature and degree of the pathologic process (Lazarus *et al* 1994.) Most chronic wounds are associated with a few clinical conditions such as venous insufficiency, diabetes mellitus, pressure necrosis and vasculitis (Eming *et al* 2007.) It is estimated that the cost to the NHS caring for patients with a chronic wound is 2.3bn-3.1bn per year; this is 3% of the total expenditure on health (Posnett and Franks 2008.) Qualitative research has shown that the presence of a chronic wound results in statistically significant lower quality of life, with depression being a frequently reported symptom (Palfreyman 2008.)

1.2.2 Pathophysiology of a chronic wound

1.2.2.1 Inflammatory changes in a chronic wound

Chronic wounds are characterised by an abnormal inflammatory profile; instead of a self limiting process the end result of inflammation is more inflammation, the normal feedback mechanisms that end the phase are disrupted leading to an uncontrolled positive feedback loop. Causes of this excessive inflammation could be due to continued recruitment and activation due to pressure, bacterial overgrowth, leukocyte trapping or ischemic reperfusion injury (Menke *et al* 2007.) Histological analysis of chronic pressure ulcers has revealed an excessive neutrophils infiltration near the surface of the wound associated with the

edema along with matrix degradation, deeper in the wound migrating neutrophils surrounded rounded epithelial cells. Cytospins created from the lavage of the ulcer surface were found to be 95% neutrophils, myeloperoxidase activity significantly correlated with neutrophils count (Diegelmann 2003.) Morphological analysis of neuropathic foot ulcers has revealed a higher prevalence of inflammatory cells that localised around small arteries invading the vessel walls (Piaggesi *et al* 2003.) This continued recruitment and activation of neutrophils leads to excessive amounts of matrix metalloproteinases. Chronic ulcers contain significantly higher levels of both collagenases MMP-8 and 1 in their active form compared to acute healing; levels of the gelatinases MMP-2 and -9 have also been reported to be significantly higher in chronic wound fluid compared to acute along with the presence of activated enzymes and pro-enzymes. Grinnell and Zhu (1996) reported high levels of neutrophils elastase in the wound fluid of chronic ulcers, high proteinase levels correlated with the degradation of fibronectin and vitronectin which could relate to the poor healing found in chronic wounds. When samples are incubated with elastase inhibitors degradation of fibronectin is blocked, this does not occur with matrix metalloproteinases suggesting that elastase is responsible. (Wysoki *et al* 1993; Grinnell *et al* 1992; Nwomeh *et al* 1999; Grinnell and Zhu 1996.) Degraded fibronectin could also act to maintain high proteinase levels by stimulating the degranulation of neutrophils and secretion of MMPs from fibroblasts (Werb *et al* 1989; Planchenault *et al* 1990.)

Expression of tissue inhibitors of matrix metalloproteinases (TIMPs) also differs in chronic wounds compared to acute. Along with higher levels of MMPs, chronic ulcers are accompanied by a differential expression of TIMP-1 which could account for the excessive collagenolytic activity within the wound (Nwomeh *et al* 1999.) TIMP-2 and -3 have also been implicated in the pathophysiology of chronic wound healing. Proliferating keratinocytes in an acute wound produce TIMP-1 and -3 but in a chronic wound there is no epidermal expression of these inhibitors. TIMP-2 localises under the migrating epithelial tongue in an acute wound; this expression is less frequent in a chronic wound which could contribute to the uncontrolled expression of MMP-2. Analysis of chronic wound fluid has also shown cleavage

products of proteinase inhibitors α2-macroglobulin (α2-M) and α1-proteinase inhibitor (α1-PI). This abnormal ratio of protective and degradative enzymes favours wound degradation therefore leading to a chronic wound state (Vaalamo *et al* 1999; Grinnell and Zhu 1996.)

Abnormal inflammatory cell levels also creates an abnormal inflammatory cytokine profile in which the inflammatory cytokines predominate with significantly higher levels of TNF-α, IL-1 and IL-6 affecting the ability of a chronic wound to heal (Mast and Schultz 1994; Trengrove *et al* 2000.)

Measurement of endogenous cytokine growth factor levels in chronic ulcers has shown huge individual variation but generally lower levels of PDGF-AB, bFGF and EGF compared to acute wounds. Cooper et al (1994) further noted that TGF-β was not expressed at all in the pressure ulcers they examined. As a result of an excessive inflammatory response, fibroblasts are unable to make progress and deposit sufficient amounts of ECM as it is degraded faster than it is synthesised.

1.2.2.2 Proliferative changes in a chronic wound

In addition to the inflammatory observations Piagessi et al (2003) also reported a distinct lack of neoangiogenesis and granulating tissue, however hyperkeratosis was reported at the edges of the ulcer and fibrosis within the dermis which disrupted the normal structure of the extracellular matrix (Piaggesi *et al* 2003.) Fibroblasts obtained from the edges of the ulcer exhibited impaired migration and appeared mis-shaped, inflated with large nuclei with the tendency to clump together when compared to fibroblasts taken from the non-ulcerated skin of the wound (Brem *et al* 2007.) Along with changes in morphology chronic wound fibroblasts have shown decreased proliferation (Stanley *et al* 1997.) This senescent characteristic of chronic wound fibroblast has been supported by data showing a higher number of β-galactosidase (a marker of senescence) -positive cells along with an inability of fibroblasts obtained from a chronic ulcer to complete DNA synthesis compared to those taken from normal dermis (Mendez *et al* 1998; Vende Berg *et al* 1998.) In further support of the increased senescence in chronic wound fibroblasts is their decreased mitogenic activity when exposed to PDGF, which decreases further with ulcer age. This suggests senescent characteristics increase with the age of the ulcer and that some fibroblasts reach the

end of their lifespan (Agren *et al* 1999.) Cook *et al* (2000) contradicted previously reported results and showed no significant difference between proliferation of wound and normal fibroblasts but the inability of wound fibroblasts to re-organise extra-cellular matrix and contract when seeded on to type-1 collagen lattices which was attributed to MMP and TIMP activity Fibroblasts taken from patients with chronic venous insufficiency (CVI) either with or without a venous ulcer showed decreased motility compared to dermal fibroblasts taken from a secondary location indicating that patient with CVI are susceptible to these changes. Furthermore, the addition of chronic wound fluid to neo-natal dermal fibroblasts lowered their motility (Rafetto *et al* 2001.)

Keratinocyte migration is dependent on the underlying matrix and cytokines released by fibroblasts and monocytes; the microenvironment within a chronic wound prevents migration from occurring resulting in a delay in re-epthelialisation which is characteristic of a chronic wound. Cytokeratin 16, a proliferation-associated antigen, is strongly expressed in chronic ulcers similar to normal wounds, and the amount and localisation of cycling cells are similar suggesting that proliferation is not the limiting factor (Andriessen *et al* 1995.) In addition in a chronic wound there is a decreased expression of α5β1 which is indicative of a non-migratory phenotype in keratinocytes further supporting that a lack in migration is key in chronic wound pathogenesis (Agren *et al* 2000.)

All these factors taken together emphasise the high degree of complexity involved in the regulation of a healing wound. There are other factors which could contribute to impair healing such as build up of necrotic tissue, bacterial infection, disease specific pathological changes and age; all these factors will amplify the cellular and molecular changes that lead to chronic wounds.

1.3 C - reactive protein

C reactive protein (CRP), initially discovered due to its precipitation in the sera of patients in the acute stage of Pneumococcus infection (Tillet and Francis, 1930), belongs to the pentraxin family of proteins and is composed of 5 non-covalently linked protomers arranged symmetrically around a central pore. The conservation and absence of a polymorphism or deficiency in man suggests an important physiological

role (Thompson *et al*., 1999). The most common function described for CRP is the opsonisation of pathogens via interaction with phosphocholine moieties in the lipid bilayer and activation of complement via interaction to the head of the C1q molecule (Thompson *et al.,* 1999). CRP concentration in the plasma increases up to 1000 fold with the onset of inflammation (Ballou and Kushner, 1992). Therefore it has long been considered a sensitive marker for inflammation and is referred to as an acute phase protein. Direct links have been made between levels of CRP and the risk of coronary heart disease (CHD) (Koenig *et al.*, 1999). High sensitivity assays detecting minimal changes in baseline levels of CRP have suggested that individuals with a level >3mg/l have an increased risk of CHD than those with <3mg/l particularly in patients with type 2 diabetes (Soinio *et al.,* 2004).

1.3.1 Dissociation of C - reactive protein

In its pentameric form CRP is termed native CRP (nCRP). However this configuration can dissociate to form free subunits known as modified CRP (mCRP) distinguished by its antigenic, electrophoretic and biological activities. Dissociation was achieved primarily in high concentrations of urea or at high temperature in the absence of calcium (Potempa *et al,.* 1983; Potempa *et al,.* 1987). *In vitro* experiments suggest the mechanism of dissociation is local; when nCRP comes into contact with areas of tissue damage and inflammation it dissociates into mCRP. Eisenhardt (2009) described this mechanism in activated platelets via lysophosphatidylcholine (LPC) (Eisenhardt *et al,.* 2009). Upon activation platelets undergo changes in morphology and composition of membrane lipids including the exposure of the bioactive lipid LPC and this interaction induces the dissociation of nCRP. LPC is also present on apoptotic cells and dissociation has been shown in apoptotic monocytic THP-1 and Jurkat cells (Eisenhardt *et al.,* 2009). A second mechanism of local expression has also been suggested as extra hepatic expression of CRP has been reported (Dong and Wright, 1996; Yasojima, *et al.,* 2001). Ciubotaru *et al.* (2005) described the expression of mCRP from differentiated stimulated macrophages with the use of antibodies specific to each isoform (Ciubotaru *et al.,* 2005).

1.3.2 Inflammatory Properties attributed to C - reactive protein

The initial stage of the wound healing process is the formation of a haemostatic plug formed when platelets come into contact with collagen or other extracellular matrix at the site of injury. (Diegelmann and Evans, 2004.) Molins et al (2011) have reported the dissociation of pentameric CRP on the surface of activated platelets which promoted further platelet recruitment. Platelets incubated with monomeric C-reactive protein showed increased aggregation surface P-selectin and CD63 exposure, and glycoprotein IIb-IIIa activation, whereas pentameric C-reactive protein was unable to produce any effect (Molins *et al* 2011). *In vitro* studies suggest that upon cleavage the free monomeric CRP subunits exert proinflammatory properties. P-selectin is up regulated in platelets treated with mCRP which leads to increased neutrophil aggregation whereas the presence of nCRP at clinically relevant concentrations caused a reduction in these changes (Khreiss *et al.,* 2004). A delay in apoptosis has been shown in neutrophils treated with mCRP compared to no detectable change in nCRP treated cells (Khreiss *et al,* 2002) and an up regulation in adhesion molecules alphaM integrin (CD11b) and integrin beta-2 (CD18) resulting in increased interaction with endothelial cells *(*Khreiss *et al.,* 2004). Modified CRP has been reported to increase interleukin 8 (Il-8) expression through peroxynitrite-mediated activation of nuclear factor kappa beta (NFκβ) and activator protein 1 (AP-1) in neutrophils (Khreiss *et al*., 2005). In comparison to nCRP, mCRP has been shown to be a more potent inducer of reactive oxygen species production in monocytes and induces Mac-1 activation (Eisenhardt *et al.,* 2009). Differences in the properties exerted by the isoforms are further highlighted in the different receptors used to recognise them. Research has shown that low affinity Fc gamma receptor III (FcγRIII) (CD16) is the main receptor for mCRP whereas low affinity Fc gamma receptor IIa (FcγRIIa) (CD32) and high affinity Fc gamma receptor I (FcγRI) (CD64) recognise nCRP (Khreiss *et al.,* 2002). Lipid raft microdomains may also be associated with mCRP recognition as interruption of these with nystatin abrogated mCRP responses including cytokine release, reactive oxygen species formation and upregulation of adhesion molecule

expression (Ji, *et al.,* 2009). Previous research has analysed the effect of CRP isoforms in inflammation involved in mainly cerebro- and cardiovascular disease but this paradigm may also applicable to inflammation in wound healing.

1.3.3 C-Reactive protein and blood vessel formation

From the most common of the inflammatory markers CRP has been shown to be the strongest univariate of the risk of cardiovascular events (Ridker, 2003.) In its pentameric form CRP has been shown to inhibit both basal and vascular endothelial growth factor stimulated angiogenesis thought to be caused by diminished NO bioactivity which could also explain a role for CRP in the clinical course of cardiovascular disease (Verma *et al* 2002) This is further supported by research which shows that CRP suppresses the expression of arteriogenic chemo-cytokines due to the up-regulations of suppressors of cytokine signalling proteins (Suh *et al* 2004.) However there is some controversy that these affects may be attributed to the presence of sodium azide in commercial CRP preparations. Liu et al (2005) provided evidence in human umbilical vein endothelial cells (HUVEC) and endothelial outgrowth cells (EOC) that when sodium azide was removed from commercial preparations of CRP via dialysis the proapoptotic, antiproliferative, antimigratory, and antiangiogenic effects of CRP were reversed. In brain tissue after acute ischemic stroke mCRP has been associated with highly angiogenic microvessels within the brain and co-localised with CD105, a marker of angiogenesis, whereas nCRP was not present. *In vitro* mCRP is angiogenic to vascular endothelial cells (Slevin *et al.,* 2009)

1.3.4 C-reactive protein and fibroblasts

Local expression of CRP has also been reported in pulmonary fibroblasts in a porcine model of a pulmonary disease with inflammatory features (Päiväniemi *et al* 2009.) CRP has been shown to increase cardiac fibrosis when added directly to cultures of cardiac fibroblasts from wild type mice significantly increased the expression of collagen 1 and 2, α-SMA, TNF-α and IL-1β mRNA and protein production of TNF-α and IL-1β (Zhang *et al* 2010.) Fibroblastic expression of CRP may not only have an effect on the cell type itself but may also effect the surrounding cells, research has shown that endothelial cells grown

in co-culture with various stromal cells such as fibroblasts can have an effect on endothelial cell function (Buckley *et al* 2005).

1.4 CRP in wound healing; Aims & Objectives

To date, CRP isoforms have not been investigated in relation to cutaneous wound healing. Previous research has shown the 2 distinct isoforms of CRP (Molins *et al* 2011) possess inflammatory properties (Eisenhardt *et al.,* 2009), along with implications in blood vessel formation (Slevin *et al.,* 2009) but this work has mainly related to cardiovascular system responses. However, cellular processes such as inflammation and tissue regeneration (including blood vessel formation) also occur during cutaneous wound repair, suggesting the CRP isoforms may also mediate acute, and more importantly, chronic wound healing states.

Aim & Objectives

To date, there has been no investigation of the presence and role of CRP in cutaneous wound healing. Therefore, the aim of this preliminary study was to investigate the temporal and spatial presence of CRP isoforms during murine acute and age-related impaired wound healing. The specific objectives of the investigation were to:

- use immunohistochemistry techniques to determine the presence of mCRP and nCRP isoforms in wax-embedded murine acute wound sections taken at 6 different time points (12 hours, 24 hours, 3 days, 7 days, 14 days and 21 days) post-wounding in order to present a temporal and spatial profile of each CRP isoform across the 3 major phases of wound healing (inflammation, proliferation and tissue remodelling).

- confirm CRP isoforms in specific inflammatory cells (neutrophils and monocytes/macrophages) using dual immunohistochemistry techniques during the inflammatory phase of wound healing.
- confirm CRP presence in both established and angiogenic blood vessels using dual immunohistochemistry techniques.
- compare CRP profile of the inflammatory phase of cutaneous healing in wounds from a murine model of age-related impaired with acute wounds from normal mice with no evidence of impaired healing.

2. Materials and Method

2.1 Immunohistochemistry

Immunohistochemistry is a technique for the detection of tissue or cellular constituents based on an antigen-antibody interaction, the interaction is identified either by direct labelling of the antibody or via a secondary labelling method (Bancroft and Gamble, 2008). The Vectastain Elite ABC kit utilises this second method of detection in which an unlabelled primary antibody is applied, followed by a biotinylated secondary antibody and then a preformed avidin and biotinylated horseradish peroxidase macromolecular complex. The horse radish peroxidase is then visualised by the development of a peroxidase substrate that produces a colour. For example, Novared produces a dark red colour in the presence of the horseradish peroxidase macromolecular complex. Prior to visualisation of an antigen with antibody labelling, a process called antigen retrieval is vital to ensure the antigen is exposed to the antibody. Antigen retrieval breaks crosslinks made between protein and fixative in the sample which could mask the antigen (Shi *et al.,* 2001). A simple antigen retrieval method is the use of high temperature and an acidic buffer. However, for comparison this study also used a 'cold' antigen retrieval method to ensure that the hot antigen retrieval was not inadvertently affecting the staining results by disrupting the pentameric form of nCRP thereby creating false-positive mCRP staining (Fig. 1)

2.2 Materials

Tris-buffered saline (TBS) adjusted to pH7.5

- Trizma Base *(Sigma Aldrich, Dorset, UK)*
- Sodium Chloride *(Sigma Aldrich, Dorset, UK)*
- Tween *(Sigma Aldrich, Dorset, UK)*

Ethylenediaminetetraacetic acid (EDTA)-Tween buffer adjusted to pH 8.0

- 0.01M EDTA *(Sigma Aldrich, Dorset, UK)*
- 0.01%Tween (*Sigma Aldrich, Dorset, UK)*

Glycine, EDTA, Tween Buffer adjusted to pH 3.5

- 0.01M EDTA *(Sigma Aldrich, Dorset, UK)*
- 0.05M Glycine *(Sigma Aldrich, Dorset, UK)*
- 0.01% Tween *(Sigma Aldrich, Dorset, UK)*

Xylene *(VWR International, Leicestershire, UK)*

3% Hydrogen Peroxide *(Sigma Aldrich, Dorset, UK)*

Methanol *(Sigma Aldrich, Dorset, UK)*

Ethanol *(Sigma Aldrich, Dorset, UK)*

Anti-CD105 antibody *(Abcam, Cambridge, UK)*

Anti-CD34 antibody *(Abcam, Cambridge, UK)*

Haematoxylin *(VWR International, Leicestershire, UK)*

Novared *(Vector Labs, Burlingame, CA)*

Vectastain Elite ABC kit (mouse IgG) *(Vector Labs, Burlingame, CA)*

- Normal horse blocking serum
- Horse anti-mouse biotinylated IgG
- Avidin-biotinylated horseradish peroxidase (HRP) complex (ABC kit reagent)

Vectastain Elite ABC kit (rat IgG) *(Vector Labs, Burlingame, CA)*

- Norrmal horse blocking serum
- Horse anti-rat biotinylated IgG
- Avidin-biotinylated horseradish peroxidase (HRP) complex (ABC kit reagent)

Pertex Mounting Media *(Cellpath PLC, Powys, UK)*

2.3Animals and wounding

Animal material was obtained from our collaborators and consisted of wax-embedded murine wound samples obtained from 10 week old Balb/C wild type mice with intact ovaries or similar mice that had undergone ovariectomy 1 month previously. Mice were anesthetized with inhaled isofluorane and the

dorsum was shaved and cleaned with alcohol. Mice were wounded on their dorsum making two equidistant 1 cm full thickness skin incisional wounds placed 1 cm from the base of the skull and 1 cm from either side of the midline, ensuring the incisions passed through the skin and panniculus carnosus muscle. Wounds were left to heal by secondary intention and subsequently removed by excision [6 mm excisional wounds] at 12 hours, 24 hours, 3 days, 7 days, 14 days or 21 days post-wounding. The excised wounds were bisected and processed such that the midpoint of each wound was used for histological analysis following an established protocol (Emmerson et al., 2009). For each time point the data represent 3 mice (n =3) and 5 separate images were collected for analysis from each wound.

2.4 Immunohistochemical Staining Protocol

Murine wound samples (n=3) from specified time points during the wound healing process (12h, 24h, D3, D7, D14, D21 post-wounding) from both wild type and ovariectomised mice were stained for nCRP and mCRP using a commercial avidin-biotin peroxidase system called Vectastain Elite ABC mouse IgG kit- (Vector Laboratories, Burlingame CA) and monoclonal mouse anti-human nCRP (2C10) and mCRP antibodies (8C10) provided by Lawrence Potempa (Hospital Universitari de Bellevitge, Barcelona). Paraffin embedded 5µm sections were cut from D3 and D7 murine wounds using a microtome and mounted on Vectabond (Vector Laboratories, Burlingame CA) coated slides. Slides were deparaffinised using xylene, rehydrated through a graded ethanol series and then incubated in 3% hydrogen peroxide (H_2O_2) in methanol to block endogenous peroxidases. Antigen retrieval involved either incubation in a 0.01M ethylenediaminetetraacetic acid (EDTA) and 0.1% Tween buffer at 60^0C for 6 hours, referred to as cold antigen retrieval: or incubated in an 0.01M EDTA 0.05M Glycine and 0.1% Tween buffer for 20 minutes after 5 minutes heating in a microwave referred to as hot antigen retrieval. After antigen retrieval slides were washed for 15 minutes in tris-buffered saline (TBS)-Tween. Slides were then incubated for one hour in normal horse serum to block non-specific binding sites and overnight at 4^0C in primary antibody (2C10 or 8C10) diluted 1:10 with TBS containing normal horse serum. Negative controls were also set up to ensure the staining seen was true and not due to non-specific binding. For the negative

control primary antibody was replaced with TBS containing normal horse serum and the staining protocol was carried out as described. After overnight incubation slides were washed in TBS-Tween for 15 minutes followed by incubation in secondary biotin-conjugated horse anti-mouse antibody for 1 hour then washed again in TBS-Tween for 15 minutes. Slides were then incubated in an avidin-biotin peroxidase reagent for 30 minutes at room temperature followed by a final wash in TBS-Tween for 30 minutes. Slides were stained with nova red (Vector Laboratories, Burlingame CA) for 2 minutes followed by counterstaining with haematoxylin (VWR International, Pennsylvania). Slides were dehydrated through a graded ethanol series before mounting in Pertex (Cell Path PLC, Hertfordshire). For each time point the data represent 3 mice (n =3) and 5 separate images were collected for analysis from each mouse.

2.4.1 Dual staining protocol.

Murine wound samples (n=3) from D3, D7 and D14 wild type mice were stained for nCRP and mCRP using a commercial avidin-biotin peroxidase system called Vectastain Elite ABC mouse IgG kit- (Vector Laboratories, Burlingame CA) and monoclonal mouse anti-human nCRP (2C10) and mCRP antibodies (8C10) provided by Lawrence Potempa (Hospital Universitari de Bellevitge, Barcelona). Sequential sections on the same slide were stained for neutrophils and macrophages using monoclonal anti-neutrophil (provide details) and MAC3 antibodies respectively (Abcam, Cambridgeshire, UK) with a commercial avidin-biotin peroxidase system called Vectastain Elite ABC rat IgG kit- (Vector Laboratories, Burlingame CA) following the previously described immunohistochemical staining protocol (2.3). The sections were stained sequentially using the primary antibodies and overlaid to determine structures within the wound which have simultaneous co-localisation of both inflammatory cell markers and CRP isoforms. The same protocol was carried out with antibodies specific to antigens (anti-CD34 and anti-CD105) that are expressed on mature and angiogenic blood vessels respectively to determine if expression of CRP isoforms co-localised with any angiogenic or mature blood vessels typical of the proliferative stage of wound healing. Negative controls were also set up for all dual staining to ensure the staining seen was true and not due to non-specific binding. For the negative control primary

antibody was replaced with TBS containing normal horse serum and the staining protocol was carried out as described. For each time point the data represent 3 mice (n =3) and 5 separate images were collected for analysis from each mouse.

2.5 Analysis

2.5.1 Inflammatory phase analysis

For histological sections at 12h, 24h, D3 and D7 post-wounding staining for both CRP isoforms was analysed from 5 pre-defined areas within the wound. Images were captured using the Axio Imager M1 microscope (Zeiss, Germany) with x630 magnification. Area 1 was representative of mid-wound and areas 2, 3, 4 and 5 represented right and left areas just under the epidermis and just above the fat layer in the dermis ensuring a representative proportion of the whole wound was analysed. The amount of inflammatory cell staining was analysed using Image J software (Version 1.6.0 Rasband W., National institutes of Health, USA). Positive staining was expressed as a ratio of total inflammatory cells providing a basis for comparison for the native and modified CRP expression. For sections at D7 post-wounding inflammatory cells were manually stratified into monocytes and neutrophils according to morphology.

2.5.2 Proliferative phase analysis

Staining in the proliferative phase was measured at D7 and D14 post-wounding via the measurement of blood vessel area that had stained positively for each CRP isoform compared to the total area of blood vessel present in the wound. Images were again captured using the Axio Imager M1 microscope (Zeiss, Germany) with x630 magnification. The amount of staining was measured from representative images captured (as described in 2.4.1); the total blood vessel area was calculated together with the area which had positively stained for CRP using Image J software (Version 1.6.0 Rasband W., National institutes of Health, USA). Area of positive staining was expressed as a ratio of the total blood vessel area.

2.5.2 Tissue remodelling phase analysis

The tissue remodelling stage was measured at D14 and D21 post-wounding via quantification of staining in fibroblasts. Images representative of the entire wound captured using the Axio Imager M1 microscope

(Zeiss, Germany) with x630 magnification were analysed using Image J software (Version 1.6.0 Rasband W., National institutes of Health, USA) as described previously in 2.4.1. Positively stained fibroblasts were expressed as a ratio of total cells providing a basis for comparison of native and modified CRP expression.

2.5.3 Statistical analysis

Statistical analysis was performed using SimFit (Version 6.2.1. Bardsley W.G., University Manchester) and included paired and unpaired t-tests for normally distributed data or Wilcoxon-Mann-Whitney U test and the Kolmorgov-Smirnov test for non linear situations. Tests were chosen depending on the distribution of each dataset generated and a p value of <0.05 was considered significant.

3. Results

3.1 Comparison of hot and cold antigen retrieval technique

Antigen retrieval is used in IHC to ensure the antigen is unmasked and available to bind to the primary antibody (Shi et al., 2001). Two methods of antigen retrieval were used in this study referred to as 'hot' or 'cold' and positively stained cell counts were compared to determine their consistency in terms of antigen retrieval and maintaining the integrity of the CRP isoforms. There was no significant difference (nCRP p=0.9056, mCRP p=0.4074) between the staining for each CRP isoform using the two different methods (Fig 1A and 1B) suggesting both methods consistently retrieve the antigen without destroying the integrity of the CRP isoforms.

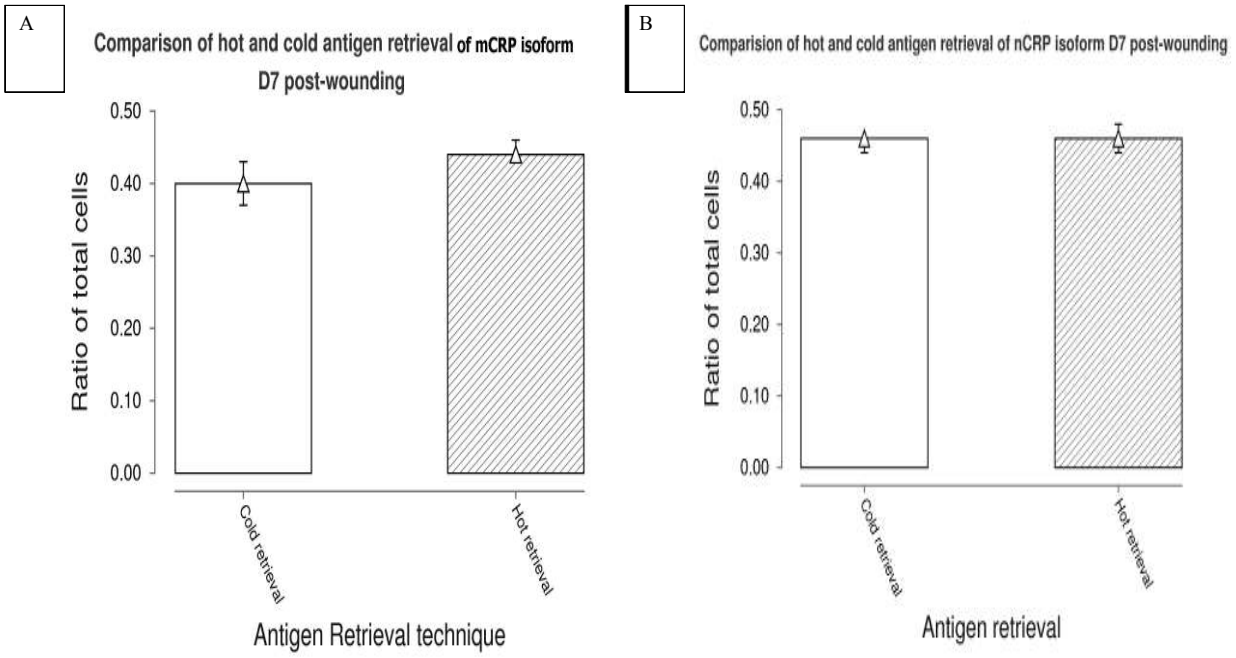

Fig. 1 Comparison of hot and cold antigen retrieval techniques **A:** Quantification and comparison of mCRP positive inflammatory cells using hot and cold antigen retrieval techniques (p=0.4074). **B:** Quantification and comparison of nCRP positive inflammatory cells using hot and cold antigen retrieval techniques (p=0.9056). Error bars represent standard error of the mean.

3.2 Presence of CRP isoforms during the inflammatory phase of wound healing

A time course indicative of early and late inflammation was examined and both CRP isoforms were detected at each time point. The predominant cell types during this phase are the neutrophil and monocyte (Kaplanski *et al*., 2003). The expression of CRP was addressed in individual cell types in the late inflammatory phase.

3.2.1 Detection of CRP isoforms in murine wound in the early inflammatory phase

3.2.1.1 12 and 24 hours post wounding

The first nucleated cell to infiltrate the wound bed is the neutrophils, this rapid response minimises the risk of infection (Dovi et al 2004.) Within the first 12-24 hours levels of this cell type within the wound will raise dramatically, levels of expression both CRP isoforms in this cell type were measured in cutaneous murine wound samples. Wounds from 12 and 24 hours post-wounding were sectioned and stained using the mCRP antibody 8C10 and nCRP antibody 2C10 (Fig 2B and C, Fig 3B and C), At 12 hours post wounding an average of 745cells/mm^2 stained positively for mCRP and similarly around 607cell/mm^2 stained positively for nCRP; this accounted for around 19% and 15 % respectively of the total cells present within the wound at 12hours post-wounding. (Fig 2A) This shows no significant difference between the amount of positively-stained cells within the wound ($p=0.1165$) and no significant difference in the percentage of cells from the total cells present staining positively for each isoform ($p=0.1113$.) At 24 hours post-wounding on average 1393cell/mm^2 stained positively for mCRP and 1164cells/mm^2 stained positively for nCRP. These cells accounted for around 45% and 40% of the total cells in the wound respectively. This again shows no significant difference between the percentage ($p=0.2393$) and amount of positively stained cells ($p=0.1310$) for each CRP isoform (Fig 3A).

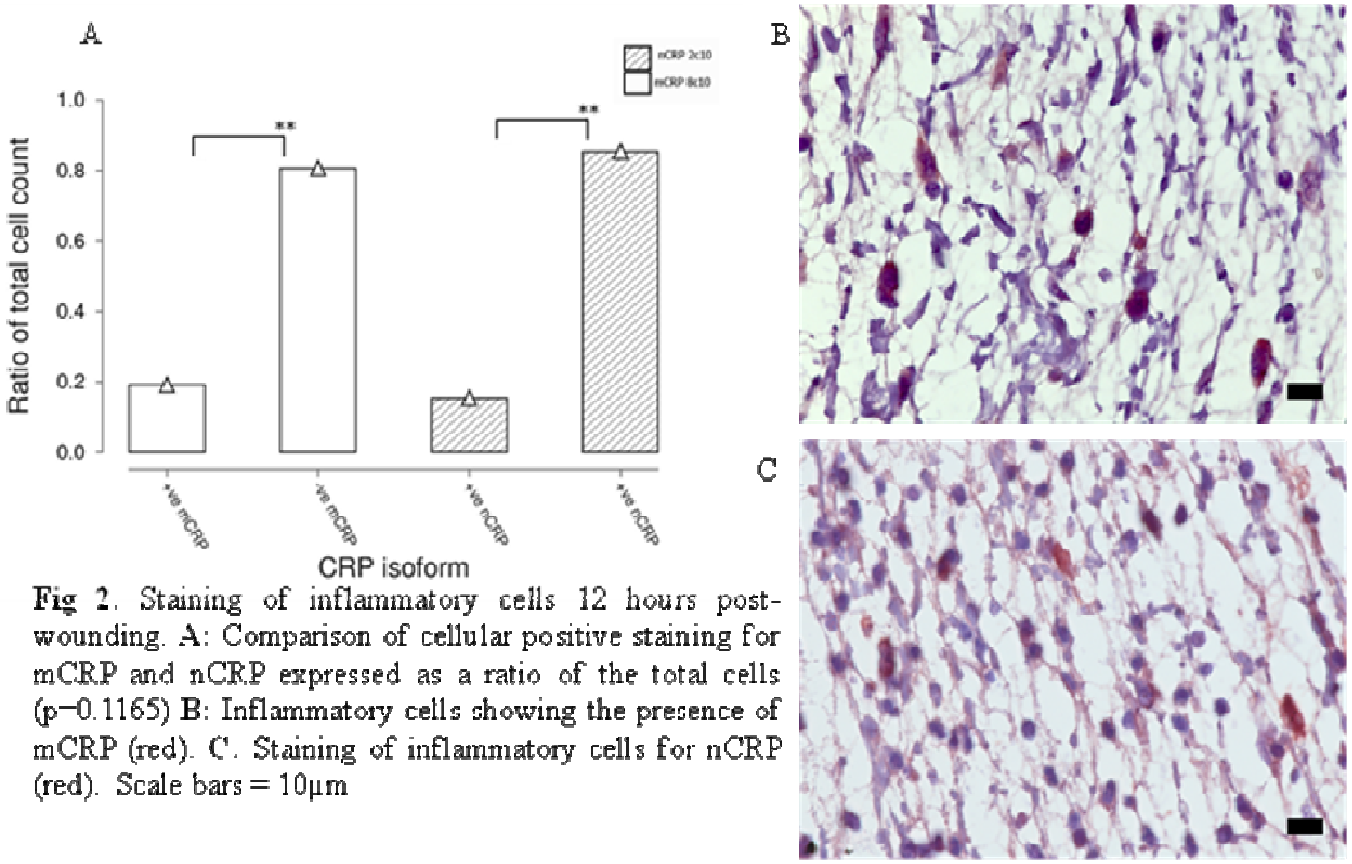

Fig 2. Staining of inflammatory cells 12 hours post-wounding. **A**: Comparison of cellular positive staining for mCRP and nCRP expressed as a ratio of the total cells ($p=0.1165$) **B**: Inflammatory cells showing the presence of mCRP (red). C: Staining of inflammatory cells for nCRP (red). Scale bars = 10µm

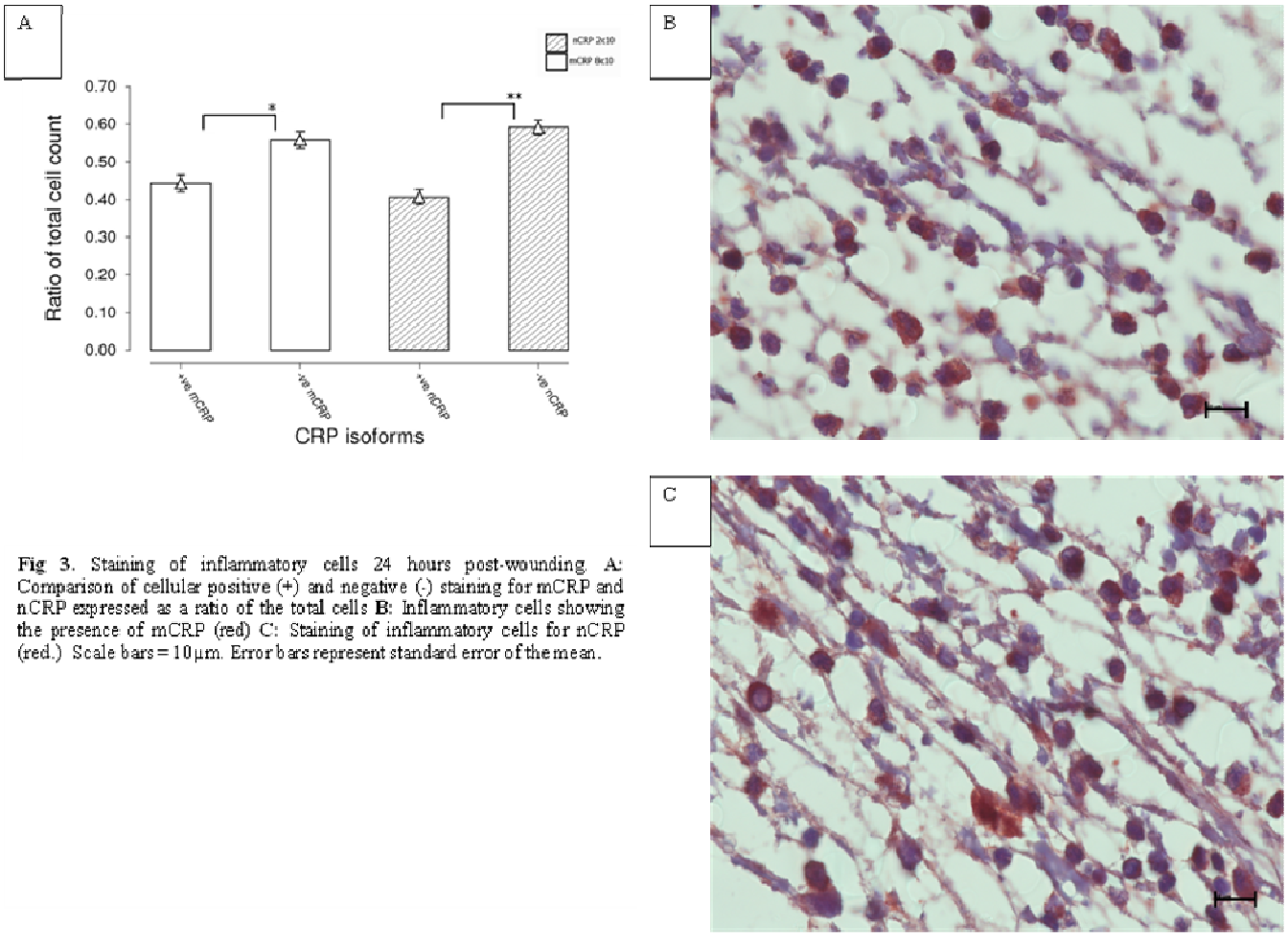

Fig 3. Staining of inflammatory cells 24 hours post-wounding. **A**: Comparison of cellular positive (+) and negative (-) staining for mCRP and nCRP expressed as a ratio of the total cells **B**: Inflammatory cells showing the presence of mCRP (red) C: Staining of inflammatory cells for nCRP (red.) Scale bars = 10 µm. Error bars represent standard error of the mean.

3.2.1.2 Comparison of 12 and 24 hours post-wounding

To analyse the temporal expression of both CRP isoforms in the very early stage of the inflammatory process levels of expression of each CRP isoform were compared. Expression of mCRP significantly increased from 19% of the total cells within the wound staining positively to 45% ($p<0.00001$) (Fig 4A). A similar trend was followed with the expression **n**CRP increasing from presence in 15% of the total cells to 40% ($p<0.00001$) (Fig.4B). This data shows that levels of expression of both CRP isoform has more than doubled in 12 hours and both are have increased by approximately the same amount showing that in the early stage of the inflammatory phase there is no differential expression of the isoforms.

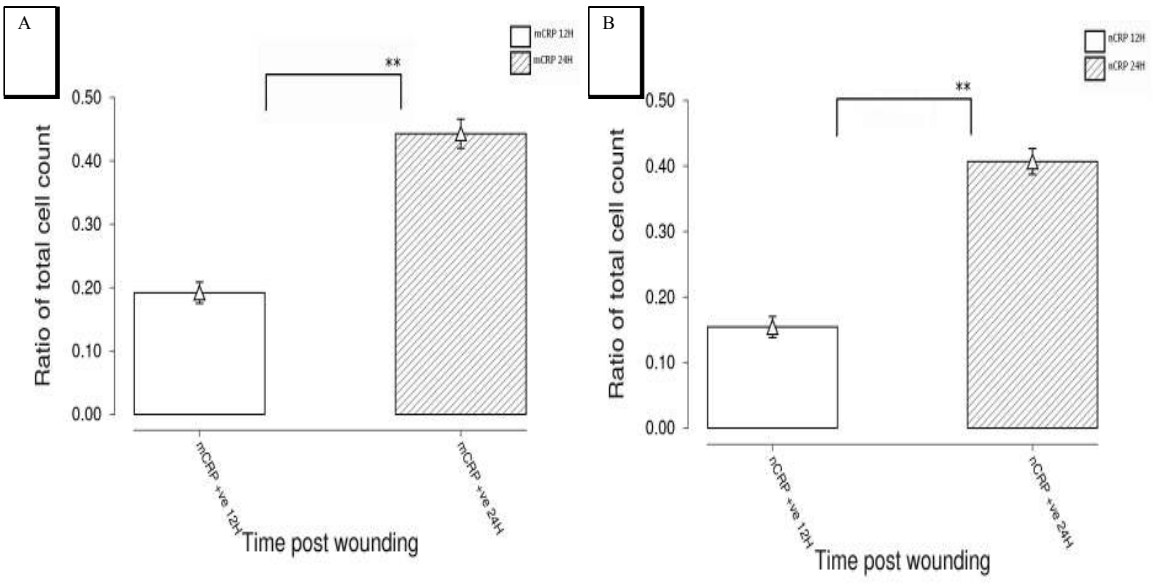

Fig 4. Comparison of positively (+) stained inflammatory cells at 12 and 24 hours post-wounding. **A:** Quantification of inflammatory cells stained positively for mCRP at 12 and 24 hours post-wounding, expressed as a ratio of total cells; **p= <0.00001. **B:** Quantification of inflammatory cells stained positively for nCRP expressed as a ratio of total cells; **p =<0.00001.

3.2.1.3 Day 3 post-wounding.

At day 3 post-wounding the predominant leukocyte subset is again the neutrophil (Kaplanski *et al*., 2003), so it was assumed that the total cell count was largely indicative of the total neutrophil count. To analyse the presence of both nCRP and mCRP in cutaneous wounds, murine wound samples D3 post-wounding

were sectioned and stained using antibodies directed towards the individual isoforms (Fig. 5B and C). The mean cell density in D3 wounds was 2467 cells/mm^2. The mean concentration of positively stained cells for mCRP was 1899 cells/mm^2 whereas only 1357 cells/mm^2 stained positively for nCRP. For comparison the results were expressed as a ratio of the total cell count (Fig. 5A). There was significantly (p=<0.00001) more mCRP staining than nCRP at D3 post-wounding, with mCRP detected in 77% of the total cells whereas nCRP was only detected in 55% of the total cells.

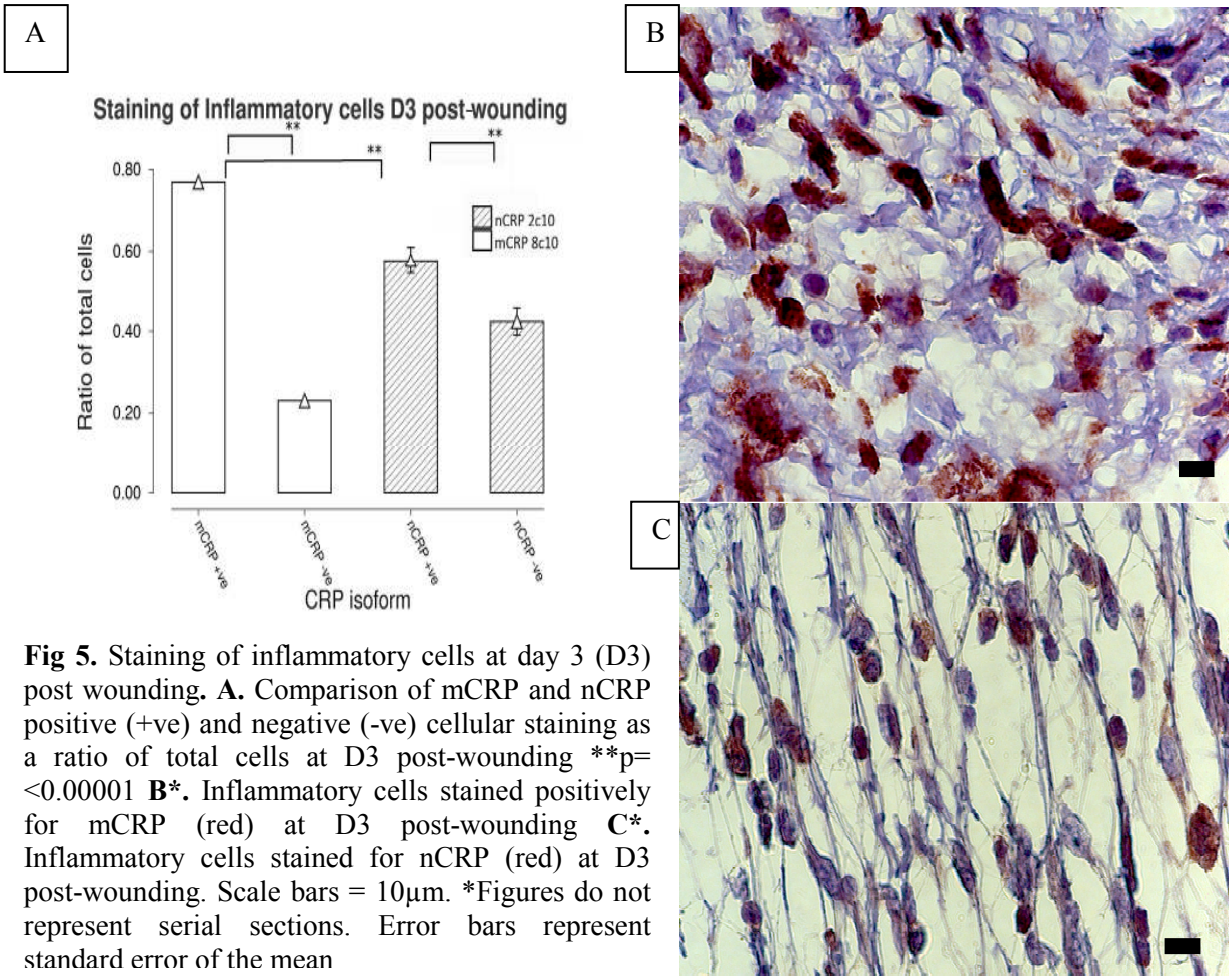

Fig 5. Staining of inflammatory cells at day 3 (D3) post wounding**. A.** Comparison of mCRP and nCRP positive (+ve) and negative (-ve) cellular staining as a ratio of total cells at D3 post-wounding **p= <0.00001 **B*.** Inflammatory cells stained positively for mCRP (red) at D3 post-wounding **C*.** Inflammatory cells stained for nCRP (red) at D3 post-wounding. Scale bars = 10µm. *Figures do not represent serial sections. Error bars represent standard error of the mean

3.2.2 Detection of CRP isoforms in murine wound in the late inflammatory phase

3.2.2.1 D7 post-wounding

To build a temporal profile for the presence of CRP isoforms during the inflammatory phase of wound healing, murine wound samples D7 post-wounding were sectioned and stained using mCRP antibody 8c10 and nCRP antibody 2c10 (Fig. 6B and C). The results (Fig. 6A) indicated no significant difference (p=0.0977) between CRP expression with approximately 40% of inflammatory cells staining positively for each isoform (42% for 8c10, 45% for 2c10) (Fig. 6A). However total cell density at D7 post-wounding increased dramatically compared with D3, rising from 2467 cells/mm^2 to approximately 5000 cells/mm^2 due to an influx of leukocyte subsets. Approximately 2100 cells/mm^2 stained positively for mCRP and 2250 cells/mm^2 stained positively for nCRP.

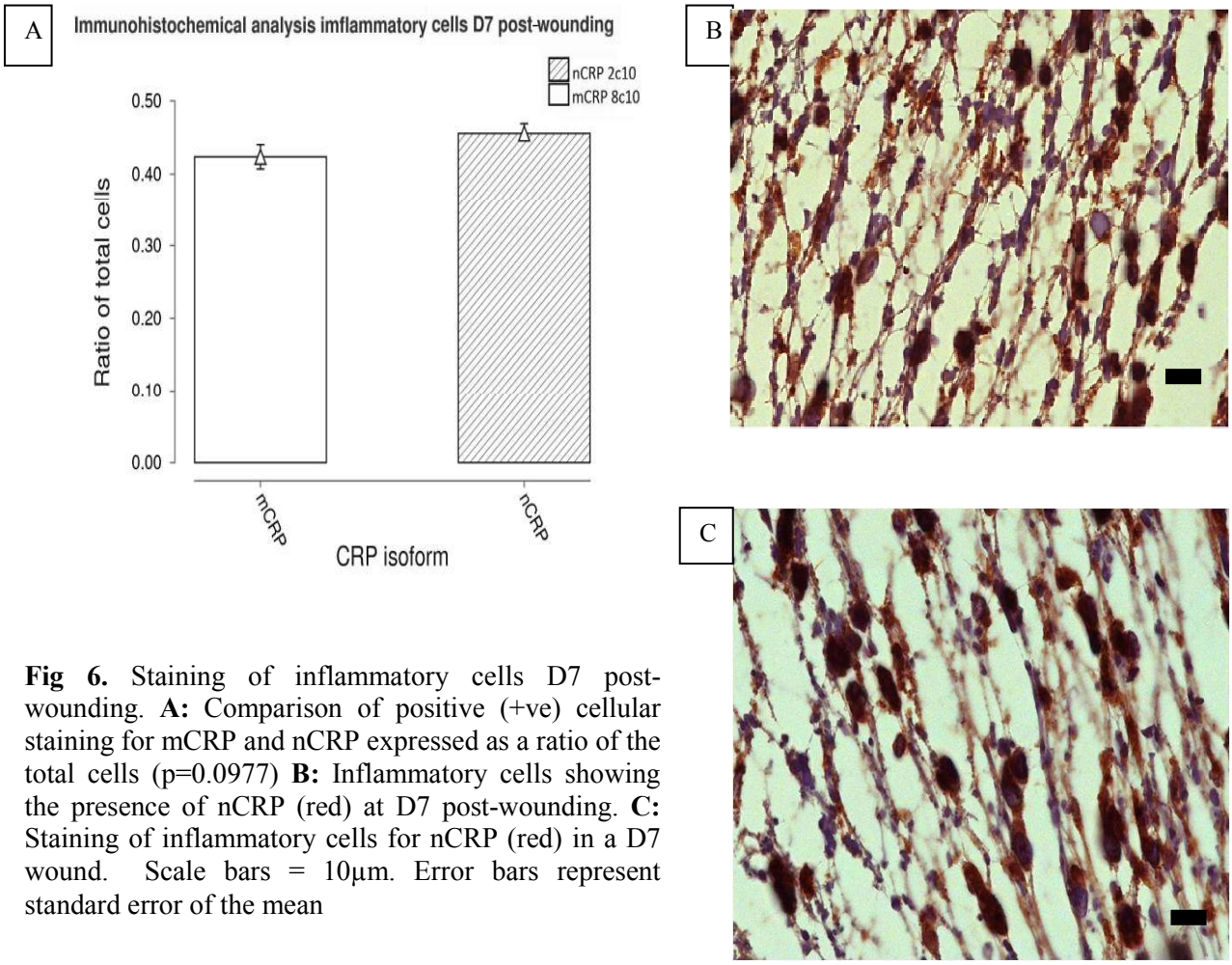

Fig 6. Staining of inflammatory cells D7 post-wounding. **A:** Comparison of positive (+ve) cellular staining for mCRP and nCRP expressed as a ratio of the total cells (p=0.0977) **B:** Inflammatory cells showing the presence of nCRP (red) at D7 post-wounding. **C:** Staining of inflammatory cells for nCRP (red) in a D7 wound. Scale bars = 10µm. Error bars represent standard error of the mean

3.2.2.2Comparison of staining in inflammatory cells D3 and D7 post-wounding

To complete the temporal analysis of the expression of CRP isoforms during the inflammatory phase a comparison of the positively stained cells for each time point and isoform, expressed as a ratio of the total cell count was evaluated (Fig. 7A and B). Expression of both isoforms had significantly decreased (p= <0.00001) at D7 post-wounding compared to D3 post-wounding. However, the expression of mCRP had decreased far more dramatically than nCRP, with mCRP reducing by 35% and nCRP by only 10%.

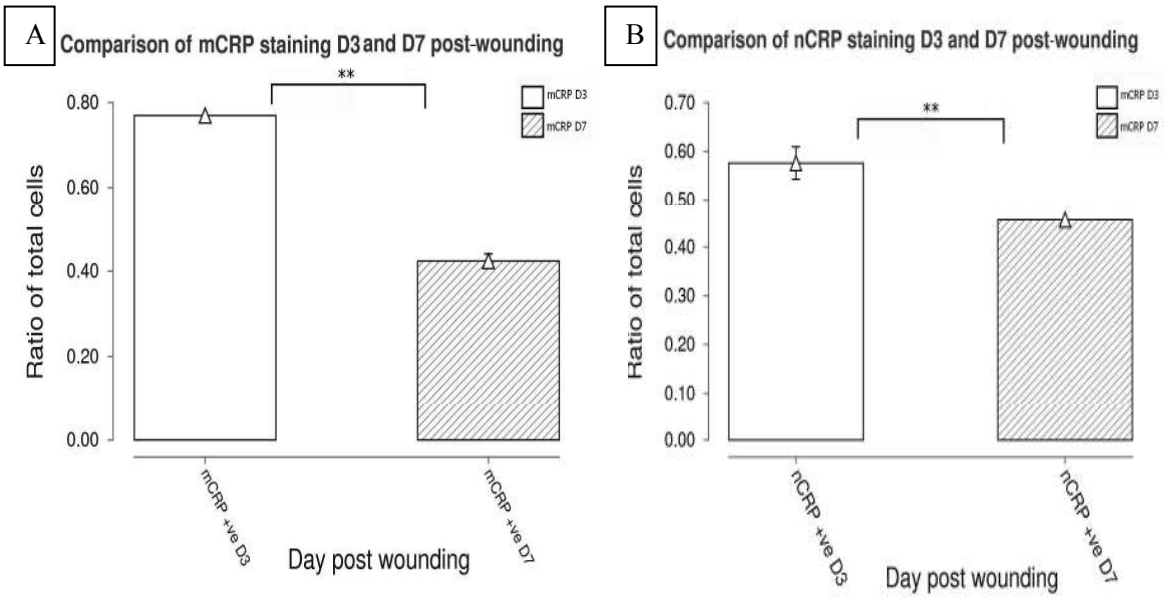

Fig 7. Comparison of positively stained (+ve) inflammatory cells at D3 and D7 post-wounding. **A:** Quantification of inflammatory cells stained positively for mCRP at D3 and D7 post-wounding, expressed as a ratio of total cells; **p= <0.00001. **B:** Quantification of inflammatory cells stained positively for nCRP expressed as a ratio of total cells; **p = <0.00001. Error bars represent standard error of the mean

3.2.2.3Monocyte expression of CRP isoforms at D7 post-wounding

At D7 post-wounding the predominant leukocyte subset is shifting from neutrophils to monocytes (Kaplanski *et al*., 2003). These cells are morphologically distinct from each other; monocytes are typically larger and more lobular in shape, ranging from 10-30µm in size (Fig 8B and C) (Trowbridge and Emling, 1997). Cell types were stratified according to morphology and counts were performed as previously described. Staining was expressed as ratio of total monocytes and comparisons were made

between CRP isoforms (Fig. 8A). There was no significant difference (p=0.6203) between staining of the 2 CRP isoforms; 80% of monocytes stained positively for mCRP and 79% for nCRP. At D7 post-wounding the mean total inflammatory cell density was approximately 5000 cells/mm^2. Total monocyte density at this time point was 2000 cells/mm^2, with 1680 monocytes/mm^2 staining positively for nCRP and 1800 monocytes/mm^2 staining positively for mCRP. This indicates that most monocytes at D7 were simultaneously expressing both CRP isoforms.

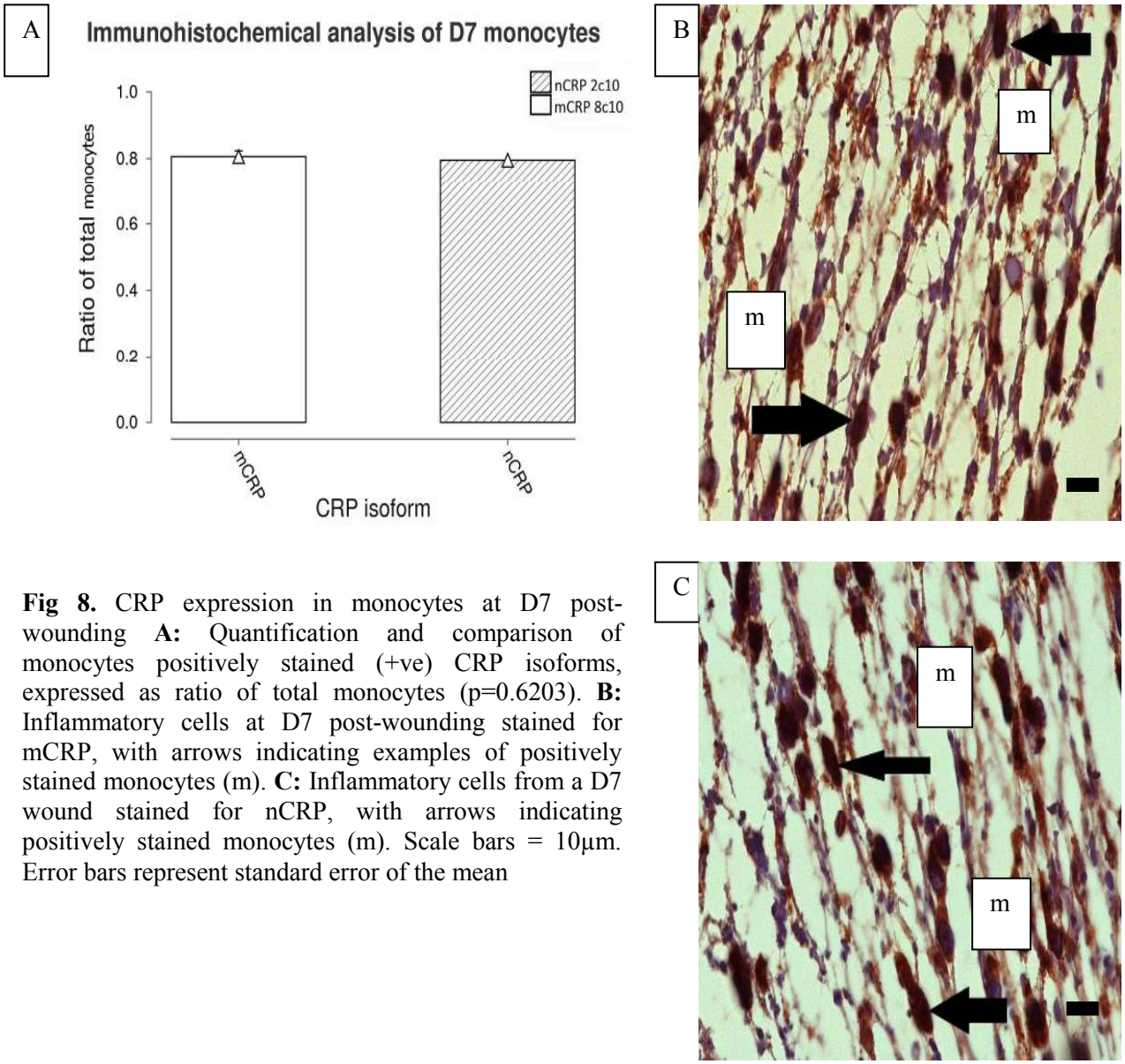

Fig 8. CRP expression in monocytes at D7 post-wounding **A:** Quantification and comparison of monocytes positively stained (+ve) CRP isoforms, expressed as ratio of total monocytes (p=0.6203). **B:** Inflammatory cells at D7 post-wounding stained for mCRP, with arrows indicating examples of positively stained monocytes (m). **C:** Inflammatory cells from a D7 wound stained for nCRP, with arrows indicating positively stained monocytes (m). Scale bars = 10μm. Error bars represent standard error of the mean

3.2.2.4 Neutrophil expression of CRP isoforms at D7 post-wounding

Neutrophils are generally smaller and rounder in shape than monocytes, ranging from 6-10μm in diameter (Trowbridge and Emling, 1997). Based on these morphological differences this cell type was distinguished from others in D7 wound sections (Fig. 9B and C). Mean total neutrophil cell density at D7 post-wounding was approximately 3000 cells/mm^2, with 602 neutrophils/mm^2 staining for mCRP and 698 neutrophils/mm^2 staining for nCRP. For comparison staining was expressed as a ratio of total neutrophils (Fig. 9A). The results indicated no significant difference ($p=0.1308$) between the staining of CRP isoforms in neutrophils at D7 post-wounding with mCRP being expressed in 28% and nCRP in 31% of neutrophils respectively. Interestingly this indicates that most neutrophils are not expressing either CRP isoform at this time point of the healing process. This is in stark contrast when compared to D3 post-wounding, where most cells stained positively for both CRP isoforms and were morphologically identified as neutrophils. Furthermore there was a significant ($p= <0.00001$) difference between staining for the 2 CRP isoforms at D3 post-wounding (Fig. 2A), with the mCRP antibody staining more neutrophils than the nCRP antibody. However at D7 post-wounding there was no significant difference ($p=0.0977$) between CRP isoform expression in neutrophils but levels had dropped by 49% for mCRP and 24% for nCRP compared to D3 wounds, despite the total number of neutrophils increasing.

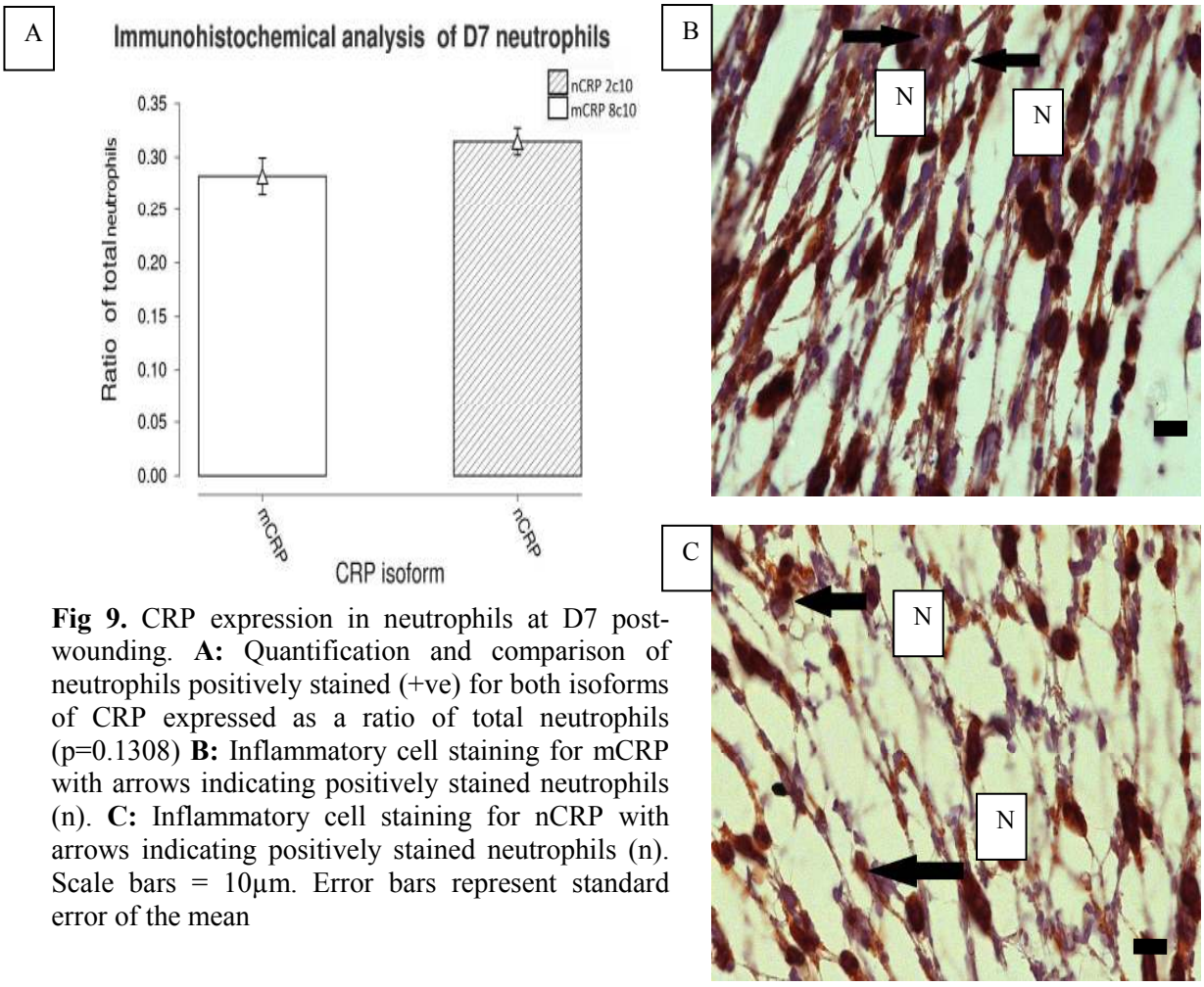

Fig 9. CRP expression in neutrophils at D7 post-wounding. **A:** Quantification and comparison of neutrophils positively stained (+ve) for both isoforms of CRP expressed as a ratio of total neutrophils ($p=0.1308$) **B:** Inflammatory cell staining for mCRP with arrows indicating positively stained neutrophils (n). **C:** Inflammatory cell staining for nCRP with arrows indicating positively stained neutrophils (n). Scale bars = 10μm. Error bars represent standard error of the mean

3.3 Presence of CRP isoforms in the Proliferative phase of wound healing

The two main events in this phase of the wound healing process are angiogenesis and fibroplasia (Li *et al.,* 2007). Previous evidence has shown that CRP isoforms exert biological effects on the main two cell types involved in these processes. To gain a temporal profile of CRP expression of both angiogenesis and fibroplasia, staining in the 2 cell types was conducted in murine wound samples from different time points in the proliferative stage

3.3.1.1 D7 post wounding analysis of angiogenesis

To analyse the expression of CRP isoforms in blood vessels early in the proliferative phase murine wound samples at D7 post-wounding we sectioned and stained for each CRP isoform (Fig 10B and C). Total area and the area of positive staining (mm^2) were measured, and the area of positively stained blood vessel was expressed as a ratio of the total blood vessel area (Fig 10A). Monomeric CRP was expressed in a larger area of blood vessel (mean sample area = $0.031mm^2$) than native CRP (mean sample area = 0.001mm2). When expressed as a ratio of total blood vessel area this showed that mCRP was expressed in a significantly (p=<0.0001) higher ratio of the total blood vessel area (27%) compared to nCRP (1%). The total blood vessel area did not differ between samples highlighting that the difference in CRP expression is not due to varying amounts of blood vessel within wound sections.

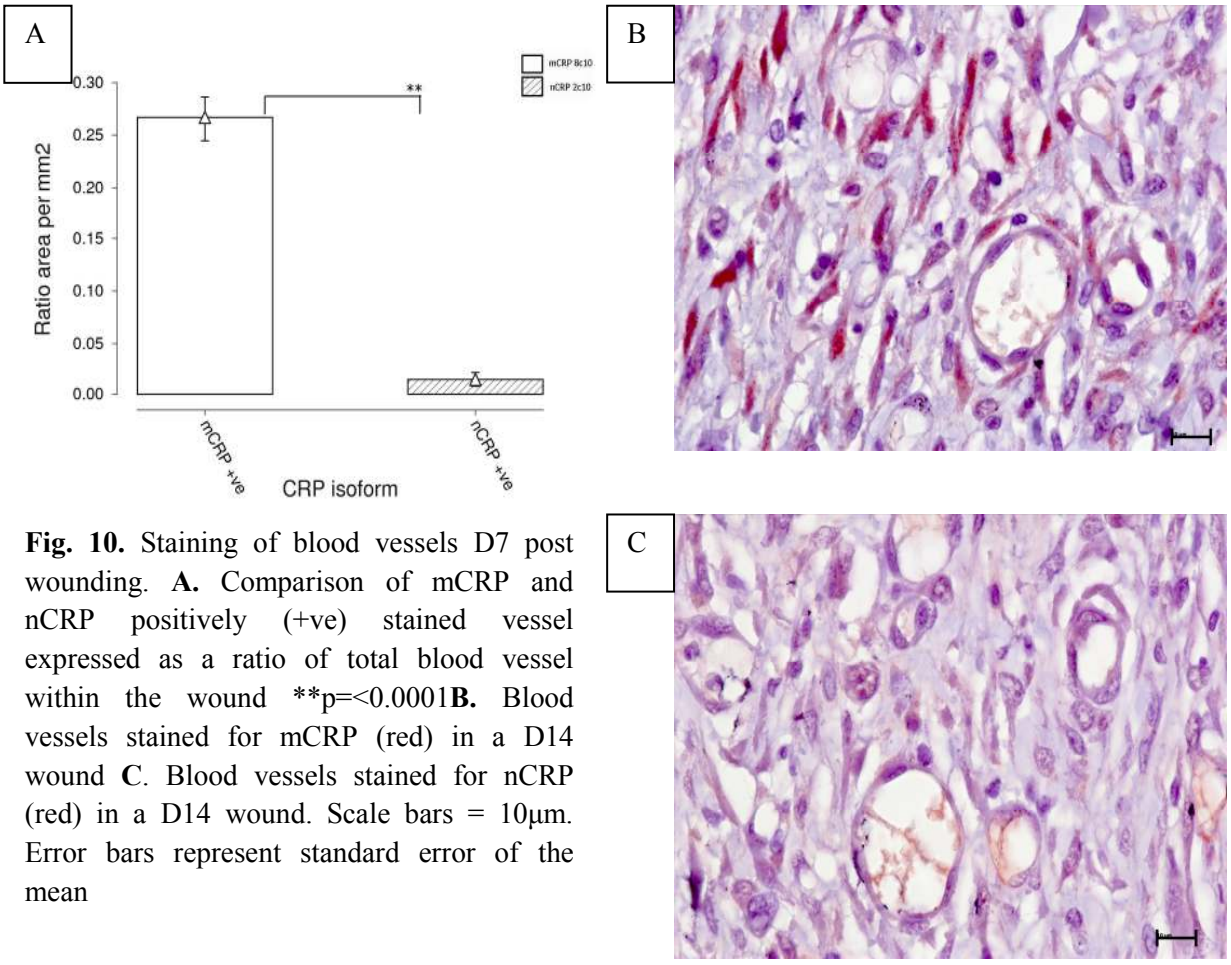

Fig. 10. Staining of blood vessels D7 post wounding. **A.** Comparison of mCRP and nCRP positively (+ve) stained vessel expressed as a ratio of total blood vessel within the wound **p=<0.0001**B.** Blood vessels stained for mCRP (red) in a D14 wound **C**. Blood vessels stained for nCRP (red) in a D14 wound. Scale bars = 10µm. Error bars represent standard error of the mean

3.3.1.2 D14 post-wounding analysis of angiogenesis

To analyse the expression of CRP isoforms in blood vessels later in the proliferative phase, sections of murine wound sample were stained with the mCRP antibody 8c10 and the nCRP antibody 2c10 14 days post-wounding (Fig 11BandC). The area of blood vessel staining positively for each CRP isoform was quantified (mm^2) and expressed as a ratio of the total blood vessel area (mm^2) (Fig 11A). Monomeric CRP was expressed in a significantly ($p>0.001$) larger area of blood vessel ($0.02mm^2$) than nCRP ($0.014mm^2$). When expressed as a ratio total area of blood vessel this showed that mCRP and nCRP were expressed in 25% and 19% of the total blood vessel area respectively, again highlighting that mCRP is expressed more frequently ($p=<0.001$) in blood vessels at D14 post-wounding and it is not simply due to a difference in the total amount of blood vessel between sections.

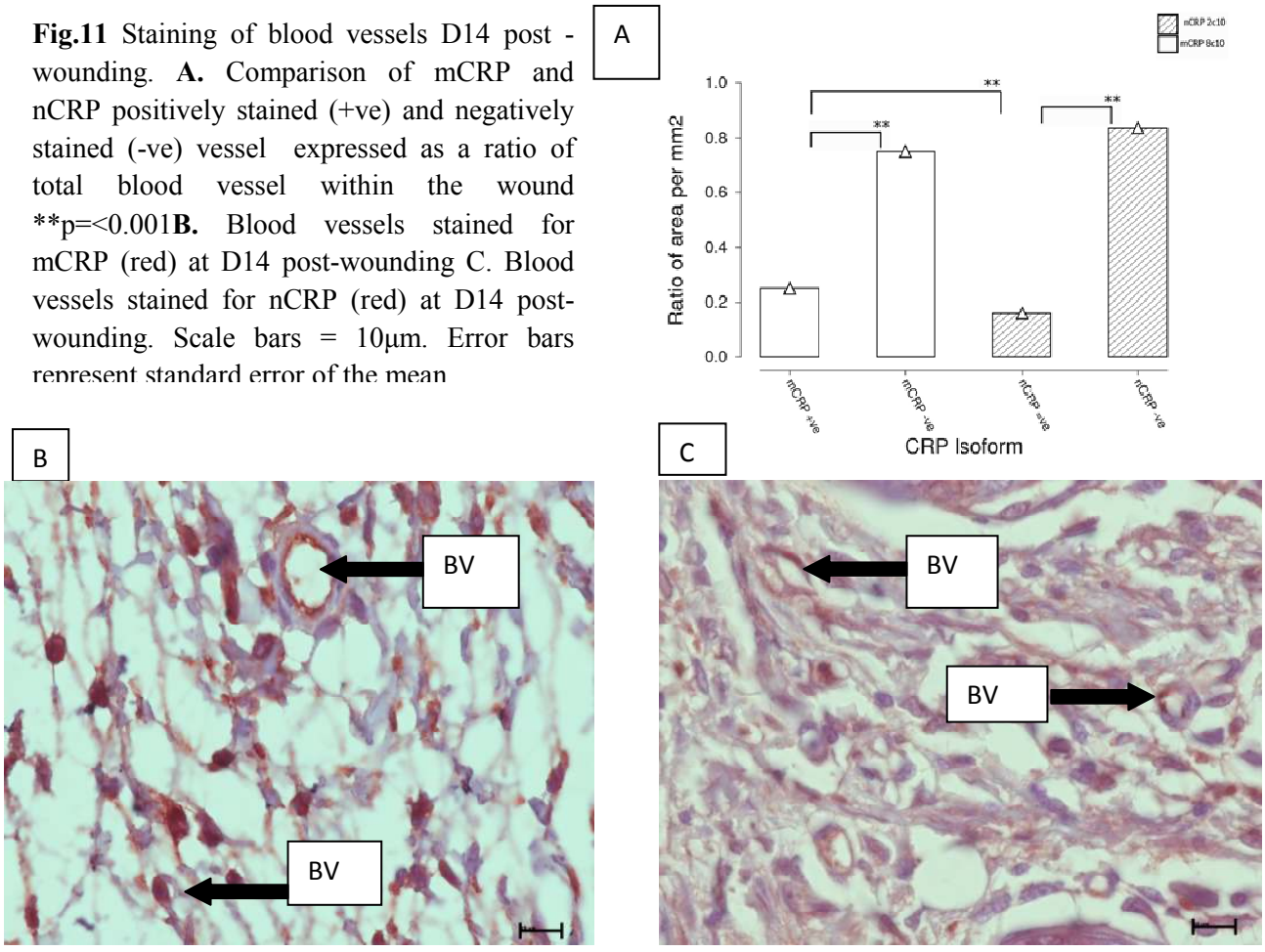

Fig.11 Staining of blood vessels D14 post - wounding. **A.** Comparison of mCRP and nCRP positively stained (+ve) and negatively stained (-ve) vessel expressed as a ratio of total blood vessel within the wound **p=<0.001**B.** Blood vessels stained for mCRP (red) at D14 post-wounding C. Blood vessels stained for nCRP (red) at D14 post-wounding. Scale bars = 10µm. Error bars represent standard error of the mean

3.3.1.3 Comparison of CRP expression D7 and D14 blood vessels

To complete the temporal profile for the presence of CRP isoforms in blood vessel during angiogenesis in the proliferative phase of wound healing a comparison between D14 and D7 post-wounding was made for each isoforms (Fig 12A and B). There was no significant difference (p=0.4472) between the staining for mCRP at D7 and D14 post-wounding. At D7 post-wounding mCRP was present in 27% of the total blood vessel count and at D14 in 25%. However nCRP staining presented a significant (p=<0.0001) difference between the time points, at D7 post-wounding nCRP antibody 2C10 stained only 1% of the total blood vessel whereas at D14 post-wounding 19% of the total blood vessel area stained positively.

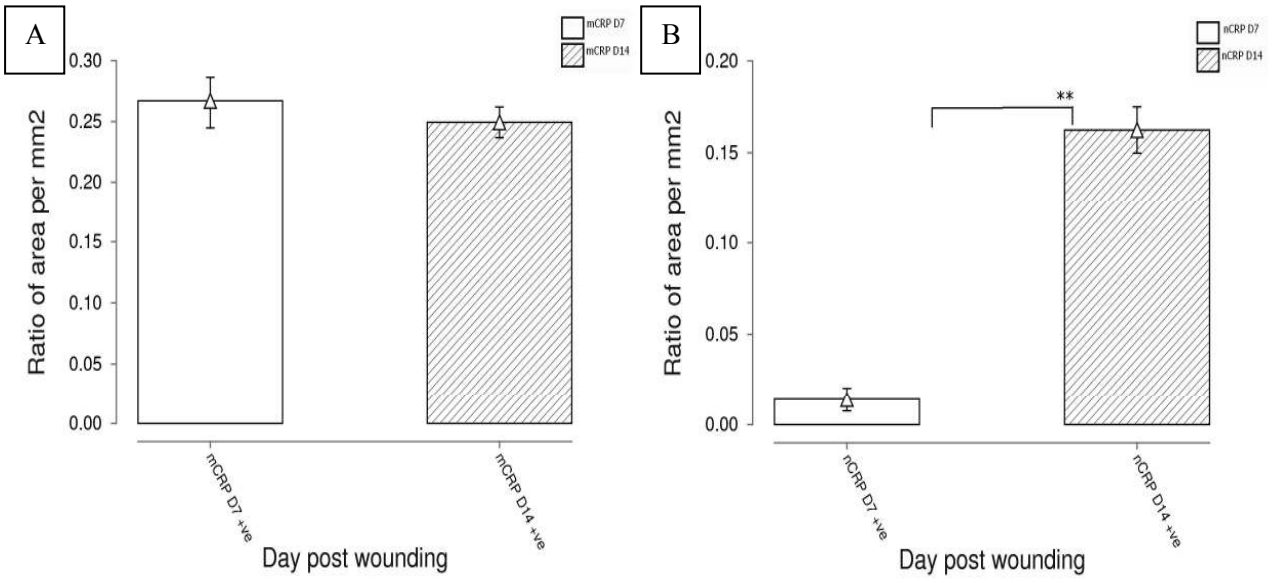

Fig.12 Comparison of positively stained (+ve) blood vessel at D7 and D14 post-wounding. **A.** Comparison of mCRP staining in blood vessels at D7 and D14 post wounding expressed as a ratio of total blood vessel within the wound p=0.4472 **B.** Comparison of blood vessels stained for nCRP in wound D7 and D14 post-wounding expressed as a ratio of total blood vessel within the wound p=<0.0001. Error bars represent standard error of the mean

3.3.2 D14 post-wounding analysis of fibroplasia

Alongside expression in blood vessels the expression of CRP isoforms in fibroblasts was also measured as they play a huge role in the proliferative stage of the wound healing process. Cells were identified via morphological analysis and quantified (Fig 13B and C), the amount of positively stained fibroblasts was enumerated and recorded and also expressed as a ratio of the total fibroblasts present. Although more cells stained positively with mCRP antibody 8C10 (598cells/mm^2) compared to those which stained positively with nCRP antibody 2C10 (449cells/mm^2) there was no significant difference (p=0.0544.) When expressed as a ratio of the total fibroblasts present the amount of mCRP positive cells represent around 50% of the total fibroblasts within the wound with nCRP positive cells representing around 49%, again highlighting no significant difference between the ratio of cells which stain positively for each CRP isoform (Fig 13A).

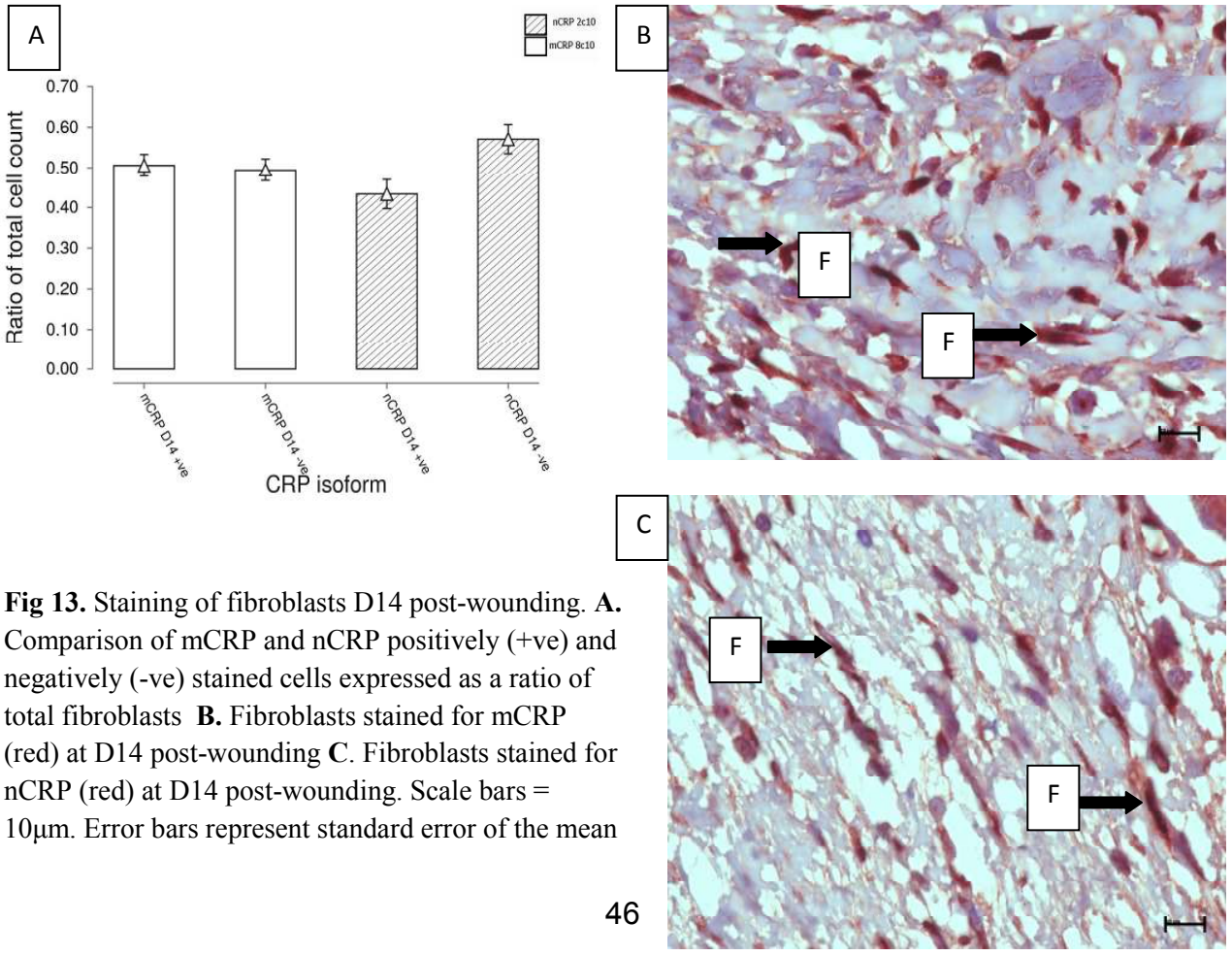

Fig 13. Staining of fibroblasts D14 post-wounding. **A.** Comparison of mCRP and nCRP positively (+ve) and negatively (-ve) stained cells expressed as a ratio of total fibroblasts **B.** Fibroblasts stained for mCRP (red) at D14 post-wounding **C**. Fibroblasts stained for nCRP (red) at D14 post-wounding. Scale bars = 10μm. Error bars represent standard error of the mean

3.4 Presence of CRP isoforms in the tissue remodelling phase of wound healing

The final stage of the wound healing process involves the remodelling of type I collagen to type III. Cells prevalent in the proliferative stage undergo apoptosis leaving mostly collagen and a few matrix proteins. To complete the temporal profile levels of CRP expression was analysed in fibroblasts at a time point indicative of the tissue remodelling phase.

3.4.1 D21 post-wounding analysis of fibroplasia

Fibroblasts in sections of murine wound samples obtained 21 days post-wounding were again identified due to their morphology quantified and positive staining for each CRP isoform (Fig 14 B and C) was enumerated, recorded and expressed as a ratio of the total fibroblast count for comparison. At this time point 424cell/mm^2 stained positively for mCRP and 373cells/mm^2 stained positively for nCRP but there was no significant difference ($p=0.4383$.) When expressed as a ratio of total fibroblasts, mCRP-positive cells made up around 27% of the total fibroblasts within the wound and nCRP positive around 24%, again highlighting the lack of difference in levels of staining for each isoform ($p=0.4841$) (Fig 14A).

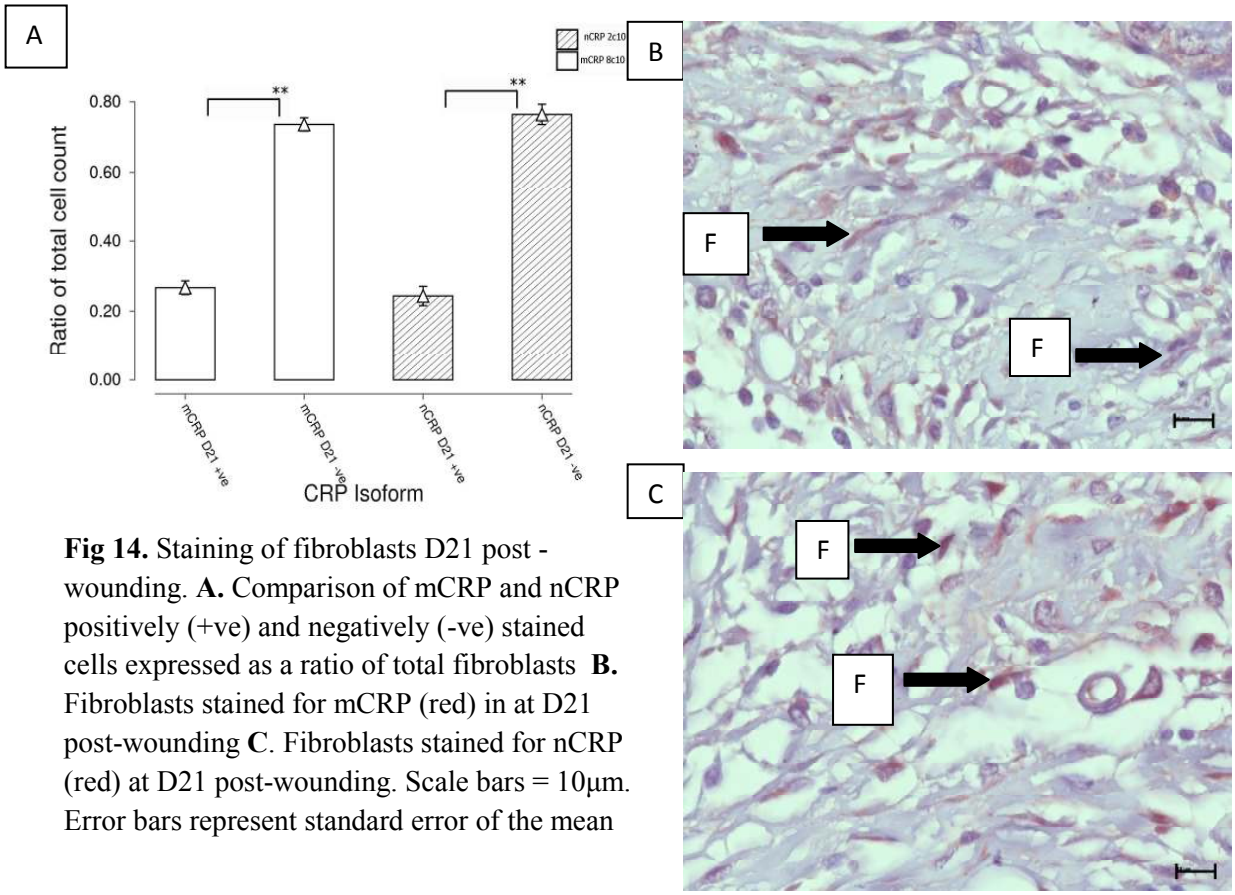

Fig 14. Staining of fibroblasts D21 post - wounding. **A.** Comparison of mCRP and nCRP positively (+ve) and negatively (-ve) stained cells expressed as a ratio of total fibroblasts **B.** Fibroblasts stained for mCRP (red) in at D21 post-wounding **C**. Fibroblasts stained for nCRP (red) at D21 post-wounding. Scale bars = 10μm. Error bars represent standard error of the mean

3.4.1.1 Comparison of CRP expression in fibroblasts at D14 and D21 post-wounding

To compare the amount of staining in fibroblasts over two different phases of wound healing the ratio of positively stained cells were compared (Fig 15 A and B). At D14 post-wounding around 50% of the total fibroblasts stained positively for the mCRP isoform. However at D21 post-wounding this had decreased significantly ($p<0.0001$) to 27%. This is also shown for nCRP, at D14 post-wounding around 49% of the total fibroblasts stained positively whereas at D21 post-wounding fibroblasts showed a significant decrease ($p<0.0001$) with only 23% of the total fibroblasts present within the wound staining positively for nCRP.

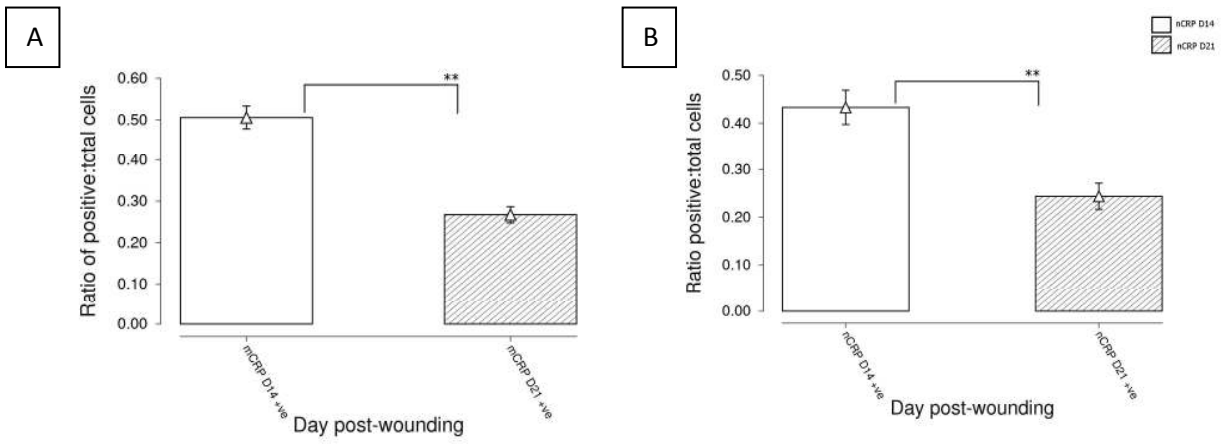

Fig 15. Comparison of positively stained (+ve) fibroblasts at D14 and D21 post wounding. **A:** Quantification of fibroblasts stained positively for mCRP at D14 and D21 post-wounding, expressed as a ratio of total cells; **p= <0.001. **B:** Quantification of fibroblasts stained positively for nCRP expressed as a ratio of total cells; **p = <0.001. Error bars represent standard error of the mean

3.5 Dual staining to compare expression of CRP isoforms in several cell types during the wound healing process

3.5.1 Dual staining to compare staining for CRP isoforms with staining for inflammatory markers

To compare the presence of CRP isoforms with the presence of known inflammatory markers serial sections were stained with antibodies specific to each CRP isoforms (2C10, 8C10) and also antibodies specific to the 2 major inflammatory cells, neutrophils and macrophages (anti-neutrophil, Mac3 respectively). Images taken from each section were overlaid and areas of dual staining were highlighted with software analysis

3.5.1.1 Dual staining to detect CRP expressing neutrophils

Dual staining revealed that at D7 post-wounding, only a relatively small proportion of inflammatory cells were neutrophils but that there was evident co-localisation of CRP expression with neutrophils (Fig 16 A and B). Cell counts were performed to quantify cells which expressed only the CRP isoform, cells which expressed only the neutrophil antigen and those which expressed both. From the total inflammatory cells stained within the wound, 22% expressed both mCRP and the neutrophil marker, 67% expressed mCRP alone and 10% were positive for just the neutrophil antibody alone. Similar results were shown for nCRP, with 25% of the total inflammatory cells expressing nCRP and the neutrophil marker, 65% staining for the nCRP antibody only and 10% staining for the neutrophil marker only. In concordance with earlier findings (3.2.2), these dual-staining results confirm the presence of other (non-neutrophilic) cell types at D7 post-wounding that express CRP isoforms.

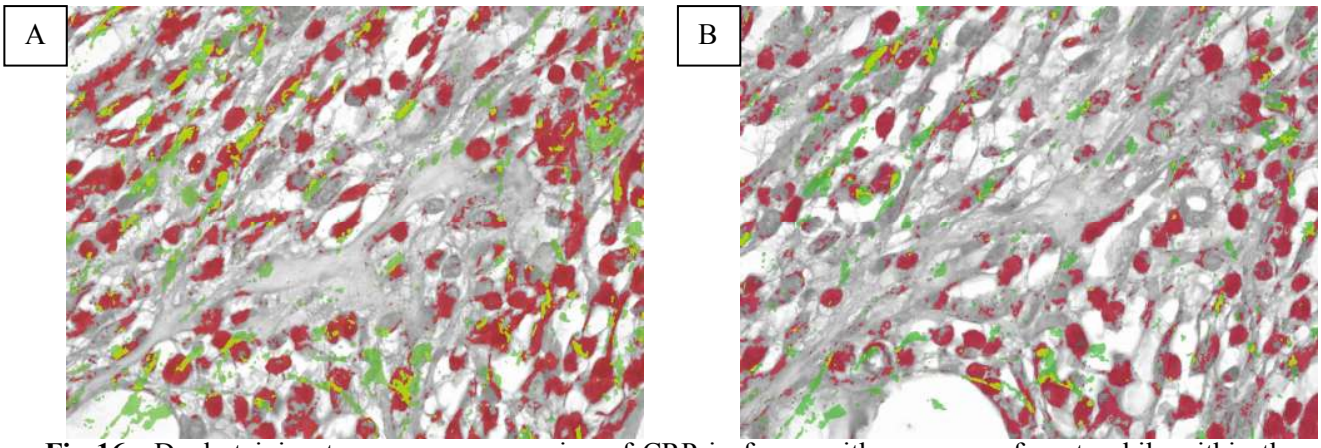

Fig 16. Dual staining to compare expression of CRP isoforms with presence of neutrophils within the wound. **A** Image of staining with mCRP antibody (8C10) (red) overlaid onto an image of staining with an antibody directed towards neutrophils (green) highlights areas of dual staining (yellow). **B** Image of staining with nCRP antibody (2C10) overlaid onto an image of staining with an antibody directed towards neutrophils highlights areas of dual staining (yellow).

3.5.1.2 Dual staining to detect CRP expressing macrophages

Dual staining with antibodies specific to CRP antibodies and an antibody specific to macrophages revealed (in concordance with previous findings) that this inflammatory cell type was predominant in the wound at D7 post-wounding with substantial co-localisation with CRP expression (Fig17 A and B). Cell counts showed that of the total inflammatory cell stained within the wound 53% expressed both mCRP and the Mac3 marker, 30% expressed only mCRP and 17% expressed the Mac3 antigen only. The nCRP isoform co-expressed with the Mac3 antigen in 41% of the total stained inflammatory cells within the wound, whereas 33% expressed nCRP only and 26% expressed the Mac3 antigen only.

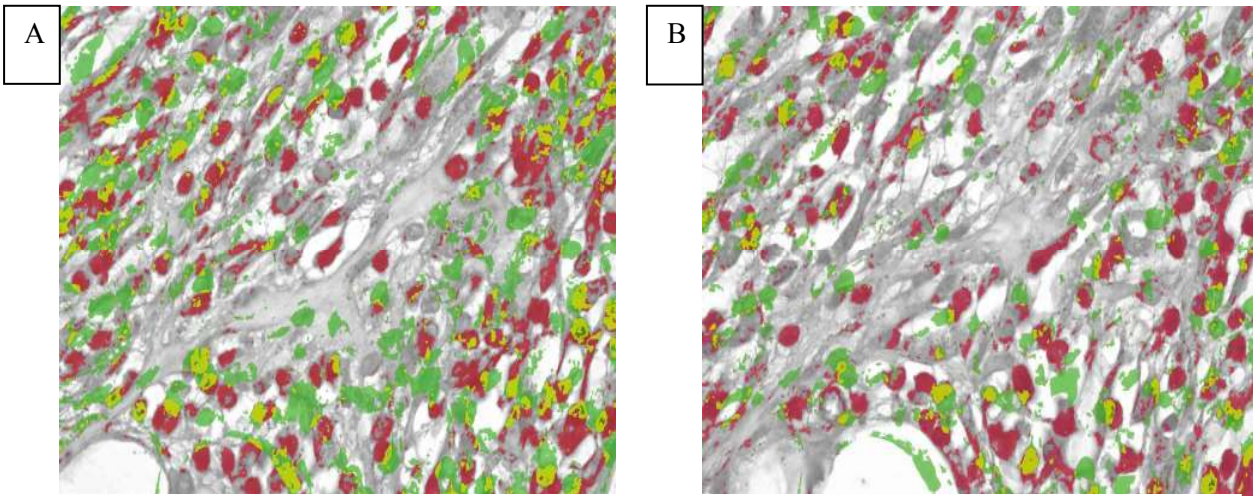

Fig 17. Dual staining to compare expression of CRP isoforms with presence of macrophages at D7 post-wounding. **A** Image of staining with mCRP antibody (8C10) (red) overlaid onto an image of staining with an antibody directed towards macrophages (green) highlights areas of dual staining (yellow). **B** Image of staining with nCRP antibody (2C10) overlaid onto an image of staining with an antibody directed towards macrophages highlights areas of dual staining (yellow)

3.5.2 Dual staining to compare staining for CRP isoforms with staining for blood vessel markers

To analyse the presence of CRP in blood vessels serial sections from D14 post-wounding were stained with antibodies specific to each CRP isoform (2C10, 8C10) and also anti-CD34 (used as a marker of blood vessels) and anti-CD105 (a marker of angiogenic blood vessels). Images taken from each section were overlaid and areas of dual staining were highlighted with software analysis

3.5.2.2 Dual staining to detect CRP- expressing blood vessels

The overlaid images revealed only a small amount of dual staining for CRP isoforms and the anti-CD34 (a marker for pre-existing blood vessels) at D14 post-wounding (Fig 18A and B). Earlier findings showed that mCRP was present in 25% of the total blood vessel at D14 post-wounding and nCRP in only 19%. Observation of the overlaid images indicated mCRP was more predominant within wound blood vessels, again corroborating earlier results.

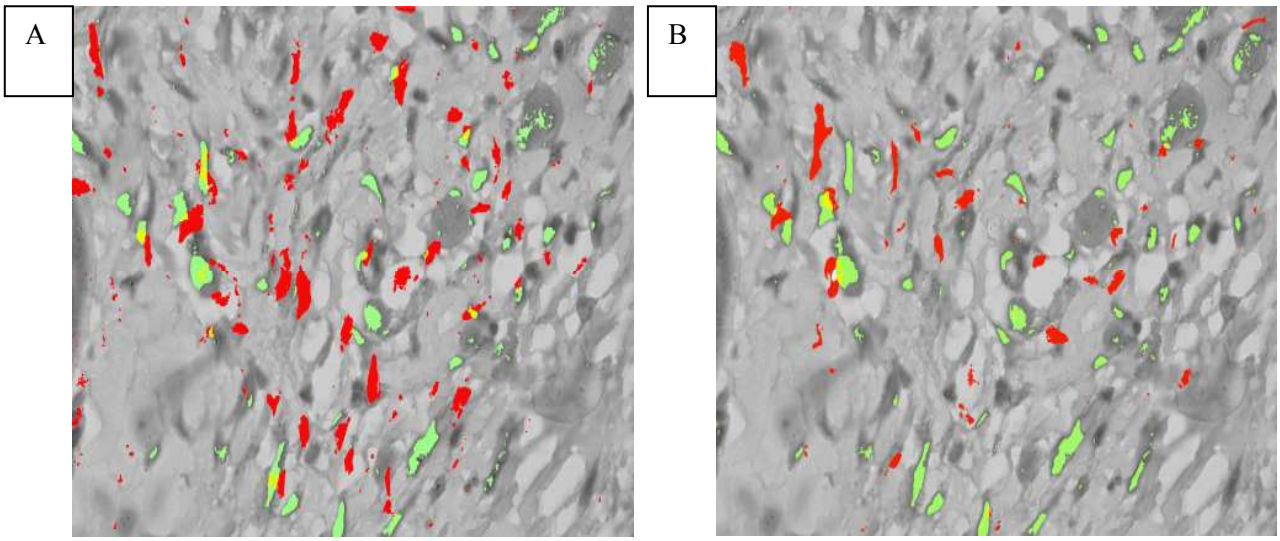

Fig 18. Dual staining to compare expression of CRP isoforms with the presence of blood vessels at D14 Post-Wounding. **A** Image of staining with mCRP antibody (8C10) (red) overlaid onto an image of staining with anti-CD34, a blood vessel marker (green) highlights areas of dual staining (yellow) **B** Image of staining with nCRP antibody (2C10) (red) over laid onto an image of staining with anti-CD34, a blood vessel marker (green) highlights areas of dual staining (yellow)

3.5.2.3 Dual staining to detect CRP expression in angiogenic blood vessels

The overlaid images revealed little staining with the CD105 antibody (green), suggesting there were few angiogenic blood vessels within the wound at D14 post-wounding. However there was some evidence of dual staining with both mCRP and nCRP, suggesting that both CRP isoforms may be expressed by angiogenic blood vessels.

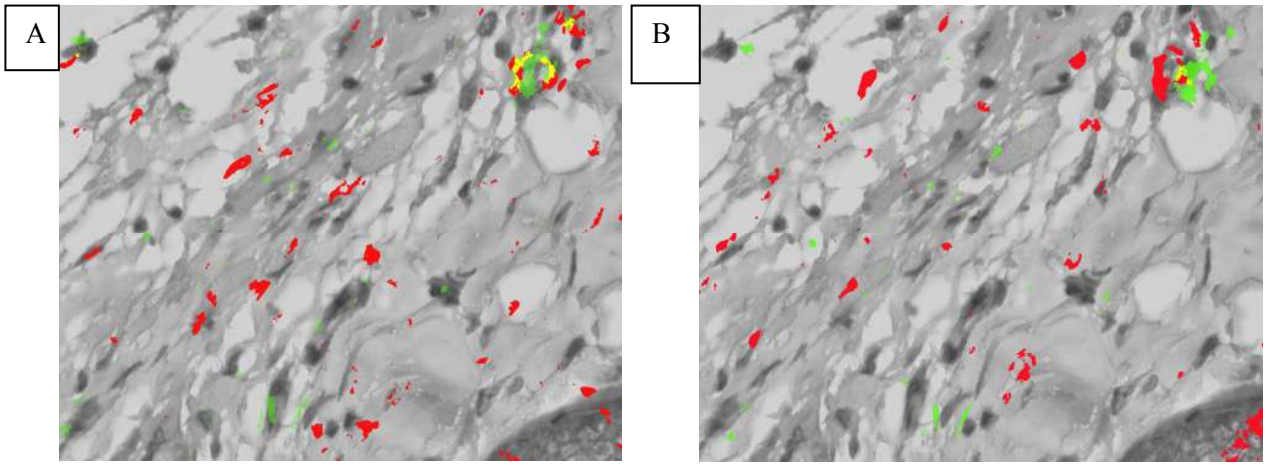

Fig 19 Dual staining to compare expression of CRP isoforms with the presence of angiogenic blood vessels at D14 Post-Wounding. **A** Image of staining with mCRP antibody (8C10) (red) overlaid onto an image of staining with anti-CD105, a marker for angiogenic blood vessels (green) highlights areas of dual staining (yellow) **B** Image of staining with nCRP antibody (2C10) (red) overlaid onto an image of staining with anti-CD105, a marker for angiogenic blood vessels marker (green) highlights areas of dual staining (yellow)

3.6. Presence of CRP isoforms during Impaired Healing

The use of ovariectomised (OVX) mice has been validated as an excellent model for delayed wound healing as they show increased inflammation, large wounds and decreased matrix deposition (Ashcroft *et al* 1997).

3.6.1 Comparison of CRP isoforms during inflammatory phase of acute wounds and a model of delayed healing

To compare the presence of CRP isoforms in an acute and impaired wound healing state, the inflammatory phase from intact (ovaries present) and ovariectomised (OVX) mice at D3 post-wounding were sectioned and stained for CRP isoforms. To minimise cross reactivity for this experiment, a new secondary staining kit was used which was specifically designed to stain for mouse antibodies on mouse tissue.

3.6.1.1 Presence of CRP isoform in an acute wound D3 post-wounding.

To ensure previous results obtained for the inflammatory phase were accurate; counts on inflammatory cells at D3 post-wounding for wound sections from intact mice were completed. In line with previous analysis murine wound samples at D3 post-wounding were sectioned and stained using antibodies directed towards the individual isoforms (Fig 20B and C). Results show the mCRP was expressed at significantly ($p=<0.05$) higher levels than nCRP, with 60% of the total cells within the wound staining positively for mCRP and only 42% of the total cell count expressing nCRP (Fig 20A), in line with previous results. However, levels of staining for both isoforms were slightly lower suggesting there could be some evidence of cross reactivity in initial findings but the difference could also be due to varying cell numbers in the different wound samples used.

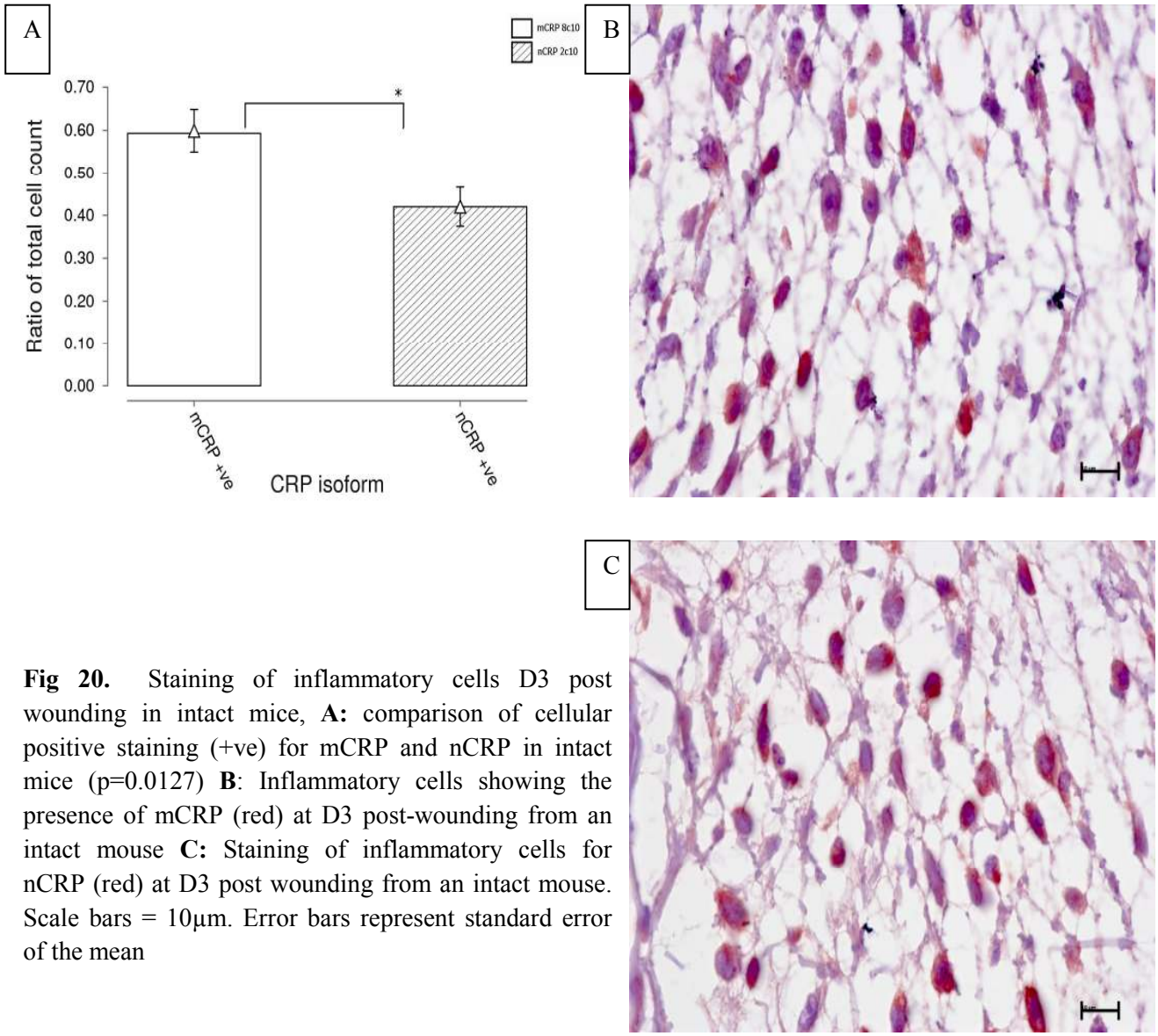

Fig 20. Staining of inflammatory cells D3 post wounding in intact mice, **A:** comparison of cellular positive staining (+ve) for mCRP and nCRP in intact mice (p=0.0127) **B**: Inflammatory cells showing the presence of mCRP (red) at D3 post-wounding from an intact mouse **C:** Staining of inflammatory cells for nCRP (red) at D3 post wounding from an intact mouse. Scale bars = 10µm. Error bars represent standard error of the mean

3.6.1.1.2 Presence of CRP isoforms in a model of age-related impaired healing D3 post-wounding

To compare presence of CRP isoforms in a model of chronic wound healing sections of a wound sample obtained from an ovariectomised mouse were stained with antibodies directed towards each CRP isoform (Fig 21B and C). Levels of staining were expressed as a ratio of the total cell count; mCRP was expressed in 49% of the total cells and nCRP in only 30% indicating that mCRP was expressed in a significantly higher amount of cells that nCRP (p=0.013).

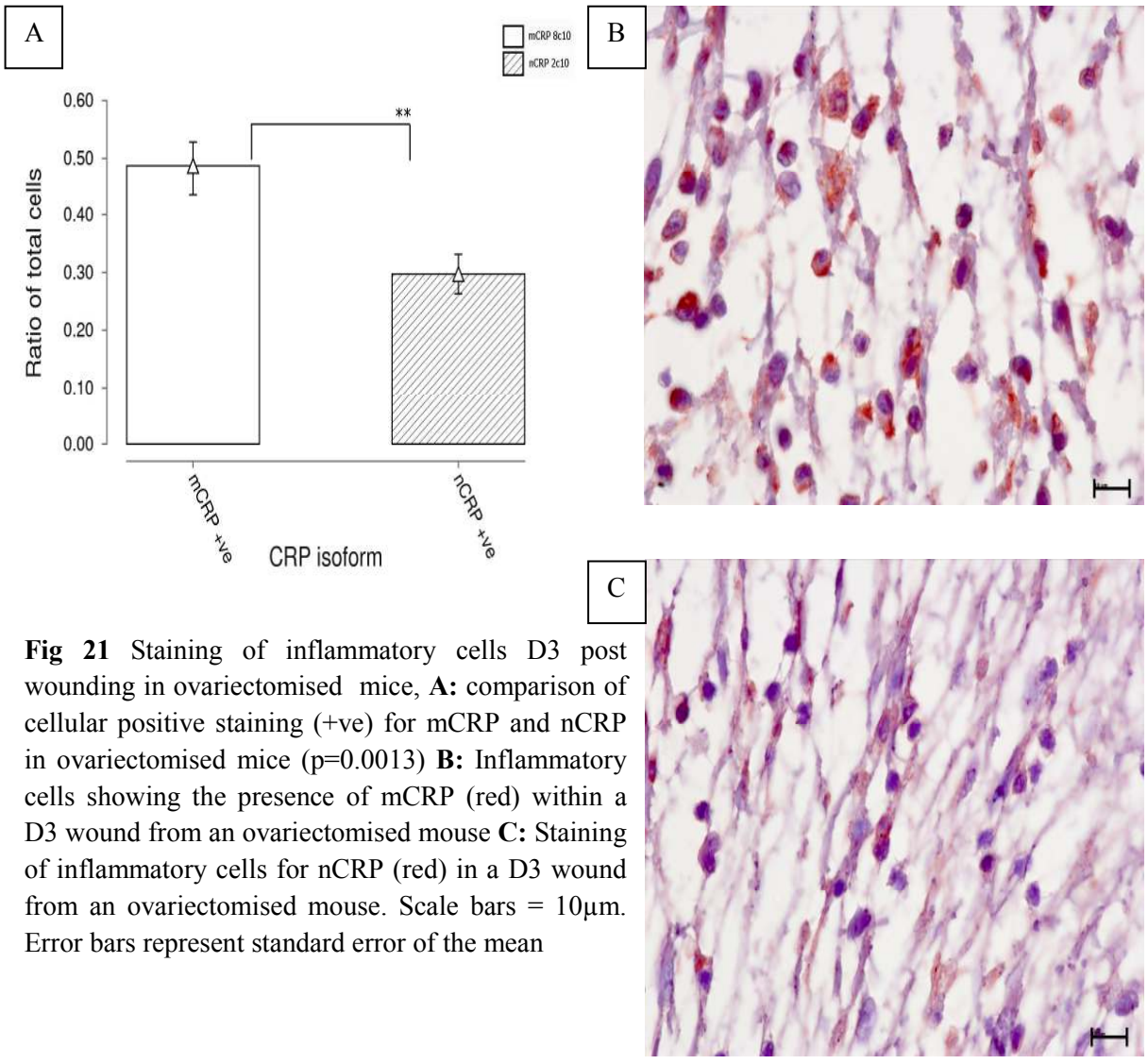

Fig 21 Staining of inflammatory cells D3 post wounding in ovariectomised mice, **A:** comparison of cellular positive staining (+ve) for mCRP and nCRP in ovariectomised mice (p=0.0013) **B:** Inflammatory cells showing the presence of mCRP (red) within a D3 wound from an ovariectomised mouse **C:** Staining of inflammatory cells for nCRP (red) in a D3 wound from an ovariectomised mouse. Scale bars = 10µm. Error bars represent standard error of the mean

3.6.2 Analysis of individual CRP isoforms in both acute and estrogen-deprived healing states

To compare the expression of CRP isoforms between acute and estrogen-deprived wound healing states the ratio of positively stained cells within the wound were compared.

3.6.2.1 Comparison of presence of monomeric CRP in acute and impaired wound healing states

Sections from wound samples from both intact and ovariectomised mice were stained with an antibody (8C10) directed again mCRP (Fig 22B and C). Although results show that levels of mCRP to be slightly higher (60%) in acute wounds compared to estrogen-deprived wounds (49%) there was no significant difference between the amount of expression of mCRP ($p=0.092$) (Fig 22A).

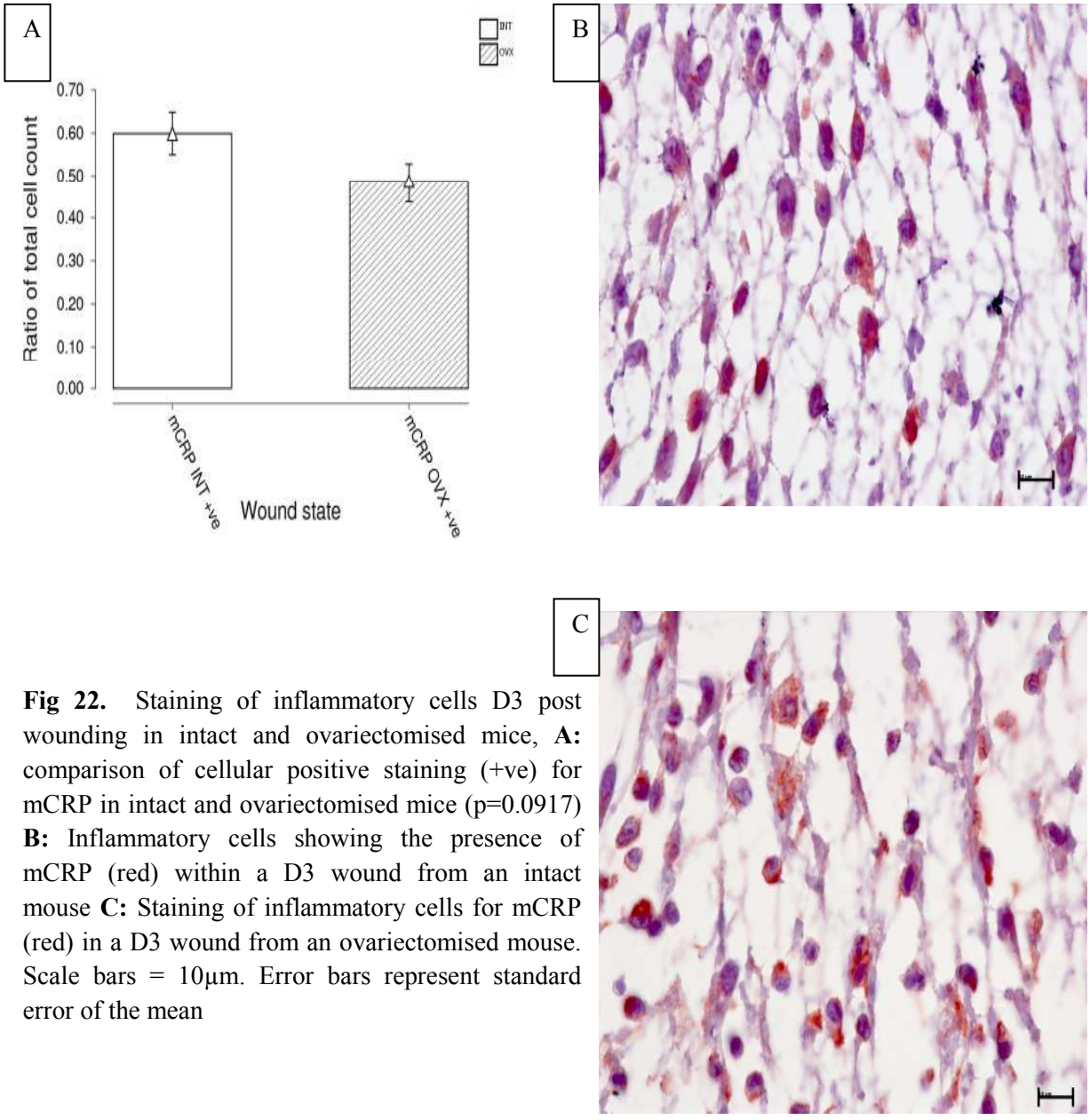

Fig 22. Staining of inflammatory cells D3 post wounding in intact and ovariectomised mice, **A:** comparison of cellular positive staining (+ve) for mCRP in intact and ovariectomised mice (p=0.0917) **B:** Inflammatory cells showing the presence of mCRP (red) within a D3 wound from an intact mouse **C:** Staining of inflammatory cells for mCRP (red) in a D3 wound from an ovariectomised mouse. Scale bars = 10µm. Error bars represent standard error of the mean

3.6.2.1.1 Comparison of presence of native CRP in acute and estrogen-deprived wound healing states

Identical to analysis for mCRP, sections of an acute and ovariectomised wounds were stained with an antibody (2C10) directed toward nCRP (Fig 23B and C), the amount of staining was quantified and expressed as a ratio of the total cell count. Results show that nCRP is expressed in a significantly higher amount of cells in acute wounds compared to estrogen-deprived wounds (p=0.0405), with 42% of the cells in intact wounds expressing the isoform and only 30% in ovariectomised wound samples (Fig 23A).

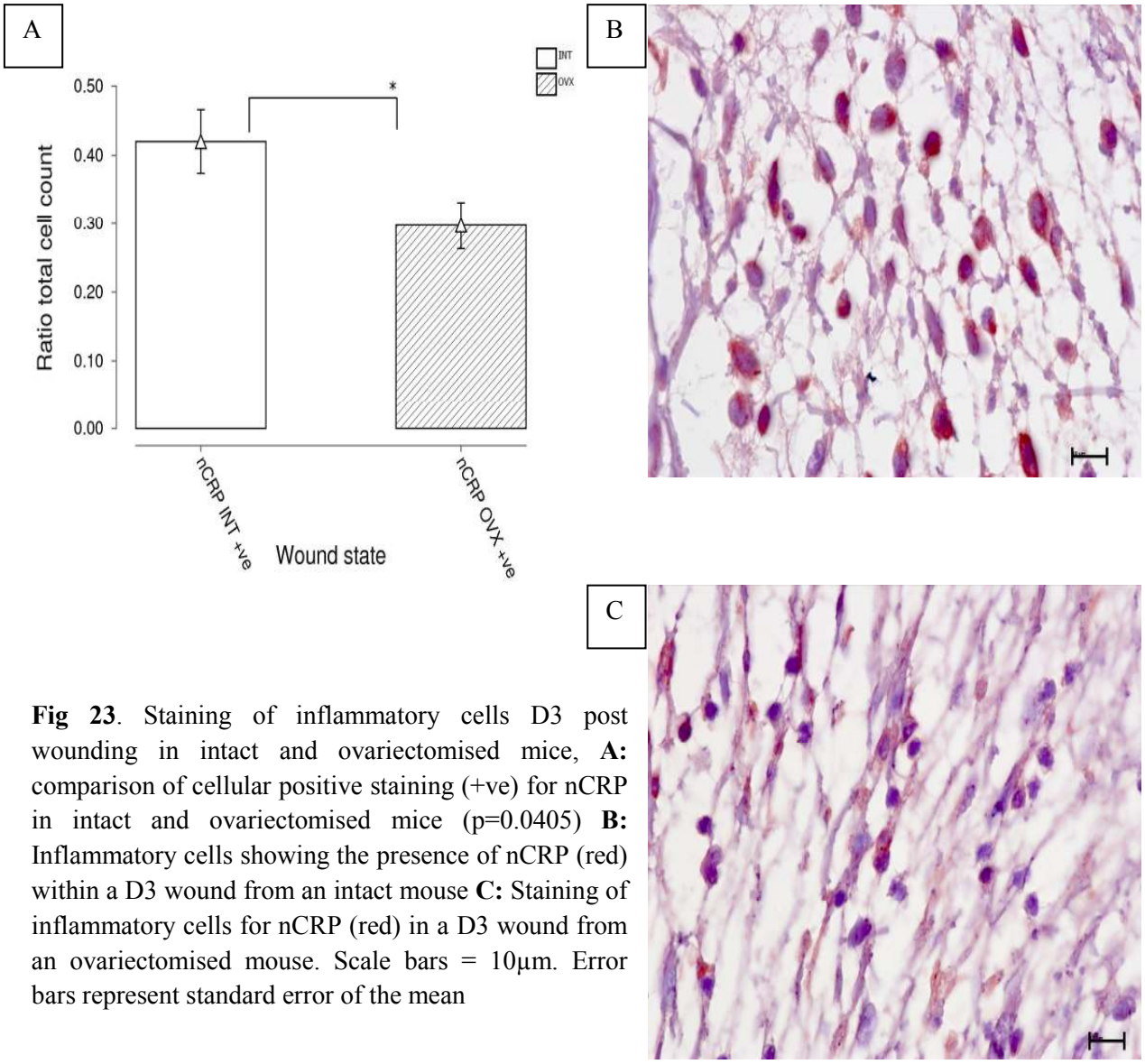

Fig 23. Staining of inflammatory cells D3 post wounding in intact and ovariectomised mice, **A:** comparison of cellular positive staining (+ve) for nCRP in intact and ovariectomised mice (p=0.0405) **B:** Inflammatory cells showing the presence of nCRP (red) within a D3 wound from an intact mouse **C:** Staining of inflammatory cells for nCRP (red) in a D3 wound from an ovariectomised mouse. Scale bars = 10µm. Error bars represent standard error of the mean

4. Discussion

To date the expression or role of CRP isoforms has never been investigated in cutaneous wound healing. These findings present a temporal profile for the expression of both CRP isoforms during the wound healing process. The primary finding of this investigation is that CRP isoforms are expressed within a wound at differing levels throughout the wound healing process. A secondary finding of his investigation is the difference between expression of CRP isoforms during the inflammatory phase of the acute and chronic wound healing processes.

Upon the onset of inflammation, levels of the acute phase protein CRP increases up to 1000 fold in the plasma (Ballou and Kushner, 1990). Therefore it has long been considered a sensitive marker for inflammation (Koenig *et al.,* 1999). Several prospective epidemiological studies have demonstrated CRP to be a strong predictor of future vascular events (Ridker, 2003). This has led to research on the effect of CRP on the inflammatory response in cardiovascular and cerebrovascular disease, and the discovery of distinct biological properties exerted by CRP isoforms. *In vitro* CRP isoforms have been shown to exert both pro- and anti-inflammatory properties upon platelets, endothelial cells, monocytes and neutrophils which all play crucial roles in the wound healing process(Khreiss et al., 2002; Khreiss *et al.,* 2004; Khreiss *et al.,* 2005; Eisenhardt *et al.,* 2009).

Upon wounding a cascade of events is initiated to minimise infection and restore integrity of the dermal barrier; this is achieved by 3 overlapping phases. The first of these phases is the inflammatory phase (Singer and Clark, 1999; Diegelmann and Evans, 2004) which is characterised by the influx of leukocyte subsets. The first predominant leukocyte to arrive at the wound is the neutrophil via diapedesis of the activated endothelium (Kaplanski *et al.*, 2003). Within the first 12-24 hours cell numbers within the wound rise dramatically, and in this study at 12 hours post-wounding 19% of the cells within the wound stained positively for mCRP and 15% stained positively for nCRP, with .no significant difference ($p=0.1165$) in expression between the two CRP isoforms. By 24 hours post-wounding this expression had increased to 45% of the cells staining positively for mCRP and 40% staining positively for nCRP, and

again there was no significant (p=0.2393) difference between the levels of expression of the two CRP isoforms. However, when compared to 12 hours, there was a significantly (p=>0.00001) higher ratio of cells stained positively for each of the CRP isoforms. The cells stained positively were calculated as a ratio so this increase was not caused by a mere increase in cell number at 24 hours post-wounding. At D3 post-wounding most cells will be neutrophilic in nature so at this time point we only investigated this cell type. Our results indicated that both isoforms of CRP are expressed in neutrophils at D3 post-wounding since 77% stained positively for mCRP and 55% stained positively for nCRP. The expression of mCRP was significantly (p=<0.00001) higher than that of nCRP at D3 post-wounding. Evidence on the opposing inflammatory properties exerted by each isoform on neutrophils may be a reason for this difference in expression. Leukocyte recruitment into inflammatory sites is achieved using constitutive or inducible adhesion molecules. The primary molecules which mediate leukocyte capture and rolling on the endothelium are L-selectin (CD62L) which is constitutively expressed on most leukocytes and P- and E-selectin which are expressed on activated endothelium (Nagaoka *et al.,* 2000). Both isoforms of CRP have been shown to down regulate L-selectin expression on neutrophils (Zouki *et al.,* 1997). However L-selectin is rapidly shed and after cell activation with a concomitant up-regulation of Mac-1 (CD11b/CD18) which is largely responsible for the tightening of the adhesion and trans-endothelial migration of neutrophils via their endothelial counter ligands ICAM-1 and ICAM-2 (Zouki *et al.,* 2001). The mCRP isoform has been shown to increase expression of Mac-1 and ICAM-1 (Khreiss *et al* 2004, Zouki *et al* 2001). Therefore the higher number of mCRP expressing neutrophils at d3 post-wounding may occur because they are able to transmigrate more efficiently due to the increase in adhesion molecule expression on their cell surface.

Neutrophils die rapidly and their survival is contingent upon rescue from cell-programmed death by signals from the environment. The life span of a mature neutrophil can be extended within the inflammatory microenvironment by bacterial products and pro-inflammatory cytokines including, interleukin-2 (IL-2), interferon-γ and granulocyte macrophage-colony stimulating factor (GMCSF) and

glucocorticoids (Khreiss *et al* 2002). The monomeric isoform of CRP has been shown to delay rather than block apoptosis in neutrophils *in vitro,* via a survival signalling pathway through extracellular signal-related kinase (ERK) and phosphatidylinositol-3 kinase/Akt. However nCRP has no significant affect on neutrophil apoptosis *(*Khreiss *et al.,* 2002). Loss of pentameric symmetry has also been associated with an increase in the pro-inflammatory cytokine IL-8 gene expression and protein secretion *in vitro* in neutrophils (Khreiss *et al.,* 2005). These factors could coincide with the significantly ($p=<0.00001$) higher levels of mCRP expressing neutrophils at an early time point (D3) post-wounding. Thus the monomeric version of CRP may be required in higher quantities to amplify the acute inflammatory response, whereas the native version may be necessary to dampen the inflammatory response to avoid prolonging the inflammatory phase longer than is necessary.

At D7 post-wounding our results showed that there are more neutrophils within the wound than at D3 post-wounding but levels of expression of both CRP isoforms had significantly ($p=<0.00001$) reduced to a similar staining level, approximately 30% of the total cell count. This indicated a larger decrease (49%) for mCRP expression than for nCRP expression (24%). The decrease coincides with the biological peak in neutrophil infiltration (Li *et al.,* 2007) and subsequent apoptosis suggesting levels of isoform expression may reduce as the effects exerted on neutrophils are not needed anymore. However our results show that of the infiltrating monocytes, 80% express both CRP isoforms suggesting that each isoform may be exerting biological effects on this leukocyte. At D7 post-wounding the predominant leukocyte subset is shifting from neutrophils to monocytes; this could be due to chemotactic factors favouring the infiltration of monocytes such as the chemokine macrophage inflammatory protein -1 and monocyte chemoattractant protein-1 levels rising within the wound (Kaplanski *et al.*, 2003); Dipietro, 1995). The nCRP isoform has been reported to stimulate monocyte chemotaxis (Torzewski et al., 2000). However mCRP has been shown to stimulate monocyte transmigration significantly stronger than nCRP (Eisenhardt *et al* 2009). Both these factors may contribute to the shift in predominant leukocyte together

with the fact that both CRP isoforms have been shown to inhibit neutrophil chemotaxis (Khreiss *et al* 2002).

Monocytes differentiate in to wound macrophages in response to interleukin-4 (IL-4), a cytokine released during tissue injury (Mosser and Edwards, 2008). Activation of the macrophage within the wound is due to several types of macrophage activating agents: tissue debris such as extracellular matrix components (e.g. fibronectin, collagen); senescent neutrophils and red blood cells (RBCs); bacterial factors; platelet derived factors (e.g. TGF-β, PAF); cell derived factors such as cytokines (e.g. IL-1, TGF-β, IFN-γ) and chemokines (e.g. MCP-1) and wound microenvironment conditions (e.g. hypoxia, lactate concentration and pH) (Dipietro, 1995). By incubating monocytes with CRP isoforms and using the activation of integrin Mac-1 as a measure Eisenhardt (2009) proposed mCRP to be a more potent stimulant for the activation of monocytes compared to nCRP. Once activated macrophages are highly phagocytic and are responsible for removing non-functional host cells, bacteria-filled neutrophils, damaged matrix, foreign debris and any remaining bacteria form the wound site (Diegelmann and Evans 2004). Reactive oxygen species (ROS) production is named the 'respiratory burst' in phagocytic cells and is critical for the bactericidal action of phagocytes. The isoform mCRP has been reported to be a more potent inductor of ROS production in monocytes than nCRP (Eisenhardt *et al.* 2009), therefore implicating mCRP in the regulation of phagocytosis. Another pro-inflammatory response reported for mCRP is the ability of the isoform to increase monocyte adhesion to a fibrinogen matrix *in vitro*. Addition of nCRP partially inhibited the effects of mCRP on monocytes and it was postulated this may be due to a regulatory attenuating function on the pro-inflammatory effects of mCRP (Eisenhardt *et al.,* 2009). This could explain the similar expression of CRP isoforms at D7 post-wounding. As the inflammatory phase comes to an end the pro-inflammatory effects of mCRP may be less important, thus causing a decrease in expression to a level similar to that of nCRP, which is exerting regulatory anti-inflammatory properties to control inflammation. However, although a high quantity of monocytes expressed both CRP isoforms at D7 post-wounding, overall inflammatory cell levels were substantially lower than at D3 post-wounding,

inferring that CRP isoforms may play more important roles at very early time points during the inflammatory phase of healing.

Further corroboration of the differing roles of CRP isoforms, which could explain the differences observed in expression in inflammation during wound healing, is the receptors that they bind in order to exert their biological function. The proposed receptor for mCRP is FcγRIII (CD16), whereas the proposed receptors for nCRP are FcγRIIa (CD32) and FcγRI (CD64) (Khreiss *et al* 2002). *In vitro* studies have also proposed mCRP interacts with lipid raft microdomains within the plasma membrane as interruption of these with nystatin abrogated mCRP responses including cytokine release, ROS formation and upregulation of adhesion molecule expression (Ji, *et al.,* 2009).

The proliferative stage of the wound healing process is characterised by angiogenesis and fibroplasia. Angiogenesis describes the formation of new blood vessels; within the context of a wound this is important to satisfy the increasing demand for oxygen and nutrients due to the high metabolic rate (Diegelmann and Evans, 2004). Previous research has focussed on the effects of CRP isoforms on angiogenesis in cardio- and cerebrovascular disease; there is currently no evidence for the presence or effect of CRP isoforms on newly formed blood vessels within the wound. This investigation has shown that both CRP isoforms are associated with blood vessels within the wound at time points indicative of the proliferative stage of the wound healing process. At D7 post-wounding mCRP is expressed in a significantly higher ratio ($p=<0.0001$) of the blood vessel than nCRP with mCRP being expressed in 27% of the total amount of blood vessel and nCRP in only 1%. Although mCRP is only expressed in a quarter of the total blood vessel the substantial difference in the expression of the two isoforms could suggest a difference in functional roles, with nCRP having limited importance in the process of angiogenesis. .

Previous research in brain tissue after acute ischemic stroke has shown mCRP to be associated with highly angiogenic microvessels within the brain and co-localised with CD105, a marker of angiogenesis, whereas nCRP was not present (Slevin *et al* 2009). This could support a role for mCRP in blood vessel development and our findings at this time point in the context of the wound healing process. Conversely

previous research which has shown nCRP to inhibit both basal and vascular endothelial growth factor stimulated angiogenesis and suppress the expression of arteriogenic chemo-cytokines due to the up-regulations of suppressors of cytokine signalling proteins (Verma *et al* 2002; Suh *et al* 2004). This could support the minimal amount of nCRP associated with blood vessel formation at D7 post-wounding in our findings. However at D14 post-wounding the expression of nCRP significantly ($p<0.0001$) increases to 19% and this could reflect the inhibitory effect nCRP exerts on angiogenic blood vessels as at this later time point angiogenesis is not the predominant biological function and therefore nCRP may be working to dampen angiogenesis as the newly formed blood vessels mature. Levels of mCRP expression decrease slightly at D14 post-wounding to 25% but this difference is not significant

Fibroplasia describes fibroblast proliferation and migration into the fibrin clot, they deposit fibronectin, interstitial collagens and proteoglycans to create granulation tissue (Trowbridge and Emling, 1997). There is currently no evidence on the effect of monomeric CRP on dermal fibroblast. This investigation has shown that both CRP isoforms are associated with wound fibroblasts at a time point indicative of the proliferative phase (D14 post-wounding) and also the tissue remodelling phase (D21 post-wounding). For each time point there was no significant difference between the ratio of total fibroblasts that stained positively for both CRP isoforms (D14 post-wounding $p=0.0544$, D21 post-wounding $p=0.4841$) However when the two time points were compared the ratio of positively stained cell for each isoform significantly decreased later in the wound healing process. At D21 post-wounding mCRP expression in fibroblasts had decreased significantly ($p=>0.0001$) from 50% to 27% of the total fibroblasts present in the wound. Expression of nCRP followed a similar pattern with levels significantly decreasing from 49% to 19% of the total fibroblasts within the wound staining positively. Local expression of CRP has been shown in pulmonary fibroblasts in a porcine model of a pulmonary disease with inflammatory features, as wound fibroblasts are primarily initiated and co-ordinated via the preceding inflammatory phase this local expression could also account for some of the expression recorded in this investigation. Native CRP has been shown to have a direct effect on the migration of human foetal lung fibroblasts to human plasma

fibronectin, the protein inhibited migration in a dose dependent fashion via the p38 MAPK pathway (Kikuchi *et al* 2009). This may be a reason why expression was higher at D14 when compared to D21 post-wounding; D14 is indicative of the proliferative stage of the wound healing process in which the fibroblasts are one of the main cell types present and with their main function being migration. Also when added directly to cultures of cardiac fibroblasts from wild type mice CRP significantly increases the expression of collagen 1 and 2, α-SMA, TNF-α and IL-1β mRNA and protein production of TNF-α and IL-1β (Zhang *et al* 2010.) Therefore the expression and association of CRP isoforms with the fibroblasts within the wound may be to control this process and ensure the cell performs its function in a timely manner. Whereas at D21 post-wounding the role of the fibroblasts is relatively diminished at this later time point, which is indicative of the tissue remodelling phase where the main event is the strengthening repaired tissue by the active remodelling of collagen from type I to type III. This could also explain the decrease in expression as the fibroblasts that are still left in the wound will be coming to the end of their lifespan and no longer carrying out their biological functions. What is not clear from our initial results is the origin of the CRP isoforms present in the wound samples. The literature proposes two mechanisms; local dissociation and local expression (Ciubotaru *et al., 2005;* Eisenhardt *et al*., 2009). *In vitro* work is necessary to derive the origin of expression in cutaneous wound.

During this investigation the levels of expression of CRP isoforms at D3 post-wounding in acute and an aged wound model was also examined. Analysis of a second acute model at D3 post-wounding further corroborated original results obtained showing a significant difference ($p=<0.05$) in staining between CRP isoforms. At D3 post-wounding 60% of the total cells within the wound were staining positively for mCRP and only 42% of the total cell count was expressing nCRP. This finding lends further support to the differing roles for each isoform within the acute wound healing process.

The definition of a chronic wound is one which has failed to proceed through the phases of healing in an orderly and timely process (Lazarus *et al* 1994), they are characterised by an abnormal inflammatory

profile. However when CRP isoform expression was profiled at D3 post-wounding in an impaired healing model, levels of expression follow the same profile. The monomeric isoform is present in significantly ($p=<0.01$) higher levels than the monomeric, with mCRP being expressed in 49% of cells but nCRP in only 30%. When these levels of expression were compared to the acute healing model there was no significant difference in levels of expression of mCRP, however results showed that levels of nCRP were significantly ($p=0.0405$) lower in wounds indicative of age-related impaired healing. The difference in expression in the native isoform of CRP between acute and age-related impaired wounds could contribute towards disrupting the self-limiting process of inflammation seen in an acute wound. However this is not entirely what we would have expected to see. We would have possibly expected to see higher levels of mCRP within an impaired healing model, to accentuate the pro-inflammatory properties exerted by this isoform, which would hinder the wound proceeding to the next phase of the healing process. However, this was just a model of age-related impaired healing which tends to have a slightly pronounced and prolonged acute wound healing and was not a chronic wound (e.g. venous ulcer) which has excessive and unabated inflammation. The significantly lower levels of nCRP within the age-related impaired healing model could play a role and contribute to the abnormal inflammatory profile and/or prolonged inflammation. We have previously discussed a role for nCRP is providing regulatory anti-inflammatory properties; this significant decrease could diminish these properties and indirectly enhance the pro-inflammatory properties of mCRP.

To date there has been no research investigating the presence or involvement of CRP during wound healing. The novel work in this study shows varying levels of CRP isoforms at different time points indicative of the 3 major phases of wound healing, with an indication of both CRP isoforms present (dual detection) in inflammatory cells that predominated the early phase of wound healing. To further explore the association of CRP isoforms with cells integral to wound healing, alternative staining methods could now be used to pinpoint the presence of CRP isoforms. Techniques such as dual fluorescence microscopy

or immunogold labelling could be adopted to enable staining of multiple proteins on a single wound section. These methods could confirm co-localization of CRP isoforms with specific cell types present in wound sections. Moreover, this study has shown for the first time that there are altered levels of CRP isoforms in a murine model of age-related impaired healing compared with acute wounds from mice with no evidence of impaired healing. These preliminary findings warrant further investigation to quantify CRP expression during wound repair and determine whether CRP isoforms induce functional effects in different cell types. This thesis has not presented any evidence to implicate CRP isoforms in the aetiology of human skin ulcers. However, it is conceivable (given emerging findings in the literature on the biological effects of each CRP isoform) that inappropriate CRP levels may contribute to impaired healing states. A combination of both *in vitro* assays and animal models would be required to confirm such biological effects and subsequently determine the precise mechanisms by which CRP isoforms may mediate wound repair processes. Although somewhat speculative at this stage, determining the precise roles CRP isoforms may play during healing may ultimately elucidate novel and focused therapeutic strategies for chronic wounds in humans.

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