MECHANISMS OF STEROID ACTION AND RESISTANCE IN INFLAMMATION

Glucocorticoid-mediated regulation of granulocyte apoptosis and macrophage phagocytosis of apoptotic cells: implications for the resolution of inflammation

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Abstract

Glucocorticoids represent one of the most effective clinical treatments for a range of inflammatory conditions, including severe acute inflammation. Although glucocorticoids are known to affect processes involved in the initiation of inflammation, the influence of glucocorticoids on the mechanisms by which acute inflammation normally resolves have received less attention. Apoptosis of granulocytes present at inflamed sites leads to their rapid recognition and internalisation by macrophages, a process which may be important for resolution of inflammation. However, if clearance of either eosinophils or neutrophils is impaired, these cells rapidly undergo secondary necrosis leading to release of pro-inflammatory mediators from the phagocyte, potentially prolonging inflammatory responses. Physiologically relevant concentrations of glucocorticoids *accelerate* eosinophil apoptosis whilst *delaying* neutrophil apoptosis during *in vitro* culture. Here we discuss key pathways regulating the granulocyte apoptotic programme and summarise the effects of glucocorticoids on monocyte differentiation and the consequent changes to apoptotic cell clearance capacity. Definition of the mechanisms underlying resolution of inflammatory responses following glucocorticoid treatment may unveil new targets for modulation of inflammatory disease, allowing co-ordinated augmentation of granulocyte apoptosis together with increased macrophage capacity for clearance of apoptotic cells.

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Introduction

Whilst the effects of glucocorticoids on the events associated with the initiation of inflammation have been studied extensively (Pitzalis *et al.* 2002, Webster *et al.* 2002), the influence of glucocorticoids on the mechanisms by which acute inflammation normally resolves have received less attention. Over the past few years, we have been studying the process of resolution of inflammation, hypothesising that definition of the underlying mechanisms may allow development of new therapeutic approaches aimed at promoting the safe resolution of inflammatory responses which underlie a heavy burden of disease in the lung and other organs (Haslett *et al.* 1994) (see Fig. 1). It is now clear that neutrophil granulocytes undergo constitutive apoptosis (Savill *et al.* 1989*a*) at inflamed sites, a process associated with the 'disabling' of their potentially injurious secretion responses and other effector functions including adhesion and phagocytosis (Whyte *et al.* 1993*a*). Surface molecular alterations, including marked down-regulation of expression of FcγRIII (Dransfield *et al.* 1994), L-selectin and uncoupling of integrins of the β2 family (Dransfield *et al.* 1995), contribute to attenuation of functional responsiveness. In addition, apoptosis-associated cell surface alterations, including phosphatidylserine exposure (Homburg *et al.* 1995), are thought to provide cues that lead to rapid recognition and internalisation of apoptotic cells by macrophages (Savill *et al.* 1989*b*). Importantly, phagocytic clearance of apoptotic granulocytes, in contrast to other phagocytic pathways, fails to promote the release of pro-inflammatory mediators from macrophages (Meagher *et al.* 1992), which may be important for the 'normal' resolution process. In addition, our studies have shown that macrophages (Savill *et al.* 1990, 1992)

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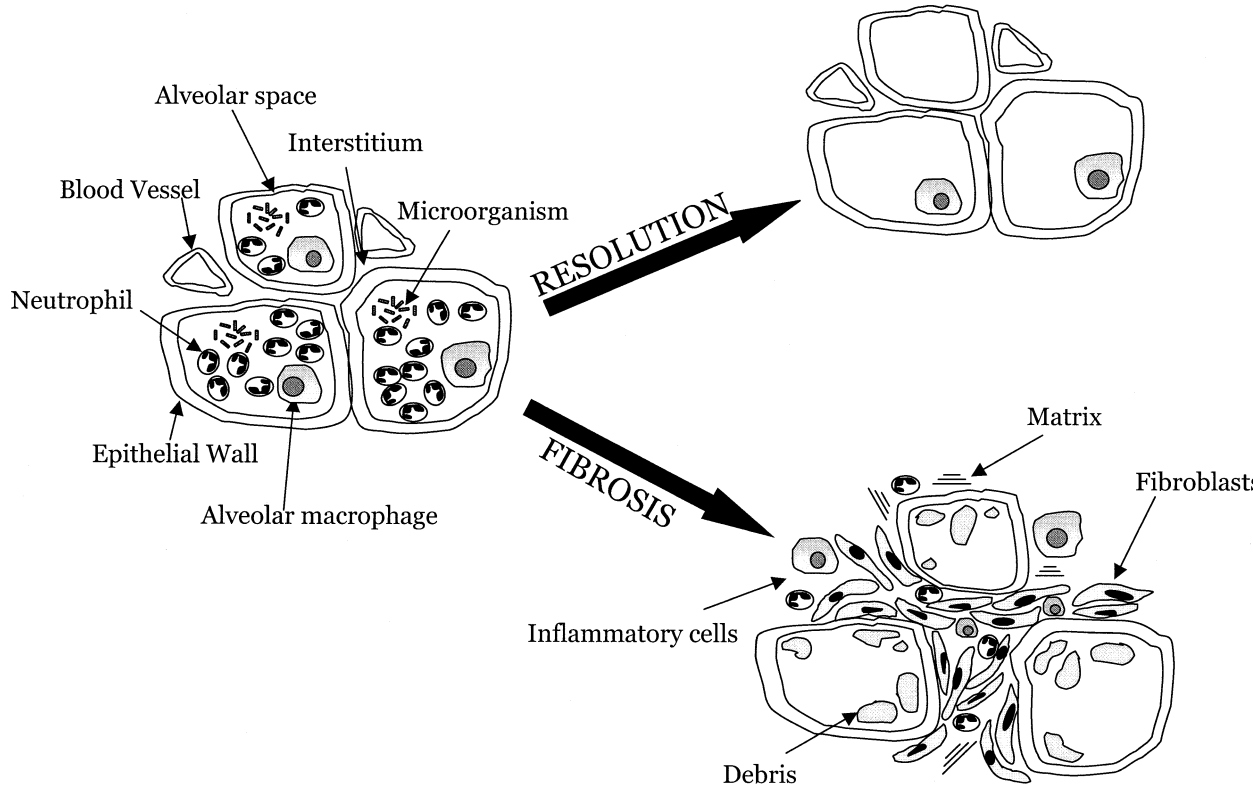

Figure 1 Schematic representation of cellular changes associated with either resolution of inflammation or development of fibrosis. Microbial infection is accompanied by infiltration of alveoli with inflammatory cells, predominantly neutrophils. Resolution of inflammation is associated with removal of microbial infection and return of tissue architecture to normal. In contrast, failure to resolve inflammation results in chronic recruitment, matrix deposition and fibroblast migration and proliferation, leading to loss of gas exchange capacity.

subsequently emigrate from the inflamed site to draining lymph nodes (Bellingan *et al.* 1996), where they may have the potential to influence activation of cells of the acquired immune system. Similarly, eosinophil granulocytes, which have been strongly implicated in tissue injury in allergic acute inflammation, are also programmed to die by constitutive apoptosis. Whilst the rate of constitutive eosinophil apoptosis is slower than that of the neutrophil granulocyte, apoptosis also leads to rapid, non-inflammatory phagocytic clearance by macrophages (Stern *et al.* 1992). However, if clearance of either eosinophils or neutrophils is impaired, these cells rapidly undergo secondary necrosis. In addition to the release of potentially toxic intracellular contents associated with necrosis, one important consequence is that macrophage phagocytosis of post-apoptotic cells leads to the release of pro-inflammatory mediators, potentially prolonging inflammatory responses (Stern *et al.* 1996).

Granulocyte deletion by apoptosis has been shown to be amenable to external regulation by lineage-specific inflammatory signals (Haslett *et al.* 1991, Stern *et al.* 1992, Lee *et al.* 1993, Whyte *et al.* 1993*b*, Hannah *et al.* 1995, Murray *et al.* 1997, Ward *et al.* 1997, Coxon *et al.* 1999, Hofman *et al.* 2000), providing an opportunity for targeted therapeutic intervention. However, if triggering of apoptosis is to be considered as a therapeutic target, failure to match the apoptotic cell load to the tissue clearance capacity at an inflamed site may have deleterious consequences in terms of resolution of inflammation. Macrophage capacity for phagocytosis of apoptotic granulocytes can be rapidly regulated by exogenous factors e.g. following ligation of extracellular matrix receptors such as CD44 (Hart *et al.* 1997, McCutcheon *et al.* 1998) or prostaglandin receptors (Rossi *et al.* 1998). Thus, co-ordinated acceleration of granulocyte apoptosis at inflamed sites together with augmentation of macrophage capacity to clear apoptotic cells may be a realistic therapeutic goal (Ward *et al.* 1999*a*).

Glucocorticoids (GCs) represent one of the most effective clinical treatments for a range of inflammatory conditions, including severe acute inflammation. GCs have profound effects, both on granulocyte apoptotic programmes (Cox 1995) and macrophage phagocytic function (Liu *et al.* 1999). In particular, physiologically relevant concentrations of GCs, acting via the GC receptor, were found to *accelerate* eosinophil apoptosis whilst

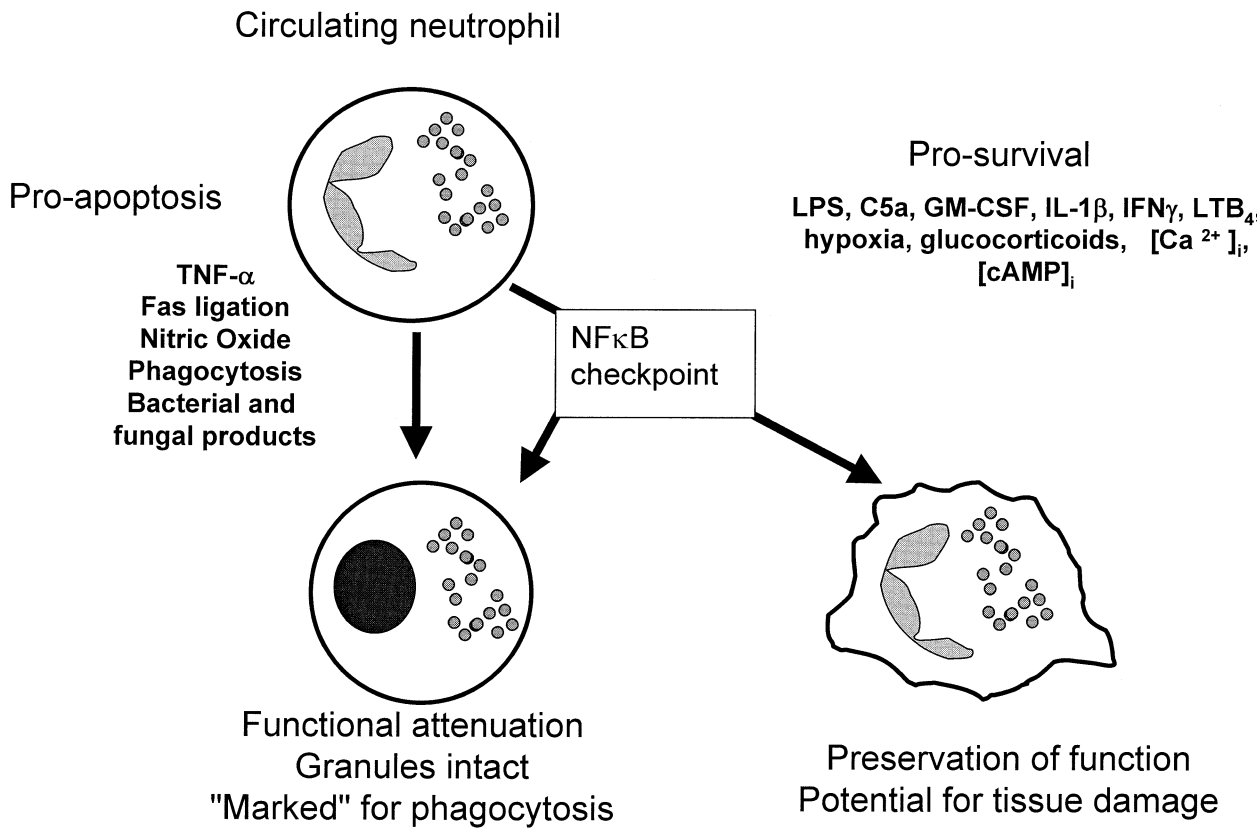

Figure 2 Neutrophils are poised on a 'knife edge' decision of life or death. A range of extracellular stimuli can promote apoptosis or survival. For those stimuli that drive survival, NFκB activation acts as a checkpoint that can result in engagement of cell death pathways, leading to functional downregulation and preservation of membrane integrity. Apoptotic cells can then be swiftly cleared by phagocytes. IFNγ, interferon γ. LTB-4, leukotriene B4.

delaying neutrophil apoptosis during *in vitro* culture (Meagher *et al.* 1996). In this article, we will discuss key pathways regulating the granulocyte apoptotic programme following treatment with GCs, and describe recent data relating to the effects of GC on monocyte differentiation and the consequent changes to apoptotic cell clearance capacity.

Granulocytes are poised on a knife edge of nuclear factor kappaB (NFκB)-mediated survival

It is now well established that at an inflammatory site there are many cytokines and growth factors present that provide survival signals for granulocytes with the potential to over-ride granulocyte apoptotic pathways (see Fig. 2). We have demonstrated important apoptosis-inhibiting effects of inflammatory stimuli that increase cyclic AMP levels in neutrophils (Rossi *et al.* 1995). The dramatic delay of the caspase-dependent apoptosis in neutrophils by dibutyryl cAMP (dbcAMP) was found to occur via a novel protein kinase A (PKA)-independent signalling pathway involving maintenance of mitochondrial potential (Martin *et al.* 2001). The survival effects of dbcAMP were independent of phosphatidylinositol-3 kinase (PI3K) and MAP kinase (MAPK) activation and our data point to a novel, reversible, transcriptionally independent mechanism of action of dbcAMP that may provide opportunities to shift the balance of pro-apoptotic and anti-apoptotic proteins and hence accelerate clearance of granulocytes from inflamed sites.

One of the most potent agents known to modulate granulocyte apoptosis is bacterial lipopolysaccharide (LPS) (Lee *et al.* 1993). Most inflammatory cells sense LPS using a complex system that involves the interaction of LBP/CD14/MD-2 and Toll-like receptor 4 (TLR4) (O'Neill & Dinarello 2000, Triantafilou & Triantafilou 2002) receptors which, when engaged, trigger inter-related signal transduction pathways, including the MAPK (ERK1/2, JNK and p38), PI3K and NFκB pathways to orchestrate innate immune responses. Detailed investigation of the role of NFκB in control of granulocyte survival has revealed that inflammatory mediators such as LPS and granulocyte macrophage colony stimulating factor

(GM-CSF) *downregulate* susceptibility of neutrophils to Fas-directed death (L Murray, S O'Dea, D Harrison, C Haslett & A G Rossi, unpublished data) implying that specific pro-apoptotic regulatory pathways are overridden by NFκB signalling pathways (Fig. 2). Although both tumour necrosis factor-α (TNF-α) and LPS act to trigger NFκB activation in neutrophils, TNFα-mediated activation of NFκB delays apoptosis in the majority of neutrophils not induced into early apoptosis (Murray *et al.* 1997, Ward *et al.* 1999*b*). The pronounced effects of protein synthesis inhibitors upon granulocyte survival supports the suggestion that NFκB-directed gene transcription and protein synthesis of anti-apoptotic factors and powerful cytokines delays apoptosis in 'inflammatory' granulocytes.

We have demonstrated that the rate of constitutive apoptosis in both neutrophils and eosinophils was greatly accelerated by the NFκB inhibitor and fungal metabolite gliotoxin. This effect was reproduced using other NFκB inhibitors and suggests TNF-α-induced activation of NFκB and production of survival proteins limits pro-apoptotic effects and may delay apoptosis at later time points. Similarly, for human eosinophils exposed to TNF-α, cytoplasmic levels of IκBα, the inhibitory subunit of NFκB are rapidly reduced and NFκB is mobilised from the cytoplasm to the nucleus (Fujihara *et al.* 2002). Inhibition of TNF-α-mediated IκBα degradation and NFκB activation by gliotoxin treatment of eosinophils reveals caspase-dependent pro-apoptotic properties of TNF-α. Selective inhibition of eosinophil NFκB activation may therefore represent an alternative target for inducing specific deletion of eosinophils in diseases including asthma and allergic rhinitis.

NFκB-dependent genes may also have a key regulatory role in the pathways responsible for the metabolism of prostaglandins (PGs) in granulocytes. Although many natural prostaglandins (e.g. PGE_2, PGA_2, PGA_1, $PGF_{2\alpha}$) act either to delay apoptosis or have no effect, PGD_2 and its metabolite PGJ_2 selectively induced eosinophil apoptosis (Ward *et al.* 2002). In contrast, the sequential PGD_2 metabolites $\Delta^{12}PGJ_2$ and 15 $dPGJ_2$ were found to induce caspase-dependent apoptosis in both eosinophils and neutrophils. Despite $\Delta^{12}PGJ_2$ and 15 $dPGJ_2$ being known activators of peroxisome proliferator-activated receptor (PPAR)-γ, apoptosis was not mimicked by synthetic PPAR-γ and PPAR-α ligands nor blocked by an irreversible PPAR-γ antagonist, suggesting a PPAR-γ-independent mechanism (Ward *et al.* 2002). We found that $\Delta^{12}PGJ_2$ and 15 $dPGJ_2$ inhibited LPS-induced IκBα degradation and NFκB activation, thereby triggering apoptosis. The powerful pro-apoptotic effects of $\Delta^{12}PGJ_2$ and 15 $dPGJ_2$ in both eosinophils and neutrophils implies that differences in the ability of eosinophils and neutrophils to process and degrade prostaglandins may be responsible for the differential effects of PGD_2 upon granulocyte survival.

One potential limitation to the effectiveness of GCs in treatment of inflammatory diseases is that they undesirably prolong neutrophil survival (Cox 1995), increasing the potential for secretion of pro-inflammatory granule contents during inflammatory episodes. We believe that definition of mechanisms by which glucocorticoid-directed survival of neutrophils may be 'disengaged' may improve the efficacy of GCs in neutrophilic inflammatory diseases. Our preliminary data indicate that glucocorticoid-mediated delay of neutrophil apoptosis is reversed by inhibition of protein synthesis and inhibited by blockade of NFκB (C Ward & A G Rossi, unpublished data). We suggest that GCs engage NFκB-directed synthesis of 'survival proteins' that may be targeted to make neutrophils respond to GCs in the same way that eosinophils do.

Macrophages can be enabled for phagocytosis of apoptotic granulocytes

Apoptotic cells have potentially toxic cellular contents and autoantigens may be revealed or generated within apoptotic cells. Thus, defects in clearance of apoptotic cells would be predicted to be associated with spontaneous and/or persistent inflammatory responses and evidence of autoimmunity to intracellular antigens (Lorenz *et al.* 2000, Beutler 2001, Botto 2001, Greidinger 2001, Stuart & Hughes 2002). In support of this suggestion, spontaneous/persistent tissue inflammation and autoimmunity is observed in mutant mice with proven and probable defects in clearance of dying cells (Botto *et al.* 1998). Indeed, some patients with systemic lupus erythematosus exhibit (as yet uncharacterised) defects in macrophage phagocytosis of apoptotic cells (Baumann *et al.* 2002). As discussed above, we would predict that upregulation of macrophage capacity for 'safe' phagocytosis of apoptotic granulocytes will represent an important aspect of therapeutic strategies aimed at promoting the resolution of inflammation.

We and others have shown that macrophage phagocytosis may be rapidly modulated in response to extracellular environmental signals (Fig. 3). For example, elevation of intracellular cAMP in human monocyte-derived macrophages using the cell permeable cAMP analogue, db-cAMP, specifically reduced the phagocytosis of apoptotic neutrophils without affecting FcR-mediated phagocytosis (Rossi *et al.* 1998). Treatment of macrophages with PGE_2 resulted in rapid, transient increases in levels of intracellular cAMP and induced PKA-dependent morphological alterations indicative of changes in the adhesive status of the macrophage, including cell rounding and disassembly of 'podosome' adhesion structures containing actin, vinculin and talin that represent points of contact with extracellular matrix (Rossi *et al.* 1998). Consistent with the suggestion that adhesive interactions may influence

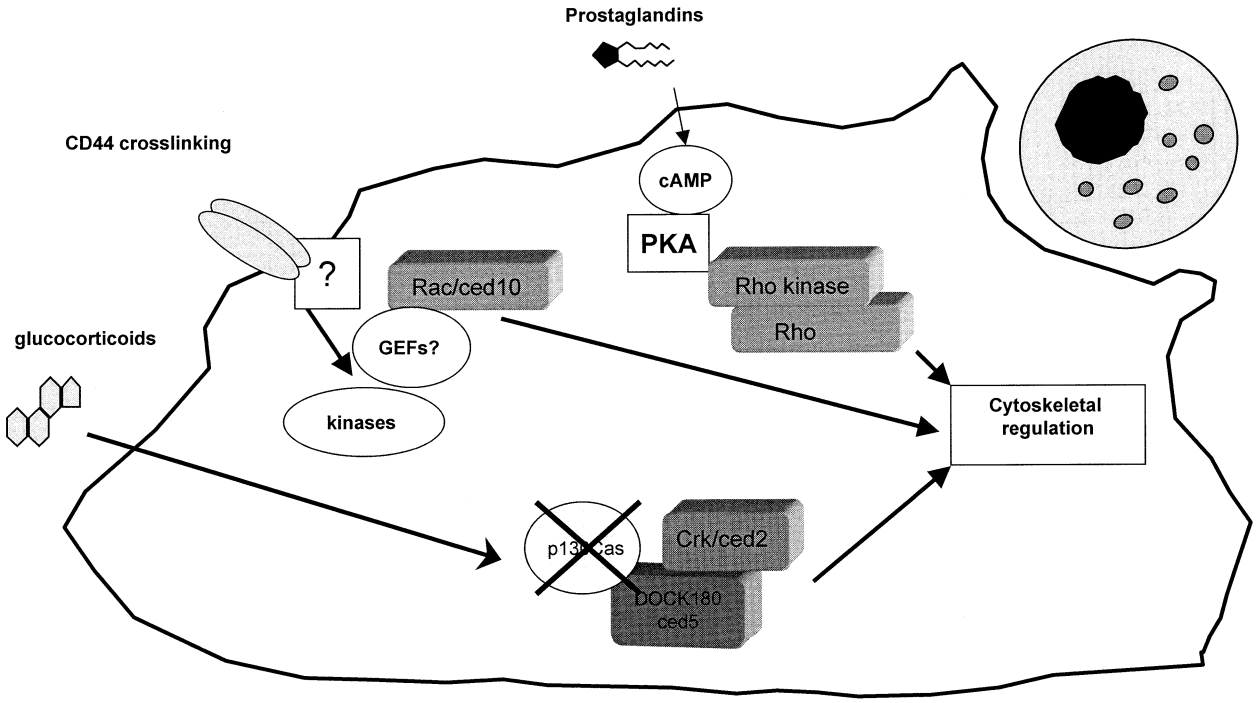

Figure 3 Schematic representation of mechanisms that regulate macrophage phagocytosis. Cross linking of CD44/matrix receptors or engagement of lipoxin receptors leads to rapid augmentation of phagocytic activity. In contrast, binding of prostaglandins to specific receptors causes cytoskeletal changes that inhibit phagocytic function. Alternatively, glucocorticoids act via glucocorticoid receptors to alter the pattern of gene expression (via either specific transactivation or transrepression) to alter cytoskeletal regulation within macrophages.

macrophage phagocytic capacity, we demonstrated that ligation of the matrix receptor CD44 rapidly and specifically increases apoptotic neutrophil internalisation (Hart *et al.* 1997). We now have very clear evidence that CD44 cross-linking is associated with the generation of intracellular signals that specifically augment clearance of apoptotic neutrophils. First, binding of Fab′ fragments of CD44 antibodies does not promote phagocytosis, indicating that these reagents do not mask sites that are normally involved in negatively regulating cellular interactions in a manner analogous to the sialomucin, CD43. Furthermore, there does not appear to be 'capping' of CD44 within the membrane e.g. within uropod-like structures following cross-linking. Detailed temporal analysis of the CD44 cross-linking effects provides evidence that CD44 acts as an 'enabler' of macrophage phagocytosis, recruiting otherwise non-responsive cells. Recent data from studies of lung injury in CD44-deficient mice adds further weight to a role for CD44 in the regulation of macrophage clearance of apoptotic neutrophils in the resolution of inflammation (Teder *et al.* 2002). Our preliminary evidence indicates that engagement of specific signal transduction events following CD44 cross-linking leads to rapid changes in cytoskeletal regulation. We are currently investigating whether CD44 initiates signals that influence cytoskeletal regulatory molecules e.g. membrane recruitment of Rac GTPase via guanine nucleotide exchange factors (GEFs) such as Tiam1 which have been shown to associate with CD44 (Bourguignon *et al.* 2000).

Glucocorticoids facilitate clearance of apoptotic cells, favouring resolution of inflammation

In contrast to the rapid effects of CD44 ligation, $[cAMP]_i$ elevation, or lipoxins (Godson *et al.* 2000), we found that exposure of macrophages to GCs for 24 hours specifically enhanced the uptake of apoptotic leukocytes by both human and murine macrophage populations (Liu *et al.* 1999). These observations establish the capacity of GCs to promote phagocytosis of cells undergoing apoptosis, raising the possibility that anti-inflammatory effects of GCs may involve pro-phagocytic effects. Importantly, glucocorticoid-mediated enhancement of macrophage phagocytosis of apoptotic cells was not achieved by costly loss of the teleogically appropriate lack of pro-inflammatory response, failing to stimulate monocyte chemoattractant protein-1 (MCP-1) production and down-regulating interleukin (IL)-8 release by the phagocyte (Liu *et al.* 1999).

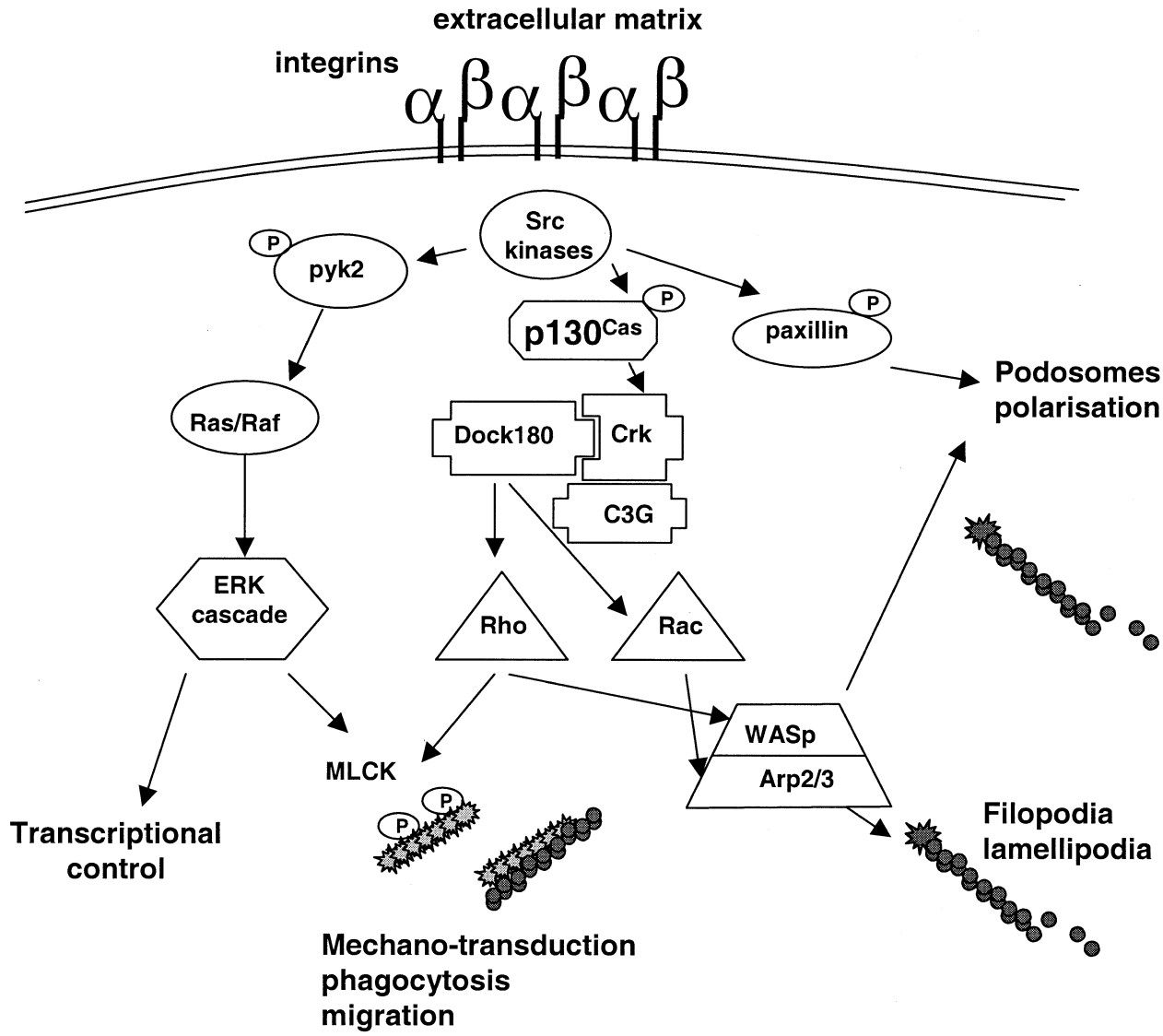

Figure 4 A central role for p130^{Cas} in adhesion signalling. Schematic representation of events occurring downstream of integrin adhesion receptor ligation. p130^{Cas} links integrin-mediated src family kinase activation to Rho family GTPases and cytoskeletal assembly necessary for adhesion, migration and phagocytosis. MLCK, myosin light chain kinase.

More recently, we have found that exposure of peripheral blood monocytes to GCs during the first 24 hours of the 5-day culture period induced a highly phagocytic monocyte-derived macrophage (MDMφ) phenotype. This GC-MDMφ phenotype was characterised by a marked morphological appearance, consisting of smaller, more 'rounded' cells with more homogeneous laser scatter properties in flow cytometric analysis (Giles *et al.* 2001). Functional and morphological homogeneity was matched by cell surface phenotype, including specific induction of expression of the haemoglobin scavenger receptor, CD163 following GC treatment. Our data indicate that GCs acting via GC receptors have the potential to re-programme monocyte differentiation towards an 'anti-inflammatory' phenotype. In light of recent studies of apoptotic cell clearance in *Caenorhabditis elegans* (Ellis *et al.* 1991, Liu & Hengartner 1998, Wu & Horvitz 1998*a*,*b*, Chung *et al.* 2000, Reddien & Horvitz 2000, Gumienny *et al.* 2001, May 2001), we next examined key intracellular components that regulate cytoskeletal coupling following adhesion. Alterations in the morphology of GC-MDMφ were mirrored by changes in

cytoskeletal organisation, with a loss of paxillin and actin containing podosome structures. Tyrosine phosphorylation of paxillin and pyk2, proteins that are recruited to adhesion contacts, were not phosphorylated in GC-MDMφ. A particularly striking change was that GC-MDMφ showed decreased expression of $p130^{Cas}$ (Giles *et al*. 2001), an adaptor protein that links integrin receptors to Rho family GTPases and the MAPK pathway (see Fig. 4). Reduced expression of $p130^{Cas}$ would be predicted to disrupt Crk/DOCK180/ELMO complexes, which together with reduced phosphorylation of paxillin and pyk2 may have implications for control of the turnover of adhesion structures in macrophages.

Specific recruitment of $p130^{Cas}$ to focal contacts following adhesion to matrix may mimic loss of $p130^{Cas}$ observed in dexamethasone-treated cells and thus influence the availability of other components to drive cytoskeletal re-organisation necessary for phagocytosis. We propose that the repertoire of adhesion receptors that are engaged on the macrophage surface might therefore control phagocytic potential indirectly by releasing or sequestering key regulatory molecules like $p130^{Cas}$ from focal adhesion complexes. Time-lapse video microscopy analysis revealed that despite the small rounded appearance of GC-MDMφ these cells are able to rapidly extend and retract cellular processes. Thus, although recruitment of proteins such as paxillin to podosome adhesion signalling complexes does not occur in the absence of $p130^{Cas}$, Rac may still drive the extension and retraction of processes required for phagocytosis. One possibility is that other $p130^{Cas}$ -like adapters such as HEF1 and Efts/Sin, present in macrophages may allow the recruitment of Rac/Crk/DOCK180 specifically to membranes in a manner that facilitates phagocytosis of apoptotic cells and possibly other particles. Importantly, these data raise the possibility that expression or phosphorylation of $p130^{Cas}$ may have a negative regulatory role upon macrophage phagocytic potential. Our recent studies examining the effect of the cytokine environment on GC-mediated monocyte differentiation have shown that the Th1 cytokine interferon-γ reverses the augmentation of phagocytosis seen in the GC-MDMφ (K M Giles, S J Heasman & I Dransfield, unpublished data). The reduction in phagocytic ability is not accompanied by morphological changes, indicating that adhesion status and the capacity for phagocytosis can be dissociated. Further understanding of the interplay between cytokine environment and GCs in inflammation may allow the tailoring of therapies that facilitate the resolution of inflammatory disease.

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