THE UNIVERSITY *of* EDINBURGH

LACTOFERRIN:

AN ANTI-INFLAMMATORY MOLECULE RELEASED BY APOPTOTIC CELLS TO INHIBIT GRANULOCYTE MIGRATION

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Doctor of Philosophy

The University of Edinburgh

2010

DECLARATION

This thesis and the research described herein is solely my own work. All work presented in this thesis was, unless otherwise acknowledged, initated and executed by myself. All sources of information in the text have been acknowledged by reference. No part of this work has been, or is submitted for any other degree qualification.

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TABLE OF CONTENTS

ABSTRACT

Apoptosis is a physiological form of cell death. It is a highly evolutionarily conserved process that is non-inflammatory or anti-inflammatory in nature. This anti-inflammatory nature of apoptosis is evident by the fact that neutrophils are histologically absent from sites where homeostatic apoptosis rates are high. The rapid phagocytosis of apoptotic cells as a means to prevent the release of noxious inflammatory compounds also accounts for the anti-inflammatory environment of such sites. However, the mechanisms that enable mononuclear phagocytes to migrate to sites where homeostatic apoptosis rates are high, and not granulocytes, the professional phagocytes that accumulate at sites of inflammation, have not been determined yet. Using Burkitt's lymphoma (BL) as a model of apoptosis, the aim of this thesis was to identify the regulatory mechanisms or factors underlying the non-phlogistic features of sites where homeostatic apoptosis rates are high and in particular, those preventing the recruitment of neutrophils -a major granulocyte subclass- to these sites.

BL is a highly aggressive B cell lymphoma that is mainly characterised by a high rate of apoptosis. By carrying out a series of *in vitro* chemotaxis assays and biochemical approaches, it was found in this thesis that BL cells actively inhibit neutrophil migration by releasing factors that were identified to be lactoferrin, a 80 kDa iron-binding glycoprotein with anti-bacterial and anti-

inflammatory properties. It was further demonstrated that lactoferrin selectively inhibited migration of granulocytes (both neutrophils and eosinophils) but not mononuclear phagocytes and this effect was irrespective of its iron saturation status and the chemoattractant used. Also, lactoferrin potently inhibited neutrophil migration, as assessed by thioglycollate-induced *in vivo* model of mouse peritonitis. This anti-inflammatory function of lactoferrin was attributed to its effect on granulocyte signalling pathways that regulate cell adhesion and motility. Finally, it was demonstrated that in cell types of diverse lineages, induction of apoptosis results in *de novo* synthesis and secretion of lactoferrin. In subsequent proliferation assays determining the *in vitro* growth of a number of BL cell types, it was demonstrated that lactoferrin is an essential component of BL cells and promotes their proliferation, as its antibody-mediated neutralisation or shRNA-mediated expression knockdown, reduced BL cell growth.

Together, the results of this thesis identified lactoferrin as one of the few characterised anti-inflammatory components of the apoptosis milieu that negatively regulate granulocyte migration. This effect may provide opportunities for broad therapeutic interventions concerning the use of lactoferrin in chronic inflammatory conditions characterised by aberrant neutrophil influx as well as atopic allergic disorders, such as asthma. Moreover, based on the tumour-supporting role of lactoferrin described in this study, targeting its

expression in tumours could lead to tumour regression and thus, be a promising therapeutic molecule in tumour immunotherapy.

INTRODUCTION

APOPTOSIS

Apoptosis is the most renowned physiological process of cell death. It is a tightly regulated and evolutionarily conserved process that was first described in 1972 by Kerr, Wyllie and Currie (Kerr *et al.*, 1972). Its term derives from the Greek words α•ό (from) and •τώσις (fall). Apoptosis is an active process that is expressed by profound alterations in the genetic component of the cell that lead to self-destruction. The importance of apoptosis is fundamental: firstly, it acts as a homeostatic mechanism to regulate the cell number in all tissues in a living organism (Majno and Joris, 1995). Secondly, it is implicated in organ and digit ontogenesis during embryonic development and later on in life in situations, such as the fashioning of the limbs, the involution of phylogenetic vestiges and the formation of interdigital clefts. In fact, the regression of *ductus arteriosus* after birth was the first typical apoptotic phenomenon described by Aristotle. Thirdly, apoptosis is involved in hormone-dependent atrophy, as first described by Wyllie in rats, during which a massive number of apoptotic cells appeared in the adrenal cortex following a reduction in adrenocorticotrophic hormone (ACTH) blood concentration (Kerr *et al.*, 1972). Apoptosis also plays a major role in cell turnover, as evidenced by the editing of exons and dendrites during brain development (low turnover rate),

the disposal of the outer segments in the eye retina, the disposal of circulating granulocytes (high turnover) as well as of gut epithelia (intermediate tissue turnover rate).

In pathological situations, apoptosis is implicated in many conditions, including stroke, myopathies, neurodegenerative conditions and malignant neoplasms. In the latter case, apoptosis is going on continuously in growing neoplasms and is affected or even exacerbated by therapeutic conditions engendered by chemotherapeutic agents, hypoxia and ionising radiation (Kerr *et al.*, 1994; Kerr *et al.*, 1972; Thompson, 1995). Of pivotal importance is also the role of apoptosis in immunity (Ekert and Vaux, 1997; Marsden and Strasser, 2003). The poorly understood apoptotic process in the germinal centres of lymphoid follicles has given rise to the notion that apoptosis is necessary for the proper functioning of the immune system. Indeed, apoptosis is necessary for the development of T and B lymphocytes, as cells not positively selected following V(D)J recombination (for B cells) or MHC recognition (for T cells) die by apoptosis. Apoptosis eliminates the self-reactive population by clonal deletion and this is particularly important to prevent autoimmune conditions. Also, cytotoxic T cell killing is mediated by an active apoptotic process. In this way, defects in the apoptosis program or inappropriate activation of apoptotic cell-death pathways could lead to autoimmune situations, immunodeficiencies and tumour progression (Poon *et al.*, 2010).

As a process, apoptosis is discerned into three phases: induction/initiation phase, effector phase and degradation. In the initiation phase, the cell receives the apoptosis-inducing stimuli, during the effector phase the apoptotic machinery is activated, while during the last (degradation) phase, the cell enters a non-reversible route of disintegration and any associated changes in cell morphology become visible (Leist and Nicotera, 1997; Majno and Joris, 1995; Susin *et al.*, 1997; Yang and Korsmeyer, 1996). The duration of the apoptotic process is estimated between 12 and 24 hours. However, it is highly dependant on the apoptotic stimulus and the affected cell type in every tissue.

Morphological Detection of Apoptosis

The first morphological description of apoptosis was described by Flemming in 1885 as cells with "ill-defined nucleus containing several small, heavily stained lumps and a pale, homogeneous cytoplasm containing what appeared to be fine fat droplets" (Flemming, 1885). This description came in accordance to what Kerr, Wyllie and Currie later described as apoptotic bodies, i.e. "small, roughly spherical or ovoid cytoplasmic fragments some of which contain pyknotic remnants of nuclei" (Kerr *et al.*, 1972). They proceeded further to divide apoptosis into two separate processes: (i) the formation of apoptotic bodies followed by (ii) their phagocytosis by other cells.

Subsequent electron microscopy analyses revealed that the morphological transformation of cells undergoing apoptosis involves the condensation of chromatin to delineated granular masses beneath the nuclear envelope, cell shrinking, convolution of the cellular outlines and finally nuclear fragmentation (Kerr *et al.*, 1994; Majno and Joris, 1995; Yang and Korsmeyer, 1996). The formed apoptotic bodies containing the nuclear remnants are rapidly removed by neighbouring macrophages. What is surprising is that during this process, the cell membrane that encases the apoptotic fragments remains intact retaining its integrity. Also, the lysosomes remain unaffected and their enzymes are not released to the surrounding tissue, enhancing in this way the role of apoptosis in tissue homeostasis, as it involves extensive cell deletion with restricted tissue disruption. Moreover, there is no inflammation and thus apoptotic cells could be described as cellular graves, in which nuclear remnants are disposed of in a non-inflammatory manner. Also, as it has been described since 1972, "the process is economical in terms of re-utilisation of cell components".

Despite the extensive involvement of apoptosis in both normal and malignant tissues, the rate of apoptosis varies depending on cell type (Kerr *et al.*, 1972). To determine apoptosis rate in a tissue section, traditional techniques are employed that are based on the characteristic morphological features of apoptotic cells. However, more refined techniques have been undertaken for tissue studies and involve the radioactive or non-radioactive labelling

of free ends of DNA (*in situ* 3'-end labelling method) or the TUNEL (terminal deoxytransferase-mediated dUTP nick-end labelling) in which terminal transferase adds labeled nucleotides into the 3' end of DNA (Ansari *et al.*, 1993; Wijsman *et al.*, 1993). Also, other recent techniques include the immunohistochemical detection of apoptosis-associated proteins, such as active caspase-3 in tissue sections. However, these techniques bear some limitations varying among research groups. This point is particularly important in the measurement of apoptotic index point to tumour tissues as the presence of resident apoptotic immune cells (e.g. macrophages, lymphocytes) might be falsely counted as apoptotic neoplastic cells.

Molecular Mechanisms of Apoptosis

Much of our knowledge about the molecular mechanisms of apoptosis arises from the nematode *Caenorhabditis elegans* (Hengartner and Horvitz, 1994; Leist and Nicotera, 1997). In this nematode, 131 somatic cells die through apoptosis out of the 1090 somatic cells that are formed during adult development. The 11 identified *C.elegans* genes that are involved in the cell death process include *CED3* and *CED4* that positively regulate apoptosis, whereas *CED9* is anti-apoptotic. As apoptosis is a highly conserved process, these *C.elegans* genes have homologous partners in mammals. The *C.elegans CED9* is highly homologous to the mammalian anti-apoptotic oncogene *BCL2*, as *BCL2* can partially substitute for *CED9* in *C.elegans* to prevent apoptosis, whereas *CED3* is homologous to cysteine proteases caspases and *CED4* has high homology to apoptosis activating factor-1 (Apaf-1), which is involved in cytochrome c dependent activation of caspase-3. Genetic studies indicate that *CED4* acts upstream of *CED3* and downstream of *CED9* (Hengartner and Horvitz, 1994).

Based on *C.elegans*, we now come to our current knowledge of apoptosis in mammals. Indeed, the apoptotic machinery can be activated by many different stimuli. Such stimuli involve DNA damage or radiation, serum withdrawal, TNF-α, 1,25-dihydroxyvitamin D3 (Haimovitz-Friedman *et al.*, 1994; Hannun, 1996) as well as the withdrawal of a growth factor or hormone, as for example, in the case of the withdrawal

of a nerve growth factor from neuronal cells pathways, in which a p38 mitogen-activated protein kinase pathway was activated to induce apoptosis (Kummer *et al.*, 1997). Apoptosis can even be initiated by changes in the cellular membrane that result to the formation of cell surface receptors, known as death receptors, which trigger apoptotic pathways. In this way, there are two main routes that have been described to induce apoptosis: the intrinsic pathway (mitochondrial-mediated apoptosis) and the extrinsic pathway that involves the activation of death receptors **(Figure 1)**. Both pathways result in the activation of caspases; however, other caspase-independent types of apoptosis have been noted (Green and Kroemer, 2004; Wolf and Green, 1999).

The role of caspases

The family of caspases involves at least 14 aspartate-specific cysteine proteases that play a key effector role in the apoptotic process (Barge *et al.*, 1997; Harvey *et al.*, 1997). They normally exist in cells as inactive proenzymes, which are proteolytically cleaved into 10 and 20 kDa subunits that constitute the active enzyme by heterodimerisation and association into tetramers. Each active caspase tetramer includes two heterodimers of a large and a small subunit. Caspase activation follows a distinct mode, in which caspases-2, -8, -9 and -10 (initiator caspases) trigger the apoptotic cascade and activate caspases -3, -6 and -7 (effector caspases) (Philchenkov, 2004; Wolf and Green, 1999).

The activity of caspases is responsible for the morphological changes that take place during apoptosis, as they act by disrupting the nuclear membrane, cleave cytoskeletal proteins, disrupt cell-cell contact and induce DNA fragmentation (Brancolini *et al.*, 1997). Indeed, caspases cleave cytoskeletal proteins such as actin, fodrin, gelsolin as well as nuclear lamins and DNA repair enzymes such as poly-ADP-ribose-polymerase (PARP) (Kothakota *et al.*, 1997; Lazebnik *et al.*, 1994; Lazebnik *et al.*, 1995). The activity of caspases is also linked to the activation of caspase-activated DNase (CAD) that triggers DNA fragmentation. Under normal conditions, the activity of CAD is inhibited by forming a complex with the Inhibitor of CAD (ICAD), which is cleaved by caspase-3 upon induction of apoptosis (Sakahira *et al.*, 1998). Other regulators of caspase activity involve the members of the Inhibitor of Apoptosis Protein (IAP) family, which include Apollon/BRUCE, c-IAP1, c-IAP2, Livin, Survivin, NAIP, ILP-2 and XIAP (X-linked IAP (Liston *et al.*, 2003).

Mitochondrial-mediated apoptosis

Mitochondrial-mediated apoptosis is elicited by the permeabilisation of the outer membrane of the mitochondrion and the subsequent formation of a permeability transition (PT) pore (Green and Kroemer, 2004; Hotchkiss *et al.*, 2009). The opening of the PT pore induces uncoupling of the oxidative phosphorylation process and entry of water to the mitochondrial matrix. This results in a consequent swelling of the mitochondrial

space and rupture of the outer mitochondrial membrane that is followed by the release of apoptosis-inducing proteins, such as apoptosis-inducing factor (AIF), cytochrome c and endonuclease G (Churbanova and Sevrioukova, 2008; van Loo *et al.*, 2001).

Cytochrome c binds to apoptosis protease activating factor-1 (Apaf-1), forming an "apoptosome" that in turn activates pro-caspase-9 that further activates pro-caspase-3. Apoptosis-inhibiting proteins like Bcl-2 and Bcl-xL counteract this apoptosis-inducing effect by binding to Apaf-1 (Zou *et al.*, 1997; Zou *et al.*, 1999). AIF is released from mitochondria into the cytoplasm and in combination with one or more factors, such as cyclophilin A, translocates to the nucleus. In the nucleus, AIF induces DNA fragmentation and chromatin condensation, as part of an endonuclease complex (Seiler *et al.*, 2008; Susin *et al.*, 1999; Wang *et al.*, 2002; Ye *et al.*, 2002). It should be noted that if the soluble 57 kDa fragment of AIF does not enter the nucleus, then apoptosis cannot be induced (Norberg *et al.*, 2010; Zhu *et al.*, 2007). Other apoptosis-inducing proteins that are released upon mitochondrial permeabilisation involve Omi/HtrA2 (high temperature requirement A2) and Smac/DIABLO (second mitochondria-derived activator of caspases/direct IAP-associated binding protein with low pI) that promote caspase activation by acting as IAP antagonists (Du *et al.*, 2000; Suzuki *et al.*, 2001).

Mitochondrial-mediated apoptosis is also induced following p53 activation by pro-apoptotic stimuli. Normally, the transcriptional activity of p53 is inhibited by human double minute-2 (HDM-2); however, once p53 becomes activated, its interaction with HDM-2 becomes disrupted and the activated p53 induces the expression of Apaf-1 and Bcl-2 family members (Brooks and Gu, 2003; Hofseth *et al.*, 2004). Moreover, it has been recently described that p53, bcl-2 family proteins and reactive oxygen species are all factors contributing to the permeabilisation of the lysosomal membrane. In fact, lysosomal membrane permeabilisation occurs following apoptosis induction; however, depending on the type of inducing agent, permeabilisation of the lysosomal membrane can stimulate the release of cathepsin early in apoptosis or can occur late in apoptosis, amplifying in this way the death signal (Johansson *et al.*, 2010).

The Bcl-2 family

Another group of proteins that play a key role during mitochondrial-mediated apoptosis is the Bcl-2 family of proteins; however, its activity is independent of the formation of the PT pore (Green and Kroemer, 2004; Reed, 1994). Members of the Bcl-2 family of proteins include both death-promoting proteins (Bax, Bak, Bad, Bid, Bik/nbk, Bcl-XS, Bim/bod, Blk and Hrk/dp5) and death-inhibiting ones (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, Bfl-1, Brag-1, A1, Boo) (Cheung *et al.*, 2005; Hockenbery, 1994; Reed, 1994). They act upstream of caspases and their activity is

competitive. For example, in apoptotic conditions, there is an excess of Bax and thus, Bax homodimers that allow apoptosis to take place are formed. By contrast, in an excess of an anti-apoptotic molecule, like Bcl-2, there are Bax/Bcl-2 heterodimers formed to lead to an inhibition of apoptosis. Moreover, there could be heterodimers formed between two apoptosis-inhibitory proteins, e.g. Bcl-2/Bcl-xL that allow the release of an apoptotic protein, e.g. Bad, something that would trigger apoptosis (Kroemer, 1997). For apoptosis induction, the apoptosis-promoting proteins (Bax, Bad etc) act by forming pores and functioning as ion channels in mitochondrial membranes that form calcium fluxes and by stimulating the release of the above mentioned apoptosis-promoting agents.

The role of Bcl-2. Bcl-2 is best known as an anti-apoptotic protein. Some characteristic examples involve its activity in inhibiting the glucorticoid-mediated apoptosis in lymphoid cells as well as oestrogen-stimulated apoptosis in breast cancer cells (Almawi *et al.*, 2004; Huang and Cidlowski, 2002; Song *et al.*, 2005). However, its activity is also linked to its oncogenic role, especially in the context of haematopoietic and lymphoid malignancies (Vaux *et al.*, 1988).

Indeed, the oncogenic activity of Bcl-2 accounts back in 1985, when the cloning of the breakpoint of *t*(14;18) chromosomal translocations in non-Hodgkin's lymphomas resulted to the identification of a juxtaposed *BCL2* gene under the control of the IgH enhancer. That position of

the *BCL2* gene accounted for its dysregulated expression (Bakhshi *et al.*, 1985; Tsujimoto *et al.*, 1985). A year later, in 1986, the identification of the amino acid sequence of Bcl-2 and its high homology to Epstein Barr Virus (EBV) and several other Herpes-family viruses provided a first clue to the possible oncogenic properties of Bcl-2 (Cleary *et al.*, 1986). This was supported by subsequent observations that *BCL2* gene rearrangements were linked to poor prognosis in large-cell non-Hodgkin's lymphoma (Armitage *et al.*, 1988; Yunis *et al.*, 1989). The involvement of Bcl-2 in oncogenesis was confirmed in 1992, when the *BCL2* gene was found to be hypomethylated in the majority of chronic lymphocytic leukemias (CLLs) (Jonveaux *et al.*, 1992; Tanaka *et al.*, 1992). Also, *BCL2* gene amplification was noted in non-Hodgkin's lymphomas (Wessendorf *et al.*, 2003). Importantly, it was until recently, in 2005, when it was revealed that specific endogenous microRNAs account for the suppression of *BCL2* gene expression in normal B cells and deletions or mutations in these microRNAs account for the majority of CLLs (Cimmino *et al.*, 2005). In summary, it is evident that apart from its anti-apoptotic role, Bcl-2 has also an oncogenic potential, linking in this way, apoptosis and malignancy (Vaux *et al.*, 1988).

Death receptor mediated-apoptosis

Death receptor mediated-apoptosis involves the activity of cell surface receptors that induce apoptosis upon ligand binding (Ashkenazi and Dixit, 1998; Hotchkiss *et al.*,

2009). Such receptors, that are all members of the TNF superfamily, include Fas (CD95; Apo1), TNFR1 (p55; CD120), TRAMP (DR3; Apo3; WSL-1; LARD), TRAIL-R1 (DR4), TRAIL-R2 (DR5, Apo2, TRICK2, KILLER) and others. Their ligands involve FasL (for Fas), TNF and lymphotoxin α (for TNFR1), Apo3L (for TRAMP) and Apo2L for TRAILR1 and TRAILR2 (Bodmer *et al.*, 1997; Dhein *et al.*, 1995; MacFarlane *et al.*, 1997).

Upon ligand binding, the intracellular death domain of each receptor interacts via the TNFR-associated death domain (TRADD) to the Fas-associated death domain (FADD). FADD then forms the death-inducing signalling complex (DISC) by interacting with procaspase-8. Caspase-8 becomes activated, which in turn induces the activation of other effector caspases (Ashkenazi and Dixit, 1998; Pan *et al.*, 1997; Tartaglia *et al.*, 1993; Thorburn, 2004).

The apoptosis-promoting activity of death receptors is regulated by the family of decoy receptors (DCR), which involves DCR1, DCR2, DCR3 and osteoprotegerin, which interact with TRAIL and FasL to inhibit their activity. Cellular FLICE-like inhibitory protein (c-FLIP) is also an antagonist of apoptosis due to its interaction with FADD (Ashkenazi and Dixit, 1998; Irmler *et al.*, 1997).

In summary, the apoptotic programme can be either mitochondrial- or death receptor-mediated and caspase-dependent or independent. The role of the members of the Bcl-2 family in the apoptotic process should be emphasised as they were demonstrated to actively regulate induction

of apoptosis. In particular, Bcl-2 acts by both suppressing apoptosis and by exerting an oncogenic effect, as mutations in the *BCL2* gene can result in lymphoma and myeloid malignancies.

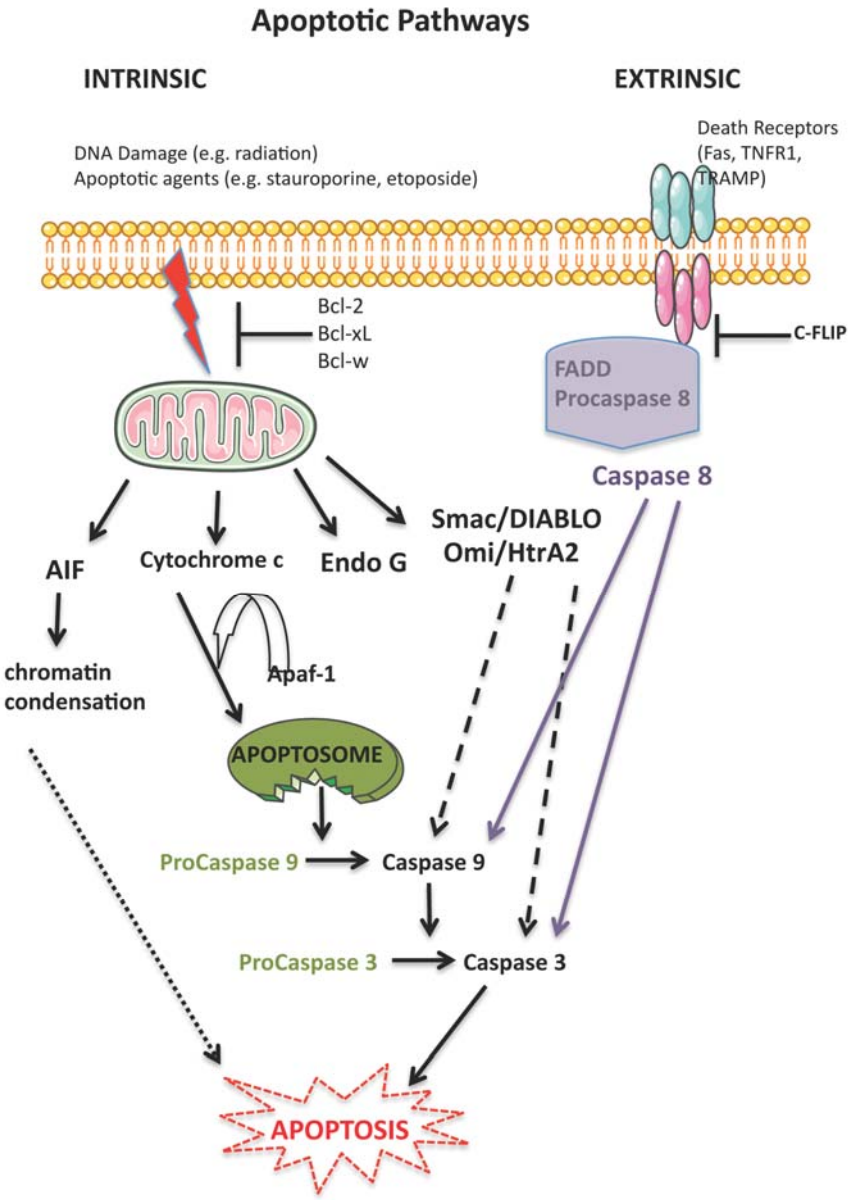


FIGURE 1: Schematic diagram of apoptotic pathways

Apoptosis is triggered via two major pathways: An intrinsic (mitochondrial-mediated) pathway that is controlled by bcl-2 family of proteins and an extrinsic (death-receptor-mediated) pathway. Both pathways result to

the cleavage and activation of pro-caspase 3 to an active caspase 3 that induces apoptosis.

The Immunosuppressive Nature of Apoptosis

Apoptosis is an anti-inflammatory form of cell death. Apart from histological evidence that neutrophils are absent from sites where homeostatic apoptosis rates are high, the anti-inflammatory nature of apoptosis is mostly due to the rapid phagocytosis of apoptotic cells to prevent the release of noxious inflammatory compounds and due to subsequent alterations in the type of cytokines (from proinflammatory to anti-inflammatory) secreted from resident immune cells (Savill *et al.*, 1993; Vaux, 1993). Indeed, failed or delayed clearance of apoptotic cells can have detrimental inflammatory consequences including the development of autoimmune pathologies.

The immunosuppressive environment of the sites where homeostatic apoptosis rates are high is greatly influenced by signals that attract monocytes to such sites in order to promote phagocytosis. These chemoattraction signals play a major role in the initiation of the resolution phase of inflammation and most of them were found to be released from late or post-apoptotic cells. These include lysophosphatidylcholine (LPC), platelet activating factor (PAF), monocyte chemotactic protein-1 (MCP-1), complement C1q, fractalkine and microparticles, which are membrane vesicles with high levels of oxidised lipids that act as a rich source of chemoattractants (Kadl *et al.*, 2004; Lauber

et al., 2003; Martinez *et al.*, 2005; Nagaosa *et al.*, 2003; Riches *et al.*, 1990). Also, the anti-inflammatory environment of the sites where homeostatic apoptosis rates are high can be greatly influenced by released anti-inflammatory cytokines, such as IL-10 (Poon *et al.*, 2010; Voll *et al.*, 1997).

This anti-inflammatory response arises independently of the apoptosis stimulus, as evidenced by apoptosis induction following bacterial LPS, ultraviolet light, γ-rays or deprivation of a growth factor. In all cases, it results in diminished IFN-γ levels and reduced inflammation (Mosmann, 1994; Paul and Seder, 1994). However, experimentally, changes in the incubation time of monocytes along with apoptotic cells have been shown to result in changes in the secreted cytokine expression pattern. Moreover, repeated exposure to the apoptotic material during tumourigenesis or viral infection results in impairments in the elicited immune responses, proving in this way that apoptosis is a tightly-regulated mechanism in terms of the molecules involved and of the association times (Hanayama *et al.*, 2004; Kang *et al.*, 1994; Meunier *et al.*, 1995). Moreover, heat-shock proteins are stress-inducing proteins, which although have been described to maintain thermotolerance, can induce apoptosis in severe stress conditions. In parallel, their role is immunomodulatory, as their expression induces the synthesis and release of various pro-inflammatory cytokines (Yokota and Fujii, 2010).

In general terms, all the above-mentioned mechanisms constitute an integral homeostatic process for the anti-inflammatory resolution of an apoptotic event. This anti-inflammatory response distinguishes apoptosis from necrosis. Necrosis has been described as a vaguely regulated, passive form of cell death (Kerr *et al.*, 1994; Majno and Joris, 1995). It is a type of cell death that is dictated by external injurious agents and not arising as part of a physiological cellular response. Indeed, there are many differences between apoptosis and necrosis that highlight the perfection in terms of the control of cell death that is observed in apoptotic tissues. Unlike apoptosis, necrosis is an inflammatory form of cell death that comprises large groups of cells in the same area, while apoptotic cells can be very few and scattered in a tissue (Leist *et al.*, 1997). In necrosis, there is a release of lysosomal compartments, which triggers an inflammatory response, whereas in apoptosis, lysosomes remain intact. In terms of cell morphology, no apoptotic bodies are formed in necrosis and chromatin is irregular and unevenly distributed as clumps with no nuclear fragmentation and cell shrinking.

Although these two forms of cell death are very distinct from each other, they do share some similarities: (a) many stimuli e.g. radiation, hypoxia, heat shock, viruses can cause both apoptosis and necrosis, (b) other external stimuli exist that could induce apoptosis at low doses, but could also trigger necrosis at high levels, (c) at a tissue level, in both normal and tumour tissues, a zone of

apoptotic cells may be surrounded by necrotic areas and (d) both apoptosis and necrosis can share a common apoptosis signalling machinery, especially caspases-8 and -10 that elicit apoptosis and Bcl-2 that has anti-apoptotic activity (Hirsch *et al.*, 1997; Leist and Nicotera, 1997). Another similarity (e) is the exposure of phosphatidylserine (PS) by both apoptotic and necrotic cells. However, there are differences in the oxidation status and physical nature of PS, as in apoptotic cells it occurs as patches. Indeed, it has been shown that specific oxidation of PS determines the recognition and engulfment of apoptotic cells (Jiang *et al.*, 2004; Tyurina *et al.*, 2004). Moreover, a recent study by Chen *et al.*, (2010b) has described that Nix/BNip3L protein that resides at the outer mitochondrial membrane and endoplasmic reticulum can induce either apoptotic or necrotic cell death via distinct signalling pathways, i.e. a classical Bax/Bak permeabilisation of outer mitochondrial membrane and mitochondrial-mediated apoptosis or via a cyclophilin D-dependent opening of mitochondrial permeability transition pore that induces necrosis.

Apoptotic cell recognition

The existing mechanism that allows the recognition of apoptotic cells by professional phagocytes is expressed in the form of surface molecules on apoptotic cells, also known as "eat-me" signals (Ravichandran and Lorenz, 2007) **(Figure 2) (Table 1)**. These signals are evolutionarily conserved and arise as alterations in the surface of

affected cells. One of the earliest signals that enable the apoptotic cell recognition and phagocytosis by macrophages involves the externalisation of phosphatidylserine (PS) (Botto, 2004; Fadok *et al.*, 1998a; Fadok *et al.*, 1992; Henson, 2005). PS is usually retained in the inner plasma membrane, but during apoptosis it becomes exposed either due to the changes in cell symmetry or due to PS oxidation. Other phospholipids such as lysophosphatidylcholine (LPC) are also exposed. Moreover, during cell death, cellular DNA becomes exposed and functions as recognition signal (Palaniyar *et al.*, 2004).

Although the presence of a PS receptor is still debatable, recent studies have identified TIM-4, stabilin-2 and BAI-1 as receptors specific for PS **(Table 2)** (Miyanishi *et al.*, 2007; Park *et al.*, 2007; Park *et al.*, 2008). These receptors are widely expressed by professional phagocytes, mainly macrophages and dendritic cells and mediate apoptotic cell engulfment via direct interactions with the exposed PS on the apoptotic cell surface. Special emphasis should also be given on the role of TIM family of genes that form a repertoire for the recognition of apoptotic cells, regulating in parallel immune responses. Its members TIM-1, TIM-3 and TIM-4 all display a specific recognition for PS, but differ in expression among cell types. This forms a control mechanism, which depending on the type of TIM molecule engaged, determines whether apoptotic cell recognition can result in immune activation or tolerance (Freeman *et al.*, 2010).

Apart from all these receptors, there are also other molecules described to bind apoptotic cells mediating their clearance by phagocytes. One of the most commonly known is thrombospondin (Savill *et al.*, 2002; Savill *et al.*, 1990; Savill *et al.*, 1992). Thrombospondin acts as a bridge molecule between apoptotic cells and CD36 and vitronectin receptor ($\alpha_v\beta_3$) on phagocytes. The use of antibodies against thrombospondin blocks apoptotic cell engulfment, highlighting, thereby the importance of this molecule in apoptotic cell recognition. Additional soluble proteins have been described to form molecular bridges between apoptotic cells and phagocytes by binding to the exposed PS on apoptotic cells. These include MFG-E8 (milk fat globule EGF-factor 8), Gas-6, protein S that is a homolog of Gas-6, annexin-1 and β_2-glycoprotein I (β_2-GPI) (Akakura *et al.*, 2004; Anderson *et al.*, 2003; Arur *et al.*, 2003; Balasubramanian *et al.*, 1997; Ishimoto *et al.*, 2000); Hall *et al.*, 2005; Hanayama *et al.*, 2004; Nakano *et al.*, 1997; (Borisenko *et al.*, 2004; Greenberg *et al.*, 2006; Jiang *et al.*, 2004). In addition to these molecules, a number of serum-derived proteins have been reported to bind apoptotic cells aiding their uptake by macrophages. These comprise serum amyloid protein (SAP), pentraxin-3 and CRP (Gershov *et al.*, 2000; Mold *et al.*, 2002; Rovere *et al.*, 2000).

Apart from the exposed PS on apoptotic cell surface, other opsonisation molecules include lectins, such as mannan-binding lectin (MBL) and proteins of the collectin family

(Kilpatrick, 2002; Lu *et al.*, 2002; Vandivier *et al.*, 2002). Also, complement proteins such as C1q and C3b that bind to apoptotic cell surfaces allow the recognition by complement receptors such as CD35 (CR1), CD11b/CD18 (CR3) and CD11c/CD18 (CR4) on macrophages (Mevorach *et al.*, 1998; Nauta *et al.*, 2002). Moreover, C1q was demonstrated to directly interact with PS in newly organised membrane patches particularly at the early stages of apoptosis (Paidassi *et al.*, 2008). Also, C1q interacts via the CD91/calreticulin complex with calreticulin, an ER protein with chaperone and calcium storage activities that is upregulated during apoptosis (Botto *et al.*, 1998; Henson and Hume, 2006).

All these signals, also known as "eat-me signals", are evolutionarily conserved and arise as alterations in the surface of affected cells. Other recently identified signals released by apoptotic cells to promote their clearance are the nucleotides ATP and UTP. Elliott *et al.* (2009) have demonstrated that these nucleotides are released by apoptotic cells and induce the directional migration of monocytes and macrophages. This is achieved via the $P2Y_2$ receptor on the surface of monocytes and macrophages, as evidenced by experiments on $P2Y_2$-deficient mice that have demonstrated an inefficient engulfment of apoptotic cells in the thymus (Elliott *et al.*, 2009; Gregory, 2009). Despite the presence of these anti-inflammatory ligand molecules, the presence of direct inhibitory molecules in the recognition and engulfment of apoptotic cells provides another clue for the regulation

of this anti-inflammatory mechanism. Such "don't eat me" signals involve CD31 that forms homotypic interactions to the CD31 on phagocytes to induce cell detachment and thus prevent engulfment (Brown *et al.*, 2002). In addition, interactions of CD47 (integrin-associated protein) with signal regulatory protein (SIRP) have been previously shown to prevent cell uptake by phagocytes (Gardai *et al.*, 2005; Oldenborg *et al.*, 2000).

In summary, the apoptotic process is a tightly regulated process in order to preserve the non-inflammatory mode of cell clearance. For this reason, several "eat-me" signals exist to stimulate monocytes or macrophages to arrive at the site of apoptosis and engulf apoptotic cells. Such signals can be in the form of soluble factors or specific receptors on the apoptotic cell surface. In this way, the physiological process of apoptosis, unlike necrosis, inhibits any host immune responses by creating an anti-inflammatory environment. Failure to do so can result to autoimmunity and inflammatory conditions. Therefore, targeting factors that regulate the apoptotic process could be therapeutically beneficial in promoting the clearance of apoptotic cells in inflammatory conditions or in triggering an immune response when required, as for example in the case of tumour.

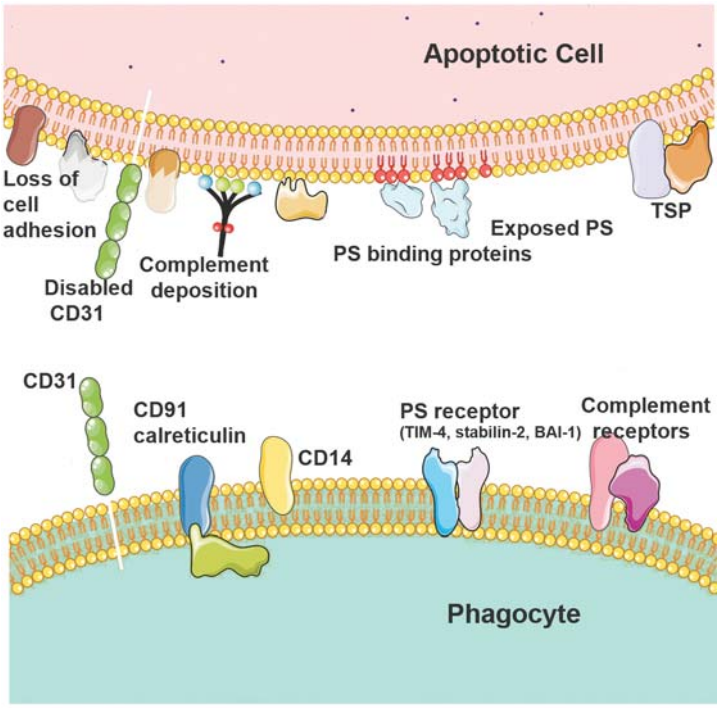


FIGURE 2: Apoptotic cell recognition

Recognition of "eat-me" signals on apoptotic cell surface to allow efficient phagocytosis by macrophages and other phagocytes. Exposed PS on apoptotic cell surface is one of the earliest signals that enable apoptotic cell recognition. Exposed PS is recognized by TIM-4, stabilin-2, BAI-1 that are specific receptors on phagocytes. Other proteins that act as ligands for PS are thrombospondin, MFG-E8, Gas-6, annexin-1 and β2-GPI. Apart from PS, other phospholipids, cellular DNA and calreticulin become exposed and function as recognition signals, whereas other molecules that opsonise apoptotic cells to enable their recognition involve lectins, proteins of the collectin

family and the complement proteins C1q and C3b. Also, CD31 becomes disabled.

Table 1.: Apoptotic Cell Recognition

"Eat-me" signals:	"Don't Eat-me" signals:
PS Externalisation	CD31
Cellular DNA Exposure	CD47
Mannan-binding lectin (MBL)	
Collectin family proteins	
Complement proteins C1q, C3b	
Secreted nucleotides ATP, UTP	

Table 2.: PS Receptors

TIM-4

Stabilin-2

BAI-1

"Bridging" molecules to macrophages:

Thrombospondin

MFG-E8

Gas-6

Protein S

Annexin-1

β_2- GPI

Serum Amyloid Protein (SAP)

Pentraxin-3

C-Reactive Protein (CRP)

Apoptosis and Malignancy

Apoptosis is a major determinant in tumour growth. At its early stages, apoptosis limits the expansion of the tumour cells; however, at the later stages apoptosis rates are relevant to tumour growth. In reality, what determines the rate of tumour enlargement is the balance between apoptotic rate and mitosis. Apoptosis rates are elevated in tumour and are highly dependent on tumour type. Also, there is the general notion that aggressive tumours display a higher apoptosis rate.

Oncogenes and apoptosis

The link between apoptosis and tumour formation is genetically reflected in the involvement of tumour promoting and tumour suppressor genes in apoptotic pathways. These include p53, Rb, Ras, Raf, myc. Of particular emphasis is the tumour suppressor gene p53, as loss of p53 contributes to the pathogenesis of tumours, especially colon, bone, brain, liver, breast, lung and leukemias (el-Deiry *et al.*, 1993; Harper *et al.*, 1993; Kastan, 1997; Lane, 1992). Under normal circumstances, p53 has a short half-life and is kept in a cell at a very low concentration making it undetectable in techniques such as immunohistochemistry. In tumours, p53 is either mutated or associates with other cellular or viral oncogenes making its detection possible by lengthening its half-life and consequently stimulating its accumulation in the nucleus. In this way, tumour-related genes that become activated

during tumour progression result in increased apoptosis rates.

Almost every tumour type is characterised by increased Myc expression, except in lymphomas, where *MYC* gene is mutated at different points, especially at the T58A and P75S hotspot. Such mutations are interrelated to apoptosis levels, as cells bearing either the T58A or the P75S mutation display a diminished apoptosis rate due to reduced Bim levels that neutralise the anti-apoptotic Bcl-2 (Evan *et al.*, 1992; Hemann *et al.*, 2005; Marsden and Strasser, 2003; Trent *et al.*, 1996). Indeed, in lymphomas with Myc Box I mutations, Bim expression was absent and in this way, tumourigenesis is enhanced due to decreased apoptosis (Dang *et al.*, 2005; Eischen *et al.*, 1999). Therefore, contrary to the general notion that increased levels of apoptosis result to enhanced, more aggressive forms of tumours, it seems that in lymphomas, specific types of mutations in the *MYC* gene can result to decreased apoptosis and enhanced tumourigenesis. However, overexpression of Myc in tumours, results in increases in p53 and Arf levels, and hence to increased apoptosis. Myc-induced apoptosis can also be triggered via other apoptosis pathways, as evidenced by redundant genetic experiments. Other genes activated by Myc include genes involved in cell cycle regulation, carbohydrate and nucleotide metabolism and mitochondrial biogenesis. Indeed, attempts towards a successful MYC-targeting therapy in different tumours are under way (Albihn *et al.*, 2010). Also, Par-4 is a protein recently characterised to

induce tumour cell-specific apoptosis upon binding to the cell surface receptor GRP78 (Shrestha-Bhattarai and Rangnekar, 2010). Moreover, abberations in the expression of Hox genes, which play a major role in apoptosis and development, were noted in abnormal development and malignancies (Shah and Sukumar, 2010). Other factors that contribute to the rise in tumour apoptotic index include the hypoxic conditions that are prevalent in the tumour, the loss of cell-cell contacts and matrix attachment that induces apoptosis and the resident immune cells that might become apoptotic (Bates *et al.*, 1995; McGill *et al.*, 1997; Meikrantz and Schlegel, 1995; Thomaidou *et al.*, 1997).

Apoptosis in tumours

In tumours, apoptosis is a well-established and generalised phenomenon; however, its extent varies between tumour types. For example, in breast carcinomas, high-grade tumours display more apoptosis (Lipponen *et al.*, 1994; Mustonen *et al.*, 1997). This is due to withdrawal of hormones, such as progesterone and oestrogen, something that elicits apoptosis. Once these hormones are present, there is stimulation of the relevant receptors and an increase of the anti-apoptotic Bcl-2. Similarly, there is an increased apoptotic rate in high-grade endometrial adenocarcinomas (Heatley, 1995; Saegusa *et al.*, 1996), as in terms of Bcl-2 expression, there is a reduction in its levels compared to normal epithelial cells in endometrium, where Bcl-2 is strongly expressed. A similar situation is observed in urogenital carcinomas, thyroid tumours and

gastric and colon carcinomas, in which apoptosis is tightly associated to high-grade tumours (Basolo *et al.*, 1997; Koshida *et al.*, 1997; Krajewska *et al.*, 1996; Staunton and Gaffney, 1995). In brain tumours, more apoptosis is evident in grade II gliomas than grade III lesions (Patsouris *et al.*, 1996), whereas in lung carcinomas, small cell lung carcinomas display a higher apoptosis index compared to non-small cell lung carcinomas (NSCLCs), despite the higher expression of the anti-apoptotic Bcl-2 (Eerola *et al.*, 1997; Ikegaki *et al.*, 1994). In lymphomas, an analogous phenomenon takes place. High-grade malignant non-Hodgkin's lymphomas have a higher apoptotic index than low-grade lymphomas and this correlates to Bcl-2 expression, which is increased in low-grade lymphomas, as well as to Bax and Mcl-1 expression (Kiberu *et al.*, 1996; Soini *et al.*, 1998b).

Finally, it should be noted that the identification of the molecular role of microRNAs has provided new insights on the pathways of apoptosis (Subramanian and Steer, 2010). To date, over 30 microRNAs are involved in apoptotic programmes, including let-7α, miR-16 and miR-21. It has been demonstrated that defects in the biogenesis of microRNAs can promote the formation of tumours, while tumour conditions are generally characterised by a downregulation of microRNAs, compared to normal situations (Kumar *et al.*, 2007; Lu *et al.*, 2005).

BURKITT'S LYMPHOMA

About Burkitt's Lymphoma

This highly aggressive B-cell lymphoma was first identified in the middle of last century by Denis Burkitt in central Africa in areas endemic for falciparum malaria (Burkitt, 1958). It was characterised by faces that were grossly distorted presenting lesions in the upper and lower jaws as well as by enlarged abdominal masses but with no affected lymph nodes. In fact, Burkitt's lymphoma (BL) had been the most common childhood tumour in that area, affecting individuals infected by the Epstein-Barr virus (EBV). Indeed, EBV was first identified in BL cells and BL remains the first identified human tumour in which a viral infection is involved in its pathogenesis (Burkitt, 1958; Burkitt, 1983).

Following the characterisation of Burkitt's lymphoma, other types of lymphomas have also been classified, especially undifferentiated lymphoma, Burkitt-like lymphoma, small non-cleaved cell lymphoma, high-grade B-cell lymphoma etc. that display a similar pathophysiology **(Table 3)** (Blum *et al.*, 2004; Magrath, 1990). According to World Health Organisation (WHO) classification, BL has three clinical variants: endemic, sporadic and immunodeficiency-associated types (Forteza-Vila and Fraga, 2010; Kelemen *et al.*, 2010). The endemic type of BL refers to affected African children (4-7 years old; male:female

ratio 2:1). It affects jaw and facial bones, gastrointestinal tract, kidneys, ovaries, breast and arises following EBV infection in all the affected cases. The sporadic type of BL has a worldwide incidence. It accounts for 40% of childhood lymphoma in the United States and Europe and 1-2% of adult lymphoma in these areas. It affects the abdomen and involves the kidneys, ovaries, breasts, terminal ileum and also displays pleural effusions or ascites. Lymph node involvement is most common in adult lymphomas. EBV infection is found in 15-30% of cases (Klein *et al.*, 2010). The immunodeficiency-associated type of BL mainly affects HIV^+ infected and immunodeficient patients as well as allograft recipients. In HIV^+ patients, this type of BL accounts for 30-40% of non-Hodgkin's lymphoma and is more commonly found in younger individuals with higher mean CD4 counts. The immunodeficiency type of BL presents the same pathogenetic features with the endemic type. It affects lymph nodes, bone marrow and abdominal sites; however HIV is not the cause of lymphomagenesis, but is involved in cytokine deregulation and reduced immune surveillance (Gaidano and Dalla-Favera, 1995; Knowles, 2003; Mbulaiteye *et al.*, 2010).

All three forms of Burkitt's lymphoma arise by translocations in the *c-myc* gene, as assessed by fluorescence *in situ* hybridisation (FISH) or long-segment polymerase reaction (Blum *et al.*, 2004; Cairo *et al.*, 2003; Gaidano and Dalla-Favera, 1995; Magrath, 1990; Peukert *et al.*, 1997; Wessendorf *et al.*, 2003). In 80% of

BL cases, there is a translocation between *c-myc* gene and IgH gene [*t*(8;14)], whereas the remaining 20% arise due to a translocation between *c-myc* and the gene for κ or λ light chain (IgL) [*t*(2;8) or *t*(8;22), respectively). In sporadic and immunodeficiency-associated BL, the *c-myc* breakpoint is between exons 1 and 2, whereas in endemic BL, the *c-myc* breakpoint is 100 kb upstream of exon 1. Differences in the breakpoint position result in differences in the neoplastic transformation of B cells, as they are affected at different mutational stages (Wessendorf *et al.*, 2003). The involvement of *c-myc* in lymphomagenesis is very important, as it consequently affects cellular functions such as cell cycle progression, cell adhesion, apoptosis and differentiation (Sanchez-Beato *et al.*, 2003). In BL, the cell of origin is the germinal centre or post-germinal memory B cell.

Also, mutations in p53 oncogene are implicated in the lymphomagenesis process, especially in the case of HIV^+-associated lymphoma. The Rb2/p130 tumour suppressor gene has also been implicated in BL disease pathogenesis (Cinti *et al.*, 2000a; Cinti *et al.*, 2000b). Rb2/p130 belongs to the retinoblastoma gene family and it is strongly involved, along with p107, in cell cycle regulation. In cases of endemic and sporadic BL, Rb2/p130 gene is mutated resulting in significant alterations in cell cycle regulation, whereas the HIV^+-related BL is associated with Rb2/p130 overexpression. In the case of HIV infection, Rb2/p130, due to its ability to physically interact with viral oncoproteins, interacts via its pocket region with

the Tat protein of HIV-1 (Huang *et al.*, 1997; Lefevre *et al.*, 1999). Consequently, the B cell clones have an uninterrupted cell cycle and proliferation. This implies that Burkitt's lymphoma subtypes arise due to different pathogenetic mechanisms.

Treatment

In recent years, efforts have been made to improve therapy, while reducing treatment-associated toxicity (Hesseling *et al.*, 2010). Short-duration, high-intensity chemotherapy regimens have been developed for this rapidly-proliferating type of neoplasm with significantly high success rate and excellent survival (>90% 5 year survival rate in localised disease; >90% complete response rate in widespread disease cases) (Kasamon and Swinnen, 2004). Usually for high-risk disease, the CODOX-M/IVAC regimen is followed; i.e. two cycles of CODOX-M (cyclophosphamide, vincristine, doxorubicin, methotrexate) alternating with IVAC (ifosfamide, etoposide, cytarabine and intrathecal therapy). For low-risk disease, three cycles of CODOX-M are sufficient for survival. However, there are treatment-associated toxicities such as myelosuppression, neuropathies, tumour lysis syndrome or even death.

Another regimen used for BL treatment involves the use of rituximab, a monoclonal anti-CD20 antibody in conjuction with hyper-CVAD (hyperfractionated, cyclophosphamide, vincristine, doxorubium and dexamethasone- 89% complete remission rate) (Jazirehi *et al.*, 2004; Vega *et al.*,

2004). Moreover, novel therapies under development involve genetic ones targeted against *c-myc* and the use of inhibitors such as DNA methyltransferase inhibitors, cyclin-dependent kinase inhibitors, proteasome inhibitors etc. (Bowman *et al.*, 1996; Magrath *et al.*, 1996).

Molecular Characteristics

In terms of cell morphology, two main variants of Burkitt's lymphoma have been characterised according to WHO: the classical form of Burkitt's lymphoma and atypical Burkitt's/Burkitt-like lymphomas (Magrath, 1990; McClure *et al.*, 2005). BL classical morphology is most characteristic in the endemic and sporadic form of childhood lymphoma. BL cells are medium-sized, uniform cells with round nuclei and multiple small basophilic nucleoli. Their cytoplasm is abundant with multiple vacuoles due to the presence of lipids. BL cells are characterised by an unusually high mitotic rate. Also, the BL stroma has a "starry-sky" pattern due to the presence of numerous admixed tingible body macrophages, also known as tumour-associated macrophages (TAMs) that phagocytose the apoptotic cell debris **(Figure 3)**. On the other hand, the atypical Burkitt's/Burkitt-like lymphoma is prevalent in sporadic adult lymphomas (Cairo *et al.*, 2003; Frost *et al.*, 2004). Burkitt-like lymphoma cells show a plasmacytoid differentiation and have a smaller number of nucleoli. Despite the pathological type of BL, both types of BL cells express the following surface antigens CD19, CD20, CD22, CD79a, CD10, Bcl-6, CD43, p53 as well as the

monotypic surface IgM and display a high proliferative activity and apoptotic rate.

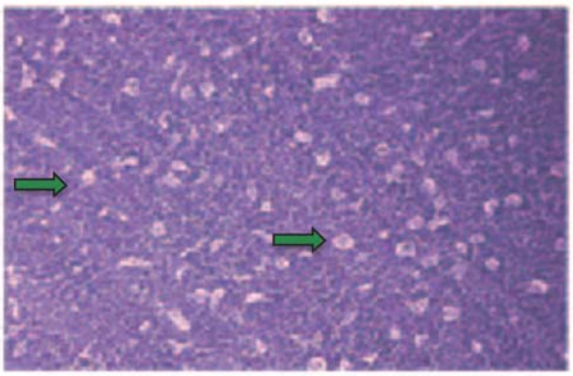


FIGURE 3: Histology of Burkitt's lymphoma

The BL stroma is composed of medium-sized, uniform cells with round nuclei and multiple small basophilic nucleoli. BL cells have an abundant cytoplasm and present a high mitotic and apoptotic rate. The BL stroma has "starry-sky" pattern due to the presence of numerous macrophages, also known as TAMs, that phagocytose the apoptotic cell debris. Figure obtained from Ogden *et al.*, 2005.

Table 3.: Types of B cell lymphomas

Lymphoma (type)	Genotypic Characteristics	Immunophenotype	Appearance
Burkitt's lymphoma and Burkitt-like lymphoma	Arise by translocations between *MYC* and *IgH* or *IgL* [*t*(8:14), *t*(2:8) or *t*(8:22)]	Bcl-2$^-$, Bcl-6$^+$, CD10$^+$, CD20$^+$, Ki67$^+$, TdT$^-$, CD5$^-$	Starry-sky pattern with uniform, medium-sized cells
Diffuse large B-cell lymphoma	Mainly due to abnormalities in *BCL2* and *BCL6*	Bcl-2$^+$, Bcl-6$^+$, CD10$^{+/-}$, CD20$^+$	Large irregular lobed nuclei; tiny cytoplasmic area
Florid follicular hyperplasia	No clonal abnormalities reported	Bcl-2$^-$, Bcl-6$^+$, CD10$^+$, CD20$^+$, Ki67$^+$	Large irregular follicles
Mantle cell lymphoma	Arises by translocations between *BCL6* and *IgH* [*t*(11:14)]	Bcl-2$^+$, Bcl-6$^-$, CD5$^+$, CD10$^-$, CD20$^+$, Cyclin D1$^+$	Large-sized pleiomorphic cells; scant cytoplasmic area
Precursor B-lymphoblastic lymphoma	Hyperdiploid; no genomic rearrangements	CD10$^+$, CD19$^+$, CD20$^-$, TdT$^+$	Cells with variable size and shape-mainly small to medium

Burkitt's Lymphoma as a Model of *in situ* Apoptosis

The characteristic "starry-sky" histological appearance of the BL stroma arises by the infiltration of monocytes that are later differentiated into tumour-associated macrophages (TAMs). Although the factors that trigger recruitment of TAMs in BL cells are not clearly identified, evidence from studies on other tumours rich in TAMs revealed that TAMs can infiltrate tumours in response to multiple chemoattractant factors such as MCP-1, MCP-2, MCP-3, GM-CSF and M-CSF (Bottazzi *et al.*, 1990; Bottazzi *et al.*, 1992; Dvorak *et al.*, 1991; Fioretti *et al.*, 1998; Fu *et al.*, 1992; Graves and Jiang, 1995; Ha *et al.*, 1999; Valkovic *et al.*, 1998). In Burkitt's lymphoma, the release of fractalkine by apoptotic BL cells has been found to be an additional chemoattractant factor that seems likely to stimulate monocyte migration to the tumour stroma (Truman *et al.*, 2008). As with other tumours, in Burkitt's lymphoma, TAMs may promote neoplastic establishment and progression by releasing factors such as IL-10 that have an immunosuppressive effect that prevent anti-tumour responses. Also, it has been demonstrated that macrophages cultured in the presence of IL-10 displayed upregulated capacity to recognise and engulf apoptotic tumour cells. This creates an anti-inflammatory environment, but also leads to increased levels of the B cell survival factor BAFF/BLyS, which in the case of a B-cell lymphoma, like Burkitt's, promotes its growth (Truman *et al.*, 2004). Although the synergy between TAMs and Burkitt's lymphoma

is still poorly defined, it can be speculated that the similar mechanisms or factors that have been identified in other tumour types to characterise TAM activity might also exist in this type of lymphoma. In other words, it can be estimated that the mechanisms that promote tumour immunosurveillance are evolutionarily conserved between tumours and only slight differences exist between different tumour types.

However, what makes Burkitt's lymphoma a model of *in situ* apoptosis is its high apoptotic level as well as the high similarity between tumour microenvironment and sites where homeostatic apoptosis rates are high. Indeed, the tumour tissue retains the anti-inflammatory characteristics of apoptosis. These characteristics are enhanced by the presence of anti-inflammatory cytokines such as IL-10 that are released following macrophage infiltration to the site and engulfment of apoptotic tumour cells. It could be argued that no adaptive immune responses are triggered in BL stroma. For example, as evidenced by histological sections of BL, there are no neutrophils present due to a till now unknown mechanism; a phenomenon also evident in sites where homeostatic apoptosis rates are high to retain their anti-inflammatory characteristics (Blum *et al.*, 2004; Burkitt, 1983; Frost *et al.*, 2004). Finally, the tumour outcome depends on the balance between mitogenic or tumourigenic mechanisms and apoptosis, something that is also relevant in all growing, proliferating tissues in an organism.

All these pose Burkitt's lymphoma as a tissue model of apoptosis, albeit a malignant tissue. Its molecular mechanisms bear strong similarities to the apoptotic process and the identification of the implicated factors would have powerful applications both in the elucidation of the anti-inflammatory nature of apoptosis as well as in the therapeutic intervention in lymphomas and types of tumour in which apoptosis is prominent.

POLYMORPHONUCLEAR LEUKOCYTES

Leukocytes are effector cells of the immune system with a pivotal role in the defense of the host organism against infections and foreign particles. These cells are distributed throughout the whole body, especially in blood and lymphatic system and under normal circumstances their number is between $4x10^{9}$ and $1.1x10^{10}$ per litre of blood. Leukocytes are subdivided into two major types based on the presence of granules in their cytoplasm: agranulocytes, which are characterised by the absence of granules and mainly include lymphocytes, monocytes as well as macrophages, and granulocytes or polymorphonuclear leukocytes, which as their name suggests, possess cytoplasmic granules and are subdivided into neutrophils, eosinophils, basophils and mast cells. However, all these subtypes originate from a multipotent haematopoietic stem cell in the bone marrow (Nathan, 2006; Weiss, 1989).

Neutrophil granulocytes represent a major class of granulocytes, as they comprise approximately 95% of circulating granulocytes as well as 70% of circulating leukocytes and in this way, they constitute the most abundant type of immune effector cell in humans. They belong to the family of polymorphonuclear cells (PMNs), as their nucleus has a multilobulated shape divided into 3-5 lobes (Ward and Lentsch, 1999; Weiss, 1989). Under normal conditions, the number of circulating neutrophils is $2.5\text{-}7.5x10^{9}$ L^{-1}; however, this number can increase up to 10-fold

under inflammatory or septic conditions. Neutrophils migrate across the endothelium hours before monocytes or lymphocytes and have a half-life of about 12 hours and a survival time into tissues of about 1-2 days following activation. All these characteristics of neutrophils suggest they are a "fighter" cell type, as neutrophil activation and migration to the sites of inflammation or infection are linked to the release of a variety of cytotoxic compounds that exert an anti-microbial effect and kill invading pathogens.

The Functions of Neutrophils

The innate effector functions of neutrophils are represented by a multi-facet model: (a) production of reactive oxygen and nitrogen species (b) degranulation to release intracellular cytotoxic components (c) phagocytosis of invading organisms (d) formation of neutrophil extracellular traps (NETs) and finally (e) regulation of cytokine synthesis to control an inflammatory response.

(a) **Production of reactive oxygen and nitrogen species**. Anti-microbial responses of neutrophils involve the generation of reactive oxygen and nitrogen species, which is initiated following the formation of NADPH-dependent oxidase complex; a process also known as oxidative burst (Babior, 1984). The NADPH oxidase complex (cytosolic $p40^{phox}$, $p47^{phox}$, $p67^{phox}$; membrane $p22^{phox}$, $gp91^{phox}$) is phosphorylated at $p47^{phox}$ upon neutrophil activation and

associates with cytochrome b558 to become activated. In this way, superoxide anion (O^{2-}) is generated, which in turn dismutates into H_2O_2, which subsequently forms hydroxyl radicals (OH^-) (Nathan, 1987). In addition, myeloperoxidase (MPO), which is normally found in the azurophil granules of neutrophils, amplifies the toxic potential of H_2O_2 by allowing the formation of by-products such as hypochlorous acid (HOCl) that chlorinates many electron-rich substrates. Superoxide production is also associated with nitric oxide (NO) forming a potent toxic nitrogen intermediate, peroxynitrite ($ONOO^-$) (Britigan *et al.*, 1986; Hazen *et al.*, 1998). Decomposition of peroxynitrite could also lead to hydroxyl radical (OH^-) formation. Although the microbicidal role of NO is obscure, NO-deficient mice formed massive abscesses rich in enteric bacteria (Shiloh *et al.*, 1999).

(b) **Degranulation.** Upon activation, neutrophils release a variety of microbicidal molecules that are packed within neutrophils in the form of granules **(Table 4)** (Borregaard and Cowland, 1997; Elsbach, 1998; Panyutich *et al.*, 1995; Spitznagel, 1990). These granules comprise the inner fighting machinery of the cell and are divided into the following categories: Azurophilic granules, also known as primary granules, emerge at the promyelocyte stage of granulocyte differentiation and contain myeloperoxidases, defensins, cathepsin G, elastase, proteinase 3, azurocidin (CAP-37) and bactericidal permeability increasing protein (BPI) (Egesten *et al.*, 1994; Fouret *et al.*, 1989). The contents of specific granules, also known as secondary

granules, include lactoferrin, cathelicidin, collagenase and gelatinase. These granules are formed at the metamyelocyte stage along with tertiary granules that contain gelatinase and cathepsins (Cowland *et al.*, 1995; Sorensen *et al.*, 1997). Secretory vesicles are another form of granule of endocytic origin that are rich in plasma proteins such as albumin and emerge at the mature stage of neutrophil development. The order of release of these granule types (exocytosis) is (1) secretory vesicles, (2) tertiary gelatinase granules, (3) specific granules and finally (4) azurophilic granules. The release of this stored material (degranulation) into the phagolysosome or in the extracellular space is a pivotal event for neutrophil functional abilities (Hoffman and Specks, 1998; Sengelov *et al.*, 1993).

(c) **Phagocytosis**. Neutrophil is a phagocyte. The opsonised particle (bacterial and viral pathogens) is engulfed by membrane protrusions that form a phagocytic cup; a process dictated by either complement receptors (CR1 and CR3) or Fcγ receptor aggregation, which induces the phosphorylation of cytoplasmic ITAM motifs and activation of Src-tyrosine kinases (Caron and Hall, 1998; Chuang *et al.*, 2000; Greenberg *et al.*, 1996). This in turn, triggers phosphoinositide 3 (PI3) and Rho protein activation. RhoA is responsible for F-actin recruitment and phagocytic cup formation, Cdc42 regulates membrane extension, while Rac-1 and PI3 kinase are involved in membrane fusion and the closure of the phagocytic cup (Cox *et al.*, 1997; Massol *et al.*, 1998). As a result, the opsonised particle becomes

engulfed and fused with neutrophil granules to activate an oxidative burst reaction.

(d) **Neutrophil Extracellular Traps (NETs).** One exciting recently described observation that enhances all the above-mentioned activities of neutrophils is the formation of neutrophil extracellular traps (NETs). These are web-like structures of DNA that are released from activated neutrophils to trap and kill pathogens extracellularly (Brinkmann *et al.*, 2004). NETs are rich in anti-microbial components and their suggested role is the creation of a physical barrier to prevent the spread of bacteria and microbes. This is particularly important in septic conditions, as NETs are formed within blood vessels to prevent exacerbation and spreading of the infection.

(e) **Regulation of cytokine synthesis by neutrophils**. Apart from their widely known phagocytic and anti-microbial properties, neutrophils constitute a central cell of immunity, as they are able to link both innate and adaptive immunity. Indeed, neutrophils have the ability to regulate cytokine synthesis. It has been reported that almost all agents that are able to activate neutrophils can also stimulate cytokine production. Neutrophils sense these signals and respond via the release of a plethora of cytokines (Cassatella, 1995; Cassatella, 1999). For example, neutrophils can release proinflammatory cytokines such as TNF-α, IL-1α, IL-1β and IL-12 (Cassatella *et al.*, 1994; Cassatella *et al.*, 1995; Dayer and Fenner, 1992; Lindemann *et al.*, 1989; Marie *et al.*, 1996). They can also

synthesise cytokines such as IL-1 (IL-1), macrophage-inflammatory protein- 1α and β (MIP-1α, MIP-1β) and growth factors such as interferon-α (IFN-α), IFN-β, granulocyte colony-stimulating factor (G-CSF), Fas ligand (FasL) and vascular endothelial growth factor (VEGF) (McColl *et al.*, 1992; Tiku *et al.*, 1986). Special emphasis should be given to IL-8 chemokine, as neutrophils form its primary cellular target, while at the same time, IL-8 is the most abundantly expressed chemokine by them (Baggiolini and Clark-Lewis, 1992; DeForge *et al.*, 1992; Gainet *et al.*, 1998; Matsumoto *et al.*, 1997; Sekido *et al.*, 1993).

Cytokine expression by neutrophils can also be modulated in both positively and negatively. More specifically, cytokine synthesis in neutrophils is positively enhanced by IFN-γ, while it is negatively regulated by IL-13 (Cassatella, 1999; Nathan, 2006).

Table 4:Types of Neutrophil Granules

Azurophilic	Specific	Tertiary	Secretory
Antimicrobial: Azurocidin Bacterial Permeability Increasing Protein (BIP) Defensins Myeloperoxidase Lysozyme	Antimicrobial: Cathelicidin Lactoferrin Lysozyme	Antimicrobial: Lysozyme	Complement receptor 1 (CR1)
Acid hydrolases: Cathepsin B Cathepsin D β-D-Glucuronidase α-Mannosidase Phospholipase A2	Neutral proteinases: Collagenase NGAL (neutrophil gelatinase-associated lipocalin)	Neutral proteinase: Gelatinase	Complement receptor 3 (CR3)
Neutral proteinases: Cathepsin G Neutrophil elastase Proteinase 3	Leukolysin	Leukolysin	N-formylpeptide receptor
Chondroitin-4-sulfate	Cytochrome b558		CD14 CD16

Neutrophil Chemotaxis

Chemotaxis, first introduced by Pfeffer in 1884, is a term that describes the guided movement of a cell towards chemical gradients. It is a tightly regulated phenomenon necessary to attract a cell at the right time to the right place for a specific purpose (Adler and Tso, 1974; Sanchez-Madrid and del Pozo, 1999). Chemotaxis is highly evolutionarily conserved between organisms. Specifically, the slime mold *Dictyostelium discoideum* and neutrophils constitute the central cells in the study of chemotaxis as they both move very fast (10-40 μm per minute) and display striking similarities in the molecular mechanisms that regulate chemotactic movements (Parent and Devreotes, 1999; Servant *et al.*, 2000; Stephens *et al.*, 2008). Indeed, genetic studies on *Dictyostelium* have revealed the presence of homologous regulatory proteins in neutrophils with a role in neutrophil chemotaxis (Parent, 2004).

Steps of chemotaxis

Neutrophils are normally found in the bloodstream. In order to reach the site of inflammation or infection, a series of events comprising the leukocyte adhesion cascade is followed. Although this cascade was originally described almost 200 years ago, recent evidence suggests that it is comprised of leukocyte rolling, strengthening of leukocyte adhesion to the endothelial wall, intravascular crawling, paracellular and transcellular migration and finally migration through the basement

membrane to reach the inflamed site **(Figure 4)** (Ley *et al.*, 2007).

All these processes initiate in response to the recognition of chemoattractant molecules, such as formylated peptides and lipopolysaccharides (LPS) present in bacteria, chemokines, mainly interleukin-8 that specifically attracts neutrophils, as well as leukotriene B_4, complement factor 5a, platelet-activating factor (PAF) produced by epithelial or dying cells (Premack and Schall, 1996; Rollins, 1997). It should be noted that there is a hierarcy in the way that neutrophils respond to these chemoattraction signals, as those expressed by the pathogens or by the mitochondria of dying cells receive a 'sensing' priority, compared to those expressed as epithelial-cell attractants (Foxman *et al.*, 1999; Kitayama *et al.*, 1997).

There are distinct regulatory processes that control each phase of the leukocyte adhesion cascade. At a first stage, leukocyte rolling is mediated by interactions between L-selectin expressed by neutrophils and E-selectin and P-selectin expressed by the endothelial cells (Muller, 2003). PSGL-1 acts as a ligand for all these three types of selectins and is expressed by both neutrophils (in glycosylated form) and endothelial cells (da Costa Martins *et al.*, 2007; Sperandio *et al.*, 2003). These selectins stably bind only in the presence of shear stress created by the bloodstream, as the rolling cells were observed to

detach when flow is stopped (Alon *et al.*, 1995; Finger *et al.*, 1996).

During rolling, neutrophil arrest is triggered by the recognition of chemoattractants and involves the binding of integrins on neutrophils to the immunoglobulin superfamily members, ICAM-1 and VCAM-1 on endothelial cells (Simon *et al.*, 2000). The most studied integrins involved in neutrophil arrest are those of the β_1-integrin family, especially β_1-integrin VLA-4 (CD49d/CD29) and of the β_2-integrin family, especially the β_2-integrin LFA-1 (CD11a/CD18; $\alpha_L\beta_2$-integrin). In order to mediate firm rolling and neutrophil adhesion, the engagement of P-selectin or E-selectin induces a subsequent conformation of LFA-1 on neutrophils to acquire intermediate affinity, which allows it to become bound to its ligand ICAM-1 on the endothelium (Chesnutt *et al.*, 2006; Salas *et al.*, 2004). Again, as in the case of selectins, the acquisition of the intermediate affinity conformation of LFA-1 under shear stress is necessary to increase its ligand-binding affinity (Astrof *et al.*, 2006). Apart from LFA-1, binding of MAC-1 (CD11b/CD18) to E-selectin is necessary to mediate firm adhesion (Dunne *et al.*, 2002).

The avidity of integrin-mediated interaction is controlled by the degree of integrin affinity, i.e. the conformational changes of integrin heterodimers and the valency of ligand binding, which corresponds to the density of integrin heterodimers (Bazzoni and Hemler, 1998; Carman and Springer, 2003). Accordingly,

fluctuations between low affinity and high affinity states determine the formation and the dissociation of molecular bonds. Other factors that determine the degree of integrin activation also involve quantitative variations in the expression levels of chemokine receptors and variations in the affinities for chemokines (Constantin *et al.*, 2000; Ley *et al.*, 2007). In all cases, firm neutrophil adhesion is mediated by high affinity forms of integrins. Special emphasis should be given on the role of talin-1, which is an actin-binding protein that regulates the level of integrin affinities, as it wedges between the α- and β-cytoplasmic tails of integrins (Tadokoro *et al.*, 2003; Wegener *et al.*, 2007). Apart from talin-1, at present, more than 47 proteins are involved in the regulation of integrin-mediated adhesion (Kinashi, 2005; Ley *et al.*, 2007). Members of the kindlin protein family were also demonstrated to bind to β-integrin cytoplasmic tails, activating in this way, integrins. Especially, the FERM domain of kindlin-1 displays great similarity to that in talin-1. Mutations in the kindlin genes are linked to inherited and acquired human diseases such as the Kindler syndrome, leukocyte adhesion deficiency and malignancies (Meves *et al.*, 2009).

The final steps in neutrophil migration to reach an inflamed site involve its intravascular crawling and its paracellular and transcellular migration through the endothelial basement membrane. During intravascular crawling, different levels of shear stress exist depending

on the cell type. Again, there are interactions between integrins on neutrophils and endothelial ligands, such as ICAM-1 and VCAM-1, which form transmigratory cups along with the cytoplasmic molecules ezrin, radixin and moesin and the cytoskeletal proteins vinculin and α-actinin (Barreiro *et al.*, 2002; Carman and Springer, 2004; Phillipson *et al.*, 2006). In order to further actively mediate transendothelial migration of neutrophils, there is an expression of endothelial junctional molecules such as PECAM-1, JAM-A, JAM-B, JAM-C, ICAM-2, CD99 and ESAM, which become bound to PECAM-1, JAM-1, MAC-1 and LFA-1 expressed on neutrophils (Vestweber, 2002). The existence of vesiculo-vacuolar organelles (VVOs) on endothelial membrane acts as a gateway for neutrophil transmigration (Dvorak and Feng, 2001).

As a final step, neutrophil transmigration through endothelial basement membrane is facilitated by the presence of regions within the membrane, which display lower than average expression of matrix proteins, such as laminin-10 and collagen IV (Wang *et al.*, 2006). In this way, neutrophil migration takes place through these regions of least resistance. Also, in parallel, such regions are more permissive to chemoattractant molecules.

Finally, once the extravasation process is complete, neutrophils migrate along a concentration gradient of chemokines and other chemoattractants secreted by cells at the site of inflammation or infection. In general, this interplay of regulatory molecules controls the migration

of neutrophils into tissues. The importance of this tightly regulated mechanism is significant, as the inappropriate or uncontrolled migration of such effector cells with their histotoxic properties may result in an unfavourable outcome such as host tissue damage.

In summary, the achievement of successful neutrophil adhesion and transmigration is a tightly-regulated multi-step process that involves an interplay of integrins based on alterations in their affinity state. Targeting integrins is an area of intensive research for the therapy of inflammatory conditions, characterised by aberrant neutrophil infiltration. For example, the use of the monoclonal antibody, natalizumab in multiple sclerosis patients targets VLA-4, while efalizumab, is a monoclonal antibody specific for LFA-1 used in individuals with psoriasis (Ley *et al.*, 2007). A very recent study by (Deban *et al.*, 2010) identified pentraxin PTX3 as a protein that dampens neutrophil recruitment by inhibiting their rolling on P-selectin, providing in this way further evidence that identification of regulatory molecules involved in the leukocyte adhesion cascade can have therapeutic implications.

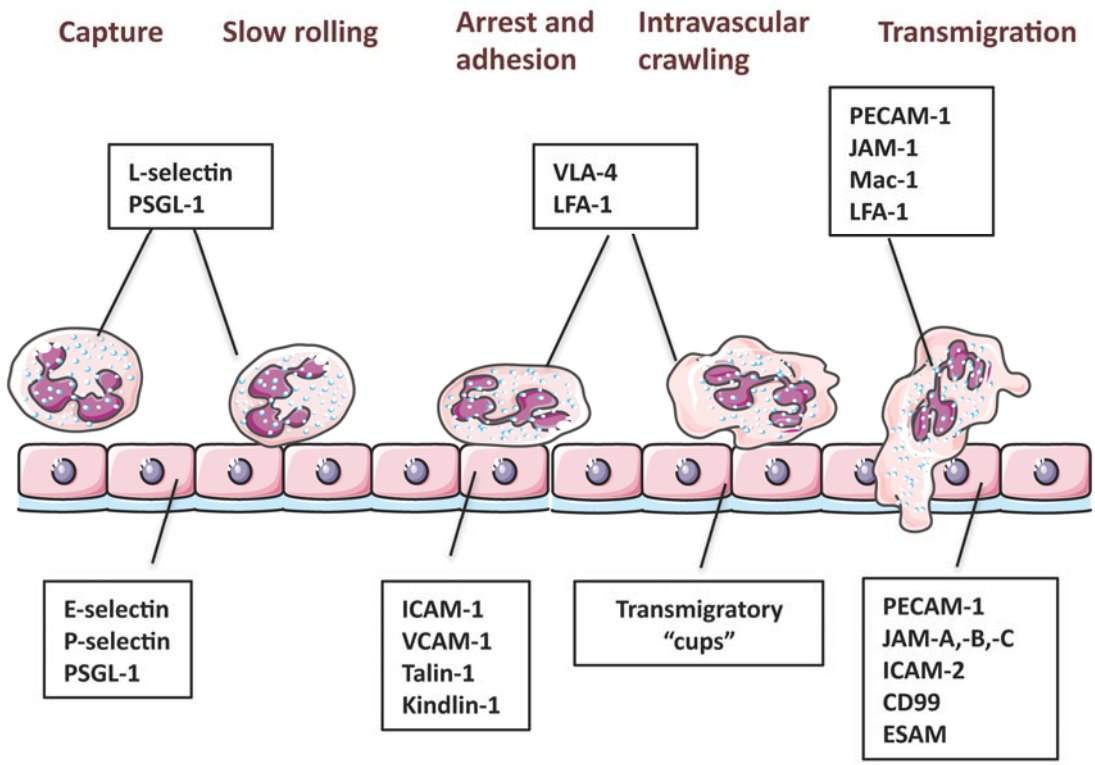


FIGURE 4: The leukocyte adhesion cascade

The steps followed so as a neutrophil to leave the bloodstream and reach the inflamed site involve: neutrophil capture, slow rolling, strengthening of adhesion to the endothelial surface, intravascular crawling and paracellular and transcellular migration. The whole process is orchestrated by the interaction between adhesion molecules expressed on the endothelial cell wall, such as ICAM-1 and their ligands (integrins) on the neutrophil surface.

Signalling pathways in chemotaxis

Almost all the chemoattractant molecules are recognised by specific seven-transmembrane receptors on neutrophil surfaces that are associated with intracellular GTP-binding heteroproteins (De Nardin *et al.*, 1991; Thomas *et al.*, 1990). Indeed, the activity of these specific receptors is modulated by their interaction with G proteins, which in the inactive state, their three subunits (α, β, γ) form heterodimers. Upon activation, a conformational change of the receptor induces the GDP (inactive) to GTP (active) exchange in the α subunit and thus the dissociation of the heterodimeric complex and the subsequent release of the α subunit from the βγ subunit complex (Jin *et al.*, 2000; Malech *et al.*, 1985; Wieland and Chen, 1999).

Based on comparative studies on *Dictyostelium,* neutrophil activation, prior to movement, is accompanied by the acquisition of a polarised morphology characterised by a leading actin-rich lamellipodium and a tail-like uropod at the distal end (Benard *et al.*, 1999; Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996) **(Figure 5A)**. In this way, the leading machinery that promotes cell movement involves cytoskeletal modifications mainly in the actin cytoskeleton **(Figure 5B)**. Modifications in the organisation or de-organisation of actin are necessary for directional neutrophil crawling along surfaces, as neutrophils need to extend their cell membrane, adhere to the surface and finally contract their body in order to

move (Parent and Devreotes, 1999). As a requisite to extend or protrude, actin assembly creates small trigger-like projections, known as lamellae (also called filopodia in *Dictyostelium*), from the leading edge of the neutrophils. Lamellae are sheet-like structures rich in actin filaments that provide a base so that the neutrophil pushes its membrane forward and in this way, moves (Jones and Sharief, 2005). The tight and well-organised regulation of this migratory mechanism creates a continuous neutrophil movement (Glogauer *et al.*, 2000). This is also enriched by the identification of actin regulating proteins, such as actin-filament capping proteins e.g. capping protein-beta 2, gelsolin and villin that regulate monomer addition in the filament end and the actin-nucleation factors e.g. JMY, mDia1 that bind to actin monomers under unfavourable conditions and promote their binding to generate new actin filaments (Pollard *et al.*, 2000; Puius *et al.*, 1998). The importance of this process is reflected by the use of the reagent cytochalasin, which binds to the growing plus ends of microfilaments and inhibits the assembly of actin filaments and the disassembly of individual monomers from the bound end; completely blocking in this way neutrophil motility (Cooper, 1987).

The whole process of actin assembly is regulated by multiple pathways. For example, the formation of a complex of actin-related proteins 2 and 3 (Arp2/3 complex) nucleates actin polymerisation by binding to profilin and to a complex of Cdc42, N-WASP (Wiskott Aldrich Syndrom

Protein family) and $PI(4,5)P_2$ (Higgs and Pollard, 2000; Machesky *et al.*, 1997; Zigmond *et al.*, 1998). The formation of this complex was demonstrated to be necessary, as by blocking one of these factors, half of the total actin assembly is also blocked. Finally, other cytoskeletal proteins that are critical for the normal migration of the neutrophils include gelsolin and lymphocyte-specific protein-1 (LSP-1) (Howard *et al.*, 1998; Janmey *et al.*, 1985; Zhang *et al.*, 2000). Gelsolin is an effector protein of Rac that binds tightly to the high-affinity end of the actin filament following its activation by calcium ions and is removed from the actin filament end when Rac is in the active GTP-bound form. LSP-1 on the other hand, is a calcium-binding protein that is associated with the cytoplasmic side of the plasma membrane and promotes the bundling of actin filaments. In fact, studies on LSP-1 deficient mice presented an enhanced migration of neutrophils and macrophages to the peritoneum (Liu *et al.*, 2005a).

Following the engagement of the chemoattractant receptor, there are subsequent downstream signalling cascades (Bokoch, 1995; Ono *et al.*, 2003; Selvatici *et al.*, 2006). Indeed, once the G-protein-$\alpha\beta\gamma$ complex becomes activated and therefore dissociated, the α subunit interacts mainly with phospholipase C (PLC) and this leads to the hydrolysis of phosphatidylinositol-4,5-bisphosphate [$PI(4,5)P_2$] to generate IP_3 (inositol-1,4,5-triphosphate) that induces $[Ca^{2+}]_i$ mobilisation and DAG (diacylglycerol)

that activates protein kinase C (PKC) (Park *et al.*, 1993). The activation of PLC is not associated with neutrophil chemotaxis *per se*, but mainly with the production of reactive oxygen intermediates. On the other hand, the association of βγ subunits with PI3 kinases plays a key role in the promotion of chemotactic movement (Clapham and Neer, 1993). In detail, PI3 kinases phosphorylate inositol phospholipids such as phosphatidylinositol-3-phosphate [PI(3)P], phosphatidylinositol-3,4-bisphosphate [$PI(3,4)P_2$] and phosphatidylinositol-3,4,5-trisphosphate [$PI(3,4,5)P_3$]. Especially the last two are strongly implicated in re-arrangements of the actin cytoskeleton and cell polarisation (Gillooly *et al.*, 2000; Li *et al.*, 2000; Rhee, 2001; Wu *et al.*, 2000). The activity of PI3 kinase signalling in chemotactic movement is also implied from the use of the PI3 kinase inhibitors, wortmannin and LY294002, which completely block (a) respiratory burst, (b) activation of phospholipase D, (c) phosphorylation of MAP/ERK2 (Extracellular-signal-regulated kinase 2) as well as (d) cell polarisation and chemotaxis (Baggiolini *et al.*, 1987; Downey *et al.*, 1996; Niggli and Keller, 1997; Reinhold *et al.*, 1990; Thelen *et al.*, 1995; Thelen *et al.*, 1994; Wu *et al.*, 2000; Wymann *et al.*, 2000). Indeed, the activity of PI3-kinase is essential for the establishment of cell polarity, as evidenced by the high concentration of $PI(3,4,5)P_3$ in the leading edge of an activated neutrophil (Gillooly *et al.*, 2000; Rameh and Cantley, 1999; Servant *et al.*, 2000; Toker and Cantley, 1997). However, studies on PI3 kinase $\gamma^{-/-}$ mice have failed to

block actin polymerisation completely, suggesting in this way the existence of a PI3 kinase-independent pathway (Hirsch *et al.*, 2000; Sasaki *et al.*, 2000).

The activity of PI3 kinases has also been linked to the activity of Ras GTPases that are small GTP-binding proteins that include Rho, Rac and Cdc42; all of which being important regulators of cell motility (Servant *et al.*, 2000). These proteins interchange between a GTP-bound active state and a GDP-bound inactive state. Their activity is also controlled by accessory proteins such as guanosine dissociation inhibitors (GDIs), GTPase activating proteins (GAPs) and guanosine exchange factors (GEFs). Vav isoforms (Vav1, Vav2 and Vav3) can also promote PI3 kinase signalling with small GTPase proteins; however, little is yet known about the exact activity of these proteins (Cherfils and Chardin, 1999; Gakidis *et al.*, 2004; Han *et al.*, 1998; Quilliam *et al.*, 1995; Servant *et al.*, 2000). Although the exact role of Rho still remains unclear, it has been proposed to interact with myosin II to induce stress fibres and cell contraction and thus forming bipolar filaments. Indeed, the activity of myosin II, as also supported by studies in *Dictyostelium*, is critical for cell contraction and release at its rear as the cell moves forward. The activity of other GTPases, such as Cdc42 and Rac, is also necessary for the stimulation of actin filament assembly, as mutations in the *RAC2* gene have resulted in ineffective bactericidal and motile function of neutrophils (Glogauer *et al.*, 2000; Zigmond *et al.*, 1998).

In summary, PI3 kinases and their effector activities are necessary to promote actin reorganisation and therefore a change in neutrophil morphology that would trigger directional migration. Genetic studies on *Dictyostelium* confirmed the regulatory role of these proteins. However, as evidenced by the genetic studies or by the use of inhibitors, the precise signalling pathway that follows chemoattractant signalling has not been fully characterised, as blocking the activity of one or more of these effector proteins fails to completely block neutrophil migration.

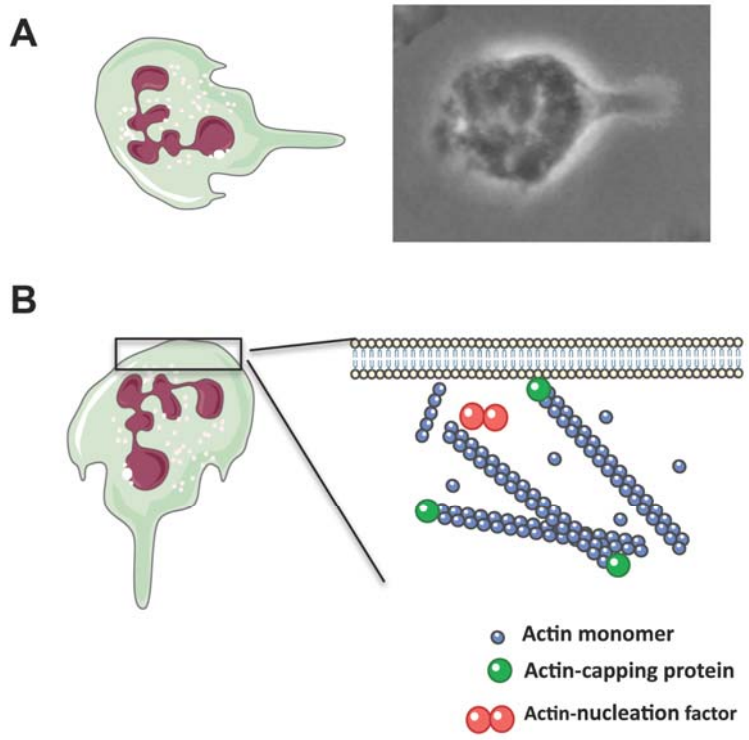


FIGURE 5: Neutrophil chemotaxis

(A) Characteristic images of an activated neutrophil. Prior to movement, neutrophils become activated and acquire a polarised morphology. Neutrophil polarisation is characterised by a leading actin-rich lamellipodium and a tail-like uropod at the distal end. Microscopy image was kindly provided by S. Bournazos. **(B)** Actin organization for directional neutrophil movement. Modifications in the organisation or de-organisation of actin in the lamellipodium are necessary for directional neutrophil crawling along endothelial surfaces. Actin assembly leads to the creation of lamellae, which are sheet-like structures rich in actin filaments. The process is controlled by actin-capping proteins that regulate monomer

addition in the filament end and actin-nucleation factors that promote the binding of actin monomers to generate new actin filaments.

Neutrophil-Associated Disorders

The fundamentally important contribution of granular proteins to the anti-microbial environment is reflected by the pathophysiology of many inherited defects: Chronic granulomatous disease (CGD) is a genetic disorder in which affected individuals display defects in any of the four subunit genes of the NADPH oxidase (Sanmun *et al.*, 2009). Phagocytes of CGD patients fail to trigger a respiratory burst, are incapable of producing reactive oxygen species (ROS) and therefore present impaired killing of ingested pathogens (Gallin *et al.*, 1992; Segal and Abo, 1993). In *in vivo* CGD mouse models, neutrophils lack NADPH activity and mice die prematurely due to recurrent bacterial and fungal infections (Dinauer *et al.*, 1999; Mardiney *et al.*, 1997). Specific granule deficiency is another genetic disorder that is marked by severe recurrent bacterial infection due to the absence of specific granules and due to abnormalities in neutrophil locomotion (Lekstrom-Himes *et al.*, 1999). Chediak-Higashi hereditary syndrome is marked by a defect in the formation of neutrophil granules, a lack of NK cell function and is manifested clinically with neutropenia and a lymphoma-like symptomatology, which can be life-threatening (Nagle *et al.*, 1996). Likewise, inherited MPO deficiencies are linked to severe infections with *Candida* as evidenced by MPO-knockout mouse models (Aratani *et al.*, 1999; Reynolds *et al.*, 1997; Reynolds *et al.*, 1999), whereas in α-1-antitrypsin deficiency, there is no inhibition of

neutrophil elastase, leading to tissue damage in the form of pulmonary emphysema.

Moreover, failure to control the migratory program of neutrophils gives rise to chronic pathological conditions. Such conditions range from vasculitis and ischaemia reperfusion injury to glomerulonephritis, rheumatoid arthritis and acute graft rejection (Weiss, 1989). Clinical manifestations of inappropriate neutrophil infiltration are more eminent in hereditary syndromes, such as familial Mediterranean fever (FMF) or familial Hibernian fever (FHF); both autosomal recessive diseases that affect serosal or synovial membranes due to the massive influx of neutrophils (Livneh and Langevitz, 2000; Williamson *et al.*, 1982). On the other hand, neutropenia is a disorder characterised by abnormally low neutrophil counts (Hsieh *et al.*, 2007). Affected individuals are highly susceptible to infections and might later develop aplastic anaemia and leukemia.

In general terms, the clinical symptoms manifested by all these disorders highlight the functional importance of neutrophils in a living organism. Moreover, they reveal the continuous presence of regulatory feedback mechanisms that control neutrophil activation and infiltration into tissues.

Neutrophils and the Resolution of Inflammation

The last step of an inflammatory response involves the resolution of the infection by eliminating the invading pathogen via phagocytosis and by spontaneously decreasing neutrophil infiltration (Dibbert *et al.*, 1999; Savill, 1997). The latter event is associated with the apoptosis and clearance of the leukocytes present at the inflammatory mileu. Indeed, it has been demonstrated that at inflammatory sites, negative signals exist that prevent neutrophil recruitment, dampen neutrophil responsiveness, and counterbalance or terminate the inflammatory response (Liles and Klebanoff, 1995; Serhan and Savill, 2005).

This anti-inflammatory program is characterised by cessation of neutrophil infiltration, as arachidonic acid-derived prostaglandins and leukotrienes are switched to lipoxins, resolvins, and protectins. In fact, the initial phase of the resolution of an inflammatory response is ultimately achieved through neutrophil apoptosis. The proinflammatory cytokines, such as MIP-2α/β that are released by neutrophils trigger the migration of professional phagocytes, such as macrophages, to enable phagocytosis, a process that leads to the release not only of anti-inflammatory cytokines but also of anti-inflammatory lipid mediators as lipoxin A_4, resolvin E1, and protectin D1 (Brown and Savill, 1999; Dibbert *et al.*, 1999; Schwab *et al.*, 2007; Serhan and Savill, 2005; Soehnlein and Lindbom, 2010). In parallel, termination of neutrophil recruitment occurs as a result of (i)

alterations in the balance of pro-and anti-inflammatory cytokines (TNF-α, IL-10, IL-4, IL-13) (Cassatella, 1999; Cox, 1996; Fadok *et al.*, 1998b; Kuhn *et al.*, 1993; Lentsch *et al.*, 1999; Wang *et al.*, 1994), (ii) the inactivation of chemoattractants, (iii) the elimination of adhesion molecules in endothelial cells (Sironi *et al.*, 1993; Vora *et al.*, 1996) and (iv) the release of molecules that actively inhibit neutrophil recruitment (Diamond *et al.*, 1999; O'Meara and Brady, 1997; Schwab *et al.*, 2007).

Negative regulators of neutrophil recruitment

Although the molecules that positively regulate neutrophil infiltration (chemoattractants) during an inflammatory process are well characterised and their mechanism of activity has been unravelled, little is known about the molecules that are synthesised within the host organism to either prevent or stop neutrophil recruitment. Two of the most well-known are: lipoxins and annexin-1. Lipoxins (lipoxin A_4 [LXA_4] and lipoxin B_4 [LXB_4]) (Fierro *et al.*, 2003) are endogenously produced eicosanoids that are enzymatically derived from arachidonic acid (ω-6 fatty acid). Lipoxins have been demonstrated to slow neutrophil infiltration to inflammatory sites, promote monocyte infiltration, enhance engulfment and phagocytosis of apoptotic neutrophils as well as reduce vascular permeability. Also lipoxins have been reported to inhibit superoxide generation and NF-κB activation. An analogous class of lipid-mediators, the resolvins, which include resolvin E1 (RvE1) and protectin D1 (PD1) (Schwab *et al.*,

2007; Serhan and Savill, 2005), are another type of lipid mediators that are derived from EPA and DHA ω-3 fatty acids. RvE1 and PD1 inhibit leukocyte infiltration both *in vivo* and *in vitro* and promote phagocytic removal by increasing macrophage recognition and engulfment of apoptotic neutrophils, as evidenced by the presence of zymosan-engulfing phagocytes in lymph nodes and spleen. Moreover, aspirin-triggered 15-epi-LX45 lipoxins (ATLs) and stable analogues of lipoxins possess similar anti-inflammatory activities in terms of neutrophil recruitment (Serhan *et al.*, 2008).

Apart from lipoxins, annexin-1 (ANXA1) is a 37-kDa glucocorticoid mediator protein that also inhibits neutrophil extravasation both *in vitro* and *in vivo* as evidenced in models of acute and chronic inflammation (Hayhoe *et al.*, 2006). The use of ANXA1-null mice and of the ANXA1 N-terminal bioactive peptide Ac2-26 revealed that ANXA1 binds to formyl-peptide receptors on neutrophil surface (FPR) or FPRs activated by lipoxin A_4 (FPRL-1/ALX), demonstrating in this way a similarity in the mode of activity of the two molecules: annexin-1 and lipoxins. Other molecular factors that also inhibit neutrophil extravasation **(Tables 5-8)** include antiflammins (inhibitors of PAF synthesis) (Camussi *et al.*, 1990), calpain (Katsube *et al.*, 2008; Noma *et al.*, 2009), cathepsin G (Tralau *et al.*, 2004), the metalloendopeptidase CD10 (Shipp *et al.*, 1991), CD47 (Chin *et al.*, 2009; Cooper *et al.*, 1995; Parkos *et al.*, 1996), C-reactive protein (Heuertz *et al.*, 1996; Mortensen and

Zhong, 2000), galectin-1 (La *et al.*, 2003), resistin (Cohen *et al.*, 2008), prostaglandin E1 and E2 (Burelout *et al.*, 2004; Mikawa *et al.*, 1994b), TGF-β1 (Shen *et al.*, 2007) as well as nitric oxide and reactive oxygen and nitrogen species (Dal Secco *et al.*, 2006; Sato *et al.*, 2000a; Sato *et al.*, 2000b). Moreover, in neural systems, leukocyte migration is inhibited by the release of molecules such as netrin-1 (Kaplan *et al.*, 2005), slit (Wu *et al.*, 2001) and sphingosine-1-phosphate (Kawa *et al.*, 1997).

Neutrophil recruitment is also affected by varying concentrations of certain cytokines. For example, high concentrations of IL-8 exert a chemorepulsive effect on neutrophils (Tharp *et al.*, 2006), while low doses of chymotrypsin exert an analogous effect (Jutila *et al.*, 1991). Semaphorins have also been characterised to exert a chemorepulsive effect (Goshima *et al.*, 2002; Klostermann *et al.*, 1998; Vianello *et al.*, 2005)

There are also other molecules reported to negatively regulate neutrophil migration. More specifically, many pharmacological agents inhibit leukocyte migration. These include non-steroidal anti-inflammatory compounds (e.g. ketoprofen, piroxicam, nimesulide; (Bizzarri *et al.*, 2001; Dapino *et al.*, 1994; Sordelli *et al.*, 1993; Tool *et al.*, 1996), corticosteroid drugs (fluticasone propionate, dexamethasone; (Hofbauer *et al.*, 1999b; Llewellyn-Jones *et al.*, 1994), antibiotics (azythromycin; (Tsai *et al.*, 2004) as well as drugs used for diabetes (gliclazide; (Okouchi

et al., 2004), rheumatoid arthritis (leflunomide; (Kraan *et al.*, 2000), inflammatory bowel disease (cyclosporine A; (Dalmarco *et al.*, 2008; Yan *et al.*, 2006), respiratory disorders (fenspiride; (Cunha *et al.*, 1993), barbiturates (thiopental, thiopentone, midazolarim; (Heine *et al.*, 1997; Nishina *et al.*, 1998), cannabinoids (McHugh *et al.*, 2008), class III antiarrhythmic methanesulfonamides (Brown *et al.*, 2007).

Furthermore, based on the knowledge of the signalling pathways that control cell migration, a number of molecules that are specifically developed to inhibit these pathways have been identified as key neutrophil inhibitory molecules. Such molecules include chemokine receptor antagonists, such as CXC chemotaxis receptor antagonist (McColl and Clark-Lewis, 1999), CXCR2 antagonist (Matzer *et al.*, 2004), SC-51146 (LTB_4 receptor antagonist) (Tsai *et al.*, 1994) and Sch527123 (CXCR1/2 R antagonist) (Chapman *et al.*, 2007). Other inhibitors used to cease neutrophil recruitment involve the carbohydrate selectin inhibitor CY-1503 (Schmid *et al.*, 1997), CI-949 allergic inhibitor (Wright *et al.*, 1991), PI3-kinase inhibitors (RO31-8220/CGP 41.251, wortmannin) (Ferrandi *et al.*, 2007; Niggli and Keller, 1997), repertaxin (IL-8 inhibitor) (Cavalieri *et al.*, 2005) (Souza *et al.*, 2004), sivelestat (neutrophil elastase inhibitor) (Hagio *et al.*, 2005) and Rho kinase inhibitors (Meyer-Schwesinger *et al.*, 2009).

All these pharmaceutical compounds and antagonists do not occur naturally, and therefore, cannot be synthesised in a

host organism. For this reason, the number of factors or molecules that are synthesised by a host organism to negatively regulate and interact with neutrophil locomotion is limited. In all cases, the existence of these negative factors accounts for the cessation of an inflammatory response and has significant therapeutic implications in conditions characterised by aberrant neutrophil infiltration.

Table 5.: Bacterial and Plant Extracts as Inhibitors of Neutrophil Chemotaxis	
Anthrax lethal toxin	(During *et al.*, 2005)
Bacteriochlorin	(Schuitmaker *et al.*, 1998)
Barleria lupulina	(Wanikiat *et al.*, 2008)
Borrelia burgdorferi	(Hartiala *et al.*, 2008)
Cryptococcal glucuronoxylomannan	(Ellerbroek *et al.*, 2002)
Epigallocatechin, green tea extract	(Hofbauer *et al.*, 1999a)
Lonchocarpus sericeus lectin	(Napimoga *et al.*, 2007)
Mikania laevigata extract	(Alves *et al.*, 2009)
Okadaic acid, shellfish toxin	(Yokota *et al.*, 1993)
Pertussis toxin (*Bordetella pertussis*)	(Thomazzi *et al.*, 1995)
Proteobacteria (α2 subgroup)	(Fumarola *et al.*, 1994)
Resveratrol, phytoallexin	(Issuree *et al.*, 2009)
Rose hip (Rosa canina)	(Larsen *et al.*, 2003)
Ryanodine, a poisonous alkaloid	(Elferink and De Koster, 1995a)
***S.aureus* β-hemolysis**	(Tajima *et al.*, 2009)
Silymarin	(De La Puerta *et al.*, 1996)
Viscolin, from *Viscum coloratum*	(Hwang *et al.*, 2006)
***Yersinia pestis* (V antigen)**	(Welkos *et al.*, 1998)

Table 6.: Chemicals and Drugs as Inhibitors of Neutrophil Chemotaxis	
15(5) hydroxyeicosatetraenoic acid	(Takata *et al.*, 1994)
2-Phenyl-2,3-dihydro-1H-imidazo	(Bruno *et al.*, 2007)
Alloxan, oxygenated pyrimidine derivative	(Diaz *et al.*, 1996)
Antifolate-10-deazaaminopterin	(Weber *et al.*, 1991)
Auranofin (arthritis drug)	(Elferink and de Koster, 1993)
Azathioprine (high dose- transplantation drug)	(Elferink *et al.*, 1997)
Azithromycin (antibiotic)	(Tsai *et al.*, 2004)
Benzodiazepines	(Finnerty *et al.*, 1991)
C3 ADP-ribosyltransferase	(Stasia and Vignais, 1995)
Cannabinoids and phytocannabinoids	(McHugh *et al.*, 2008)
Chlorinated hydrocarbon insecticide	(Miyagi *et al.*, 1998)
Class III antiarrhythmic methanesulfonanilides	(Brown *et al.*, 2007)
Cyclosporin A	(Dalmarco *et al.*, 2008; Yan *et al.*, 2006)
Dapsone hydroxylamine (haemolytic agent)	(Wozel *et al.*, 1997)
Dexamethasone (glucocorticoid)	(Hofbauer *et al.*, 1999b)
Emedastine difumarate (agent for allergic conditions)	(el-Shazly *et al.*, 1996)
Fenspiride (respiratory disorders)	(Cunha *et al.*, 1993)
Fluticasone propionate (corticosteroid)	(Llewellyn-Jones *et al.*, 1994)
Furosemide (diuretic)	(Hofbauer *et al.*, 2002)
Gabexate mesilate (protease inhibitor)	(Mikawa *et al.*, 1994a)
Gliclazide (anti-diabetic agent)	(Okouchi *et al.*, 2004)
Glucocorticoids	(O'Leary and Zuckerman, 1997)
Hg^{2+} small sized polyethylene glycols	(Loitto and Magnusson, 2004)
Iron complexes	(Sengoelge *et al.*, 2003)
Itraconazole (antifungal agent)	(Steel and Anderson, 2004)
Ketamine (recreational drug; binds to opoid receptor)	(Nishina *et al.*, 1998; Zahler *et al.*, 1999)
Ketoprofen isomers (non-steroidal anti-inflammatory agents)	(Bizzarri *et al.*, 2001)
Lansoprazole/ omeprazole (GI reflux drug)	(Capodicasa *et al.*, 1999)
Leflunomide (for rheumatoid arthritis)	(Kraan *et al.*, 2000)
Mercuric chloride	(Nordlind and Liden, 1990)
Methotrexate (chemotherapy)	(Kraan *et al.*, 2000; Liu *et al.*, 1996; Okuda *et al.*, 1996)
Metipranolol (β-blocker)	(Djanani *et al.*, 2003)
Midazolam (for insomnia)	(Nishina *et al.*, 1998)
Nedocromil sodium (ophthalmic drug)	(Bruijnzeel *et al.*, 1989; Bruijnzeel *et al.*, 1990)
Nicotine	(Huston *et al.*, 2009)
Nimesulide (non-steroid anti-inflammatory)	(Dapino *et al.*, 1994; Tool *et al.*, 1996)
Opiate	(Grimm *et al.*, 1998)

Pentoxifylline	(Dominguez-Jimenez *et al.*, 2002)
Piroxicam-copper complexes (anti-inflammatory)	(Sordelli *et al.*, 1993)
Potassium iodide	(Honma *et al.*, 1990)
Pravastatin (statin)	(Dunzendorfer *et al.*, 1997)
Salicylates	(Pierce *et al.*, 1996)
Salmeterol (for asthma, COPD)	(Tool *et al.*, 1996)
Sodium cromoglycate (for asthma, allergy)	(Bruijnzeel *et al.*, 1989; Bruijnzeel *et al.*, 1990)
Spiromolactone (diuretic)	(Hofbauer *et al.*, 2002)
Sufentanil (opioid analgesic drug)	(Hofbauer *et al.*, 1998)
Thimerosal (mercury containing organic compound)	(Elferink and de Koster, 1998)
Thiopental (barbiturate)	(Nishina *et al.*, 1998)
Thiopentone (barbiturate)	(Heine *et al.*, 1997)
Tolfenamic acid (non-steroidal anti-inflammatory)	(Kankaanranta *et al.*, 1991)
Troxipide (GI reflux treatment)	(Kusugami *et al.*, 2000)

Table 7.: Inhibitory Molecules and Antagonists as Inhibitors of Neutrophil Chemotaxis	
α7 cholinergic receptor activation	(Giebelen *et al.*, 2007)
Adenosine kinase inhibitors	(Rosengren *et al.*, 1995)
Adenylate cyclase-activating peptide	(Kinhult *et al.*, 2001)
BN52021 (PAF antagonist)	(Liu *et al.*, 1996)
C1 inhibitor	(Cai *et al.*, 2005)
Cannabinoid agonist WIN55.212-2	(Nilsson *et al.*, 2006)
Carbohydrate selectin inhibitor CY-1503	(Schmid *et al.*, 1997)
CCR1 antagonist (CP-Y81.715)	(Gladue *et al.*, 2006)
CHS-111 (2-benzyl)	(Chang *et al.*, 2009)
CI-949 (allergic inhibitor)	(Wright *et al.*, 1991)
CXC chemokine receptor antagonist	(McColl and Clark-Lewis, 1999)
CXCR2 antagonist	(Matzer *et al.*, 2004)
GLEPP1 protein tyrosine phosphatase phi inhibitors	(Gobert *et al.*, 2009)
mAb 137-26	(Fung *et al.*, 2003)
Neurokinin 1 receptor antagonist	(Rittner *et al.*, 2007)
PAF antagonists (WEB-2086)	(Hakansson and Venge, 1990)
PI3-kinase inhibitor	(Ferrandi *et al.*, 2007)
PKC inhibitors (RO31-8220/ CGP 41.251)	(Keller and Niggli, 1994)
PKI55 –protein kinase C modulator	(Selvatici *et al.*, 2008)
Repertaxin –IL-8 inhibitor	(Cavalieri *et al.*, 2005; Souza *et al.*, 2004)
Rho kinase inhibitors	(Meyer-Schwesinger *et al.*, 2009)
SC-51146 (LTB_4 receptor antagonist)	(Tsai *et al.*, 1994)
Sch527123 (CXCR1/2 R antagonist)	(Chapman *et al.*, 2007)
Sivelestat –neutrophil elastase inhibitor	(Hagio *et al.*, 2005)
Timolol –non-selective β-adrenergic receptor blocker	(Djanani *et al.*, 2003)
Wortmannin (PI3 kinase inhibitor)	(Niggli and Keller, 1997)

Table 8.: Cytokines and Enzymes as Inhibitors of Neutrophil Chemotaxis	
Annexin-1	(Hayhoe *et al.*, 2006)
Antiflammins –inhibit PAF synthesis	(Camussi *et al.*, 1990)
Calpain (Ca^{2+}-dependent protease)	(Katsube *et al.*, 2008; Lokuta *et al.*, 2003; Noma *et al.*, 2009)
Cathepsin G	(Tralau *et al.*, 2004)
CD10 (metalloendopeptidase)	(Shipp *et al.*, 1991)
CD47	(Chin *et al.*, 2009; Cooper *et al.*, 1995; Parkos *et al.*, 1996)
Chymotrypsin (low dose)	(Jutila *et al.*, 1991)
C-reactive protein (CRP)	(Mortensen and Zhong, 2000)
C-reactive protein (CRP) peptides	(Heuertz *et al.*, 1996)
Elastase-generated IgG fragments	(Eckle *et al.*, 1991)
Endothelin-3	(Elferink and de Koster, 1995b)
Fibrinogen	(Higazi *et al.*, 1994)
Galectin-1	(La *et al.*, 2003)
Guanine nucleotides	(Boonen *et al.*, 1991)
Heme oxygenase	(Freitas *et al.*, 2006)
Heme-binding protein (F2L)	(Gao *et al.*, 2007; Lee *et al.*, 2007)
Hexa- and hepta-peptides	(Hayashi *et al.*, 1995)
Hirudin C-terminal fragments	(Rowand *et al.*, 1992)
Human activated protein C	(Elphick *et al.*, 2009; Nick *et al.*, 2004)
Human GMCSF	(Kownatzki *et al.*, 1990)
Human leukocyte elastase	(Tralau *et al.*, 2004)
IL-8 high doses	(Tharp *et al.*, 2006)
Intravascular lectin	(Sakamoto *et al.*, 1998)
Leumedin (anti-inflammatory compound)	(Jorens *et al.*, 1994)
Lipocortin -1	(D'Amico *et al.*, 2000)
Lipoxin A9 and aspirin lipoxins	(Fierro *et al.*, 2003)
L-selectin shedding	(Hafezi-Moghadam *et al.*, 2001)
Netrin-1	(Kaplan *et al.*, 2005)
Neuropeptide a-MSH	(Catania *et al.*, 1996)
Neutrophil inhibitory factor	(Lo *et al.*, 1999; Zhou *et al.*, 1998)
Nitric oxide	(Dal Secco *et al.*, 2006; Tavares-Murta *et al.*, 1998)
Nonleukoreduced red blood cell	(Ottonello *et al.*, 2007)
Pig seminal plasma	(Rozeboom *et al.*, 2001)
Plasma from burned patients	(Piccolo and Sannomiya, 1995)
Prostaglandin E1 and E2	(Burelout *et al.*, 2004; Mikawa *et al.*, 1994b)
Protectin D1	(Schwab *et al.*, 2007)
P-selectin glycoprotein ligand-1	(Hicks *et al.*, 2003)
Reactive oxygen and nitrogen species	(Sato *et al.*, 2000a; Sato *et al.*, 2000b)
Resistin (cysteine rich proteins secreted from adipose tissue)	(Cohen *et al.*, 2008)

Resolvin E1	(Campbell *et al.*, 2007; Ishida *et al.*, 2009; Schwab *et al.*, 2007)
Retinol binding protein	(Cohen and Horl, 2004)
RGDS peptide	(Moon *et al.*, 2009)
S100A8/ S100A9 (member of S100 family Ca^{2+}-binding proteins)	(Ryckman *et al.*, 2003)
Slit	(Wu *et al.*, 2001)
Sphingosine-1-phosphate	(Kawa *et al.*, 1997)
Spinorphin (opioid agent)	(Yamamoto *et al.*, 1997)
Sulfated/nonsulfated cholecystokinin-octapeptides	(Carrasco *et al.*, 1997)
TGF-β1	(Shen *et al.*, 2007)

Eosinophils: Another granulocyte subclass

Eosinophils, though they constitute only about 1-6% of leukocytes, are important in defense against invading parasites and play prominent effector roles in several inflammatory disorders (Rothenberg, 1998). Normally, eosinophils are essentially limited to the digestive tract and are absent from other tissues (Kato *et al.*, 2001). However, the inflammatory responses observed in bronchial asthma, allergic rhinitis, dermatitis or in allergic gastrointestinal diseases are characterised by the local accumulation of eosinophils within the affected tissues (Wardlaw *et al.*, 1995). The presence of eosinophils is associated with the release of eosinophil-derived toxic inflammatory mediators, such as major basic protein, eosinophil cationic protein, eosinophil-derived neurotoxin and eosinophil peroxidase, all of which are stored in intracellular granules within eosinophils (Gleich *et al.*, 1986; Rothenberg and Hogan, 2006). The release of these products, evolved to deal efficiently with invading parasites, is also believed to contribute to the pathophysiology of the underlying inflammatory disorders (Simon *et al.*, 2004).

As in the case of neutrophils, recruitment of eosinophils to inflammatory sites is tightly regulated and occurs in response to chemokines, especially eotaxin, and cytokines expressed at sites of allergic inflammation, such as IL-1 and TNF-α, along with the Th2 cytokines, IL-5, IL-4, IL-13

and IL-9 that promote eosinophil recruitment and activation either directly or by regulating local IL-5 and eotaxin synthesis and by suppressing IFN-γ production. Leukotrienes and prostaglandins, mainly LTB_4 and PGD_2 respectively, along with PAF, are also implicated in promoting eosinophil chemotaxis (Pinho *et al.*, 2005; Rothenberg and Hogan, 2006; Rothenberg *et al.*, 1995; Simon *et al.*, 2004).

Although such positive chemoattractant signals have been well characterised (Lukacs *et al.*, 1996; Numao and Agrawal, 1992; Roviezzo *et al.*, 2004), little is known about the mechanisms or the presence of regulatory molecules that negatively influence eosinophil migration and thus, may contribute to the paucity of eosinophils in normal tissues. Indeed, agents that can influence eosinophil infiltration and activation have been applied in the treatment of allergic disorders and for this reason several therapeutic agents that target multiple mechanisms underlying eosinophilia are being investigated. For example, glucocorticoids limit eosinophil cytokine production and promote eosinophil apoptosis (McColl *et al.*, 2007), leukotriene antagonists and inhibitors interfere with eosinophil recruitment, while therapeutic antibodies against IL-5 and eotaxin-1 are under development to treat allergic conditions (Rothenberg and Hogan, 2006). Recently, it has also been found that lipoxin A_4 blocks eosinophil trafficking (Bandeira-Melo *et al.*, 2000), while IL-6, IL-11 and IL-12 cytokines inhibit Th2 cytokine expression, VCAM-1 expression and

eosinophilia (Wang *et al.*, 2000a; Zhao *et al.*, 2000) (Davoine *et al.*, 2006).

LEUKOCYTES AND TUMOUR

Neutrophils and Tumour

Role of neutrophils in tumour

Although many leukocyte types have been characterised to be present in the tumour stroma and promote the neoplastic process, the role of neutrophils in tumour progression has been characterised as controversial. There has been an ever increasing number of studies over the last decade that support the anti-tumour effect of neutrophils over other inflammatory cells due to their cytostatic effects (Dallegri and Ottonello, 1992). However, there are also tumours such as gliomas, gastric carcinomas and melanomas that have been reported to display a strong correlation between tumour grade and the extent of neutrophil infiltrate, proposing in this way a tumour enhancing role of neutrophils (Fossati *et al.*, 1999; Schaider *et al.*, 2003; Takematsu *et al.*, 1994).

In a very recent study by Fridlender *et al.* (2009) it was shown that TGF-β within the tumour microenvironment allows the generation of a population of specialised $CD11b^{+}/Ly6G^{+}$ TANs (tumour-associated neutrophils) that exhibit a pro-tumour phenotype. However, once TGF-β becomes blocked as a means of tumour therapy to slow tumour growth and increase neutrophil-attracting chemokines, an influx of TANs was induced, which were hypersegmented, more cytotoxic to

tumour cells and generally, expressed an anti-tumour phenotype. These findings compare TANs with TAMs and support two populations of TANs: N1 polarised neutrophils that in the absence of TGF-β induced increased neutrophil recruitment and anti-tumour effects and a second population that has a N2 polarisation phenotype, which in the presence of neutrophils promote tumour progression. These two populations reveal for another time the controversial role of neutrophils in tumours and how they can exert tumour regression (Hicks *et al.*, 2006; Nozawa *et al.*, 2006). Moreover, preliminary studies from our group have shown that co-culture of neutrophils with BL cells had cytostatic effects on BL cell growth by decreasing both viable and apoptotic cell populations (I. Bournazou, unpublished data).

In this way, a new model of cancer immunotherapy could be proposed that is based on the cytostatic effects of neutrophils as a new approach (Allendorf *et al.*, 2005). For example, picibanil, an extract from *Streptococci*, has been widely utilised to treat malignant ascites and its anti-tumour mechanism involves complement-mediated neutrophil activation (Yang *et al.*, 1992). Also, murine granulocyte infiltration to SCID mice was demonstrated to delimit the human tumour mass and treatment of such mice with anti-murine granulocyte antibody markedly improved the growth of human tumour cell lines of different origin by suppressing the host granulocyte reaction (Lozupone *et al.*, 2000). Moreover, the use of replicating viruses for cancer therapy (virotherapy) was predominantly

characterised by an influx of neutrophils that in turn, slowed tumour progression considerably. For example, measles virus was found to be oncolytic for lymphomas *in vivo* (Grote *et al.*, 2003; Grote *et al.*, 2001; Peng *et al.*, 2001; Peng *et al.*, 2002).

Based on the anti-tumour effect of neutrophils combined with substantial histological evidence that reports the absence of neutrophils from the stroma of several tumour types, it could therefore be proposed that negative signals are likely to exist to prevent their infiltration to the tumour site. These outcomes might also probably arise by the distortion of the balance of the type and of the amount of cytokines, e.g. IL-1, IL-2, IL-10, IL-12, TNF-α, TNF-β, TNF-γ, G-CSF released by tumour cells to suppress the massive local inflammatory response triggered by the degree of recruited neutrophils as well as by neutrophil cytostatic effects (Musiani *et al.*, 1996; Musiani *et al.*, 1997). This overall phenomenon seems highly dependent on the heterogeneity of the tumour, as in most cases neutrophils do not constitute a component of the inflammatory milleu of the tumour stroma. In this way, tumour itself seems to actively suppress neutrophil effector activity and render them hypofunctional, as in the case of other inflammatory cells resident in the tumour as part of its cancer immunoediting process (Dunn *et al.*, 2002; Dunn *et al.*, 2004; Lollini and Forni, 2003).

Despite the difficulties to identify the exact mechanisms or the factors involved in the prevention of neutrophil

migration to the tumour site, it could be proposed that this process is orchestrated by the dynamic tumour microenvironment itself. There are many possibilities that for instance, (i) tumour cells actively inhibit neutrophil migration via the release of several factors; (ii) the tumour microenvironment stimulates or abrogates neutrophil migration via a regulation of adhesion molecules on tumour or pericapillary endothelial cells and finally, (iii) the whole process is orchestrated by the intratumoural cytokine network. Indeed, upregulation of the expression of endothelial adhesion molecules in tumours was shown to trigger the recruitment of neutrophils intratumourally and thus, elicit an anti-neoplastic response (Colombo *et al.*, 1996; Kushner and Cheung, 1992). For example, tumour cells transfected to release G-CSF, IL-2, IL-4, IL-12 and TNF-α also displayed an upregulation of ICAM-1 in blood vessels as well as elevated production of chemokines such as GRO/KC (growth-related oncogene/cytokine-induced neutrophil chemoattractant; a murine functional homologue of IL-8) (Cavallo *et al.*, 1999; Di Carlo *et al.*, 2000; Hirose *et al.*, 1995; Musiani *et al.*, 1996). As a result, integrin-mediated adhesion and extravasation of neutrophils to the tumour site was elevated with oncolytic effects. On another study of transfected TSA-LEC (liver expressed chemokine), LEC expression is chemotactic for dendritic cells, monocytes, but not neutrophils; however, when triggered by IL-10, LEC became upregulated and had oncolytic effects for the transfected tumours accompanied by the recruitment of neutrophils, $CD8^+$ lymphocytes and

natural killer cells (Giovarelli *et al.*, 2000; Hedrick *et al.*, 1998). Moreover, as evidenced by several studies using cytokine-engineered tumour cells, some types of cytokines found in the tumour microenvironment, such as IL-2, IL-3, IL-4, IL-7, IL-10, IFN-α, IFN-β, IFN-γ and TNFα, when sustainedly released by engineered tumour cells, resulted in the rejection of tumour cells and the establishment of a massive anti-tumour immune reaction (tumour immunoprevention) (Hirose *et al.*, 1995). In most cases, this was accompanied by a decreased intratumoural microvessel density, increased infiltration of neutrophils, augmented $CD4^+$ and $CD8^+$ T cell proliferation and survival and finally induction of cell death in the tumour microenvironment. For example, in recombinant IL-2 infusional therapy of cancer patients, there was a marked activation of neutrophils and a subsequent interaction with tumour and endothelial cells that results to tumour cell lysis (Blay *et al.*, 1990; Gemlo *et al.*, 1988). Another case is that of a 56-year-old patient with advanced hepatocarcinoma that was cured after treatment with granulocyte colony-stimulating factor (G-CSF), a key regulator of neutrophil production, providing in this way merit for the use of G-CSF in anti-cancer therapies (Lopez-Lazaro, 2006). In mice, tumour cells engineered to release G-CSF were directly killed by neutrophils, which were observed histologically forming prominent cytoplasmic projections in close contact with dead tumour cells well before any vascularisation was evident (Colombo *et al.*, 1992).

Eosinophils also possess an anti-tumour efficacy (Kataoka *et al.*, 2004). In particular, IL-4 transfection in tumour cell lines of various histologic types appeared to prevent tumour formation via the infiltration of eosinophils and macrophages to the tumour site, while tumour formation reoccurred despite the presence of macrophages in eosinophil-depleted animals that received the IL-4 transfected cancer cell lines (Rothenberg *et al.*, 1995; Tepper *et al.*, 1992; Tepper *et al.*, 1989). This phenomenon was attributed to the cytotoxic activity of eosinophils and is further supported by the localised expression of eotaxin, an eosinophil-specific chemoattractant. Analogous results were observed in models of hepatocellular carcinoma, in which the co-operative expression of IL-5 and eotaxin induced the antitumour activity of eosinophils. Because of the scarcity of these cell types in circulation, the exact tumour-killing mechanisms remain largely unknown.

To sum up, it is evident that tumour establishment and progression, as it is until now evidenced by studies investigating the anti-neoplastic potential of neutrophils, is orchestrated by the intratumoural cytokine network (Coussens and Werb, 2002; Frederick and Clayman, 2001). Moreover, the identification of the adhesion molecules participating in the tumour-PMN interaction will be very important for the exploitation of neutrophil effector systems as a form of immunotherapy (Allendorf *et al.*, 2005). Indeed, it has been recently identified that PMNs increase the adhesion of circulating tumour cells to

microvascular endothelium via ROS production and in this way, set a first step to prevent metastasis by preventing tumour recurrence at distant sites (Ten Kate *et al.*, 2007). In addition, emphasis should be given on the fact that tumour progresses using a low immunogenicity dynamic equilibrium as a basis. Any distortion in the level of local inflammation from moderate to acute by disturbing the intratumoural cytokine availability seems to have detrimental effects at a first stage on endothelial adhesion markers, which in turn control inflammatory cell recruitment.

The tumour lytic mechanism of neutrophils

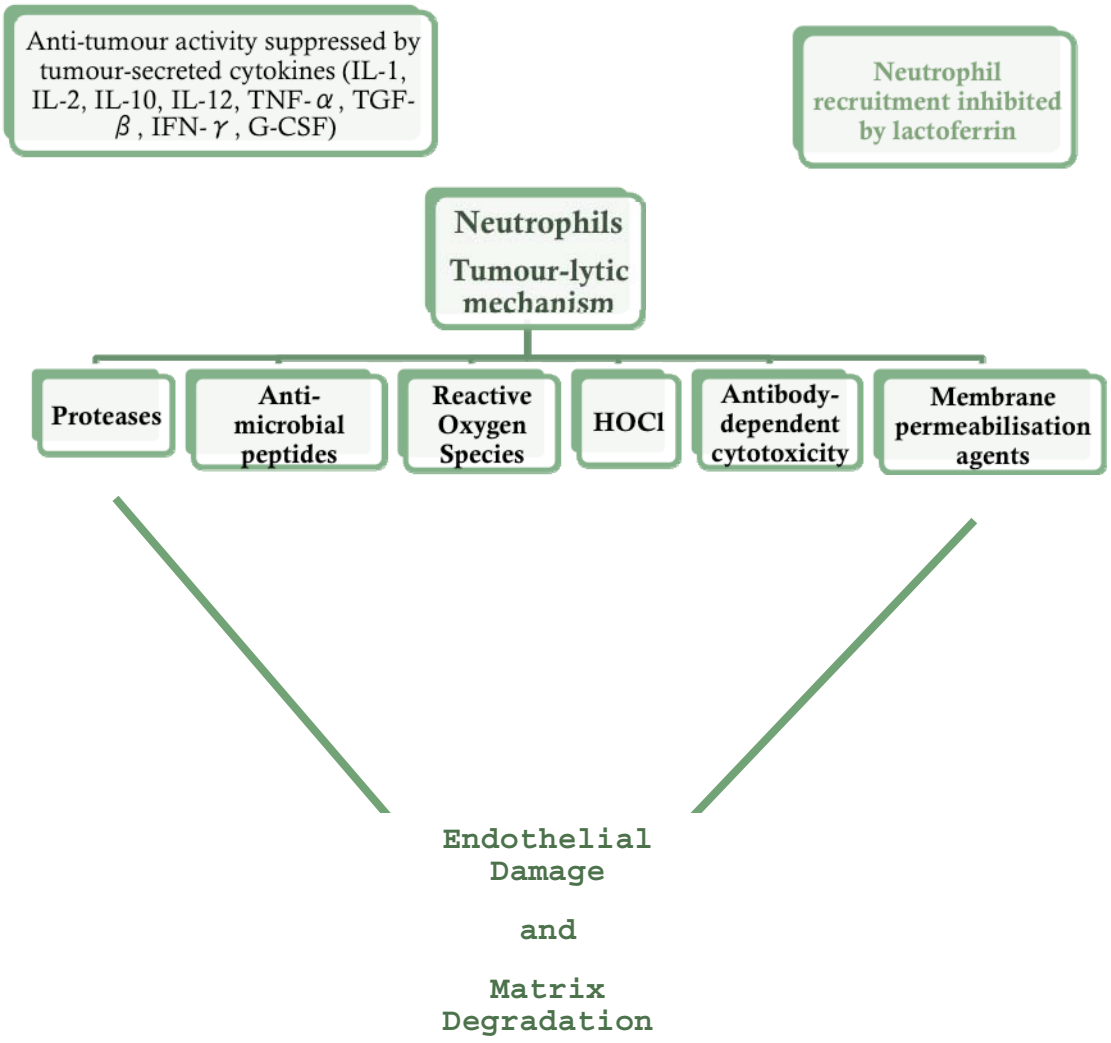
Uncontrolled neutrophil influx is a characteristic of chronic inflammatory diseases that range from vasculitis and ischaemia reperfusion injury to glomerulonephritis, rheumatoid arthritis and acute graft rejection. In tumours though, uncontrolled neutrophil influx switches the balance from tumour progression to tumour rejection via the release of proteases, reactive oxygen species, membrane-permeabilisation agents and soluble mediators of cell lysis such as IFN-γ and TNF-α. Tumour cell lysis is also mediated by the release of hypochlorous acid (HOCl) from activated neutrophils that in turn reacts with primary amines to form chloramines with immunostimulatory properties (She *et al.*, 1993) **(Table 9)**.

Accumulation of proteases and antimicrobial peptides has also been presented to lead to endothelial damage and matrix degradation. For instance, defensins, a family of antimicrobial peptides, are highly toxic against several tumours, whereas elastase and cathepsin G cause severe injury to endothelial cells (Ganz *et al.*, 1985; Okrent *et al.*, 1990; Westlin and Gimbrone, 1993). Another mechanism that accounts for neutrophil-mediated tumour lysis is antibody-dependent cell-mediated cytotoxicity (ADCC) that is augmented by GM-CSF administration in cases of melanoma and colorectal cancer (Kindzelskii and Petty, 1999). Moreover, as evidenced by histological analyses, neutrophil-mediated tumour cell killing is also associated to a local ischemic and/or haemorrhagic necrosis

(Hainsworth, 2000). Thus, unlike other inflammatory cells, neutrophil effector activities are not affected by the tumour immunoediting process and remain competent to exert their cytostatic effects once recruited to the tumour stroma.

Based on these observations, recent anti-neoplastic studies have been directed to the identification of molecules or strategies that would stimulate neutrophil migration to the tumour stroma so as to increase local inflammation and consequently elicit an innate anti-tumour response (Di Carlo *et al.*, 2001; Giovarelli *et al.*, 2000; Hainsworth, 2000; Lollini and Forni, 2003).

Table 9: The Tumour Lytic Mechanism of Neutrophils



Neutrophils and tumour immunotherapy

In general, over the last decade, there has been an emerging effort to identify new anti-neoplastic approaches or increase the potency of the existing ones. Especially, new directions have focused on the use of agonists or blockers of TNF-α (Jackson, 2007), cyclooxygenase-2 (COX-2) (Ragel *et al.*, 2007), transcription factor activator protein 1 (AP-1) (Matthews *et al.*, 2007), naturally-occuring cytokines (van de Vosse and van Agtmael, 2007), tissue factor (TF) (Lwaleed *et al.*, 2007), cJun-N-terminal kinases (Salh, 2007) as well as DNA vaccines in order to stimulate cytotoxic T lymphocyte activity (Lollini and Forni, 2002; Lollini and Forni, 2003; Stevenson *et al.*, 2002). However, neutrophils seem to represent the most promising and successful type of tumour therapy, as evidenced by an ever-increasing number of studies based on the anti-tumour effector activities of neutrophils. In most, if not all of these studies, there has been a complete eradication of the tumour (Dranoff *et al.*, 1993; Suttmann *et al.*, 2006). Notably, the exploitation of neutrophils as anti-neoplastic immune cells in these studies has a potent advantage over other inflammatory cells, as according to Carl Nathan (2006), "neutrophils might be especially valuable as anti-tumour effector cells when one considers their enormous numerical preponderance over tumour-specific cytoxic T cells".

Some of the most recent strategies directed to trigger neutrophil recruitment to the tumour site are presented

below. Apart from the studies using cytokine engineered tumour cells as described before that aim at the distortion of intratumoural cytokine balance to elicit an inflammatory response, other relevant attempts have been made to create human tumour cells engineered to release neutrophil chemoattractant molecules such as IL-8, human MIP-1α and GROα (Hirose *et al.*, 1995). For example, protein intratumoural transfer of neutrophil chemotactic molecules and other molecules such as FasL, 4-IBBL and TNF-α in the L5178Y lymphoma model resulted in a strong anti-tumour response and the acquisition of a long-term systemic anti-tumour immunity (Liu *et al.*, 2007). This was accompanied by an increase of tumour-infiltrating neutrophils, IL-12-producing DCs, IFN-γ- producing $CD4^+$ and $CD8^+$ T cells and a decrease of intratumoural IL-10 producing Treg cells. Furthermore, knockout of IL-8 using RNA interference also was found to exert potent effects on tumour establishment and growth in oestrogen receptor (ER) positive and ER negative breast cancer models correlating to neutrophil influx (Yao *et al.*, 2007).

A dramatic transcriptional activation of neutrophil chemoattractant genes *CXCL1* and *CXCL5* was also found following the *in vivo* tumour killing activity of two oncolytic viruses: vesicular stomatitis virus (VSV) (Breitbach *et al.*, 2007) and vaccinia virus. This effect was accompanied by an infiltration of neutrophils after chemokine induction, according to histological evidence from infected tumours. Based on this example, special

emphasis should be given on the use of bacterial and viral agents. Their use in anti-neoplastic therapy has been widely accepted as a mean to trigger neutrophil infiltration to the tumour site. For instance, systemically administered *Salmonella choleraesuis* in the ML-1 orthotopic tumour model was associated to increased infiltration of neutrophils and was marked by significant prolongation of animal survival, reduction of tumour size and upregulation of IFN-γ and IL-10 production (Lee *et al.*, 2008). *Bordetella pertussis* was also used recently as a vaccine vector in a tritherapy model and led to potent therapeutic immune responses against TC1 tumours in a murine model of cervical carcinoma. Again, tumour regression was marked by a large expansion of splenic neutrophils (Berraondo *et al.*, 2007). In addition, the immunotherapeutic potential of tumour-specific phages to treat established solid tumours in a mouse model of melanoma (B16-F10, B16/ATK) resulted in a massive infiltration of neutrophils and a complete tumour regression and long-term survival in 50% of the mice (Eriksson *et al.*, 2007).

Recently, several molecular-based gene therapy approaches have also been developed. A characteristic example includes the overexpression of the C-terminal fragment of the human telomerase reverse transcriptase (hTERC27) via intratumoural injection of an adenoassociated virus that inhibited the growth and tumourogenicity of human cervical cancer HeLa cells as well as the growth of established human glioblastoma xenografts in nude mice (Ng *et al.*,

2007). In both cases, this type of treatment was marked by profound necrosis, elevated infiltration of neutrophils and reduced microvessel density. Other examples involve the regulation of the levels of the apoptosis-inducing Fas ligand (FasL) in a variety of human cancers. Ectopic expression of FasL in experimental tumours triggered a neutrophil-mediated inflammatory response and tumour rejection, while FasL (low) tumours were found to grow faster in parental cells in mice that had acquired tumour-specific immunity (Wada *et al.*, 2007).

Accordingly, in support of these studies, ongoing research using various chemotherapeutic agents highlighted the requirement of neutrophils to elicit an anti-tumour response. For example, topical treatment with the new anti-cancer agent ingenol-3-angelate (PEP005) induced the presence of neutrophils that were in turn required to prevent relapse of skin tumours (Challacombe *et al.*, 2006). In detail, PEP005 activated human endothelial cells in order to enable neutrophil adhesion and the release of tumouricidal reactive oxygen intermediates. Similarly, the use of anti-angiogenic molecules in the eradication of tumours has also been extensively studied. Plasminogen kringle 5 (K5) suppressed cancer growth in tumour xenograft models and on an immune competent model of breast cancer (Perri *et al.*, 2007). Its activity involved the anti-tumour role of T lymphoid cells, accompanied by a significant decrease in tumour-associated microvessel length and density as well as a robust neutrophil

infiltration due to the neutrophil chemotactic activity of K5.

All these studies indicate that further research is required to unravel the exact molecular mechanisms that enable neutrophil anti-tumoural activity and identify the molecules that tumour cells employ in order to prevent neutrophil recruitment. It is evident that the manipulation of the underlying mechanisms that induce neutrophil migration and their cytostatic responses can be very successful in order to decrease the rate of tumour proliferation and finally succeed in the total eradication of the tumour itself.

Leukocyte Infiltration in Tumours

The concept that inflammation promotes oncogenesis has long been established (Balkwill and Mantovani, 2001; Coussens and Werb, 2002; Frederick and Clayman, 2001; Monks *et al.*, 2002; Talmadge *et al.*, 2007; Wouters, 2005). Virchow, back in 1863, first hypothesised that the infiltration of leukocytes to tumours is a pivotal step for the origin of tumourigenesis and its link to inflammation. However in the last decade, novel approaches in tumour immunotherapy have revealed that inflammation can exert anti-tumour effects (Allendorf *et al.*, 2005; Giovarelli *et al.*, 2000; Lollini and Forni, 2002). Indeed, there is an ever-increasing number of studies that demonstrate that the presence of leukocytes within tumour tissues could elicit both pro- and anti-tumour mechanisms (Lehrnbecher *et al.*, 2008; Lin and Pollard, 2004; Yu and Rak, 2003). In fact, the type and number of the immune cells infiltrating to the tumour stroma have mild anti-inflammatory potential and indeed, they establish a symbiotic relationship to the tumour-contrary to inflammatory lesions- via the release of a plethora of proinflammatory cytokines as well as vascular epithelial growth factors.

Therefore, contrary to inflammatory lesions, tumours do not display self-limiting processes for the clearance of the infiltrating immune cells and are thus fairly characterised as wounds that never heal, as they selectively direct the immune system in favour of their

development (cancer immunoediting) (Dunn *et al.*, 2002; Lollini and Forni, 2003). This tight association of inflammation and tumourigenesis placed the presence of an immune response as a prerequisite for tumour establishment, as virtued by histological evidence on a wide range of solid tumour types, in which a high density of leukocytic infiltration is linked to increased prognosis (Dinarello, 2006; Graves *et al.*, 1989; Lehrnbecher *et al.*, 2008; Lin and Pollard, 2004; Yu and Rak, 2003). This is further strengthened by the fact that chronic inflammatory conditions or infections account for many malignancies e.g. colon carcinogenesis and inflammatory bowel disease (Crohn's disease), thyroiditis and papillary thyroid carcinoma, *H.pylori* and stomach cancer, hepatitis C and liver cancer (Blaser *et al.*, 1995; Crowe, 2005; Penn, 1999). Also, cells with DNA alterations due to chemical carcinogens do not allow tumour formation until exposed to a secondary stimulus such as chronic irritants or inflammatory agents. Perhaps the best evidence for the coupling of the chronic inflammatory condition with tumourigenesis is the reduced risk of colon, lung, oesophagus and stomach cancers in long-term users of aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) (Baron and Sandler, 2000; Garcia-Rodriguez and Huerta-Alvarez, 2001).

All these examples strengthen the assumption that immunocompromised individuals are protected from cancer. However, both animal and human studies have abolished this hypothesis, as immunity is necessary to combat a tumour at

its earlier stages, especially if this tumour arises as a result of a viral infection e.g. increased incidence of lymphoma in AIDS patients (Boshoff and Weiss, 2002; Gatti and Good, 1971). This forms the cornerstone of the "cancer immunosurveillance" theory and constitutes a protective response of a host organism towards malignant mutations. However, immunity turns out to be Damocles' sword in favour of tumour establishment. This is supported by the principle of cancer immunoediting, as tumour cells are initially eliminated at the immunosurveillance stage and undergo selective pressure to establish tumour variants with low immunogenicity (Dunn *et al.*, 2004). This process sculpts the immunogenic phenotypes of tumours and allows low immunogenicity tumours to establish and finally direct the immune cells towards malignant favour. In this way, the tumour enters a dynamic equilibrium, in which it not only avoids anti-inflammatory cytotoxic effects, but also manipulates immune cells via the secretion of chemokines, cytokines and growth factors (such as IL-10, VEGF) that promote vascularisation and tissue remodelling, reduced expression of MHC, NK and NKT cell recognition, rendering T cell anergy (Condeelis *et al.*, 2005; Dunn *et al.*, 2002; Smyth *et al.*, 2001; Subramanian *et al.*, 2007). Therefore, recent immunotherapies are orientated towards the distortion of this equilibrium and the switch of the nature of the immune system from tumour promoting to tumour regressing (Colombo and Mantovani, 2005; Lokich, 1997; Rosenberg, 2001).

Tumour-associated macrophages (TAMs)

In a developing neoplasm, the inflammatory component comprises a diverse immune cell population, e.g. macrophages, mast cells, dendritic cells and lymphocytes, all of which provide a diverse set of cytokines, metalloproteinases and growth factors that promote cellular proliferation, tissue remodelling, angiogenesis and influence DNA stability. Especially macrophages derived from circulating monocytes represent a major component of the infiltrated leukocyte population (Condeelis and Pollard, 2006; Murdoch *et al.*, 2008).

Tumour-associated macrophages (TAMs) are derived from circulating monocytes and constitute a key component of the infiltrating leukocytes in the neoplastic tissues **(Table 10)**. Although their content in tumours varies between tumour types, the mechanisms that trigger monocyte recruitment to the tumour stroma remain common and sometimes involve even danger signals from apoptotic tumour cells. One well-characterised monocyte chemotactic factor is monocyte chemotactic protein-1 (MCP-1), a 12-kDa protein that is released by tumour cells and induces the synthesis of gelatinase, urokinase type plasminogen-activator (uPA) that binds to the uPA receptor in monocytes, as well as of IL-1 and IL-6 by monocytes. Indeed, in human ovarian carcinomas there is a significant correlation between TAMs and MCP-1 expression (Bottazzi *et al.*, 1992; Hildenbrand *et al.*, 1999; Opdenakker and Van Damme, 1992; Valkovic *et al.*, 1998; Zachariae *et al.*,

1990). Other mediators that chemoattract monocytes to the tumour stroma involve MCP-2 (Graves and Jiang, 1995), MCP-3 (Fioretti *et al.*, 1998), GM-CSF (Fu *et al.*, 1992), M-CSF (Bottazzi *et al.*, 1990), vascular permeability factor (VPF) (Dvorak *et al.*, 1991) and IL-12p40 (Ha *et al.*, 1999). CSF-1 (colony stimulating factor-1) is also implicated in macrophage recruitment to tumours, as in CSF-1 null mice, a failure to recruit macrophages to the tumour stroma has been noted. Restoration of CSF-1 expression in CSF-1 null/PyM2 mice was linked to restoration of macrophage recruitment and tumour development and metastasis (Lin *et al.*, 2002). CCL5/RANTES is another chemokine involved in TAMs infiltration, as the use of the Met-RANTES antagonist resulted in reduced macrophage infiltration and decreased tumour growth in a model of 410.4 mammary carcinoma (Robinson *et al.*, 2003).

Though the exact mechanism of action is still under investigation, the regulation of the activation phenotype of resident intratumoural macrophages is a characteristic example of the immunoediting process occurring upon tumour establishment. In the tumour microenvironment, infiltrated macrophages display an M2 phenotype (Biswas *et al.*, 2006; Gordon, 2003; Karin and Greten, 2005; Mantovani *et al.*, 2004; Wyckoff *et al.*, 2004). This type of phenotype, also known as alternatively activated macrophages, presents low IL-12, low IL-23 and high IL-10 levels and arises upon stimulation with IL-23, IL-4 and glucocorticoids. M2 macrophages are tumour-friendly and accumulate mainly in the hypoxic areas of the tumour mainly in response to an

upregulation of hypoxia-dependent chemokine receptor 4 (CXCR4) (De Palma *et al.*, 2005; Mantovani *et al.*, 2002; Murdoch *et al.*, 2004). Classically activated macrophages (M1 phenotype) that mediate resistance against tumours are efficient producers of ROS and nitrogen intermediates, IL-1 and IL-6, but have not been reported in the tumour environment (Verreck *et al.*, 2004). This is further supported by a study using mice deficient in Src homology-2-containing inositol 5-phosphate (SHIP) that displayed increased proliferation of transplanted tumours in association to spontaneous establishment of the M2 polarisation phenotype (Rauh *et al.*, 2005). TAMs produce pro-angiogenic growth factors, cytokines and proteases that have a positive effect on neoplastic progression, whereas when they are activated by IL-21 and IFN-γ, they promote immune responses that lead to neoplastic cell destruction (Reddy *et al.*, 2003). In general, the presence of TAMs in tumours, such as breast and lung cancer, has been linked to increased angiogenesis, increased tumour growth and poor prognosis. In other words, there is a correlation between TAMs' infiltration and malignant outcome, posing in this way the involved cytokines or chemokines as potential target in tumour therapeutics (Ali and Lazennec, 2007; Frederick and Clayman, 2001; Singh *et al.*, 2007).

Although no direct association between tumour apoptosis rate and the level of macrophage infiltration has been established, the role of apoptosis should not be undervalued as tumour cells have been shown to release

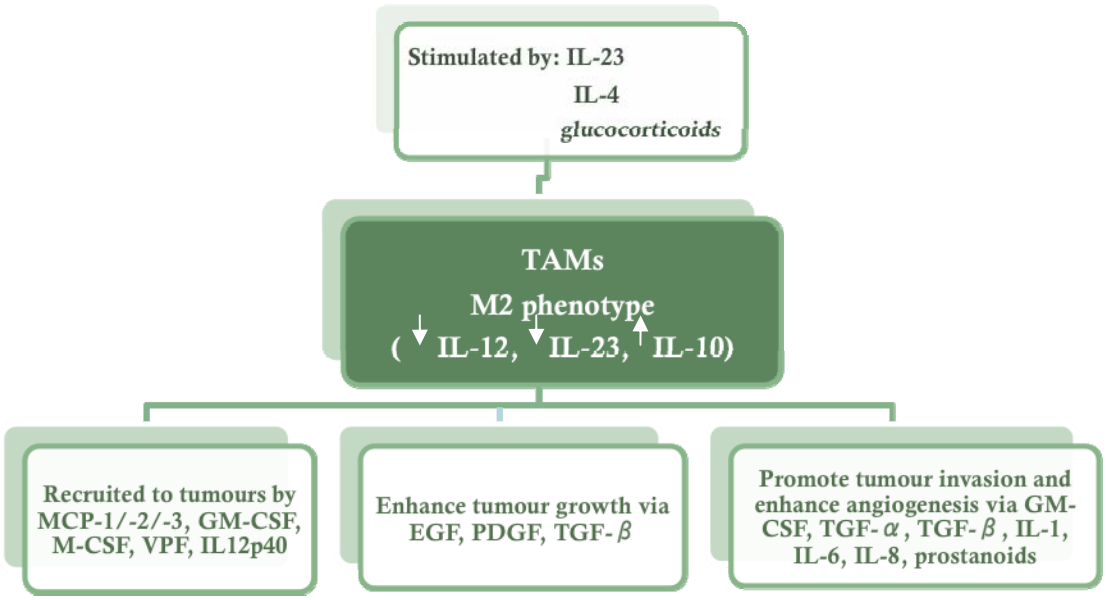
factors upon apoptosis induction that act as chemoattractants for monocytes. One of such factors is fractalkine, a glycoprotein that was recently identified to be released by apoptotic lymphoma cells and stimulate monocyte recruitment to the tumour stroma *in vitro* (Truman *et al.*, 2004). Therefore, it seems that the regulation of the released cytokines could be a major determinant in the final outcome that promotes tumour growth or tumour regression. TAMs are pivotal players in both of these processes. They enhance tumour growth via the release of growth factors, such as EGF, PDGF and TGF-β, promote tumour invasion and dissemination, and enhance angiogenesis through the production of various cytokines (GM-CSF, TGF-α, TGF-β, IL-1, IL-6, IL-8) and the release of prostanoids (Leek *et al.*, 1996; Salvesen and Akslen, 1999; Takanami *et al.*, 1999). Prostanoids, such as prostaglandins E2 (PGE2) have also been demonstrated to pose an immunosuppressive effect to evade anti-tumour responses via cell-mediated immune mechanisms. Also, the fact that PGE2 was found to inhibit neutrophil recruitment, augments the role of this prostanoid in tumour immunosurveillance. In addition, TGF-β acts by inhibiting Th1 activity and thus cytotoxic T cell responses as well as NK cell and LAK (lymphokine-associated killer) cell cytotoxicity (Akdis and Blaser, 1999; Metzger *et al.*, 1980; Young *et al.*, 1992).

By contrast, when infiltrated or resident macrophages become classically activated, they can be cytostatic or

cytotoxic for tumour growth. For example, phorbol myristate acetate (PMA) when used in combination with the calcium ionophore A23/8771 has been found to prime peritoneal macrophages and elicit an anti-tumour response (van Hilten *et al.*, 1990). In general, the mechanism that classically-activated macrophages might promote tumour regression is via antibody-dependent cellular cytotoxicity (Fc receptor CD16) (Bonta and Ben-Efraim, 1993; De Young and Gill, 1984; Mantovani, 1994). They can also act synergistically with secretory products with cytostatic activity such as the eicosanoids PG, LTs, IL-1 and TNF-α cytokines to kill a range of tumour cells. Moreover, the release of free radicals (ROI, NO) can exert analogous tumour-regressive effects. It should be noted that in the case that macrophages impose such an inhibitory effect on tumour growth, the tumour itself activates a rescue homeostatic mechanism. Such mechanism involves the release of a plethora of tumour-derived molecules, such as TGF-β, macrophage-deactivating factor, PGE2, M-CSF, p15E, IL-4, IL-6 and IL-10; all of which suppress the cytotoxic effects of macrophages. Indeed, the activity of IL-18 and IL-12, which are macrophage-derived anti-tumour cytokines is modulated and compromised (Brunda, 1994; Micallef *et al.*, 1996; Romagnani, 1992). A similar effect is observed with GM-CSF that might act as macrophage activator and proliferation molecule, as tumour macrophages become hyporesponsive to GM-CSF and stimulated to release IL-10 that in turn, inhibits GM-CSF synthesis (Kato *et al.*, 1990; Sunderkotter *et al.*, 1994; Walker *et al.*, 1994). In

general terms, within a tumour site complementary mechanisms exist that subvert the host immune responses to promote tumour progression.

Table 10.: Characteristics of Tumour-Associated Macrophages



Other tumour-associated leukocytes

In essence, the tumour itself orchestrates the inflammatory response and the nature of the infiltrating immune cells reaching its stroma. Apart from the direction of the immunogenicity of resident macrophages, tumours also have a direct effect on other cell types such as B, T lymphocytes, NK, NKT and dendritic cells. In the case of B cells, it has been shown that these cells contribute to oncogenesis in many animal models as well as in human studies in which tumour-specific antibodies were identified in cancer patients (Ammirante *et al.*, 2010; de Visser *et al.*, 2005; DeNardo *et al.*, 2009; Gallimore and Simon, 2008; Karin, 2006; Mosser, 2003). Based on these studies, it was shown that B cells do not infiltrate the tumour lesion but are remote regulators of the intratumoural infiltration of other immune cell types via the deposition of immune complexes in the extracellular matrix of the tumour.

Concerning other lymphocytes, there is a positive correlation between T lymphocytes in tumour stroma, especially CD8+ T lymphocytes and increased patient survival (Clark *et al.*, 1989; Clemente *et al.*, 1996; Kolbeck *et al.*, 1992; Matsui *et al.*, 1999; Naito *et al.*, 1998; Ohtani, 2007; Shimizu *et al.*, 1999). Although this observation depends on tumour heterogeneity and varies among tumour types, a characteristic example is that of a model of skin tumourigenesis induced by 7, 12-dimethylbenzantracene (DMBA) and 12-O-

tetradecanoylphorbol-13-acetate (TPA) (Girardi *et al.*, 2001). In this model, γ/δ $T^{-/-}$ cell mice were more susceptible to tumour formation and papilloma-to-carcinoma progression than wildtype mice, while α/β $T^{-/-}$ cell did not present such an effect. A similar discrepancy also occurs in different types of NKT cells, as type I NKT cells enhance anti-tumour responses and type II NKT cells suppress these responses (Ambrosino *et al.*, 2007; Kronenberg, 2005; Park *et al.*, 2005; Smyth *et al.*, 2000; Subleski *et al.*, 2006).

This paradoxical role of NKT cells is evident in a study using $CD1d^{-/-}$ mice (deficient in both type I and type II NKT cells) and $Ja18^{-/-}$ mice (deficient in type I NKT cells only) (Terabe *et al.*, 2005). $CD1d^{-/-}$ mice were resistant to tumour growth, while $Ja18^{-/-}$ mice behaved similar to wild-type mice; an effect abrogated upon anti-CD4 treatment. These results indicated that the promotion of oncogenesis is established specifically by $CD4^{+}$ type II NKT cells contrary to type I NKT cells.

The ability of tumours to direct the local immune response away from an anti-tumour response is further validated by the systemic effect of the tumours on dendritic cells (DCs). Although DCs normally bridge innate with adaptive immunity, DCs in cancer patients display impaired function (Bell *et al.*, 1999; Gabrilovich, 2004). Immature DCs are found in more than 90% of breast cancers, whereas in 60% of them, mature DCs are confined to the peritumoural areas surrounded by T cell clusters. Again, tumours interfere

with DC immunogenicity by means of the expression of tumour glycoproteins, e.g. mucin-1, carcinoembryonic antigen, arachidonic acid metabolites by viable and apoptotic tumour cells as well as by elevated VEGF and IL-10 production that interferes with DC maturation rendering them tolerogenic and inducing tumour-specific anergy (Aarnoudse *et al.*, 2006; Cambi and Figdor, 2005; Vlad *et al.*, 2004).

Moreover, apart from all the above-mentioned cell types, the role of mast cells and eosinophils in tumourigenesis has also been elucidated in recent years. Many types of cancer, such as non-small-cell lung cancer, colorectal cancer, breast cancer, pulmonary adenocarcinoma, basal cell carcinoma and colonic epithelial tumours are characterised by a vast infiltration of mast cells that become activated via CD30L and stimulated to migrate to the tumour site by the production of stem cell factor from tumour cells (Coussens *et al.*, 1999; Diaconu *et al.*, 2007). Surprisingly, unlike the other types of immune cells, the presence of eosinophils in the tumour stoma is very scarce. Indeed, eosinophils have been reported to be present in some types of tumours such as Hodgkin's lymphoma and colonic carcinoma however, there has been evidence supporting their anti-tumour role, as IL-4 transfection in tumour cell lines of various histologic types prevented tumour formation via the infiltration of eosinophils to the tumour site (Rothenberg, 1998).

LACTOFERRIN

Structural Characteristics

First discovered in milk almost 70 years ago, lactoferrin is an 80-kDa glycoprotein with non-haem iron-binding properties and is part of the transferrin family of proteins (Ward *et al.*, 2005). It is mainly expressed in the secondary granules of neutrophils as well as in epithelial cells of the digestive and respiratory tracts and is found in exocrine fluids such as colostrum, bile, saline, tears, gastrointestinal fluids, vaginal fluid, semen, nasal secretions and urine **(Figure 6A)**. In fact, it is the second most abundant protein in milk after caseins. It is secreted in the apo (non-iron saturated) form and its concentration varies from 1 g L^{-1} (mature milk) to 7 g L^{-1} (colostrum). In plasma, its concentration under normal conditions is as low as 0.4 – 2 ng L^{-1}, whereas in septic conditions it can increase up to 0.2 g L^{-1} (Bennett and Kokocinski, 1978; Houghton *et al.*, 1985; Masson *et al.*, 1969).

The lactoferrin gene is highly evolutionarily conserved in mammals and displays extensive homology. At the chromosomal level, it is located at chromosome 3 in humans, while in mouse at chromosome 9. It is organised in 17 exons, 15 of which are identical among species (Teng *et al.*, 1987). The length of the gene varies widely, but it ranges from 23 kb to 35 kb, mainly due to deletions and

other mutations in the stop codon. Additionally, there are many regulatory molecules that control the expression of the lactoferrin gene, especially the ERE (estrogen response element) region located in the 5' flanking region of the gene (Liu and Teng, 1992). The location and expression of ERE overlaps the binding site of the COUP transcription factor that acts as a negative regulator of oestrogen. Within a species' mammary gland, lactoferrin gene activity is dependent on prolactin expression (Green and Pastewka, 1978), whereas the uterine lactoferrin gene expression is highly dependent on 17β-oestradiol treatment (Pentecost and Teng, 1987; Teng *et al.*, 1989). The cDNA sequence for neutrophil human lactoferrin displays a 99.7% agreement to that of human mammary gland (Powell and Ogden, 1990; Rado *et al.*, 1987).

As a protein, lactoferrin encodes a mature protein of 692 amino acid residues with a 19-amino acid signal peptide (Rejman *et al.*, 1989; Rey *et al.*, 1990). Among species, bovine lactoferrin is highly homologous to human lactoferrin (77%), mouse lactoferrin (72%), human transferrin (68%) and porcine transferrin (67%). In general, lactoferrin displays a very high homology (over 85%) to other mammals, moderate homology to other vertebrates (e.g. 55% homology to zebrafish and 68% to chicken) and low or non-homology to nematodes and insects. However, the iron-binding domain of lactoferrin is evolutionarily conserved to invertebrates. Despite its high homology, its concentration among mammalian species varies (Masson and Heremans, 1971). For example, human,

pigs and mice milk are high in lactoferrin, while cow and other ruminant's milk is low in lactoferrin content. Rat milk on the other hand has no lactoferrin at all. Lactoferrin is also even produced by fish, as evidenced by analysis of rainbow trout eggs.

As defined by X-ray crystallographic analysis, lactoferrin consists of two highly homologous symmetrical globular lobes (N-lobe and C-lobe) that are linked by a protease-sensitive α-helix at amino acids 333 and 343, which provides flexibility to the molecule **(Figure 6B)**. In the polypeptide chain, amino acids 1-332 correspond to the N lobe, whereas amino acids 344-703 correspond to the C lobe (Anderson *et al.*, 1987; Anderson *et al.*, 1989). Each lobe structurally consists of an α-helix and β-pleated sheet structure with two domains (I and II). The binding region of each lobe incorporates iron in its ferric (Fe^{3+}) form and one atom of bicarbonate (CO_3^{2-}), an anion that is important for metal binding as its incorporation facilitates iron saturation. The metals that bind include iron (Fe^{2+} or Fe^{3-4+} ions), Cu^{2+}, Zn^{2+} and Mn^{2+} ions; however, the binding of lactoferrin to iron is 2000-times more stable and stronger than to any other metal (Lonnerdal *et al.*, 1985). The conformation of each lobe is different and the iron-affinity slightly varies. Lactoferrin can exist in its iron-free form (apo-LTF) or associated with Fe^{3+} (holo-LTF). Apo-LTF presents an open conformation, while holo-LTF is a closed molecule. In its apo-form (iron-free) the change in the conformation of the lobes has

consequences to the sensitivity of the molecule to proteases. The amino acids found in each lobe that are directly involved in the binding of iron include Asp, Tyr and His, while Arg is responsible for the interaction with the carbonate ion (CO_3^{2-}) (Anderson *et al.*, 1989).

Also, what is characteristic of lactoferrin as a protein is its proteolytic resistance, as it can survive digestion by pepsin and pancreatic enzymes in the gastrointestinal tract and is excreted in the intact form (Brines and Brock, 1983; Brock *et al.*, 1976; Goldblum *et al.*, 1989; Hutchens *et al.*, 1991). It is glycosylated at two sites, possesses intramolecular disulfide bonds and no free sulfhydryl groups. Distinct poly-*N*-acetyllactosaminic glycans are contained in both human milk-derived and neutrophil-derived lactoferrin.

In comparison to human transferrin, human lactoferrin has a 60% identity as a protein with major differences in the surface-exposed sequences (Harrington, 1992; Metz-Boutigue *et al.*, 1984; Park *et al.*, 1985). Transferrin is an acidic molecule with a pI of 6.5, whereas lactoferrin has a pI of 8.5-9 and is thus a basic protein. There are also differences in terms of iron stability of these two proteins as lactoferrin has a higher affinity and stability by retaining iron to pH values as low as 3, while transferrin releases iron at a pH of 5. The pepsin digestion of human lactoferrin between residues 1-19 and 20-37 results in the generation of lactoferricin (LFcin), a peptide arising from domain N1 (Wakabayashi *et al.*,

2003). LFcin has a β-sheet-α-helical structure of basic amino acid residue repeats, has a highly cationic nature and accounts for most of the bactericidal, anti-microbial and anti-inflammatory properties of the protein (Gifford *et al.*, 2005; Valenti and Antonini, 2005). Lactoferrampin is another peptide close to LFcin that also possesses bactericidal activity (van der Kraan *et al.*, 2004).

Apart from its bacteriostatic properties that are attributed to its iron-binding activities, lactoferrin is a pleiotropic molecule with a wide spectrum of functions in a living organism (Ward *et al.*, 2005). Its physiological roles range from regulation of iron homeostasis and host defense against microbial microorganisms to a plethora of anti-inflammatory functions, activity as a growth factor and against tumour development and metastasis (Baker and Baker, 2005; Ward and Conneely, 2004).

A

Fluid	Concn (μM)		Underlying condition
Colostrum	100	8 mg/ml	Normal
Milk	20	1.6 mg/ml	Normal
	40	3.2 mg/ml	Normal
	60	4.8 mg/ml	Normal
Tears	25	2 mg/ml	Normal
Seminal fluid	1.4	112 μg/ml	Normal
Vaginal fluid	2.0	160 μg/ml	Just after menses
	0.1	8 μg/ml	Just before menses
	< 0.25	20 μg/ml	Oral contraceptive users
Saliva	0.11	8.8 μg/ml	Normal adults
	0.05	4 μg/ml	Normal children
	0.25	20 μg/ml	Children: cystic fibrosis
Amniotic fluid	0.02	1.6 μg/ml	Non-infected
	0.04	3.2 μg/ml	Infected
Cerebrospinal fluid	0.00	0	Normal children
	0.01	0.8μ g/ml	Children: aseptic meningitis
	0.13	10.4 μg/ml	Children: bacterial meningitis
Synovial fluid	0.014	1.12 μg/ml	Non-inflammatory
	0.338	27 μg/ml	Inflammatory arthritis
Serum	0.005	0.4 μg/ml	Normal
	2.5	200 μg/ml	Acute sepsis

B

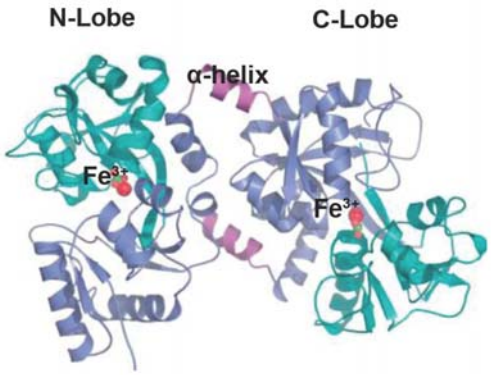


FIGURE 6: Lactoferrin

(A) Concentrations of lactoferrin under physiological and septic conditions. **(B)** Crystal structure of lactoferrin. Lactoferrin consists of two highly-homologous symmetrical globular lobes [N-lobe (aa 1-332) and C-lobe (aa 344-703)] that are linked by a protease-sensitive α helix (aa 333-343). Each lobe consists of an α-helix and β-pleated sheet structure with two domains (I and II). The

binding region of each lobe incorporates iron in its ferric (Fe^{3+}) form and one atom of bicarbonate (CO_3^{2-}).

Functional Characteristics

The most well-known activity of lactoferrin is the regulation of iron homeostasis (Ward *et al.*, 2005). Indeed, excessive free iron in tissues turns out to be harmful, as it promotes microbial growth and cellular damage caused by free radicals. Lactoferrin is an iron-binding molecule and thus, iron saturation restricts microbial multiplication. One of its roles is also the regulation of iron absorption, as evidenced by the high concentration of lactoferrin in breast milk, the absorption of iron in the neonatal intestine as well as the fact that lactoferrin survives the proteolytic mechanisms in the digestive tract and is excreted intact. Indeed, infants fed on lactoferrin-rich breast milk displayed a lower rate of iron absorption compared to those fed lactoferrin-free breast milk (Davidson and Lonnerdal, 1988). This observation supports the notion that lactoferrin transferred to the neonate via lactation acts primarily by controlling free iron in the gut and in this way limiting microbial pathogenesis and cellular oxidative damage. Lactoferrin is released from the circulation in its apo-form and this regulation of iron overload should be evaluated in neurodegenerative diseases that are characterised by increased deposits due to oxidative stress (Bullen, 1972; Fillebeen *et al.*, 2001; Sanchez *et al.*, 1992).

The bacteriostatic ability of lactoferrin is attributable not only to its iron-binding ability, but also to its ability to bind directly to the outer surface of the Gram negative bacteria; this induces the rapid release of LPS and increases membrane permeability and damage (Ellison *et al.*, 1988). The similar and more potent effect is more evident by the peptide lactoferricin (Lfcin) that exerts negative effects on Gram negative, Gram positive bacteria, yeast, fungi, viruses etc. (Bellamy *et al.*, 1992). Characteristically, lactoferrin prevents the biofilm formation of *Pseudomonas aeruginosa* by inhibiting bacterial twitching and attachment to epithelial cell surface (Singh *et al.*, 2002). Moreover, oral administration of lactoferrin has been demonstrated to present host protective effects during septic shock, lethal bacteraemia and oral candidiasis (Takakura *et al.*, 2003; Valenti and Antonini, 2005). In a similar way, lactoferrin inactivates proteins required for the survival and colonisation of *E.coli*, *H influenza*, *S.flexneri* and others (Gomez *et al.*, 2003; Ochoa *et al.*, 2003; Plaut *et al.*, 2000). In the case of viral infections, lactoferrin binds directly to glycosaminoglycan receptors, especially for heparin sulfate (HS) on viral surface (van der Strate *et al.*, 2001). This binding prevents the contact between virus and host cell and thus prevents infection. This effect extends from human immunodeficiency virus and respiratory virus to hepatitis C and rotavirus (Groot *et al.*, 2005; Seganti *et al.*, 2004). Furthermore, treatment with bovine lactoferrin of guinea pigs that were infected

with the fungus *Trichophyton mentagrophytes* resulted in an apparent reduction of fungal infection on the skin of the back (tinea corpus) and limbs (tinea pedis) (Wakabayashi *et al.*, 2000). Other studies show binding of human lactoferrin to the intracellular parasite *Toxoplasma gondii* and in this way, inhibiting the intracellular growth of the parasite within host cells (Dzitko *et al.*, 2007).

Also, the bacteriostatic property of lactoferrin is enhanced by the anti-inflammatory effects of the protein and the stimulation of an immune response. For example, human lactoferrin transgenic mice displayed a better bacterial clearance than congenic littermates; something accompanied by inhibition of *S.aureus* growth as well as activation of the Th1 response (Guillen *et al.*, 2002). The anti-inflammatory effect is mediated by changes in the cytokine profile expression as well as by promotion of T cell proliferation, as lactoferrin can act as an iron donor for maturing T cells. For instance, there was a marked upregulation in CD4 antigen expression following lactoferrin interactions with Jurkat T cells (Dhennin-Duthille *et al.*, 2000). Moreover, lactoferrin enhances phagocytosis against several bacteria and promotes the release of proinflammatory molecules such as TNF-α, IL-8 and nitric oxide (Gahr *et al.*, 1991; Kai *et al.*, 2002; Shinoda *et al.*, 1996; Sorimachi *et al.*, 1997). It has also been shown to exert an adjuvant effect on delayed-type hypersensitivity as well as in the T cell response stimulated by BCG (Bacille Calmette-Gurein) vaccine (Hwang

et al., 2005; Zimecki *et al.*, 2002). In herpes simplex virus I type infection in mice, lactoferrin enhances cytokine responses, whereas in hepatitis C infection lactoferrin stimulates Th1-response that favours the eradication of the infection based on IFN therapy (Wakabayashi *et al.*, 2004).

Lactoferrin as an Anti-inflammatory Molecule

A key question that arises is: given that lactoferrin is classically considered to be a marker of inflammation then how can it also be considered an anti-inflammatory effector molecue? In fact, lactoferrin secretion by neutrophils is a key event during an inflammatory response as well as in chronic inflammatory conditions. For example, in rheumatoid arthritis, there are increased lactoferrin levels in synovial fluid, whereas severe acute respiratory syndrome (SARS) infection is associated with a strong upregulation of lactoferrin gene expression (Caccavo *et al.*, 1999; Reghunathan *et al.*, 2005). Faecal lactoferrin is a marker of intestinal inflammation and therapeutic response in chronic inflammatory bowel disease and Crohn's disease (Buderus *et al.*, 2004; Kane *et al.*, 2003). Moreover, during neurodegenerative diseases that are linked to an activation of dopaminergic neurons, there is also an increased PMN degranulation and therefore, a dramatic increase in lactoferrin secretion (Kawamata *et al.*, 1993; Leveugle *et al.*, 1994).

However, the increase in lactoferrin levels is in parallel linked to the activation of an anti-inflammatory machinery that would eventually promote the resolution of the inflammatory condition. Indeed, lactoferrin has a suppressive effect on the synthesis of proinflammatory cytokines e.g TNF-α, IL-1 and IL-6 by resident activated monocytes or macrophages following LPS stimulation (Crouch *et al.*, 1992; Mattsby-Baltzer *et al.*, 1996). Simultaneously, lactoferrin stimulates the production of anti-inflammatory cytokines, such as IL-10 and IL-4 (Togawa *et al.*, 2002a). Interaction of lactoferrin with soluble CD14 inhibits IL-8 secretion (Elass *et al.*, 2002), while suppression of TNF-α production further downregulates the secretion of the proinflammatory IL-6 (Mattsby-Baltzer *et al.*, 1996). For example, in adjuvant-stimulated arthritis rat models, oral administration of lactoferrin inhibited TNF-α and stimulated IL-10 secretion (Hayashida *et al.*, 2004). The interaction of lactoferrin with LPS and CD14 complex influences the activation of adhesion molecules on endothelial cells, such as E-selectin, ICAM-1 and thus challenges neutrophil activation properties (Baveye *et al.*, 2000). It has also been suggested that lactoferrin competes with IL-8 and other chemokines to bind to proteoglycans (Elass *et al.*, 2002). On a similar basis, while lactoferrin is overexpressed in allergic patients, it can also inhibit an IgE-induced histamine release from human colon mast cells (He and Xie, 2004). Other reported anti-inflammatory properties of lactoferrin involve NK cell activation, activation of

monocytes, activation of GM-CSF, stimulation of antibody-dependent cell cytotoxicity and macrophage cytotoxicity as well as maturation of splenic B cells (Legrand *et al.*, 2005).

Lactoferrin as a Growth Factor

In normal tissues, lactoferrin expression is not organ-specific, but in contrast, it is expressed by all organs. Its broad spectrum of secretion and its bacteriostatic activity place lactoferrin as a pivotal component of a homeostatic, anti-microbial mechanism. Indeed, lactoferrin is found in all tissues such as breast, stomach, pancreas, salivary gland, endometrium, spleen, prostate, liver and blood (Siebert and Huang, 1997). However, its expression is upregulated under specific conditions that are part of the normal function of a living organism and involve organ morphogenesis, lactation and involution of the mammary gland (Close *et al.*, 1997; Masso-Welch *et al.*, 2000; Rossiello *et al.*, 1984; Wilde *et al.*, 1997).

The effect of lactoferrin on organ morphogenesis is illustrated by the regulatory role of lactoferrin in bone formation. More specifically, lactoferrin induces the development of osteoblast cells by promoting their proliferation and decreasing their apoptosis. This mitogenic effect of lactoferrin is mainly mediated via low density lipoprotein receptor related protein-1 (LRP-1) signalling (Cornish *et al.*, 2004; Grey *et al.*, 2004). *In vivo*, in mice in which lactoferrin (4 mg) had been

administered subcutaneously for 5 days, a marked 4-fold increase in bone mass was noted (Cornish *et al.*, 2004). This mitogenic effect also implicates the p42/44 MAPK signalling pathway as an LRP-1 independent pathway and even extends to enterocyte formation, B and T lymphocyte expansion as well as macrophage development (Grey *et al.*, 2006). Although the physiological relevance of this mitogenic activity is still under investigation, it can be suggested that lactoferrin is an important contributor in terms of organ formation and bone development, based on its high levels of expression in a foetus.

Lactoferrin and apoptosis

During lactation, there is a variation of lactoferrin mRNA expression during the different lactation stages (Wang *et al.*, 2005). On day 1 of lactation, lactoferrin is strongly expressed, whereas there is a gradual decrease in its expression from day 9 to day 17 of lactation. This is followed by a marked increase of lactoferrin expression on day 25. A cDNA microarray analysis on the expression of genes during the involution of bovine mammary gland further revealed a decreased mRNA expression of the milk protein genes of β-, κ-casein, α-lactalbumin and β-lactoglobulin and a dramatic increase of the expression of lactoferrin 192 h post-milking (Singh *et al.*, 2008). As the involution of the mammary tissue is dominantly characterised by elevated levels of apoptosis, the associated increase in the levels of lactoferrin provided initial insights that link lactoferrin to apoptosis.

Subsequent studies have demonstrated that lactoferrin induces apoptosis in hepatic epithelial cells via an iron-dependent lysosomal death pathway in a process necessary for caspase-3 activation (Gorria *et al.*, 2008). Similarly, in hepatocyte apoptosis induced by D-galactosamine, the levels of caspase-3 were elevated in the apoptotic liver cytoplasm and were accompanied by elevated serum transaminases (Katunuma *et al.*, 2006). Although the involved molecular pathways are under investigation, it has been noted that the retinoid signalling cascade in cell growth and apoptosis in mammary cells upon lactoferrin treatment is implicated (Baumrucker *et al.*, 2006b).

All these studies clearly demonstrate that lactoferrin is involved in the apoptotic process; however, they do not state that lactoferrin induces apoptosis. For example, in Jurkat T leukaemias, lactoferrin induced apoptosis via the regulation of c-Jun-N-terminal kinase (JNK) activity and via the activation of caspase-9 and caspase-3 (Lee *et al.*, 2009). An apoptosis-like cell death was also observed in *Candida albicans* that was characterised by PS externalisation, chromatin condensation, DNA degradation and increased ROS production (Andres *et al.*, 2008). Similarly, lactoferrin peptides, like lactoferricin, exert a more potent effect as they promote apoptosis in oestrogen non-responsive MDA-MB-435 breast cancer cells (in association to C6 ceramide or tamoxifen) and in Jurkat T-leukaemic cells by sequential permeabilisation of the cell membrane and targeting of mitochondria. The observed

effect of lactoferricin was not attributed to the iron-binding properties of the protein. This effect of lactoferricin extends to other human leukemias and carcinoma cell lines (Freiburghaus *et al.*, 2009; Furlong *et al.*, 2006; Iigo *et al.*, 2004; Mader *et al.*, 2007; Mader *et al.*, 2005; Richardson *et al.*, 2009). Also, the native Lf-B derived peptide (Pep1 –residues 17-34) that was highly cationic induced both apoptotic cell death and necrotic cell death in the leukemic cell line HL-60 depending on its concentration (Onishi *et al.*, 2008). Additional studies that support the role of lactoferrin in the induction of apoptosis include the apoptotic effect of pepsin-digested bovine lactoferrin in oral cancer cells via JNK/SAPK activation (Sakai *et al.*, 2005) as well as the activation of the apoptosis-promoting Fas, Bid and Bax in the colon of azoxymethane-treated rats following lactoferrin treatment (Fujita *et al.*, 2004a; Fujita *et al.*, 2004b).

Lactoferrin and tumour

In comparison to healthy tissues, lactoferrin is also expressed in malignant tumours. Although its exact role in tumour development and metastasis is still under investigation, it is believed that it plays a key role in the regulation of the apoptosis levels observed in the tumour tissue, as well as in the proliferation of the tumour itself. For example, there is enhanced lactoferrin expression in human normal and neoplastic bone tissues, mainly in chondroblastomas, giant cell tumours (GCTs) and

osteoid osteomas (Ieni *et al.*, 2009). Also, lactoferrin expression was observed in human sporadic renal cell carcinomas (Giuffre *et al.*, 2007), in human astrocytomas and multiform glioblastomas (Tuccari *et al.*, 1999), in colon carcinomas (Brock *et al.*, 1994), in human breast cancer (Campbell *et al.*, 1992), in Hodgkin's disease and Burkitt's lymphoma (Hoffer *et al.*, 1979) and during the neoplastic transformation of the endometrium (Walmer *et al.*, 1995) and of the endocervix (Giuffre *et al.*, 2006); in the case of endocervix neoplasms, neoplastic formation correlates with downregulation of lactoferrin expression (Farley *et al.*, 1997). Lactoferrin mRNA was also shown by RT-PCR analysis to be expressed by the promyelocytic leukaemia HL-60 cell line, T-lymphoblastic leukaemia Jurkat, breast tumour T41D, Burkitt's lymphoma Raji, colorectal adenocarcinoma SW490 and erythroleukaemia K562 cell lines (Hoffer *et al.*, 1979; Siebert and Huang, 1997). Some forms of these tumour cells and tissues are characterised by a downregulation of lactoferrin levels and this is possibly attributable to differences in the methylation pattern of the lactoferrin gene and downregulation of its expression (Shaheduzzaman *et al.*, 2007; Teng *et al.*, 2004). Moreover, its alternative splice variant, delta (Δ) lactoferrin, is not expressed in most of the tumours, implying in this way, that tumours evade host regulatory mechanisms in order to become established (Benaissa *et al.*, 2005; Goldberg *et al.*, 2005). The phenomenon of alternative splicing is also common in the

regulation of transcription factors such as c-Myc, GATA-1, prostate protein probastin and Pit1.

The strong association of lactoferrin and development of normal and malignant tissues has given rise to the application of lactoferrin as a molecule that triggers anti-tumour responses. Indeed, oral administration of lactoferrin in a mouse intestinal mucosa inhibits carcinogenesis and metastasis (Iigo *et al.*, 2004) as well as azoxymethane (AOM)-induced rat colon tumour formation and aberrant crypt foci development (Sekine *et al.*, 1997a; Sekine *et al.*, 1997b; Tsuda *et al.*, 1998; Tsuda *et al.*, 2000). Specifically, when administered at doses of 100 or 300 mg/kg/day, lactoferrin exerts an inhibitory effect on metastasis and tumour formation (Kuhara *et al.*, 2000). Oral lactoferrin has also been widely reported to inhibit head and neck squamous cell carcinomas *in vivo* (McKeown *et al.*, 2006; Wolf *et al.*, 2007; Xiao *et al.*, 2004). Injections of human lactoferrin were also found to inhibit the growth of solid tumours induced by v-ras transformed mice fibroblasts and methylcholanthrene-induced fibrosarcomas. Additionally, using B16-F10 melanoma cells, lactoferrin substantially reduced lung colonisation and experimental metastasis (Bezault *et al.*, 1994). A similar effect was reported *in vivo* in colon carcinoma (Shimamura *et al.*, 2004) and lung metastasis (Iigo *et al.*, 1999) as well as in the Apc (Min) mouse model that is characterised by both familial adenomatous polyposis and sporadic colon cancer; in all cases, lactoferrin exerted an inhibitory effect on tumour growth (Ushida *et al.*, 1998).

This effect of lactoferrin also extends to the stimulation of a chemopreventive effect. More specifically, iron-saturated lactoferrin was found to be a potent natural adjuvant for augmenting cancer chemotherapy (Kanwar *et al.*, 2008). Mice bearing EL-4 lymphoma, Lewis carcinoma or B16 melanomas that were fed with lactoferrin for 6 weeks prior to chemotherapy showed a complete rejection of tumours within 3 weeks following a single injection of the chemotherapeutic agent. On an analogous basis, in head and neck squamous cell carcinomas, combination therapy with oral recombinant human lactoferrin and cis-platinum resulted in a 79% growth inhibition of tumour; a statistically significant improvement compared to the use of each agent alone (Varadhachary *et al.*, 2004). Moreover, lactoferrin was reported to reduce methotrexate-induced small intestinal damage.

The anti-tumoural activity of lactoferrin is even conserved in the properties of lactoferrin-derived peptides. Treatment with lactoferricin resulted in an *in vitro* and *in vivo* decrease in the rate of cell proliferation of Caco-2 colon cancer cells as well as in a regression of MethA fibrosarcoma, B16 F10 melanoma and C26 colon carcinomas. A similar effect was also observed by other lactoferrin-derived peptides such as L12 (Yang *et al.*, 2004) that were active against the tumour cell lines MethA, HT-29 and MT-1 (Yang *et al.*, 2002). Moreover, the intracellular delivery of the antimicrobial core of lactoferricin (RRWQWR –Lfcin B6) was reported to kill T-leukemia cells (Richardson *et al.*, 2009).

Despite the observed efficacy of lactoferrin and lactoferrin-derived peptides to trigger an anti-tumoural response in established tumours (Iigo *et al.*, 2009; Yoo *et al.*, 1998), there are attempts currently made to unravel the underlying molecular mechanisms. In most of the studies performed, lactoferrin activity has been shown to interfere with the cell cycle and induce a cell cycle arrest at the G1/S checkpoint. This negative effect on cellular proliferation mainly affects cell cycle regulatory proteins such as cyclin E, p21 and p27 Cdk inhibitory proteins that are associated with the Akt and MAPK pathways (Damiens *et al.*, 1999; Xiao *et al.*, 2004; Zhou *et al.*, 2008). The apoptosis-promoting effect of lactoferrin might also molecularly account for its observed anti-tumoural effect by interfering for example with the Fas signalling pathway (Fujita *et al.*, 2004a). Another recently identified mechanism for lactoferrin activity involves the activation of the NF-κB pathway and of the tumour suppressor p53, Mdm2 and p21 genes (Oh *et al.*, 2004).

Furthermore, the anti-tumoural efficacy of lactoferrin has broad implications on the activity of adaptive immune cells such as $CD8^+$ and $CD4^+$ T lymphocytes as well as NK cells; all of which become activated upon lactoferrin administration (Cao *et al.*, 1999; Shau *et al.*, 1992). For example, Balb/c mice transgenic for the rat *neu* (ErbB2) oncogene (BALB-*neu* T) showed tumour growth inhibition that was marked by an increase in intestinal mucosal IFN-γ

production along with an increase in Peyer's patch cellularity. The overall effect was accompanied by an expansion of NK T cells and $CD8^+$ lymphocytes and an enhancement of their cytotoxicity (Damiens *et al.*, 1998; Spadaro *et al.*, 2007; Wang *et al.*, 2000b). This effect of lactoferrin is also mediated by IL-18, a key regulatory interleukin in tumour pathology, as lactoferrin upregulates IL-18 expression parallel to the activation of caspase-1 that is required to enzymatically cleave the active form of IL-18 (Cao *et al.*, 1999; Iigo *et al.*, 2004). More importantly, the pepsin hydrolysate of bovine lactoferrin (bLfH) has been shown to upregulate IL-18 mRNA expression in mouse small intestine, which was also evident in the activity of bovine lactoferricin (bLfcin). In the same model, tumour growth and metastasis were inhibited by a simultaneous activation of the IFN-α/IL-7 effector pathway (Iigo *et al.*, 2009). Moreover, lactoferrin inhibits tumour-initiated angiogenesis both *in vitro* and *in vivo*; something that possibly accounts for the anti-angiogenic properties of IL-18 (Shimamura *et al.*, 2004). Specifically, in terms of the restriction of tumour angiogenesis, lactoferricin has been shown to inhibit basic fibroblast growth factor and vascular endothelial growth factor 165-induced angiogenesis by competing for heparin-like binding sites on human umbilical vein endothelial cells (HUVECs) (Mader *et al.*, 2006).

Lactoferrin Receptors

Lactoferrin is a highly cationic protein. Its cationic nature in combination with its high binding capacity as a protein, make lactoferrin receptors very difficult to identify definitely. Indeed, researchers over many years have attempted the detailed characterisation of lactoferrin receptors from various cell types with only partial success and many candidate receptors have been proposed for different cell types. In fact, different cell types or different tissues express their own lactoferrin receptors and their characteristics vary accordingly. Also, the involvement of "pseudo-receptors" should not be excluded. Something that should be noted is that the molecular structure and function of these different lactoferrin receptors have not been elucidated yet. Elucidation of their characteristics would provide new insights on the exact role of lactoferrin in tissues. However, the lactoferrin receptors that have been characterised till now are expanded to the whole organism from brain to breast, small intestine, liver etc.

Intestinal lactoferrin receptors

Intestinal lactoferrin receptors (SI-LfRs) were first discovered in brush border membrane vesicles (BBMVs), to which human or bovine lactoferrin were found to bind in several organisms such as rats, rabbits and others with a dissociation constant (Kd) of 1 μM. Lactoferrin binding to

BBMVs was abrogated following addition of EGTA, whereas addition of Ca^{2+} enhanced the binding effect. Analogous studies on BBMVs in macacques showed that all forms of lactoferrin (apo, partial, holo lactoferrin) were able to bind to their specific receptor irrespective of its iron saturation however, holo-lactoferrin received a higher priority of binding. In mouse SI-LfR, the optimum pH for human lactoferrin binding was 5.5 and the binding process was Ca^{2+}-dependent, while in human infant BBMVs, the binding of human lactoferrin had an optimum pH of 6.5-7.5 and was saturable at protein concentrations of 0.2 to 16 µM. Also, Kd was about 1 µM. Using HT-29 cell line as a model of human small intestine revealed a Kd of 60 nM (for high affinity binding sites) and 700 nM (for low affinity binding sites). Structurally, SI-mLfR had a molecular weight of 130 kDa, a pI of 5.8 and was composed of a single polypeptide chain with no subunits. On the contrary, human SI-LfR had a molecular weight of 114 kDa and several subunits of a molecular weight of 38 kDa. In terms of function, all SI-LfRs facilitate iron absorption by mediating the apical transport and not the basolateral transport of lactoferrin in the small intestine. In adult tissues, SI-LfRs are distributed in higher affinity in salivary glands, heart, adrenal glands, pancreas and skeletal muscle, whereas in fetal tissues, they are found exclusively in abundant amounts in the small intestine.

Liver lactoferrin receptors

Lactoferrin receptors in the liver (liver LfR1) were identified based on the rapid clearance of lactoferrin after an intravenous injection. In the liver, bound lactoferrin becomes internalised by hepatocytes and transported to lysosomes where it is degraded. Studies on rat parenchymal liver cells showed that the receptor in the liver for lactoferrin is LRP (LDL receptor-related protein) and is responsible for the binding of apolipoprotein E. Lactoferrin interacts with the extracellular domain of LRP via the arginine residues found in the sequence of lactoferrin. More specifically, LRP is comprised of an extracellular 515-kDa heavy chain and an 85-kDa light chain that passes through the cell membrane. The extracellular domain consists of four binding clusters (I-IV) and lactoferrin interacts with the second and fourth cluster. The binding of lactoferrin to LRP is Ca^{2+}-independent and has also been implicated in bone formation and osteoclast survival. Apart from LRP, a Ca^{2+}-dependent binding of lactoferrin also takes place; something that suggests the presence of a second lactoferrin receptor in the liver, the LV-LfR2, which was identified to be the RHL-1 subunit of the asialoglycoprotein receptor.

Lymphocyte lactoferrin receptor

Lymphocyte lactoferrin receptor (LC-LfR) was first identified in mitogen-activated lymphocytes and has a size of 105 kDa. The optimal binding pH was between pH 6.5 and

7.5 and its degree is irrespective of the iron saturation status of lactoferrin. The binding site is located in N-terminal domain 1 and its function accounts for the effect of lactoferrin in B and T cell activation. A lactoferrin receptor is even characterised in megakaryocytes (PL-LfR) that was also involved in inhibition of platelet aggregation. Detailed characterisation proved that PL-LfR was identical to LC-LfR.

Monocyte lactoferrin receptors

Monocyte lactoferrin receptors (MC-LfRs) were first identified in mouse peritoneal macrophages and are implicated in iron turnover. The ability of lactoferrin to bind to soluble CD14 clearly proposed CD14, a 55-kDa glycoprotein, as an additional receptor on macrophages. Human lactoferrin binds to soluble CD14 with a high affinity (Kd: 16 ± 7 nM). The implication of CD14 possibly accounts for the anti-inflammatory effects of lactoferrin on monocytes. Moreover, using THP-1, a monocytic leukemia cell line as a model, four lactoferrin binding proteins were identified; 35 kDa, 35-37 kDa, 50 kDa and 80 kDa on the membrane of THP-1 cells; detailed structural characterisation of these proteins has not been carried out yet.

In addition, the accumulation of high concentrations of human lactoferrin in neurons of Parkinson's disease patients supported the involvement of a brain lactoferrin receptor that allows lactoferrin to pass the blood-brain barrier. Binding studies using bovine lactoferrin have

shown a high affinity binding site (Kd: 40 nM) and a low affinity one (Kd: 2 µM). Apart from brain capillary endothelial cells, lactoferrin receptors were also identified in human mammary epithelial cells from SV-40 immortalised cell lines in non-malignant breast tissue, benign tumours and breast carcinomas as well as in the bovine mammary epithelial cell line MAC-T. Studies on BEAS-2B, a respiratory epithelial cell line of bronchial epithelium, also revealed the presence of lactoferrin receptors (BE-LfR). The upregulation of receptor expression by metals, such as iron, vanadium etc. supported the role of this receptor in reducing oxidative stress in the respiratory tract by forming complexes with the active, toxic metals.

Neutrophil lactoferrin receptors

In neutrophils, no distinct receptors for lactoferrin have been characterised so far. Scatchard analyses provided by Dr. Simon B. Brown (University of Edinburgh) confirmed previously published literature (Maneva *et al.*, 1983; Spik *et al.*, 1994) on the identification of two types of lactoferrin receptors on neutrophils. According to these results, it was evident that purified lactoferrin can directly associate with neutrophils, as scatchard binding analysis of ^{125}I-labelled apo-lactoferrin indicated that lactoferrin binds to neutrophils via two classes of receptors that differ in affinity and number of binding sites per cell (Bournazou *et al.*, 2009). The higher affinity receptors were determined to be expressed at a

density of 9,100 ± 2,500 binding sites per cell with an affinity of 350 ± 65 nM and the lower affinity receptors to be expressed at a density of $2.5x10^6$ ± $0.7x10^6$ per cell with an affinity of 20 ± 10 μM. Moreover, on eosinophils two classes of lactoferrin receptors were identified using ^{125}I-labelled lactoferrin with dissociation constants of 47 nM and 260 nM (Thomas *et al.*, 2002). However, in all cases, the detailed characterisation of the relative receptors as well as the molecular mechanisms underlying neutrophil and eosinophil inhibition by lactoferrin, require extensive further work.

THESIS AIMS

Based on the characterisation of lactoferrin as a pleiotropic molecule, the aim of this thesis was to examine the effect of lactoferrin on granulocyte chemotaxis, how its expression is related to the proliferation of Burkitt's lymphoma and how it is associated with apoptosis induction.

Moreover, the next aim of this project was to place lactoferrin in an inflammatory context by linking its function to apoptotic and necrotic programs. The arising conclusions would provide clear insights on the role of lactoferrin in cellular proliferation and would allow the evaluation of its therapeutic use in tumour and inflammatory conditions.

MATERIALS AND METHODS

Antibodies, Reagents and Culture Media

The following antibodies were used in this study: rabbit polyclonal anti-human lactoferrin IgG antibody (cat. no L3262; Sigma-Aldrich; Poole, Dorset, UK), mouse monoclonal anti-human lactoferrin antibodies (clone LF-2B8, murine IgG1, AbD Serotec, Kidlington, Oxford, UK; clone imab75, mIgG1, ImmunoSolv, Edinburgh, UK and clone imab77, mIgG1, ImmunoSolv), rabbit polyclonal IgG negative control (Dako Cytomation, Ely, Cambridgeshire, UK), mouse monoclonal IgG1 isotype control (MOPC21; Sigma-Aldrich), phycoerythrin (PE)-conjugated rat anti-mouse Ly-6G (GR1; RB6-8C5; eBioscience, Hatfield, UK), rat anti-mouse Ly-6G (GR1; rat IgG2b; eBioscience), PE-conjugated rat IgG2b isotype control (eBioscience), rat IgG2b isotype control (eBioscience), allophycocyanin (APC)-conjugated anti-human CD11b (ICRF44; BD Biosciences, Oxford, Oxfordshire, UK), fluorescein isothiocyanate (FITC)-conjugated anti-human CD62L (DREG56; BD Biosciences), APC-conjugated mouse IgG1 negative control (MOPC21; BD Biosciences), FITC-conjugated mouse IgG1 negative control (AbD Serotec), mouse monoclonal anti-MAPK activated (diphosphorylated ERK1 and ERK2) antibody (MAPK-YT; Sigma-Aldrich), mouse monoclonal anti-human ERK2 (sc-81457; Santa Cruz Biotechnology, Heidelberg, Germany), horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin (Ig) (eBioscience) , rabbit monoclonal anti-human IκBα (E130;

Abcam, Cambridge, UK) and HRP-conjugated goat anti-rabbit Ig (Dako Cytomation).

Unless otherwise stated, all reagents were obtained from Sigma-Aldrich. Nucleic acid extraction reagents were purchased from Qiagen (Crawley, West Sussex, UK) and magnetic separation reagents were from Miltenyi Biotech (Bergisch Gladbach, Germany) or from Qiagen. All SDS-PAGE reagents were purchased from Invitrogen, ECL and nitrocellulose membrane were from GE Healthcare (Bucks, Buckinghamshire, UK) and primers were obtained from MWG Biotech (Ebersberg, Germany). All PCR reagents were obtained from Promega (Southampton, Hampshire, UK) and reverse-transcription enzymes from Invitrogen. Unless otherwise stated, all reagents for immunohistochemistry were purchased from Dako Cytomation and Vector Laboratories (Orton Southgate, Peterborough, UK). Nucleocounter cassettes were from Chemometec (Epsom, Surrey, UK), Flow-Counter beads from Beckman Coulter (High Wycombe, Buckinghamshire, UK), Sepharose Fast Flow™ beads from Sigma-Aldrich, DNase I from Qiagen, FITC-conjugated recombinant human annexin V/propidium iodide (Anx/PI) from Arcus Biologicals (Modena, Italy), Fura 2/AM from Calbiochem (Beeston, Nottingham, UK) and Lipofectamine™ from Invitrogen. For leukocyte chemotaxis and activation, the following reagents were used: purified formyl-methionyl-leucyl-phenylalanine (fMLP), recombinant human complement 5a (C5a), recombinant human interleukin 8 (IL-8), phorbol myristate acetate (PMA) and leukotriene B4 (LTB_4) from Sigma-Aldrich, recombinant human eotaxin from

PeproTech (London, UK) and human TNF-α from R&D Bioscience (Abingdon, Oxfordshire, UK).

Purified milk-derived human lactoferrin, recombinant human lactoferrin (partially iron saturated), purified bovine lactoferrin and purified human transferrin were all purchased from Sigma-Aldrich, purified neutrophil-derived human lactoferrin from Athens Research and Technology (Athens, Georgia, USA), while recombinant human apo-lactoferrin (0.05 mg iron *per* g of protein) and recombinant holo-lactoferrin (1.4 mg iron *per* g of protein) were purchased from ProSpec Bio (Rehovot, Israel).

Cell culture media and reagents were from Gibco Invitrogen (Paisley, Renfrewshire, UK) and PAA Laboratories (Pasching, Austria). X-vivo 20™ medium was purchased from BioWhittaker Cambrex (Cambridge, UK) and foetal bovine serum (FBS) from BioWest (West Sussex, UK). For bacterial cell culture, terrific broth (TB) and selection antibiotics were obtained from Sigma Aldrich. Luria Bertani (LB) media were prepared in house using reagents from Sigma Aldrich.

Cell Isolation and Culture

Cell lines and culture

Unless otherwise stated, all cell lines were cultured at 37°C (5% CO_2) in a humidified atmosphere. The EBV-positive BL lines, Mutu and Wan, the EBV-negative BL lines, BL2 and

L3055 as well as the lactoferrin shRNA-expressing BL2 cell line and its shRNA negative control were cultured in RPMI 1640 growth medium supplemented with 2 mM L-glutamine, 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 10% FBS. These cell lines retain the features of the parental tumour cells and display a characteristic cell surface phenotype of $CD10^{+}$ $CD77^{+}$ $CD23^{-}$ $CD39^{-}$. Also, they lack Bcl-2 protein expression and display a high apoptosis rate. BL2/bcl-2 cell line is a cell line stably transfected to express Bcl-2 protein and therefore displays a reduced apoptosis rate; however it was cultured in the same mode as the Mutu and BL2 cell lines.

For proliferation assays, the EBV-positive BL cell lines Mutu and Wan and the EBV-negative BL cell lines BL2 and L3055 were used and were cultured in serum-free medium (1:1 X-vivo™ 20/RPMI 1640) supplemented with 2 mM L-glutamine, 100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Culture in serum-free medium (1:1 X-vivo™ 20/RPMI 1640) occurred gradually by increasing its ratio vs. RPMI supplemented with 10% FBS.

MCF7-caspase-3 (MCF7-C3) is an adherent breast cancer cell line transfected with caspase-3, so as cells to be able to undergo classical apoptosis. MCF7-C3 cell line was cultured in high-glucose DMEM (Dulbecco's modified Eagle medium) supplemented with 2 mM L-glutamine, 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 10% FBS until 75-80% confluent. Jurkat cell line is a T lymphoblastic leukaemia cell line cultured in RPMI 1640 growth medium

supplemented with 2 mM L-glutamine, 100 IU ml^{-1} penicillin, 100 µg ml^{-1} streptomycin and 10% FBS.

A549 lung cancer cell line is an adherent cell line cultured in high-glucose DMEM supplemented with 2 mM L-glutamine, 100 IU ml^{-1} penicillin, 100 µg ml^{-1} streptomycin and 10% FBS until 75-80% confluent.

293FT cell line used for the generation of lentiviral particles was cultured in high-glucose DMEM supplemented with 2 mM L-glutamine, 100 IU ml^{-1} penicillin, 100 µg ml^{-1} streptomycin and 10% FBS.

Neutrophil isolation

Leukocytes were isolated according to the Royal Infirmary of Edinburgh (Scotland) Lothian Local Research Ethics Committee (approval 1702/95/3111 and 08/S1103/38). All volunteers were healthy and provided oral or written informed consent. Peripheral venous blood was drawn using a 19-gauge needle from an antecubital vein, immediately transferred to polypropylene tubes containing 12.9 mM sodium citrate and gently mixed. Citrated blood was centrifuged at 350*g* for 20 min in order to separate blood plasma from the cellular component. Platelet-rich plasma was then removed and used to prepare autologous serum in sterile glass tubes by recalcification of the plasma ($CaCl_2$ 22 mM final concentration) and 1 h incubation at 37°C. Autologous serum was then stored at 4°C and used as required.

Leukocytes in the remaining plasma-free blood component were then separated from erythrocytes by dextran sedimentation (0.6% w/v) (Pharmacia, Buckinghamshire, UK). After a 20-min incubation at room temperature, two separate layers were formed. The upper layer (leukocyte-rich) was collected and cells were pelleted by centrifugation at 350*g* for 6 min. Three isotonic Percoll™ gradient fractions (50%, 61%, 73%, Pharmacia) were prepared in phosphate buffered saline (PBS) (Ca^{2+}/Mg^{2+}-free) and once complete, the leukocyte-rich pellet was resuspended in the 50% Percoll gradient fraction and added on top of the two other isotonic gradients 61% and 73%. Following a 20-min centrifugation at 720*g*, mononuclear leukocytes were harvested from the 50%/ 61% interface, whereas the PMN leukocytes from the 61% /73% interface and washed twice in PBS (230*g*; 6 min). Cells were >99% viable, as assessed by trypan blue exclusion and unless otherwise stated, isolated cells were immediately used.

Neutrophils represented >95% of isolated PMN cells, as assessed routinely by flow cytometry and cytospin analysis.

Eosinophil isolation

For eosinophil isolation, eosinophils (about 2-15% of total PMN population) were separated from freshly-isolated PMN leukocytes by negative selection using an immunomagnetic separation step with sheep anti-mouse IgG-Dynabeads (Dynabeads M-450™, Invitrogen, UK) coated with the human CD16 antibody (clone 3G8), as described by

(Rossi *et al.*, 1998). Dynabeads were coupled with the anti-CD16 antibody following incubation with the supernatant from 3G8 hybridoma cells ($8x10^6$ beads ml^{-1} of supernatant) at 4°C for 14 h. Unbound antibody was removed by washing the beads with ice-cold PBS (Ca^{2+}/Mg^{2+}-free). Freshly-isolated PMN leukocytes co-incubated with anti-CD16 coupled beads were washed with PBS (Ca^{2+}/Mg^{2+}-free) three times. Isolated eosinophils were >98% pure and >99% viable, as assessed by cytospin analysis and trypan blue staining.

Monocyte isolation

Monocytes (>90% $CD14^+$ cells) were positively selected from freshly-isolated mononuclear leukocytes using CD14 magnetic beads (Miltenyi Biotec). In detail, mononuclear cell suspension was centrifuged at 300*g* for 10 min. Cell pellet was resuspended in PBS pH 7.2 containing 0.5% (w/v) bovine serum albumin (BSA) and 2 mM EDTA. CD14 MicroBeads were added and incubated for 15 min on ice. Cells were washed with PBS containing 0.5% (w/v) BSA and 2 mM EDTA and $CD14^+$ cells were separated using the MACS magnetic column. Isolated cells were >90% $CD14^+$ monocytes, as assessed by flow cytometry. Cell viability was >98%, as assessed by trypan blue exclusion. Isolated cells were used immediately for subsequent experiments.

Generation of human monocyte-derived macrophages (HMDMs)

In order to obtain human monocyte-derived macrophages, mononuclear leukocytes (8-25% monocytes) were suspended at $4x10^6$ ml^{-1} in IMDM and incubated at 37°C for 1 h. Non-

adherent cells were removed by vigorous washing with Hanks' buffered salt solution (HBSS) (with Ca^{2+}/Mg^{2+}) medium. Adherent cells were cultured in IMDM containing 10% autologous serum at 37°C for 6 days. Culture medium was replaced every 48 h. Monocyte-derived macrophages were phenotypically assessed by microscopy and/or by flow cytometry ($CD14^{+}$ cells).

Biochemical Analysis

Size fractionation

To determine the molecular weight of the inhibitory factor(s), size fractionation of the BL supernatant was performed using filters with specific molecular weight cut-off sizes [Amicon Bioseparations Centriplus Centrifugal filters YM-50 (50 kDa cut-off point) and YM-100 (100 kDa cut-off point) Millipore (Watford, Hertfordshire, UK)]. The BL conditioned medium was obtained from BL2 cells ($2x10^{6}ml^{-1}$) that were incubated in assay medium for 12 h at 37°C. Once incubation complete, BL cells were centrifuged at 400*g* for 10 min. For the size fractionation analysis, the BL conditioned medium was placed on the sample reservoir of the filtration unit and centrifuged at 3000*g* for 15 min. Filtrate (supernatant that passed through the filter, i.e. proteins that had lower MW size than filter) and retentate (supernatant that remained on top of the filter, i.e. higher MW size than filter) fractions were collected and used immediately for chemotaxis assays.

Ion Exchange Chromatography

To determine the pI of the inhibitory factor(s), an ion exchange chromatography was carried out using Sepharose Fast Flow beads (Sigma-Aldrich) with either positive (Q beads) or negative (S beads) charge. Prior to use, each bead type was washed with PBS and neutralising buffer (10 mM Tris; pH 7.0) four times. BL supernatant or control medium (RPMI 1640 supplemented with 2 mM L-glutamine, 100 IU ml^{-1} penicillin, 100 µg ml^{-1} streptomycin) was mixed with the beads and incubated at room temperature for 5 min under continuous agitation so as anionic or cationic proteins to stick to the surface of the corresponding beads (Q and S, respectively). Then, samples were centrifuged (300*g*, 5 min) and resulting supernatants were kept. Bound proteins were then eluted by adding the corresponding elution buffer (for S beads: 10 mM Tris, 0.5 M NaCl; pH 10; for Q beads: 10 mM sodium acetate, 0.5 M NaCl; pH 4). Following a 5-min incubation at room temperature, beads were centrifuged (300*g*, 5 min) and supernatants were collected and analysed. Prior to chemotaxis analysis, the supernatants were diluted (1:100) and pH was adjusted to 7.0.

MALDI-TOF Analysis

Supernatants from BL2 cells that were incubated in serum-free medium (RPMI 1640, 2 mM L-glutamine, 100 IU ml^{-1} penicillin, 100 µg ml^{-1} streptomycin) for 12 h were collected, centrifuged at 400*g* for 10 min and their protein content was precipitated using trichloroacetic

acid (TCA). TCA (100% w/v; 100 µl) was added in 1 ml conditioned medium at 4°C. Samples were centrifuged at 20,000*g* and the pellets washed in ice-cold acetone. The pellet was resuspended in sample buffer (25 µl; non-reducing conditions –NuPAGE, Invitrogen). Proteins were resolved by SDS-PAGE electrophoresis and selected proteins were identified by peptide mass fingerprinting using MALDI-TOF mass spectrometry. The procedure was carried out by the Scottish Instrumentation and Resource Centre for Advanced Mass Spectrometry (SIRCAMS), School of Chemistry, University of Edinburgh.

In vitro Chemotaxis Assay

In vitro leukocyte chemotaxis was determined based on a well-established transfilter migration assay using polyvinyl uncoated Transwell™ inserts (5 µm pore size, Costar Corning, Lutterworth, Leicestershire) (Truman *et al.*, 2004). Leukocytes ($1x10^5$ cells -except for monocytes, $2x10^5$ cells) were added on top of the transwell filter and stimulated to migrate towards BL cells (at the indicated concentrations), conditioned cell media or lactoferrin (10 µg ml^{-1}, unless otherwise stated) that were placed in the lower chamber of the well (600 µl) in the presence or absence of a chemoattractant. Chemotactic agents used in the study, included fMLP (100 nM; Sigma-Aldrich), C5a (6.25 ng ml^{-1}; Sigma-Aldrich), IL-8 (100 nM; Sigma-Aldrich), LTB_4 (100 nM; Sigma-Aldrich) and eotaxin (100 nM; PeproTech). Time of incubation (37°C; 5% CO_2) varied for cell type (neutrophils: 60 min; eosinophils: 60 min;

monocytes: 90 min; macrophages: 4 h). For neutralisation experiments, rabbit polyclonal anti-human lactoferrin IgG antibody or mouse monoclonal anti-human lactoferrin antibodies, rabbit polyclonal IgG negative control and mouse monoclonal IgG1 isotype control were used. Cells that had migrated through the filter were fixed in methanol and stained with Diff-Quick II (Dade, Germany). The number of migrated cells was determined by counting ten random high-power fields (magnification 400x) using an inverted microscope (Axiovert 25, Zeiss, Welwyn Garden City, Hertfordshire, UK) attached to a JVC KYF55B camera.

Lactoferrin Absorption Experiments

For lactoferrin absorption experiments, each chemoattractant [fMLP (100 nM), C5a (6.25 ng ml^{-1}), IL-8 (100 nM)] was incubated with purified human milk-derived lactoferrin (10 µg ml^{-1}) in assay medium at 37°C for 15 min. A mouse monoclonal anti-human lactoferrin antibody (10 µg ml^{-1}; LF-2B8; AbD Serotec) or isotype control [mouse monoclonal IgG1 isotype control (MOPC21; Sigma-Aldrich)] was added and samples were incubated at 4°C for 20-30 min. Samples were then added to the BioMag goat anti-mouse IgG particles (Qiagen) to completely remove the antibody. It should be noted that prior to use, BioMag particles were washed 2-3 times in assay medium. Samples were incubated at 4°C for 15 min under continuous agitation. Tubes were then applied to the magnetic separator for 5-10 min. Once separation was complete, the supernatant was carefully removed without disturbing the pellet and used in a

chemotaxis assay. The efficiency of antibody removal was assessed by analysing the pre-absorbed chemoattractants by anti-mouse IgG ELISA.

Short Hairpin RNA (shRNA)-Mediated Lactoferrin Expression Knock-Down

Bacterial Plasmids

The following shRNA MISSION lentiviral vectors (pLKO.1-puro vectors; ampicillin- and puromycin-resistant; Sigma-Aldrich) were used in this study so as to downregulate lactoferrin expression: **TRCN0000047028** –**Recognising sequence** 5'-CGGTGCAGATAAAGGACAGTT-3'; **TRCN0000047029** 5'-CCTGATCCTAACTGTGTGGAT-3'; **TRCN0000047030** 5'-GCAGGCATTACTAATCTGAAA-3'; **TRCN0000047031** 5'-GCCATCCAGAACTTGAGGAAA-3'; **TRCN0000047032**- 5'-CCCTACAAACTGCGACCTGTA-3'. Also, the plasmids pLP1, pLP2, pLP/VSV-G (ViraPower™ Lentiviral packaging mix; Invitrogen) were included for the generation of lentiviral particles. All plasmid maps and sequences are presented in the Appendix.

Plasmid Extraction

Plasmid extraction was performed using Qiagen Plasmid Maxi kit™ (Qiagen), based on manufacturer's instructions. In detail, a single *E.coli* colony was inoculated in LB medium (5 ml; 37°C) that contained carbenicillin (100 μg ml^{-1}) as a plasmid selection antibiotic. Cultures were incubated for 8 h (37°C; 250 rpm) and further diluted (1:500) into

selective LB medium containing carbenicillin (100 µg ml^{-1}). After a 15-h incubation at 37°C (250 rpm), culture was centrifuged (6000*g*; 15 min; 4°C) and bacterial pellets were resuspended in ice-cold Buffer P1 (10 ml containing RNase A). Buffer P2 (10 ml; Lysis buffer) was further added and cells mixed well several times by inversion. After a 5-min incubation at room temperature, ice-cold Buffer P3 (10 ml) was added in order to neutralise the lysis reaction. Lysates were incubated on ice for 20 min and then centrifuged (6000*g*; 30 min; 4°C) to remove cell debris. Supernatant was carefully transferred to new polypropylene tubes and further centrifuged (3400*g*; 15 min; 4°C) to remove any remaining cell debris completely.

Supernatant was further applied onto a Qiagen-tip™ 500 column. Prior to use, the column was equilibrated by adding Buffer QBT (10 ml). Once supernatant applied, the column was thoroughly washed by applying Buffer QC (60 ml). Buffer QF (elution buffer) was further applied to elute the plasmid DNA. Isopropanol (10 ml) was added to the eluate and mixed thoroughly. Isopropanol-containing plasmid DNA was centrifuged (4000*g*; 30 min; 4°C), the pellet was resuspended in 70% (v/v) ethanol (5 ml) and further centrifuged for 10 min (4000*g*; 4°C). Supernatant was decanted and the DNA pellet air-dried and gently resuspended with sterile, nuclease-free water. Plasmid DNA was quantified spectrophotometrically and the OD_{260}/OD_{280} ratio was used to assess purity. Plasmid quality was further assessed by 1% (w/v) agarose gel electrophoresis

(UltraPure™ agarose; Invitrogen). Values of 1.9 were considered optimal. DNA was stored at -20°C.

Generation of lentiviral particles

The production of lentiviral stocks was performed following an optimised transfection protocol, as suggested by the manufacturer (Invitrogen). In detail, the day before transfection (Day 1), 293FT cells ($5x10^6$ cells in 10 ml of DMEM growth medium containing 10% FBS) were plated in a 10 cm tissue culture plate so as to achieve 90-95% confluency on the day of transfection. No antibiotics were included in the medium.

The following day (Day 2), the culture medium was removed from the 293FT cells and replaced with 5 ml of Opti-MEM I™medium containing serum, but no antibiotics. For each shRNA transfection sample, a separate pDNA-Lipofectamine™ LTX complex was prepared as follows: In a sterile 5 ml tube, 9 µg of the ViraPower™ Packaging Mix and 3 µg of shRNA plasmid DNA were diluted in 1.5 ml of serum-free Opti-MEM I medium and mixed gently. In a separate sterile 5 ml tube, Lipofectamine™ LTX (36 µl) was diluted in 1.5 ml of serum-free Opti-MEM I medium. Sample was mixed gently and incubated for 5 min at room temperature. Then, the diluted DNA and the diluted Lipofectamine™ LTX samples were combined, mixed gently and incubated for 20 min at room temperature to allow the DNA-Lipofectamine™ LTX complexes to form. The DNA-Lipofectamine™ LTX complexes were then added dropwise to each plate of cells and mixed gently by rocking the plate back and forth. Cells were

incubated overnight at 37°C in a humidified 5% CO_2 incubator.

The next day (Day 3), the medium containing the DNA-Lipofectamine™ LTX complexes were removed and replaced with 10 ml complete culture medium (without antibiotics). After 48 h post-transfection (Day 4), the virus-containing supernatants were harvested and placed into a 15 ml sterile tube. Supernatants were centrifuged (400*g*; 15 min; 4°C) and stored in aliquots at -80°C.

Lentiviral infection of BL2 cells

BL2 cells were removed from culture, washed twice in PBS and resuspended in culture medium (RPMI 1640 growth medium supplemented with 2 mM L-glutamine, 10% FBS, 100 IU ml^{-1} penicillin and 100 µg ml^{-1} streptomycin) at a concentration of $2x10^6$ cells ml^{-1}. Then, BL2 cells were plated on a 48-well plate ($2x10^5$ BL2 cells per well). The supernatant containing the lentiviral particles was then added to the BL2 cells at a dilution of 2:1 (200 µl of supernatant) and 1:1 (100 µl of supernatant). Infected BL2 cells were then incubated at 37°C (5% CO_2). Stable cell lines were then generated by selection with puromycin (2 µg ml^{-1}; Sigma-Aldrich).

Reverse-Transcription PCR (RT-PCR) Analysis

RNA extraction

Total RNA was extracted by using the RNeasy™ Mini-Kit (Qiagen), following manufacturer's instructions. In

detail, $1x10^7$ cells in suspension, as in the case of BL2, BL2/bcl-2 and Jurkat cells, were centrifuged at 300*g* for 5 min, supernatant was removed by aspiration and pellet was disrupted by adding 350 µl of RLT Buffer [+ 1% β-mercaptoethanol (β-ME)]. In adherent cells (A549 and MCF7-C3; $5x10^6$ cells), cell culture medium was aspirated, cells washed twice with PBS and disrupted by adding 600 µl of RLT Buffer (+1% β-ME) directly to the cell culture flask.

In all types of cells, the resulting lysate was homogenised by passing the lysate at least five times through a 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe. Ethanol (70% v/v) was added to the homogenised lysate (350 µl for cells in suspension; 600 µl for adherent cells) and mixed well by pipetting. Sample was transferred to an RNeasy spin column and centrifuged at 14,000*g* for 15 s. To increase RNA purity and eliminate genomic DNA contamination, an on-column DNase digestion process was carried out. Buffer RW1 (350 µl) was added to the RNeasy spin column and column was centrifuged for 15 s at 14,000*g* to wash the spin column membrane. DNase I stock solution (10 µl) was added to 70 µl Buffer RDD and mixed by gently inverting the tube. This DNase I incubation mix (80 µl) was then added directly to the RNeasy spin column membrane and samples were incubated for 15 min at room temperature. After 10 min, column was washed with Buffer RW1 (350 µl) and further centrifuged for 15 s at 14,000*g*. Column was subsequently washed with Buffer RPE (500 µl) twice. RNA was eluted from the column by the addition of

RNase-free water (50 µl) and a further centrifugation at 14,000*g* for 1 min. Resulting RNA was quantified spectrophotometrically and the OD_{260}/OD_{280} ratio was calculated. Values of 1.9-2.0 were considered optimal. RNA was stored at -70°C.

RNA reverse-transcription

RNA (2 µg) was reverse-transcribed using Superscript™ III Reverse Transcriptase (Invitrogen) by the following reaction: 2 µg of RNA was added along with 3.8 mM oligodT (Promega, Southampton, Hampshire, UK), 0.78 mM dNTP mix (dATP, dTTP, dCTP, dGTP; Promega) and nuclease-free water to make a final reaction amount of 13 µl. Samples were incubated at 65°C for 5 min and immediately transferred on ice and incubated for at least 1 min. After a brief centrifugation, RNA was reverse-transcribed using Superscript III reverse transcriptase (Invitrogen), following manufacturer's instructions. In each 13-µl sample, the following components were added: 5x First Strand Buffer, 7.6 mM DTT, RNase OUT recombinant RNase inhibitor and Superscript III reverse transcriptase (200 U μl^{-1}). Reactions were mixed gently and incubated at 50°C for 75 min. Reaction was terminated by heat-inactivation of the enzyme by incubating at 90°C for 10 min. Samples were stored at -20°C until used for PCR amplification analysis.

PCR amplification

Resulting cDNAs were used as template in PCR experiments in 50 µl (for GAPDH) or 25 µl (for lactoferrin) PCR amplification reactions. For GAPDH, the following primer

pair sequence was used: forward primer (5′-CGACAGTCAGCCGCATCTTCTTTTGCGTCG-3′); reverse primer (5′-GGACTGTGGTCATGAGTCCTTCCACGATAC-3′). For lactoferrin: forward primer (5′-TGTCTTCCTCGTCCTGCTGTTCCTCG-3′); reverse primer (5′-CTGCCTCGTATATGAAACCACCATCAA-3′). PCR amplification reactions for GAPDH were performed in 50 µl reaction volume containing: 1x GoTaq® Flexi Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, cDNA (2 µl), 0.2 µM of each forward and reverse primer pair and GoTaq® Flexi DNA polymerase (2.5 U μl^{-1}). PCR amplification reactions for lactoferrin were performed in 25 µl reaction volume containing: 1x GoTaq Flexi Buffer, 1.5 mM $MgCl_2$ (1.5 µl), 0.2 mM dNTP mix, cDNA (2 µl), 0.4 µM of each forward and reverse primer pair and GoTaq Flexi DNA polymerase (2.5 U μl^{-1}). Cycle parameters (for lactoferrin: 40 cycles; GAPDH: 28 cycles) involved a primer annealing temperature at 67°C (lactoferrin) or 50°C (GAPDH) for 1 minute, and extension at 72°C for 45 s. All PCR amplification conditions included an initial denaturation step at 94°C for 7 minutes and a final extension step at 72°C for 5 minutes. PCR reactions were run on an MJ Thermal Cycler (Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK). PCR products were then analysed by agarose (2% w/v) gel electrophoresis. Gels were stained with ethidium bromide and visualised by UV illumination.

DNA sequencing

PCR products were purified using QIAquick™ gel extraction kit (Qiagen), according to manufacturer's instructions. In

detail, the DNA fragment was excised from agarose gel with a clean, sharp scalpel. Excised gel fragments were dissolved by incubation at 50°C for 10 min in the presence of Buffer QG (3 volumes of buffer for 1 volume of gel). Isopropanol was then added (1:1 volume). Samples were mixed thoroughly and applied to a QIAquick column. Column was sequentially washed with Buffer QG (0.5 ml) and Buffer PE (0.75 ml) and centrifuged for an additional 1 min at 14,000*g* to completely remove residual wash buffer. DNA was eluted with nuclease-free sterile water and incubated at room temperature for 1 min before centrifugation (14,000*g*; 1 min). Purified PCR products were then sequenced by the Sequencing Service of the School of Life Sciences, University of Dundee, using Applied Biosystems BigDye 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequence analyser. For sequencing, the same set of primer pairs were used as those used for PCR amplification, i.e.forward primer (5′-TGTCTTCCTCGTCCTGCTGTTCCTCG-3′); reverse primer (5′-CTGCCTCGTATATGAAACCACCATCAA-3′). Chromatograph files were viewed and analysed using the Sequence Scanner Software v.1.0 (Applied Biosystems).

In vivo BL Tumour Xenografts

All animal procedures were carried out under a UK Home Office Animals (Scientific Procedures) Act 1986 project licence. For the BL tumour xenografts, six- to 10-week-old BALB/c SCID mice were injected i.p. with 10^7 BL2 cells.

Tumours developed i.p. within 2 months of injection. Mice were sacrificed and tumours excised.

Immunohistochemistry

An immunohistochemical staining to detect the presence of neutrophils on frozen slides of BL2 tumours in SCID mice ($GR1^+$ cells) was carried out. In detail, the following protocol was carried out: slides (stored at -20°C) were allowed to reach room temperature for approximately 1 h prior to staining. Then, sections were washed in PBS and incubated in 0.1% (v/v) H_2O_2 for 30 s. Once loaded to the chamber, 3 drops of Vector Avidin Block were added per slide for 10 min followed by a PBS-wash and a further addition of 3 drops of Vector Biotin Block for 10 min. Dako serum-free Protein Block (3 drops) was further added followed by the addition of the primary antibody affinity purified rat anti-mouse Ly-6G (1:100; GR1; RB6-8C5; eBioscience) or affinity purified rat IgG2b isotype control (eBioscience) and a subsequent incubation for 12 h at 4°C. Slides were then washed twice with PBS and anti-rat biotin vector secondary antibody (125 µl) diluted in Dako ChemMate Antibody Diluent was added at a 1:200 concentration and slides were incubated for 30 min at room temperature. Slides were then washed twice in PBS, 3 drops of Vector RTU Vectastain Elite ABC reagent were added and slides were further incubated for 30 min. After two wash steps with PBS, 125 µl of Dako DAB chromagen were added per slide and once complete, slides were removed from the chamber and counterstained with haematoxylin for 30 s.

Three wash steps with PBS were then carried out followed by a last step with distilled H_2O. Tumour sections were dehydrated through incubation in alcohol (70%, 90%, 100%; 5 min each) and finally soaked in histoclear (10 min) and mounting histoclear (10 min). Slides were mounted and allowed to dry for 12 h prior to their analysis by microscopy (Zeiss Axioskop 2 microscope).

In vivo Model of Peritonitis

For the peritonitis model, 8- to 12-week-old female C57BL/6 mice (n = 7 per group) were injected i.p. with purified human lactoferrin or transferrin (500 ng in saline/0.1% (w/v) BSA; Sigma Aldrich) or saline/0.1% BSA alone followed by a second i.p. injection with 1% thioglycollate (500 μl) or saline/0.1% (w/v) BSA after 20 min. Recruited leukocytes were harvested after 4 h by peritoneal lavage with ice-cold saline containing 2 mM EDTA. Harvested cells were counted using a NucleoCounter™ (ChemoMetec), which excluded non-nucleated cells. Counting results were also confirmed using Flow-Count beads™ (Beckman Coulter). Red blood cells were lysed under hypotonic conditions. Harvested cells were suspended in PBS to the original lavage volume, mixed with pre-defined numbers of flow-count beads and analysed by flow cytometry. Cytospin slides were also prepared and the number of neutrophils was assessed microscopically. In addition, the percentage (%) of recruited mouse neutrophils was determined by GR1 immunolabelling.

Harvested cells were pelleted by centrifugation at 300*g* for 5 min and pellet was resuspended in PBS (Ca^{2+}/Mg^{2+}-free) containing 1% FBS and either PE-conjugated rat anti-mouse Ly-6G (GR1; RB6-8C5; eBioscience) or PE-conjugated rat IgG2b isotype control (eBioscience) was added. After a 20-min incubation on ice, the number of $GR1^{+}$ cells was recorded from the % of total cells by flow cytometry analysis (BD FACSCalibur). Data were analysed by CellQuest (BD Biosciences) or FlowJow (Treestar, Ashland, Oregon, USA).

Time-lapse Video Microscopy

For video microscopy, freshly isolated neutrophils (10^{6} ml^{-1}) were suspended in assay medium (RPMI 1640, 2 mM L-glutamine, 100 IU ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 0.1% bovine serum albumin) and incubated in the presence or absence of purified milk-derived human lactoferrin (10 µg ml^{-1}; Sigma-Aldrich) for 40 min at 37°C under continuous agitation. Prior to video microscopy analysis, fMLP (1 µM) was added and neutrophils were incubated at 37°C for 10 min. Images were captured every 5 s over a 1-h time course. During the time of video microscopy analysis, room temperature was stable at 37°C. Videos were created and analysed by QuickTime player. Criteria for neutrophil activation included the increased locomotion of neutrophils around the substratum, increased movement as well as changes in neutrophil morphology as activated cells retain a polarised morphology.

Measurement of $[Ca^{2+}]_i$

Measurement of $[Ca^{2+}]_i$ was performed according to the published protocol (McMeekin *et al.*, 2006). Freshly isolated neutrophils were suspended (10^7 ml^{-1}) in HBSS (Ca^{2+}/Mg^{2+}-free) and were incubated with 2 μM Fura2/AM (Calbiochem) at 37°C for 30 minutes. The cells were washed twice, resuspended at 2×10^6 ml^{-1} in HBSS (with Ca^{2+}/Mg^{2+}), incubated for an additional 30 min at 37°C in the presence of lactoferrin (10 μg ml^{-1}), and then stimulated with fMLP (1 nM or 10 nM). $[Ca^{2+}]_i$ levels were determined based on 340:380 nm dual wavelength excitation in a PerkinElmer luminescence spectrometer at 37°C with constant stirring. Calibration was performed after each experiment using Triton X (R_{max}) and EGTA (R_{min}) (R_{max} and R_{min} are the maximum and minimum 340:380 fluorescence ratios, respectively). $[Ca^{2+}]_i$ was calculated based on the 340:380 nm fluorescence ratio.

Immunolabelling and Flow Cytometry

Neutrophil activation status was assessed by quantifying the expression levels of two activation markers, CD11b and CD62L by flow cytometry. Neutrophils ($5x10^6$ per condition) were centrifuged at 300*g* for 5 min and incubated in the presence or absence of purified milk-derived human lactoferrin (10 μg ml^{-1}) in PBS (Ca^{2+}/Mg^{2+}) for 40 min at 37°C under continuous agitation. Agonists were then added, samples were mixed gently and further incubated at 37°C for 30 min. Agonists used included fMLP (Sigma-Aldrich), TNF-α

(R&D Bioscience, Abingdon, Oxfordshire, UK) and PMA (Sigma-Aldrich) at the indicated concentrations. Cells were immunolabelled with APC-conjugated anti-human CD11b (ICRF44; BD Biosciences) or FITC-conjugated anti-human CD62L (DREG56; BD Biosciences) and incubated for 30 min on ice. APC-conjugated mouse IgG1 negative control (MOPC-21; BD Biosciences) and FITC-conjugated mouse IgG1 negative control (AbD Serotec) were used as isotype controls. Samples were analysed by flow cytometry (BD FACSCalibur) and data analysis was performed using CellQuest (BD Biosciences) or FlowJow (Treestar).

Proliferation Assay

To determine BL cell proliferation, BL cells (control, shRNA-transfected for lactoferrin, shRNA-transfected negative control) were suspended in serum-free medium (1:1 X-vivo 20/RPMI 1640) at a concentration of $0.5x10^{6}$ ml^{-1} and incubated at 37°C (5% CO_2) for 48 h. Unless otherwise stated, rabbit polyclonal anti-human lactoferrin IgG antibody, mouse monoclonal anti-human lactoferrin antibody (clone LF-2B8; mIgG1; AbD Serotec), rabbit polyclonal IgG negative control, mouse monoclonal IgG1 isotype control or purified milk-derived human lactoferrin were added at a final concentration of 10 µg ml^{-1}. Cells were counted using a NucleoCounter (ChemoMetec).

Direct ELISA (Enzyme-Linked Immunosorbent Assay) for Lactoferrin

A direct ELISA specific for lactoferrin was carried out by plating purified milk-derived human lactoferrin, purified bovine lactoferrin or purified human transferrin (Sigma-Aldrich) at standard concentrations of 1.5 ng ml^{-1} to 200 ng ml^{-1} in a 96-well plate (100 µl per well). Plate was incubated for 14 h at 4°C and washed twice with PBS (Ca^{2+}/Mg^{2+}-free) containing 0.1% (v/v) Tween-20™ and further blocked with 0.5% (w/v) BSA/0.5% (v/v) Tween-20™ for 1 h at room temperature. A variety of monoclonal mouse anti-human lactoferrin antibodies were added per well and the plate was incubated for 1 h at room temperature. The plate was then washed twice with PBS (Ca^{2+}/Mg^{2+}-free) containing 0.1% (v/v) Tween-20™. HRP-conjugated goat anti-mouse IgG (eBioscience) was then added (1:1000 dilution) for 30 min at room temperature. Plate was then washed twice and developed with 3,3'5,5' tetramethylbenzidine (TMB) containing 3% (v/v) H_2O_2 solution was added. Reaction was stopped with 1 M KCl and absorbance values (OD_{450}) were recorded.

Immunoblot Analysis

To prepare neutrophil lysates, neutrophils ($5x10^6$ cells ml^{-1}) were incubated in the presence or absence of purified milk-derived human lactoferrin (10 µg ml^{-1}) for 40 min at 37°C, prior to their activation with fMLP (100 nM). Neutrophils were further incubated at 37°C under continuous agitation at the indicated time points. Neutrophils were

then centrifuged at 14,000*g* for 1 min and lysed with Tris buffered saline (TBS) containing protease inhibitor cocktail (Sigma-Aldrich), aprotinin, leupeptin, pepstatin A, 4-(2-aminoethyl)benzenesulfonyl fluoride, sodium orthovanadate, benzamidine, levamisole, and β-glycerophosphate for 10 min on ice. After 10 min, NP-40 was added to a final concentration of 1% (v/v) and samples were further incubated for 10 min on ice. Samples were then centrifuged (13,000*g*; 4°C, 20 min) and supernatants resuspended in 25 µl sample buffer (NuPAGE™, Invitrogen) and denatured under reducing conditions at 95°C for 10 min. Samples were then stored at 4°C and were used within 24 h. For TCA-precipitation of conditioned cell media, trichloroacetic acid (TCA; 100% w/v; 100 µl) was added in 1 ml conditioned medium and samples were centrifuged for 10 min at 13,000*g*; 4°C. Pellets were washed in ice-cold acetone and finally suspended in sample buffer (25 µl; non-reducing conditions –NuPAGE™, Invitrogen).

For immunoblotting, samples were resolved by SDS-PAGE using 4-12% Bis-Tris gels (NuPAGE™; Invitrogen) and proteins were then electroblotted onto a nitrocellulose membrane (NuPAGE™; Invitrogen), blocked with 3% (w/v) BSA in PBS containing Tween-20 (0.1% v/v) for 1 h at room temperature and probed with mouse monoclonal anti-MAPK activated (diphosphorylated ERK1 and ERK2) antibody (1:1000; Sigma-Aldrich), polyclonal mouse ERK2 (1:1000, Santa-Cruz Biotechnology) or mouse monoclonal anti-human lactoferrin antibody (1:100; LF-2B8, AbD Serotec) in 0.5% (w/v) BSA/0.5% (v/v) Tween-20 overnight at 4°C. Membrane

was further washed with PBS containing 0.1% (v/v) Tween-20 for 30 min at room temperature and incubated with secondary antibody (HRP-conjugated goat anti-mouse Ig 1:2000 in 0.5% (w/v) BSA/0.5% (v/v) Tween-20 (Amersham, Little Chalfont, Buckinghamshire, UK) for 1 h at room temperature. After subsequent wash-up steps for 30 min, membrane was visualised using enhanced chemiluminescence (ECL, GE Healthcare).

For IκB immunoblot analysis, same protocol was followed; however, rabbit monoclonal anti-IκBα (1:2,500; E130; Abcam) was used as a primary antibody followed by HRP-conjugated goat anti-rabbit Ig (1:2,500; Dako Cytomation).

Induction of Apoptosis and Primary Necrosis

Cell lines were induced to undergo classical apoptosis, according to the following protocol: Staurosporine (1 µM; Sigma-Aldrich) was added to BL2, BL2/bcl-2 and Jurkat cells that were suspended at a concentration of $1x10^6$ cells ml^{-1} in serum-free medium (RPMI 1640 supplemented with 2 mM L-glutamine, 100 IU ml^{-1} penicillin and 100 µg ml^{-1} streptomycin). Cells were gently mixed and incubated at 37°C (5% CO_2) for the indicated time points. In MCF7-C3 and A549 cells (60-70% confluent), apoptosis was induced by suspending the cells into serum-free high glucose DMEM medium (containing 2 mM L-glutamine, 100 IU ml^{-1} penicillin and 100 µg ml^{-1} streptomycin) and by adding etoposide (100 µM; Sigma-Aldrich) or staurosporine (1 µM). Cells were incubated at 37°C (5% CO_2) for 20 h (etoposide-induced) or

1 h (staurosporine-induced apoptosis). In order to prevent apoptosis in A549 cells, the pan-caspase inhibitor zVAD-fmk (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone; Sigma-Aldrich) was added in serum-free high glucose DMEM medium (containing 2 mM L-glutamine, 100 IU ml^{-1} penicillin and 100 µg ml^{-1} streptomycin) at a concentration of 100 µg ml^{-1}. Cells were then incubated at 37°C (5% CO_2) at the indicated time points.

BL2 cells were induced to become necrotic by suspending them ($1x10^6$ cells ml^{-1}) in serum-free medium and incubating them at 56°C for 1 h. Apoptosis or necrosis were assessed by trypan blue exclusion and/or annexin V/propidium iodide (Anx/PI) staining.

Quantification of Apoptosis and Cell Cycle Analysis

Apoptosis in leukocytes or cell lines used in this study was determined by annexin V/propidium iodide (Anx/PI) staining. Cells ($1x10^5$) were centrifuged at 400*g* for 10 min and pellet resuspended in the Anx/PI buffer provided by Arcus Biologicals. Annexin V was added to the cell suspension and incubated for 10 min on ice. Anx/PI buffer (0.5 ml) was then added to the cell suspension and propidium iodide (PI; 10 µl) was added prior to flow analysis (BD FACSCalibur).

For cell cycle analysis, harvested cells were washed twice with PBS (Ca^{2+}/Mg^{2+}-free) and pellets were fixed in cold 70% (v/v) ethanol that was added dropwise to the cell pellet to minimise clumping. Cells were fixed for 30 min at 4°C or

stored for 2-3 days in the cold room. At the day of the cell cycle analysis, cells were washed twice in PBS (Ca^{2+}/Mg^{2+}-free) and further treated with ribonuclease by adding 50 µl of RNase (100 µg ml^{-1}; Sigma-Aldrich) to ensure that only DNA was stained. Before flow analysis, 200 µl of propidium iodide (50 µg ml^{-1}; Sigma-Aldrich) was added to each sample. Samples were analysed by flow cytometry and data were analysed by CellQuest (BD Biosciences) or FlowJow (Treestar).

Statistical Analysis

Unless otherwise stated, results from multiple experiments are presented as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was performed to test for differences in the mean values of quantitative data and where statistically significant effects were observed, Bonferroni post-hoc test was performed. In all cases, p values of 0.05 or less were considered statistically significant. Data analysis was performed with GraphPad Prism software (GraphPad, La Jolla, USA).

RESULTS

Apoptotic cells actively release factors that inhibit neutrophil migration

The initial aim of this work was to address whether apoptotic cells influence the migratory activity of neutrophils, based on the observation that neutrophils are absent from sites where homeostatic apoptosis rates are high accounting in this way for the anti-inflammatory nature of the apoptotic program (Kerr *et al.*, 1972; Serhan and Savill, 2005). To this end, a series of *in vitro* Boyden-type chemotaxis assays were carried out in order to investigate neutrophil migration towards Burkitt's lymphoma cells. As already mentioned, Burkitt's lymphoma was initially employed as a model tissue for apoptosis, as this class of tumour cell population displays high levels of apoptosis, a property that is retained constitutively in the tumour-derived cell lines (Frost *et al.*, 2004; Gregory *et al.*, 1991; Soini *et al.*, 1998b). As at all sites where homeostatic apoptosis rates are high, in Burkitt's lymphoma, there is a marked infiltration of macrophages that engulf the apoptotic cells, giving rise to the typical "starry sky" histological appearance of this tumour (Truman *et al.*, 2004). Macrophage infiltration is marked by the synthesis and secretion of cytokines that are commonly expressed by sites where homeostatic apoptosis rates are high and include fractalkine, IL-10 and others (Ogden *et al.*, 2005; Truman *et al.*, 2008).

However, as evidenced by an immunohistochemical staining for the detection of GR1 granulocytic marker in **Figure 7**, no neutrophils are present in the BL stroma.

In order to assess the effects of BL cells on the migratory activity of neutrophils *in vitro*, chemotaxis assays were carried out **(Figure 8A)**, in which neutrophils were added to the top compartment of a transwell filter and stimulated to migrate towards the lower chamber containing BL cells (Mutu that are EBV^+ or BL2 that are EBV^-) at a concentration of $2x10^6$ ml^{-1} in the presence of the powerful neutrophil chemoattractant formyl-methionyl-leucyl-phenylalanine (fMLP -100 nM) (mean number of migrated neutrophils: 8.1 ± 1.68 towards Mutu cells, mean number of migrated neutrophils: 9.4 ± 1.31 towards BL2 vs. 97.1 ± 6.68 towards fMLP control) **(Figures 8B and 8C)**. Also as shown in **Figure 8D**, neutrophil chemotaxis was significantly inhibited in a concentration-dependent manner towards BL2 cells that were placed at the lower chamber of the filter at varying concentrations ($0.5x10^6$ cells ml^{-1}, $1x10^6$ cells ml^{-1} and $2x10^6$ cells ml^{-1}). In all cases, 100 nM of fMLP were added to stimulate neutrophil migration (36.8% inhibition in neutrophil migration towards $0.5x10^6$ cells ml^{-1}; 50.8% towards $1x10^6$ cells; 63.8% towards $2x10^6$ cells).

Subsequent chemotaxis assays were carried out using BL-conditioned medium. In detail, BL2 or Mutu cells ($2x10^6$ cells ml^{-1}) were washed in PBS, resuspended in chemotaxis assay (serum-free) medium and incubated at 37°C (5% CO_2)

for 12 h. Cells were then centrifuged at 400*g* for 10 min and supernatant was collected and used in chemotaxis assays. It was found that fMLP-stimulated neutrophils failed to migrate towards both types of BL supernatants at levels comparable to fMLP positive control (mean number of migrated neutrophils: 28.8 ± 1.6 towards BL2 medium; 27.6 ± 1.62 towards Mutu medium vs. 102.9 ± 3.6 towards fMLP) **(Figure 9A)**. Similarly, a marked inhibition in neutrophil migration was also evident in further chemotaxis assays using supernatants obtained from BL2 cells over a 7-hour time course (17.4% inhibition in neutrophil migration (1 h), 32.2% inhibition (3 h), 45.2% (5 h), 50.4% (7 h)) **(Figure 9B).** The release of the inhibitory factor appeared to be linked to the levels of apoptosis in the BL cell population, since the inhibitory activity was significantly lower in further chemotaxis assays using BL-conditioned medium derived from cells overexpressing the apoptosis inhibitor Bcl-2, as compared with that of parental cells **(Figure 9C)**. In detail, at the 5 h time point, the mean number of migrated neutrophils was 62 ± 2.5 towards BL2 conditioned medium vs. 111.5 ± 3.7 migrated neutrophils towards BL2/bcl-2 conditioned medium. In summary, it was evident that neutrophils are absent from the BL stroma due to an active mechanism and in particular, due to factors released by BL cells. The release of such factors seems to be related to the apoptosis levels of the cells.

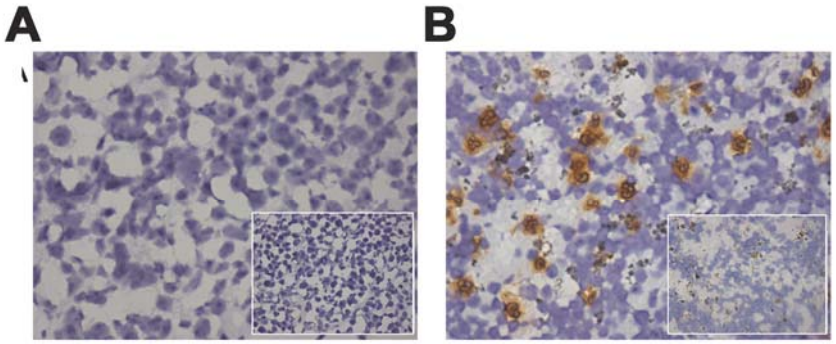


FIGURE 7: Neutrophils are absent in Burkitt lymphoma

Immunohistochemical analysis of neutrophils in **(A)** BL and **(B)** spleen (positive control) sections. Slides were stained to detect the presence of GR1 (granulocyte marker; affinity purified rat anti-mouse Ly-6G). Inset images represent isotype control (rat IgG2b isotype control). Original magnification: x400. It was revealed that neutrophils are absent from the BL stroma.

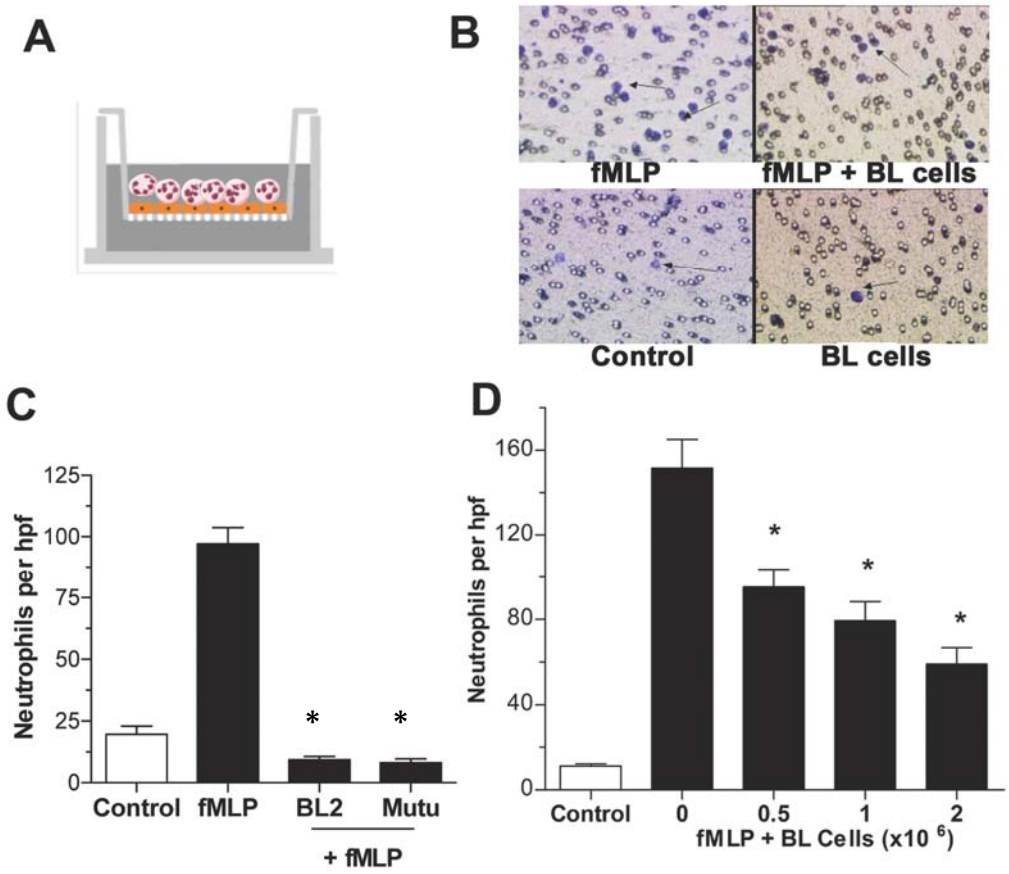


FIGURE 8: BL cells actively inhibit neutrophil migration

(A) In a chemotaxis assay, neutrophils are added on top of a transwell filter and stimulated to migrate towards a chemoattractant or medium alone added on the bottom well of the assay **(B)** Representative images of stained Transwell filters **(C)** Neutrophil chemotaxis towards Mutu (EBV^+ cell line; $2x10^6$ cells ml^{-1}) and BL2 (EBV^- cell line; $2x10^6$ cells ml^{-1}) assessed in the presence of fMLP (100 nM) n=3; *p<0.01 vs. fMLP control. **(D)** Neutrophil chemotaxis towards increasing concentrations of BL cells ($0.5x10^6$ cells ml^{-1}; $1x10^6$; $2x10^6$) assessed in the presence of fMLP (100 nM) n=3; *p<0.05 vs. concentration 0. Original magnification: x400; fMLP, formyl-methionyl-leucyl-

phenylalanine; hpf, high power field. Error bars indicate SEM.

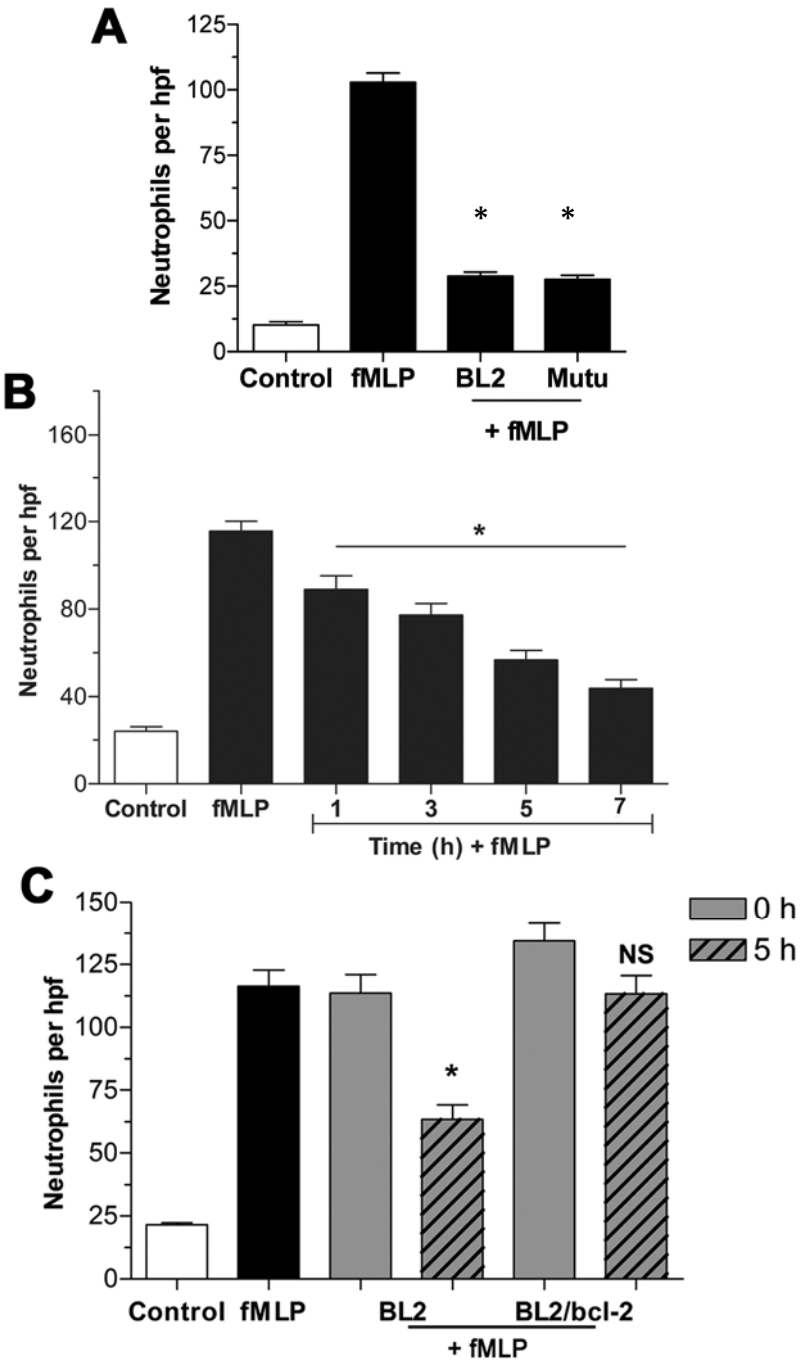

A
Neutrophils per hpf
125
100
75
50
25
0
*
*
Control
fMLP
BL2
Mutu
+ fMLP
B
Neutrophils per hpf
160
120
80
40
0
*
Control
fMLP
1
3
5
7
Time (h) + fMLP
C
Neutrophils per hpf
150
125
100
75
50
25
0
0 h
5 h
NS
*
Control
fMLP
BL2
BL2/bcl-2
+ fMLP

FIGURE 9: Apoptotic cells release factor(s) that inhibit neutrophil migration.

(A) Neutrophil chemotaxis towards conditioned media obtained from BL2 and Mutu cells [$2x10^6$ cells ml^{-1} cultured in serum-free medium; 12 h; 37°C (5% CO_2)]. n=3; *p<0.05 vs. fMLP control. **(B)** Neutrophil chemotaxis towards conditioned media obtained from BL2 cells ($2x10^6$ cells ml^{-1}) at the indicated time points n=3; *p<0.05 vs. fMLP **(C)** Neutrophil chemotaxis towards fMLP was analysed in the presence of conditioned media from BL2 control or *BCL2*-transfected BL2 cells obtained following a 0- and 5-h incubation at 37°C (5% CO_2) n=3; *p<0.05 vs. BL2 0 h; NS=non-significant vs. BL2/bcl-2 0 h; fMLP, formyl-methionyl-leucyl-phenylalanine; hpf, high power field. Error bars indicate SEM.

Biochemical characterisation of the neutrophil migration inhibitory factor(s)

Having established that BL cells actively release factor(s) that inhibit neutrophil chemotaxis, a series of assays were performed in order to biochemically characterise the inhibitory factor(s). Initially, it was determined whether the neutrophil migration inhibitory factor was heat-labile. In detail, BL2-conditioned medium ($2x10^6$ cells; 12 h incubation) was heat-treated (incubation at $100^{\circ}C$ for 30 min) and subsequently used in a chemotaxis assay to determine neutrophil migration. As shown in **Figure 10**, heat inactivation completely abrogated all chemotaxis inhibitory activity in BL cell-conditioned medium, suggesting that the inhibitory factor(s) were most likely protein or heat-sensitive lipid mediators in nature. In an attempt to gain further insight into the biochemical nature of the factor(s) that BL cells secrete in order to exclude neutrophils from their environment, we then estimated the molecular weight range of the inhibitory factor(s) by using filters with molecular weight cut-off points of approximately 3, 10, 30, 50, and 100 kDa. BL-conditioned media obtained after 24-hour incubation were fractionated and each fraction was examined *in vitro* using the neutrophil chemotaxis assay described above. The results revealed that fractions containing molecules of less than 50 kDa failed to display any inhibitory effect on neutrophil migration **(Figure 11A)**. In fact, the >50 kDa fraction retained the ability

to inhibit neutrophil migration (3.44 ± 1.31 mean number of migrated neutrophils; 56.52 ± 3.87 for fMLP control) and thus, it makes it clear that at least one factor had a molecular weight that ranged between 50 and 100 kDa. However, the use of 100 kDa filters revealed that both fractions (>100 kDa and <100 kDa) displayed an inhibitory effect on neutrophil migration (<100 kDa fraction: 32.6 ± 1.5 migrated neutrophils; >100 kDa 11.2 ± migrated neutrophils vs. 62.3 ± 2.6 towards fMLP control; 82.0% inhibition for >100 kDa; 52.6% inhibition for <100 kDa) **(Figure 11B)**, indicating that at least one factor has a molecular weight that ranges between 50 and 100 kDa. The presence of inhibitory activity in the filtrate of the 100 kDa cut-off membrane is likely to result from (a) imprecise molecular weight cut-off of molecules in the 50-100 kDa range, (b) complex formation through multimerisation of the 50-100 kDa factor or through interaction with other molecules, or (c) the existence of a distinct inhibitory activity of greater than 100 kDa. It should be noted that the selected isolation approach is skewed in favour of proteins and that additional low molecular weight (for example, lipid) mediators of neutrophil migration inhibition would not be identified by these procedures.

To investigate further the biochemical properties of the retentate and filtrate of the 100 kDa cut-off membrane, the charge (pI value) of the migration-inhibitory activity was first determined by means of an ion exchange analysis of BL-conditioned media. In detail, specialised ion-

exchange beads were used that were either positively (Q Sepharose beads) or negatively (S Sepharose beads) charged. In this way, proteins found in the BL supernatant were distinguished according to their charge, based on their ability to become bound to the oppositively charged beads.

At a first stage, supernatants of BL cells cultured for 24 h were analysed using negatively charged beads (S beads). Two fractions of the BL supernatants were obtained; S1: negative charged fraction that failed to become bound to S beads and S2: positively charged fraction of molecules bound to and eluted from the beads. These two fractions were used in neutrophil chemotaxis assays and it was revealed that neutrophil migration was significantly reduced compared to their corresponding control (43.5% inhibition for S1; 50.0% for S2) **(Figure 12A)**. In summary, these findings clearly indicated the presence of a positively charged (cationic) or a negatively charged (anionic) inhibitory factor present in the BL supernatant. However, the possibility of an inhibitory factor of a neutral charge (pI about 7.0 –8.0) could not be excluded.

At a second stage, based also on these observations, subsequent ion-exchange analyses of both fractions of the 100-kDa filter were carried out. More specifically, positively charged beads were used (Q beads) in this case and analyses of the <100 kDa fraction presented an inhibitory effect in both the supernatant (positive charge) and the eluant (negative charge) of the Q beads.

In detail, the cationic fraction (Q1) showed a 50.3% inhibition, whereas the inhibition in the anionic one (Q2) was 29.0% compared to their corresponding controls **(Figure 12B)**. By contrast, analyses of the retentate (>100 kDa fraction) revealed that only the negatively charged eluant displayed significant activity in inhibiting neutrophil migration (51.5%), clearly indicating in this way that the inhibitory factor in the >100 kDa fraction possesses a negative charge.

Collectively, these results indicate that at least two moieties with neutrophil migration-inhibitory activity were present in BL-conditioned medium: one of 50-100 kDa with positive pI and a second of 100 kDa or more and negatively charged. We then analysed the proteins released from BL cells in viable and apoptotic states by protein fingerprinting. Polypeptide bands of greater than 50 kDa were excised from a 10% SDS polyacrylamide gel. Tryptic peptides were gel extracted and a matrix-assisted laser desorption ionisation–time of flight (MALDI-TOF) mass spectrometric analysis was carried out **(Figure 13 and Table 11)**. Also, given the crude biochemical characteristics described above, a candidate approach was then undertaken based on the proteins released from apoptotic BL cells. The factor released by BL cells that prevented neutrophil chemotaxis was identified to be lactoferrin. The identification of lactoferrin supported the findings of the biochemical approach carried out in this study, as lactoferrin is heat-sensitive, has a

molecular weight of 80 kDa, which is over 50 kDa, and is cationic in nature with an isoelectric point of 8.0.

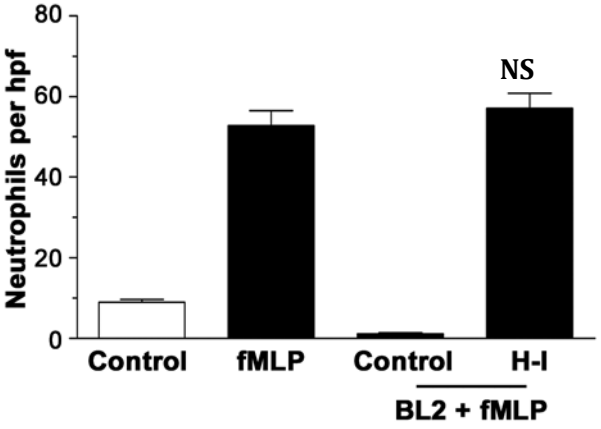


FIGURE 10: Heat-inactivation of BL medium abrogated the observed inhibitory effect on neutrophil migration.

Chemotaxis assay of neutrophils towards BL-conditioned medium ($2x10^6$ BL2 cells ml^{-1}; 12 h incubation in serum-free medium) that was heat-inactivated (100°C; 30 min). Results showed that no inhibition in neutrophil migration was evident towards heat-inactivated BL medium, compared to non-heat-inactivated control. n=3; NS vs. fMLP control; fMLP, formyl-methionyl-leucyl-phenylalanine; H-I, heat-inactivated; hpf, high power field. Error bars indicate SEM.

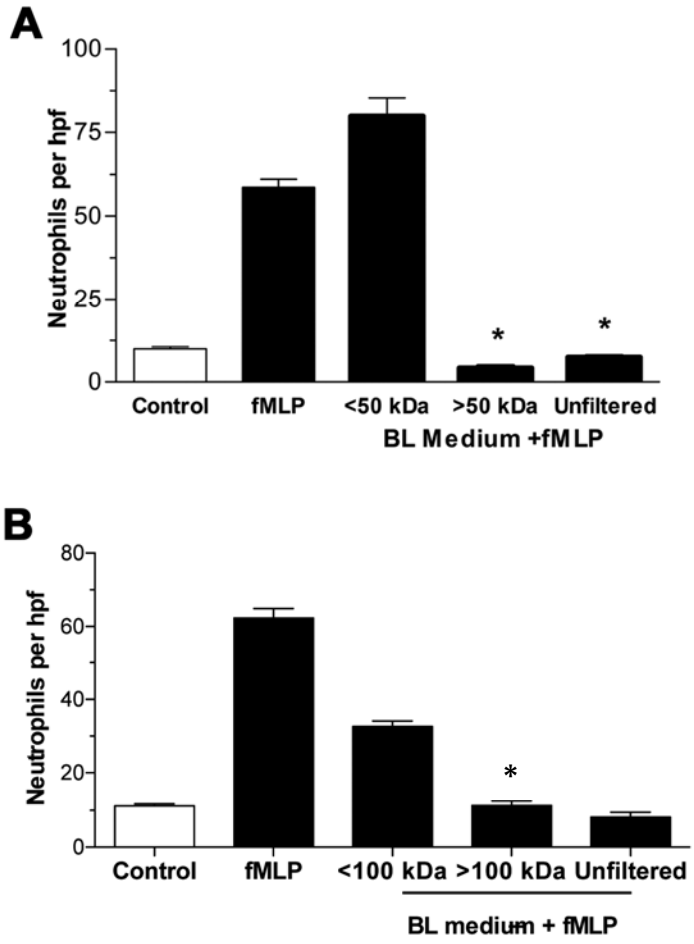


FIGURE 11: Biochemical characterisation of the inhibitory factor (molecular size analysis).

Conditioned media from BL2 cells cultured for 12 h were size fractionated using filters with **(A)** 50 kDa and **(B)** 100 kDa molecular weight cut-off sizes. Unfiltered medium was included as control. The >50 kDa fraction retained the ability to inhibit neutrophil migration n=3 *p<0.001 vs. fMLP; fMLP, formyl-methionyl-leucyl-phenylalanine; hpf, high power field. Error bars indicate SEM.

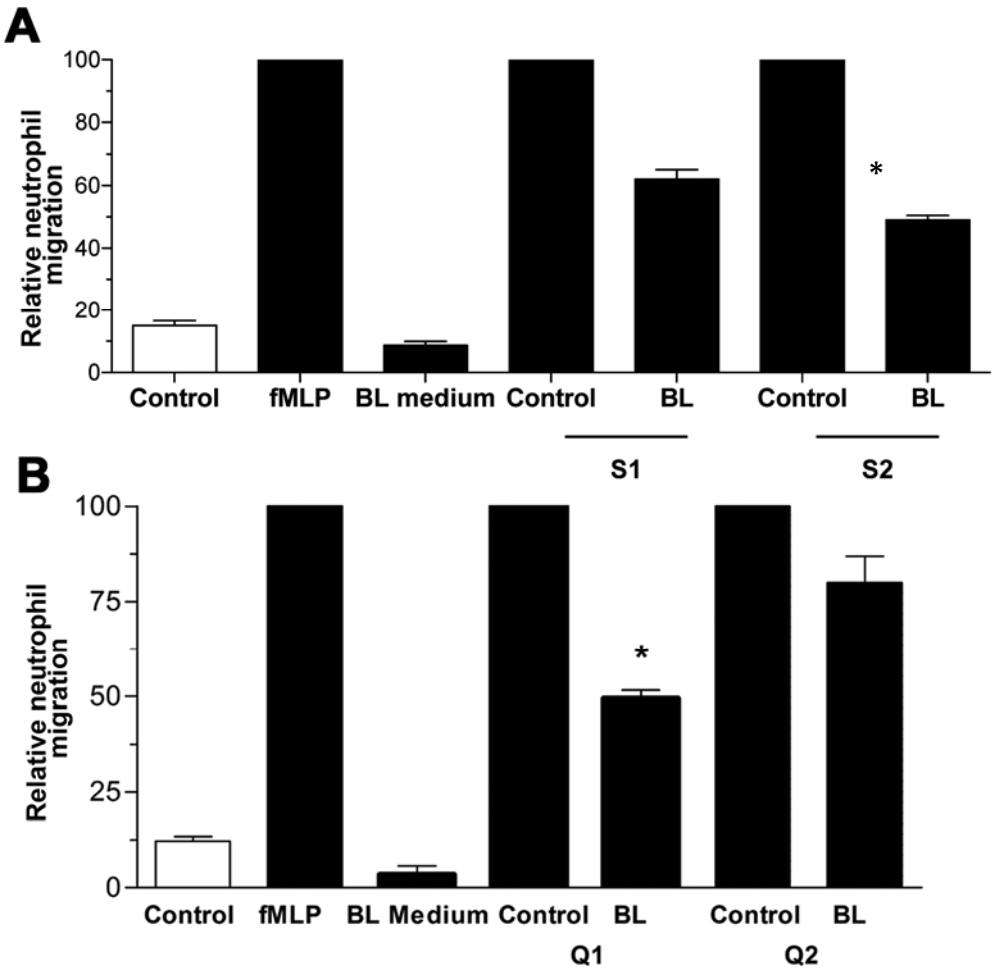

A
Relative neutrophil migration
100
80
60
40
20
0
Control
fMLP
BL medium
Control
BL
Control
BL
*
S1
S2
B
Relative neutrophil migration
100
75
50
25
0
Control
fMLP
BL Medium
Control
BL
Control
BL
*
Q1
Q2

FIGURE 12: Biochemical characterisation of the inhibitory factor (Ion exchange analysis).

Ion exchange analysis included the use of **(A)** S (negatively charged) and **(B)** Q Sepharose beads (positively charged) in order to distinguish positively and negatively charged molecules in the whole BL medium and in the <100 kDa fraction respectively. Unbound molecules (S1 and Q1 fraction) were collected, whereas bound molecules (S2 and Q2 fraction) were eluted from the beads. Neutrophil migration toward these fractions in the presence of fMLP (100 nM) was assessed. S and/or Q fractions of serum-free medium (no BL) were included as control n=2 for both A and B, *$p<0.05$ compared with the corresponding control; ANOVA was performed based on the mean number of migrating neutrophils per hpf for each condition; fMLP, formyl-methionyl-leucyl-phenylalanine; hpf, high power field. Error bars indicate SEM.

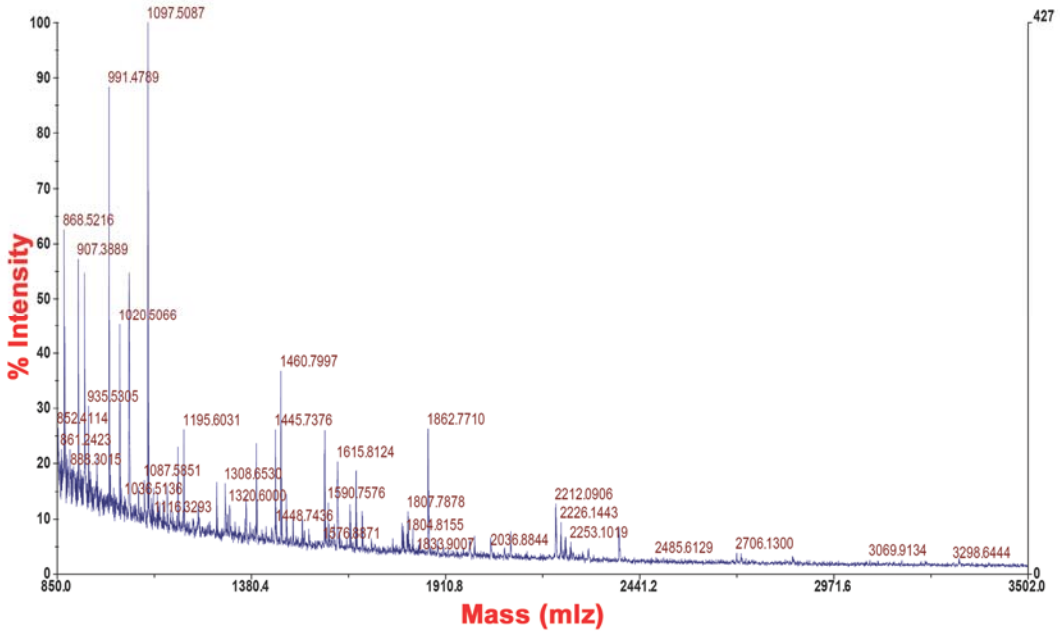


FIGURE 13: Profile of MALDI-TOF analysis

MALDI-TOF mass spectrum for the tryptic digest of the peptide bands released from BL cells that were induced to become apoptotic (1 μM staurosporine). MALDI-TOF analysis was performed by Scottish Instrumentation and Resource Centre for Advanced Mass Spectrometry (SIRCAMS).

Table 11.: Protein Hits from MALDI-TOF	
PLSL_HUMAN	Plastin-2 (L-plastin) (Lymphocyte cytosolic protein 1) (LCP-1) (LC64P)
KPYM_HUMAN	Pyruvate kinase isozymes M1/M2 (EC 2.7.1.40) (Pyruvate kinase muscle isozyme) Cytosolic thyroid hormone-binding protein) (CTHBP) (THBP1)
CH60_HUMAN	Heat shock protein 60) (HSP-60) (Mitochondrial matrix protein P1) (P60 lymphocyte protein)
TBB2C_HUMAN	Tubulin β-2C chain (Tubulin β-2 chain)
ACTA_HUMAN	Actin, aortic smooth muscle (α-actin-2)
LDHA_HUMAN	L-lactate dehydrogenase A chain (EC 1.1.1.27) (LDH-A) (LDH muscle subunit) (LDH-M)
HNRPK_HUMAN	Heterogeneous nuclear ribonucleoprotein K (hnRNP K)
TCPZ_HUMAN	T-complex protein 1 subunit ζ (TCP-1-ζ) (CCT-ζ) (CCT-ζ-1) (Tcp20) (HTR3)
EF1A1_HUMAN	Elongation factor 1-α 1 (EF-1-α-1)
ALDOA_HUMAN	Fructose-bisphosphate aldolase A (EC 4.1.2.13) (Muscle-type aldolase)
ENOA_HUMAN	A-enolase (EC 4.2.1.11) (2-phospho-D-glycerate hydro-lyase) (Non-neural enolase) (NNE) (Plasminogen-binding protein)
TBAK_HUMAN	Tubulin α-ubiquitous chain (Tubulin K-α-1)
UGPA1_HUMAN	UTP--glucose-1-phosphate uridylyltransferase 1 (UDP-glucose pyrophosphorylase 1)
TCPW_HUMAN	T-complex protein 1 subunit ζ-2 (TCP-1-ζ-2) (CCT-ζ-2) (TCP-1-ζ-like) (CCT-ζ-like)
PDIA3_HUMAN	Protein disulfide-isomerase A3 precursor (Disulfide isomerase ER-60)
SERA_HUMAN	D-3-phosphoglycerate dehydrogenase (3-PGDH)
K22E_HUMAN	Keratin, type II cytoskeletal 2 epidermal
TCPD_HUMAN	T-complex protein 1 subunit δ (TCP-1-δ)
PDCD4_HUMAN	Programmed cell death protein 4 (Nuclear antigen H731-like)
TCPQ_HUMAN	T-complex protein 1 subunit θ (TCP-1-θ)
NP1L4_HUMAN	Nucleosome assembly protein 1-like 4
BASP_HUMAN	Brain acid soluble protein 1 (BASP1 protein)
HS90A_HUMAN	Heat shock protein HSP 90-α (HSP 86)
F10A1_HUMAN	Putative tumour suppressor ST13 (Protein FAM10A1) (Progesterone receptor-associated p48 protein)
TRFL_HUMAN	Lactotransferrin precursor (Lactoferrin) (Talalactoferrin alfa)
HSP7C_HUMAN	Heat shock cognate 71 kDa protein (Heat shock 70 kDa protein 8)
HS90B_HUMAN	Heat shock protein HSP 90-β (HSP 84) (HSP 90)
HS90A_HUMAN	Heat shock protein HSP 90-α (HSP 86) (Renal

	carcinoma antigen NY-REN-38)
ACTB_HUMAN	Actin, cytoplasmic 1 (β-actin)
EZRI_HUMAN	Ezrin (p81) (Cytovillin) (Villin-2)
EF2_HUMAN	Elongation factor 2 (EF-2)
HSP76_HUMAN	Heat shock 70 kDa protein 6 (Heat shock 70 kDa protein B')
TBAK_HUMAN	Tubulin α-ubiquitous chain (α-tubulin ubiquitous) (Tubulin K-α-1)
TRAP1_HUMAN	Heat shock protein 75 kDa, mitochondrial precursor (HSP 75)
MOES_HUMAN	Moesin (Membrane-organizing extension spike protein)
VPS35_HUMAN	Vacuolar protein sorting-associated protein 35 (Vesicle protein sorting 35) (hVPS35)
SAHH_HUMAN	Adenosylhomocysteinase (S-adenosyl-L-homocysteine hydrolase)
NUCL_HUMAN	Nucleolin
EF1B_HUMAN	Elongation factor 1-β (EF-1-β)
TBB5_HUMAN	Tubulin β chain (Tubulin β-5 chain)
DDEF2_HUMAN	Development and differentiation-enhancing factor 2

Lactoferrin is actively released from BL cells and specifically inhibits neutrophil chemotaxis *in vitro* and *in vivo*

Lactoferrin is a glycoprotein of approximately 75-80 kDa that belongs to the transferrin family of proteins due to its iron-binding properties (Ward *et al.*, 2005). It is a well-characterised component of neutrophil secondary granules, lacrimal fluid, colostrum, saliva and mucosal secretions, in which it confers antibacterial activity. Apart from its role in iron metabolism, lactoferrin is a pleiotropic molecule, whose properties range from anti-inflammatory to anti-microbial and anti-tumoural.

In this study, lactoferrin was identified as a factor that inhibits neutrophil migration, as evidenced by chemotaxis assays, in which anti-human lactoferrin antibody was added to conditioned media from BL2 and/or from MCF7-C3, a breast cancer cell line, which is a caspase-3 transfectant of MCF7, known to constitutively express lactoferrin (Turner *et al.*, 2003). In detail, addition of rabbit polyclonal anti-human lactoferrin IgG antibody abrogated the inhibitory effect compared to isotype control (mean number of migrated neutrophils towards BL medium+antibody: 78.1 ± 1.32 vs. 8 ± 0.21 mean number of migrated neutrophils towards BL medium+isotype control in the presence of fMLP (100 nM); 92 ± 1.28 towards fMLP control) **(Figure 14A)**. BL-conditioned medium was obtained following incubation of $2x10^{6}$ cells ml^{-1} under serum-free conditions for 12 h. An analogous effect was also observed using

conditioned media from MCF7-C3 cells ($2x10^6$ cells incubated in 3 ml serum-free medium -mean number of migrated neutrophils: 98.1 ± 2.21 towards MCF7-C3 +antibody vs. 30.3 ± 0.01 mean number of migrated neutrophils towards MCF7-C3 medium+isotype control in the presence of fMLP (100 nM); 110.8 ± 1.36 towards fMLP control), indicating in this way that the neutrophil migration-inhibitory activity is not restricted to BL cell-derived lactoferrin **(Figure 16A)**.

Moreover, subsequent chemotaxis assays were carried out using mouse monoclonal anti-human lactoferrin antibodies (LF-2B.8; AbD Serotec) as well as imab75 and imab77 (Immunosolv), which also abrogated the inhibitory effect on neutrophil migration towards BL-conditioned medium. For LF-2B.8: mean number of migrated neutrophils towards BL medium + LF-2B.8 antibody: 72.1 ± 3.1 vs. 8.5 ± 0.21 mean number of migrated neutrophils towards BL medium + isotype control in the presence of fMLP (100 nM); 93.7 ± 4.2 towards fMLP control **(Figure 14B)**. When LF-2B.8 monoclonal antibody was added to MCF7-C3 medium: mean number of migrated neutrophils towards MCF7-C3 medium + LF-2B.8 antibody was 109 ± 2.9 vs. 38 ± 1.2 mean number of migrated neutrophils towards MCF7-C3 medium+isotype control in the presence of fMLP (100 nM); 115 ± 2.6 towards fMLP control **(Figure 16B)**.

A similar effect was observed using imab75 and imab77 antibodies that were provided by ImmunoSolv and selected from a panel of monoclonal mouse anti-human lactoferrin

antibodies, based on their specificity to human lactoferrin in comparison to human transferrin and bovine lactoferrin, as assessed by a direct ELISA assay **(Table 12)**. For imab 75: mean number of migrated neutrophils towards BL medium + imab75 antibody: 88.8 ± 5.60 vs. 22.1 ± 1.21 mean number of migrated neutrophils towards BL medium+isotype control in the presence of fMLP (100 nM); 88.3 ± 4.12 towards fMLP control **(Figure 15A)**. For imab 77: mean number of migrated neutrophils towards BL medium+ imab77 antibody: 102.3 ± 1.26 vs. 21 ± 0.81 mean number of migrated neutrophils towards BL medium+isotype control in the presence of fMLP (100 nM); 118.7 ± 6.3 towards fMLP control **(Figure 15B)**. Therefore, when imab75 or imab77 were added to the BL-conditioned medium, an abrogation of the inhibitory effect in neutrophil chemotaxis was observed vs. BL-medium control in the presence of fMLP.

In order to provide additional support for the specificity of the observed lactoferrin effect, MCF7-C3 and BL2 cell lines were created that were transfected with a shRNA vector specific for human lactoferrin or with a mismatch control **(Figures 17A and 17B)**. Thus, it was found that neutrophil chemotaxis towards supernatants obtained from BL2 cells transfected with shRNA vectors targeted against lactoferrin [$2x10^6$ cells ml^{-1}; serum-free conditions for 12 h at 37°C (5 % CO_2)] was higher (mean number of migrated neutrophils: 87 ± 2.6 towards supernatants from LTF shRNA BL2 cells; mean number of migrated neutrophils: 53 ± 2.8 towards shRNA mismatch (mm) control; 108 ± 2.3 towards fMLP control) **(Figure 17C)**. A similar effect was observed

in chemotaxis assay towards conditioned medium obtained from shRNA MCF7-C3 cells transfected for lactoferrin (mean number of migrated neutrophils: 108 ± 1.2 towards supernatants from LTF shRNA BL2 cells; mean number of migrated neutrophils: 49 ± 2.8 towards shRNA mm control; 119 ± 1.2 towards fMLP control) **(Figure 17D)**.

In an additional attempt to verify these results and provide further evidence on the role of lactoferrin in the regulation of neutrophil chemotaxis, the effect of purified milk-derived human lactoferrin towards fMLP-induced neutrophil migration was *in vitro* assessed. In detail, varying concentrations of human lactoferrin were used ranging from 10 pg ml^{-1} to 100 μg ml^{-1} and neutrophil migration towards them was measured. It was observed that lactoferrin inhibited neutrophil chemotaxis in a bell-shaped manner. Maximum neutrophil inhibition was observed at 10 μg ml^{-1} (84.8% inhibition), but even at a 10^{6}-fold lower concentration (10 pg ml^{-1}), an inhibition in neutrophil chemotaxis was also evident **(Figure 18A)**.

Apart from milk, lactoferrin is also found in the secondary granules of polymorphonuclear neutrophils. Although both types of lactoferrin (milk-derived and neutrophil-derived) have been reported to possess no difference in terms of their functional properties, neutrophil-derived lactoferrin structurally lacks terminal fucose residues in the glycal chains necessary for binding to macrophages. In chemotaxis assays, it was found that the neutrophil migration-inhibitory effect was also

displayed by lactoferrin purified from human neutrophils (mean number of migrated neutrophils: 18.3 ± 0.12 towards neutrophil-derived lactoferrin; 17.2 ± 0.1 towards milk-derived lactoferrin in the presence of fMLP vs. 119.2 ± 2.1 migrated neutrophils towards fMLP control) **(Figure 18B)**. It should be noted that both types of purified lactoferrin used in this study were free of endotoxin contamination, as assessed by Limulus assay, and the observed inhibitory effect did not appear to be due to any lactoferrin-associated molecules such as LPS. Furthermore, the inhibitory effect in neutrophil migration by shRNA-transfected BL cells- further supports the specificity of lactoferrin in promoting this inhibitory effect on neutrophils. In addition, lactoferrin exerted no toxic effects on neutrophils, as assessed by Anx/PI staining of control and lactoferrin-treated neutrophils (> 98% cell viability).

Iron and iron-associated molecules have been previously shown to play an important role in many immunomodulatory functions. Indeed, suppression of IL-1 release by monocytes is observed by purified iron-saturated lactoferrin, whereas an inhibition of GM-CSF activity production by monocytes and macrophages correlated with the iron saturation status of lactoferrin (Broxmeyer *et al.*, 1978; Broxmeyer *et al.*, 1986; Zucali *et al.*, 1989). Therefore, it was further examined whether differences in the iron saturation profile of lactoferrin affect its ability to inhibit neutrophil migration. Chemotaxis assays to determine neutrophil migration towards iron-depleted

(apo-form), partially iron-saturated, or fully iron-saturated (holo-form) lactoferrin revealed that the level of iron saturation was not responsible for the observed inhibition in neutrophil migration, as in all cases, an inhibition of neutrophil chemotaxis was noted **(Figure 19)**. Also, as lactoferrin belongs to the transferrin family of proteins sharing 74% sequence homology with transferrin (both of them are ~80 kDa cationic iron-binding glycoproteins) (Harrington, 1992; Metz-Boutigue *et al.*, 1984; Park *et al.*, 1985), it was reasoned that, if the underlying neutrophil migration-inhibitory mechanism of lactoferrin was rooted in its ability to chelate iron, transferrin might show similar effects on neutrophil migration. To explore this possibility, chemotaxis assays were performed in which neutrophils were induced to migrate toward fMLP in the presence of partially iron-saturated transferrin. The results showed that transferrin, unlike lactoferrin, had no effect on fMLP-induced neutrophil chemotaxis (mean number of migrated neutrophils: 19.2 ± 0.8 towards purified lactoferrin + fMLP vs. 152 ± 1.8 towards purified transferring + fMLP) **(Figure 19)**. These findings support the conclusion that the observed neutrophil migration-inhibitory effect is both lactoferrin specific and not related to the iron-binding properties of the protein.

Having established the inhibitory effects of lactoferrin on neutrophil chemotaxis *in vitro*, a murine peritonitis model was then used to assess the effect of lactoferrin on leukocyte recruitment *in vivo*. Lactoferrin and transferrin

were tested for their ability to affect thioglycollate-induced leukocyte recruitment to the peritoneal cavity. At a first stage, the peritonitis model was optimised in terms of the concentration of thioglycollate to be used. For this reason, the effect of 1%, 2.5%, 5% and 10% thioglycollate was checked by injecting C57BL/6 mice with thioglycollate at 500 µl final volume. As shown in **Figure 20**, thioglycollate caused a rapid recruitment of leukocytes following a dose-dependent mode compared to negative control and the recruited leukocytes were predominantly neutrophils (88.0%), as assessed by GR1 marker expression. To test the ability of lactoferrin in this *in vivo* model, mice were injected with human lactoferrin (10 µg ml^{-1}), human transferrin (10 µg ml^{-1}) or saline alone, followed by a second injection with 1% thioglycollate, 20 min after the first injection. In the presence of lactoferrin, the total number of neutrophils recruited to the peritoneal cavity was reduced by 52.0% compared with control, whereas transferrin had no effect ($3.6x10^6$ ± 2.5 recruited cells harvested from lactoferrin-treated mice; $8.3x10^6$ ± 5.1 recruited cells from transferrin-treated vs. $7.2x10^6$ ± 3.8 recruited cells in thioglycollate control) **(Figure 21A)**. Lactoferrin reduced specifically the proportion and number of neutrophils migrating into the cavity but did not affect recruitment of other types of leukocytes in response to thioglycollate ($2.1x10^6$ ± 1.8 recruited $GR1^+$ cells harvested from lactoferrin-treated mice; $6.1x10^6$ ± 5.3 recruited $GR1^+$ cells from transferrin-treated vs. $4.7x10^6$ ± 2.2 $GR1^+$ cells

in thioglycollate control) **(Figures 21B and 22)**. These results demonstrate that, similar to its effect on neutrophil chemoattraction *in vitro*, lactoferrin is a potent inhibitor of neutrophil migration *in vivo*.

It has been recently demonstrated that chemokines or factors that are known to attract neutrophils, like IL-8, also exert a chemorepulsive effect on them when used at low concentrations (Tharp *et al.*, 2006). In order to investigate whether lactoferrin is one of such factors that promote neutrophil repulsion or acts by inhibiting neutrophil migration, chemotaxis assays were further carried out, in which lactoferrin (10 µg ml^{-1}) was added to the upper chamber along with neutrophils. It was revealed that when added with neutrophils, neutrophils failed to migrate towards fMLP (100 nM) or even control medium at significantly lower levels, compared to neutrophils stimulated to migrate in the absence of lactoferrin **(Figure 23A)**. This result also suggested that lactoferrin exerts a direct effect on neutrophils by inhibiting their migratory ability and not forcing them to migrate in all directions away from the chemoattractant; an activity that would have been predicted to result in a statistically higher number of migrated neutrophils in this assay.

Moreover, pre-treatment of neutrophils with 10 µg ml^{-1} lactoferrin (serum-free medium; 20 min; 37^{o}C) followed by removal of lactoferrin via washing with PBS, resulted in the maintenance of the inhibitory effect of lactoferrin, despite its absence, and thus, a reduced number of

migrating neutrophils towards fMLP, compared to non-lactoferrin treated control cells (mean number of migrated neutrophils: 31 ± 1.5 towards fMLP following lactoferrin-pre-incubation; vs. 118.2 ± 3.9 mean number of migrated neutrophils towards fMLP, pre-incubated in the absence of lactoferrin) **(Figure 23B)**. The fact that the inhibitory effect on neutrophil migration was evident after 20 min suggests that lactoferrin possibly acts on the intracellular signalling cascade of the cell by affecting NF-κB pathway or stimulating new protein synthesis.

In summary, it was found that lactoferrin specifically inhibited neutrophil migration both *in vivo* and *in vitro*, as evidenced by neutralisation experiments, by shRNA-mediated knockdown of lactoferrin expression in BL2 and MCF7-C3 cells as well as by experiments on a murine model of peritonitis.

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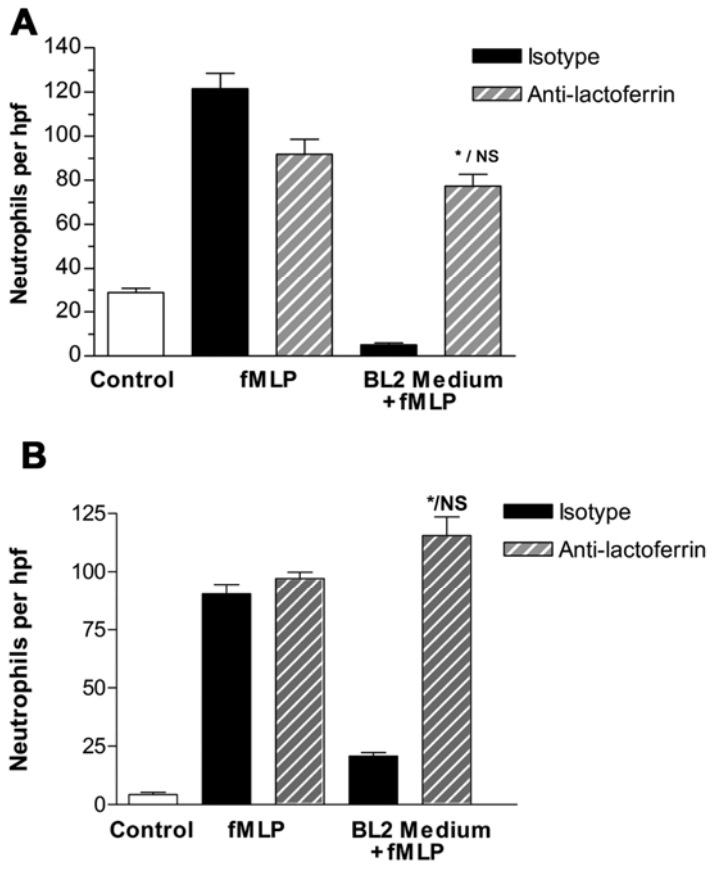

FIGURE 14: Lactoferrin specifically inhibited neutrophil chemotaxis.

Neutralisation experiments to determine neutrophil chemotaxis in the presence of **(A)** rabbit polyclonal anti-human lactoferrin IgG antibody; n=3; *p<0.05 vs. isotype control; NS vs. fMLP anti-lactoferrin control **(B)** mouse monoclonal anti-human lactoferrin IgG1 antibody (clone LF-2B8); or isotype control [rabbit polyclonal IgG negative control or mouse monoclonal IgG1 (MOPC21) isotype control] using media from BL2 cells [serum free-medium; 12 h incubation at 37°C (5% CO_2)]; n=3; *p<0.05 vs. isotype control; NS vs. fMLP anti-lactoferrin control. In all cases, addition of anti-human lactoferrin antibody

abrogated the inhibitory effect on neutrophil migration. fMLP, formyl-methionyl-leucyl-phenylalanine; hpf, high power field. Error bars indicate SEM.

Table 12.: ELISA Results from in house prepared antibodies			
Clone No	**Human Lactoferrin**	**Bovine Lactoferrin**	**Human Transferrin**
1	+	+	+
2	+	-	-
3	-	-	-
4	-	-	-
5	-	-	-
6	+	-	-
7	-	-	-
8	+	-	-
9	-	-	-
10	-	-	-
11	-	-	-
12	+	-	+
13	-	-	-
15	-	-	-
16	-	-	-
17	-	-	-
18	-	+	-
19	-	-	-
20	+	+	+
21	+	+	+
22	+	+	-
23	+	+	-
24	+	-	-
25	+	+	+
26	-	-	-
27	-	-	-
28	-	+	-
29	+	-	-
30	-	-	-
31	+	-	-
32	+	-	-
33	+	-	-
34	+	-	-
35	+	-	-
36	+	-	-
37	+	-	-
38	+	-	-
39	+	-	-
40	+	-	-

41	+	-	-
42	-	-	-
43	-	-	-
44	+	-	-
45	+	+	+
46	+	-	-
47	-	-	-
48	-	-	-
49	-	-	-
50	-	-	-
51	+	-	-
52	-	-	-
53	+	-	-
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66	-	-	-
67	+	-	-
68	-	-	-
69	+	-	+
70	-	-	-
71	-	-	-
72	+	+	+
73	-	-	-
74	+	+	-
76	+	+	+
77	-	-	-
78	-	-	-
79	+	+	+
80	-	-	-
81	-	-	-
+ = positive reactivity - = negative reactivity			

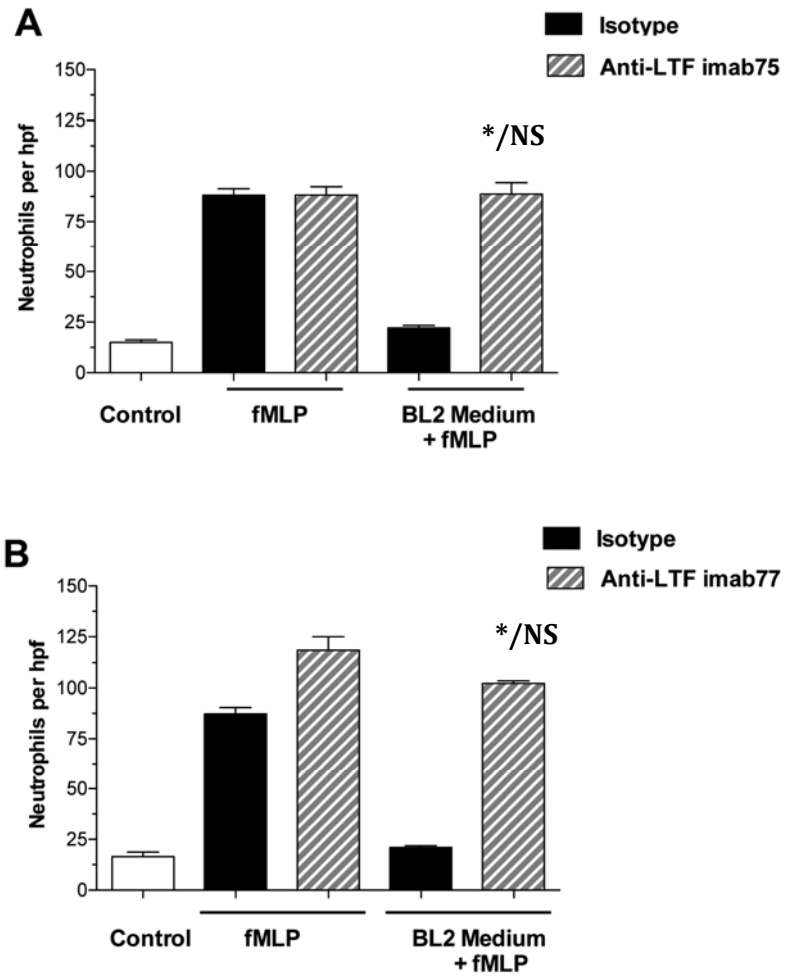


FIGURE 15: Lactoferrin specifically inhibited neutrophil chemotaxis.

Neutralisation experiments to determine neutrophil chemotaxis in the presence of **(A)** monoclonal anti-human imab75; n=3; *p<0.05 vs. isotype control; NS vs. fMLP anti-lactoferrin control and **(B)** imab77 IgG1 (in house prepared antibodies) or isotype control [mouse monoclonal IgG1 (MOPC21) isotype control] using media from BL2 cells [serum free-medium; 12 h incubation at 37°C (5% CO_2)]; n=3; *p<0.05 vs. isotype control; NS vs. fMLP anti-lactoferrin control. In all cases, addition of anti-human lactoferrin antibody abrogated the inhibitory effect on neutrophil

migration fMLP, formyl-methionyl-leucyl-phenylalanine; LTF, lactoferrin; hpf, high power field. Error bars indicate SEM.

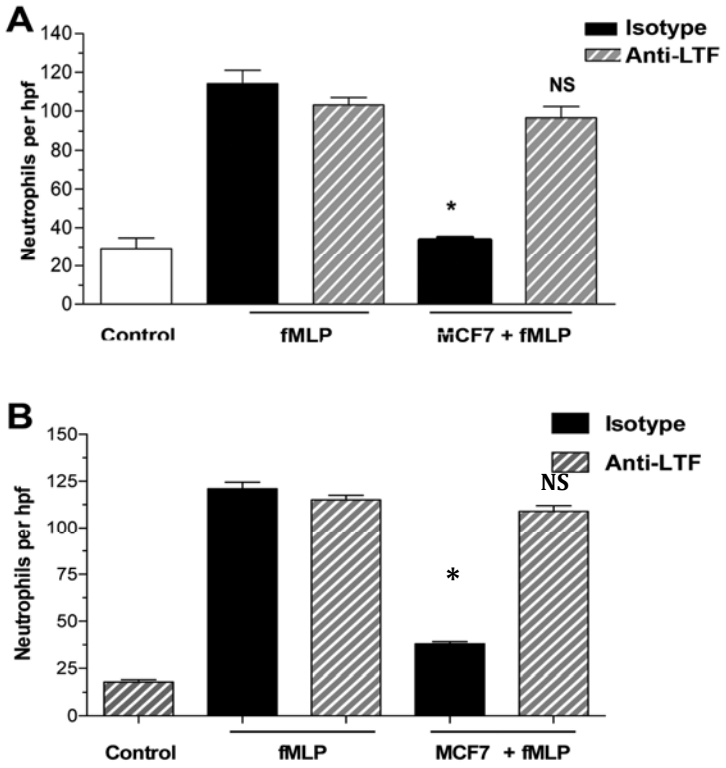


FIGURE 16: Lactoferrin specifically inhibited neutrophil chemotaxis.

Neutralisation experiments to determine neutrophil chemotaxis in the presence of **(A)** rabbit polyclonal anti-human lactoferrin IgG antibody; n=3; *p<0.001 vs. fMLP/isotype control; NS vs. fMLP/anti-lactoferrin control **(B)** mouse monoclonal anti-human lactoferrin IgG1 antibody (clone LF-2B8) or isotype control [rabbit polyclonal IgG negative control or mouse monoclonal IgG1 (MOPC21) isotype control] using media from MCF7-C3 cells [serum free-medium; 12 h incubation at 37°C (5% CO_2)]; n=4; *p<0.001 vs. fMLP/isotype control; NS vs. fMLP/anti-lactoferrin control. In all cases, addition of anti-human lactoferrin antibody (10 µg ml^{-1}) abrogated the inhibitory effect on neutrophil migration; fMLP, formyl-methionyl-leucyl-

phenylalanine; LTF, lactoferrin; hpf, high power field. Error bars indicate SEM.

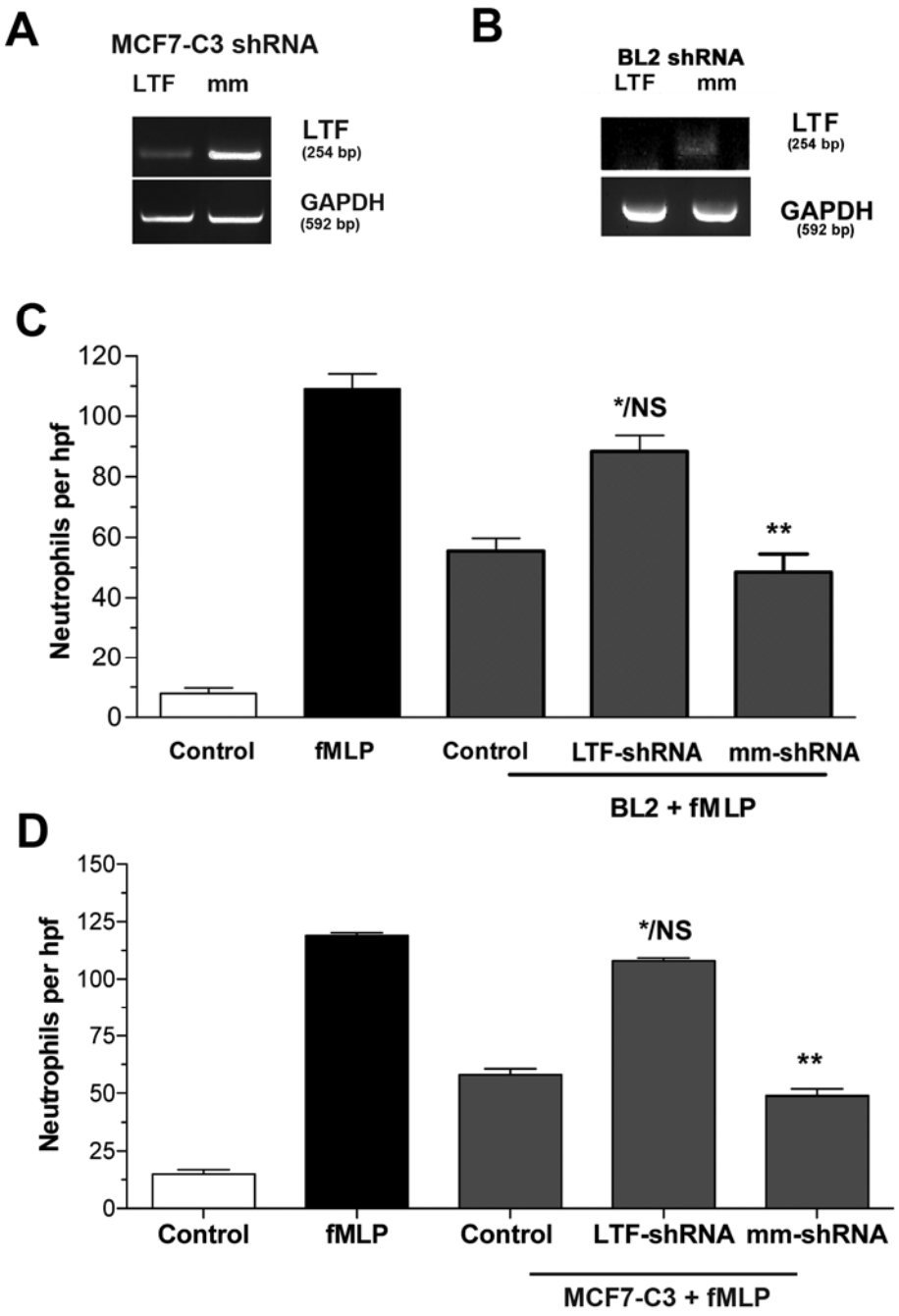

A
MCF7-C3 shRNA
LTF
mm
LTF
(254 bp)
GAPDH
(592 bp)
B
BL2 shRNA
LTF
mm
LTF
(254 bp)
GAPDH
(592 bp)
C
Neutrophils per hpf
120
100
80
60
40
20
0
*/NS
**
Control
fMLP
Control
LTF-shRNA
mm-shRNA
BL2 + fMLP
D
Neutrophils per hpf
150
125
100
75
50
25
0
*/NS
**
Control
fMLP
Control
LTF-shRNA
mm-shRNA
MCF7-C3 + fMLP

FIGURE 17: shRNA-mediated knockdown of lactoferrin expression abrogated its inhibitory effect on neutrophil migration.

RT-PCR analysis to assess lactoferrin expression in BL cells **(A)** and MCF7-C3 **(B)** stably expressing lactoferrin shRNA and mock-transfected (mm) cells that were induced to become apoptotic (BL2: 1 μM staurosporine; MCF7-C3: 100 μM etoposide; 37°C). Images are representative of three independent experiments. **(C)** Chemotaxis assay to determine neutrophil migration towards conditioned media obtained from control, lactoferrin shRNA and mock-transfected BL cells (88.7 ± 1.2 migrated neutrophils towards LTF-shRNA vs. 53.7 ± 1.8 towards mm-shRNA control) n=3, **p<0.05 compared with fMLP; NS=non-significant compared with fMLP control. **(D)** Chemotaxis assay to determine neutrophil migration toward conditioned media obtained from control, lactoferrin shRNA and mock-transfected MCF7-C3 cells n=3, **p<0.05 compared with fMLP; NS=non-significant compared with fMLP control; It was revealed that depletion of lactoferrin by an shRNA-mediated approach in both BL2 and MCF7- C3 cell line resulted in an abrogation of the observed inhibitory effect on neutrophil migration. fMLP, formyl-methionyl-leucyl-phenylalanine; LTF, lactoferrin; mm, mismatch control; hpf, high power field. Error bars indicate SEM.

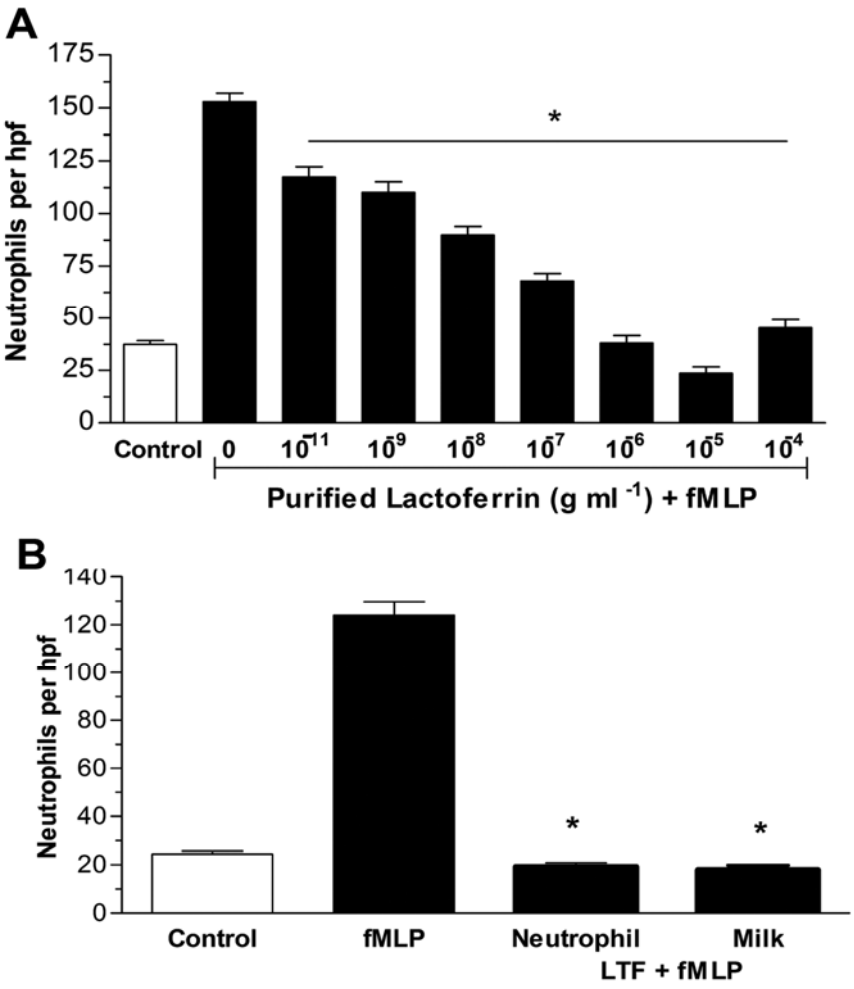


FIGURE 18: Lactoferrin specifically inhibited neutrophil chemotaxis irrespective of its source.

(A) Dose-response analysis of purified milk-derived human lactoferrin n=3 *p<0.05 vs. 0 g ml^{-1} purified lactoferrin +fMLP. **(B)** Neutrophil chemotaxis towards milk-derived or neutrophil-derived purified lactoferrin (10 µg ml^{-1}), assessed in the presence of fMLP n=3 **p<0.001 vs. fMLP; fMLP, formyl-methionyl-leucyl-phenylalanine; LTF, lactoferrin; hpf, high power field. Error bars indicate

SEM.

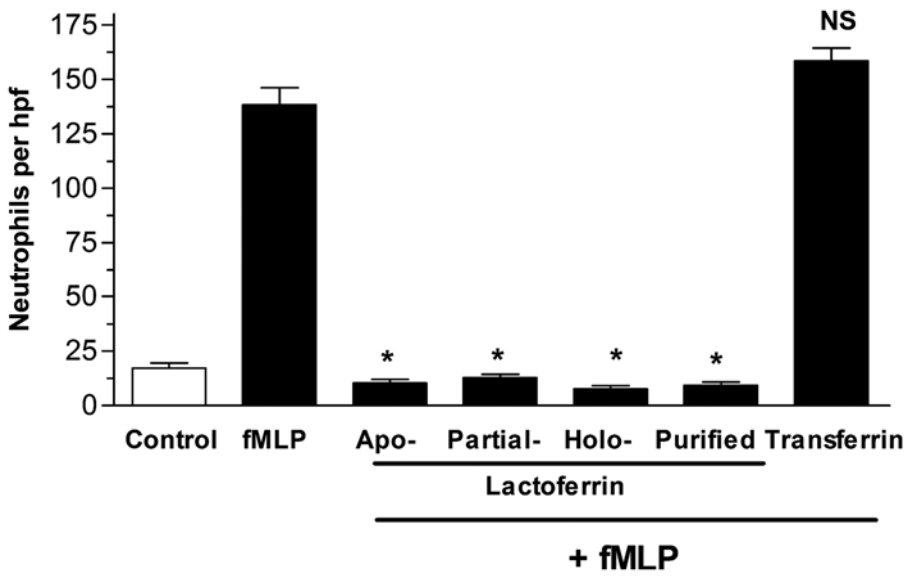


FIGURE 19: Inhibition of neutrophil chemotaxis towards lactoferrin was irrespective of its iron saturation status.

Chemotaxis assay to determine neutrophil migration towards purified recombinant iron-depleted (apo-), partially iron-saturated and fully iron-saturated (holo-) recombinant lactoferrin (10 µg ml^{-1}). Milk-purified lactoferrin and partially iron-saturated transferrin (10 µg ml^{-1}) were used as control. In all cases, neutrophil chemotaxis was inhibited towards lactoferrin in the presence of fMLP, despite its iron saturation status. However, neutrophil migration towards transferrin, a homologous protein, was not inhibited. n=4; *p<0.001 compared to fMLP control; fMLP, formyl-methionyl-leucyl-phenylalanine; LTF,

lactoferrin; hpf, high power field. Error bars indicate SEM.

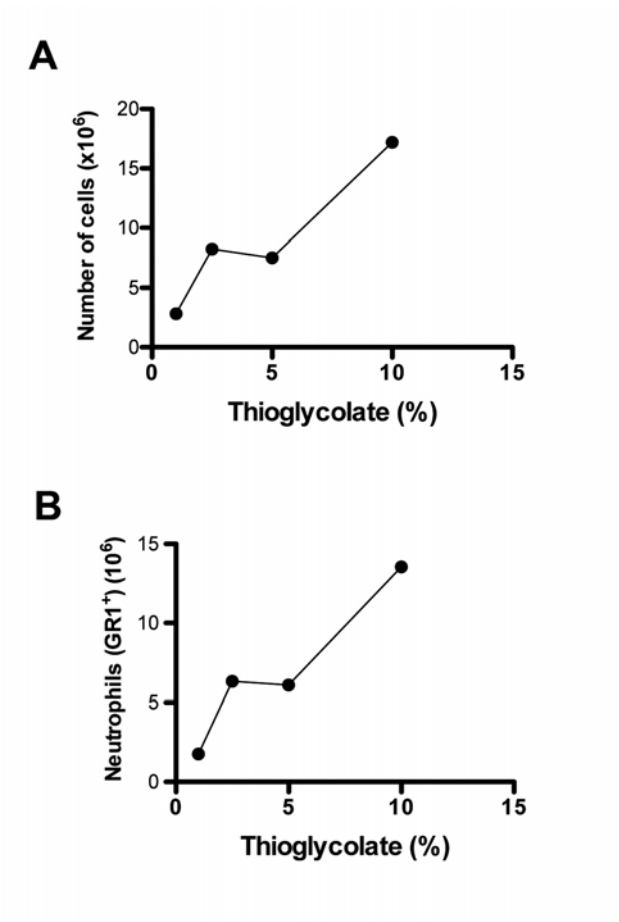


FIGURE 20: Thioglycollate elicited an inflammatory response in vivo.

Total cell number harvested from peritoneal lavage after stimulation with different doses of thioglycollate (1%, 2.5%, 5% and 10%). Mice (n=2 per thioglycollate dose) were injected with the indicated doses of thioglycollate and recruited leukocytes were harvested after 4 h by peritoneal lavage. **(A)** Total cell number was determined using a NucleoCounter™, which excluded non-nucleated cells and results were confirmed using Flow-Count beads™; n=1 **(B)** Neutrophil number ($GR1^+$) was determined by cytospin staining and GR1 immunolabelling (PE-conjugated rat anti-

mouse Ly-6G or PE-conjugated rat IgG2b isotype control) n=1.

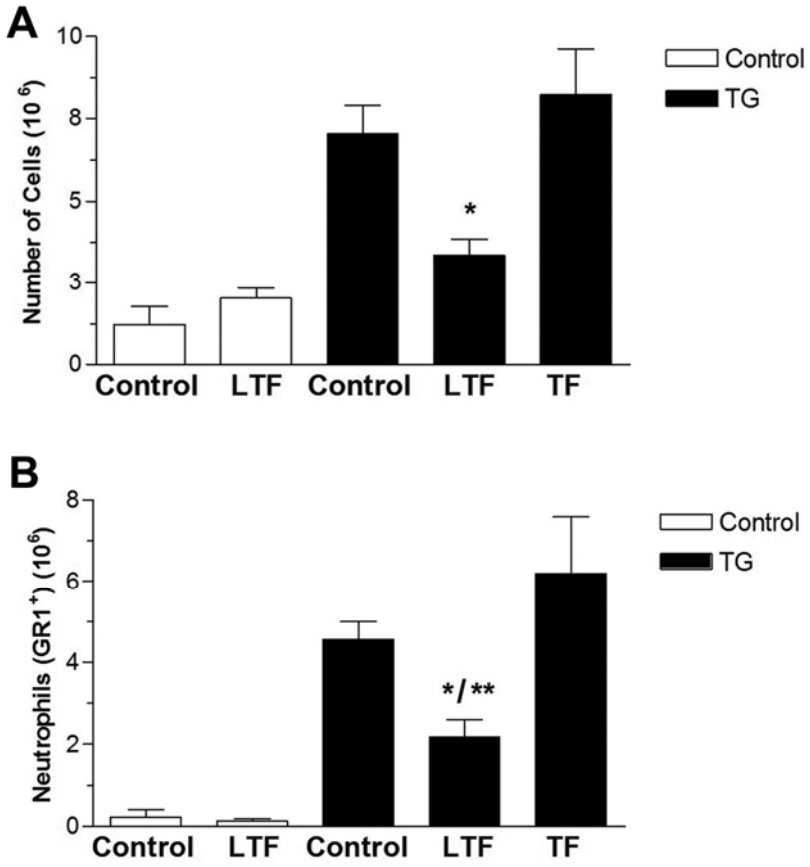


FIGURE 21: Lactoferrin inhibited neutrophil migration in vivo.

(A) Total cell or **(B)** neutrophil number ($GR1^+$) obtained from peritoneal lavage following pre-treatment with lactoferrin. In detail, mice (n=7 mice per group) were injected with human lactoferrin (10 µg ml^{-1}), human transferrin (10 µg ml^{-1}) or saline alone (control), followed by a second injection with 1% thioglycollate 20 min after the first injection. Recruited leukocytes were harvested after 4 h by peritoneal lavage. Lactoferrin reduced specifically the proportion and number of neutrophils ($GR1^+$) migrating into the peritoneal cavity *p < 0.05 vs. transferrin; **p < 0.01 vs. transferrin

control. TG, thioglycollate; LTF, lactoferrin; TF, transferrin. Error bars indicate SEM.

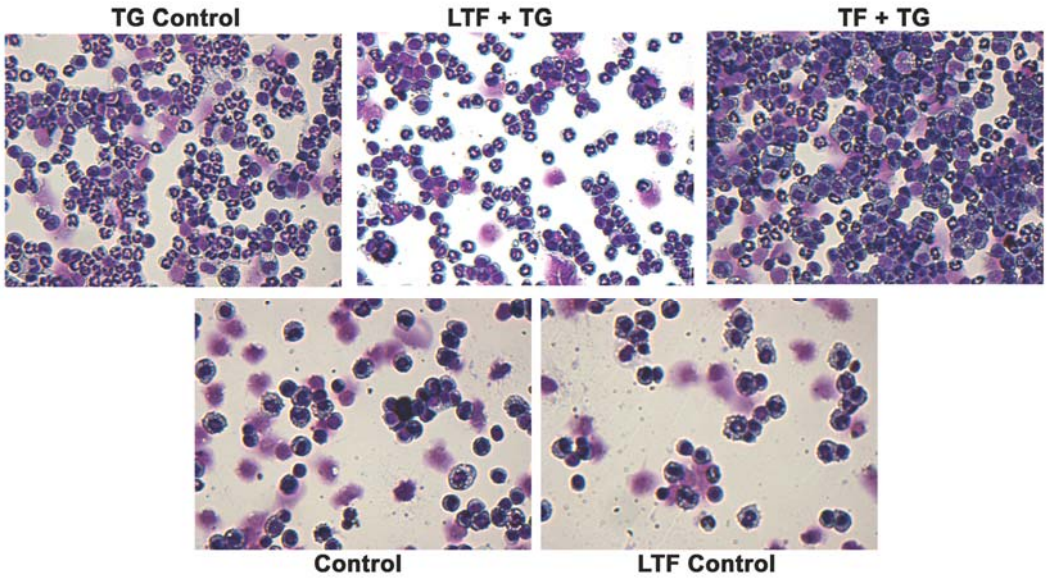


FIGURE 22: Lactoferrin inhibited neutrophil migration in vivo.

Characteristic cytospin images obtained from peritoneal lavage. Mice (n=7 mice per group) were injected with human lactoferrin (10 µg ml^{-1}), human transferrin (10 µg ml^{-1}) or saline alone (control), followed by a second injection with 1% thioglycollate 20 min after the first injection. Lactoferrin reduced specifically the proportion and number of neutrophils migrating into the peritoneal cavity but did not affect recruitment of other types of leukocytes in response to thioglycollate. TG, thioglycollate; LTF,

lactoferrin; TF, transferrin. Original magnification, ×400, top; ×200, bottom.

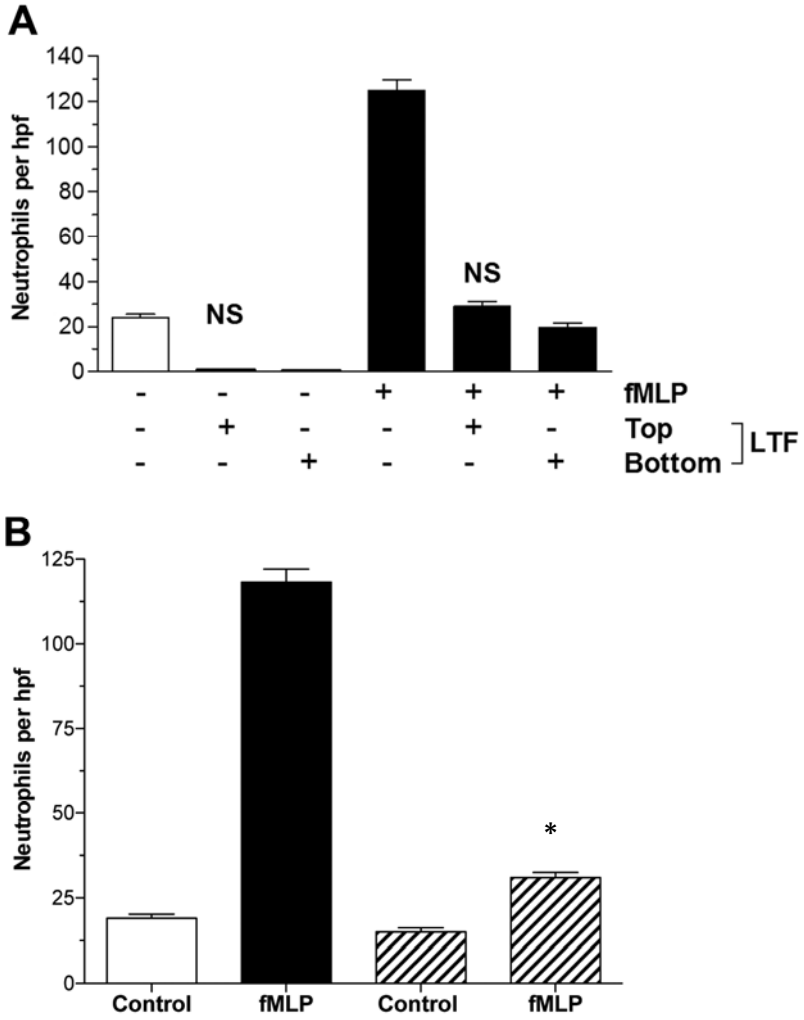


FIGURE 23: Lactoferrin acted by inhibiting neutrophil migration and not exerting a chemorepulsive effect.

(A) Chemotaxis assay to determine neutrophil migration in the presence of lactoferrin (10 µg ml^{-1}) in the top or bottom compartment of the Transwell insert n=3; NS vs. corresponding lactoferrin (bottom+) controls. **(B)** Chemotaxis assay towards fMLP or negative control to determine the migration of neutrophils that were pre-incubated in the presence (dashed columns) or absence of milk-derived lactoferrin (10 µg ml^{-1}; 40 min; 37°C) and washed with PBS prior to use in the assay n=3 *p<0.001 vs. fMLP. Results revealed that lactoferrin did not exert a chemorepulsive effect on neutrophils and the observed inhibitory effect was maintained in lactoferrin pre-treated neutrophils following the removal of lactoferrin. fMLP, formyl-methionyl-leucyl-phenylalanine; LTF, lactoferrin; hpf, high power field. hpf, high power field. Error bars indicate SEM.

Lactoferrin inhibits neutrophil chemotaxis towards a range of chemoattractants without interfering or impairing the chemoattractant activity

Till now, the results of this study suggested that lactoferrin binds to neutrophils and inhibits their ability to undergo chemotaxis. To exclude the possibility that the observed inhibitory activity of lactoferrin was due to its ability to bind to chemoattractants modulating their activity, additional chemotaxis assays were performed to assess the effect of lactoferrin on neutrophil migration in the presence of other chemoattractants such as C5a, IL-8 and LTB_4. It was revealed that with all these chemoattractants, lactoferrin inhibited neutrophil migration, strongly suggesting that it does not directly interfere with the chemoattractant activity **(Figure 24A)**. Moreover, to examine whether lactoferrin functionally neutralises the chemoattractant efficiency, additional chemotaxis assays were performed using chemoattractants (fMLP, C5a, IL-8) that were preabsorbed with lactoferrin. To achieve this, chemoattractants were pre-incubated with lactoferrin for 30 min at 37°C in serum-free chemotaxis medium. Subsequently, anti-lactoferrin antibody was used to remove the lactoferrin with the aid of IgG-coated magnetic beads that capture lactoferrin. As shown in **Figure 24B**, no difference in neutrophil chemotactic activity was observed between the control and lactoferrin-absorbed chemoattractants, which excludes the possibility that

lactoferrin binds to and alters the activity of the chemoattractants.

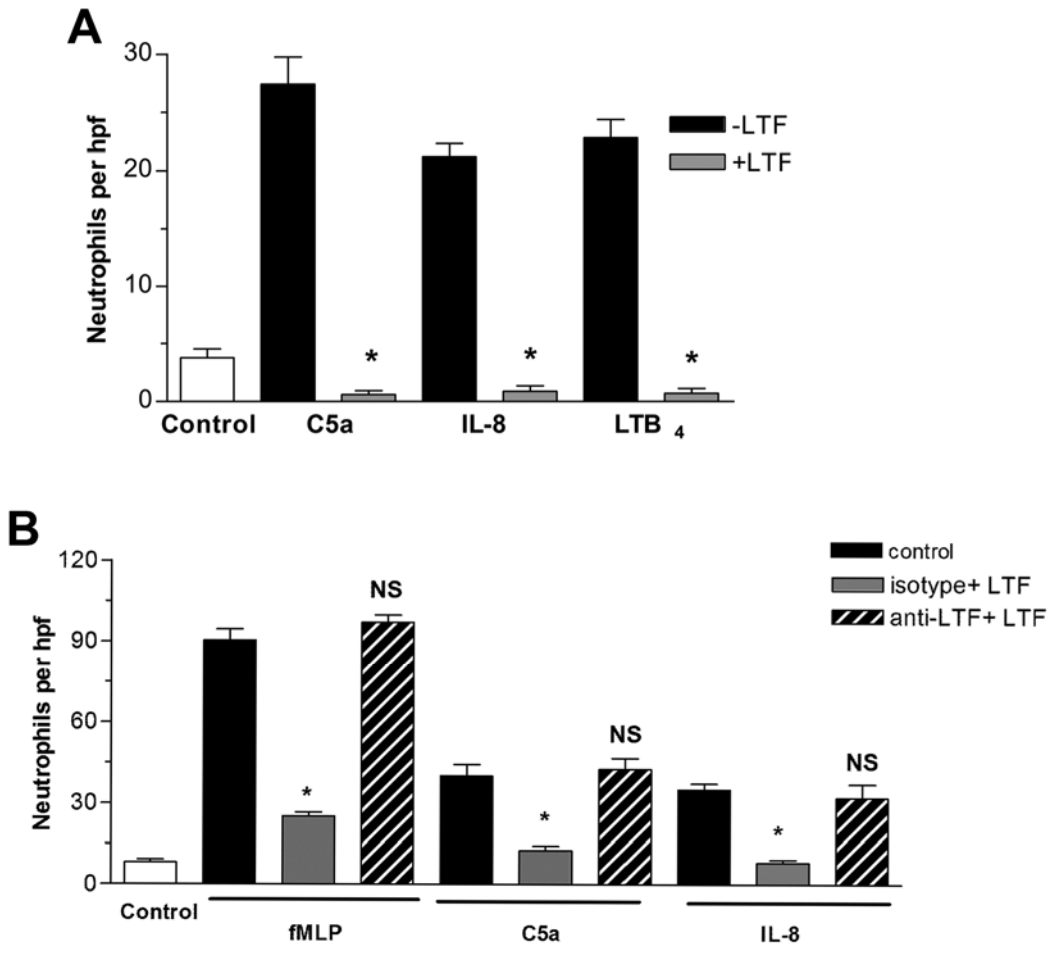


FIGURE 24: Neutrophil chemotaxis towards lactoferrin was irrespective of the chemoattractant used.

(A) Neutrophil chemotaxis towards different chemoattractants: C5a (726 nM), IL-8 (100 nM), LTB_4 (50 nM). n = 3; *p< 0.05. **(B)** Neutrophil chemotaxis toward chemoattractants (control) or chemoattractants that were incubated with lactoferrin (10 μg ml^{-1}) followed by the addition of isotype or anti-lactoferrin monoclonal antibody (10 μg ml^{-1}). Antibodies were removed using magnetic IgG beads. n = 3; *p < 0.05, NS= non-significant vs. chemoattractant control; fMLP, formyl-methionyl-leucyl-phenylalanine; C5a, complement 5a; IL-8, interleukin-8;

LTB_4, leukotriene B_4; LTF, lactoferrin; hpf, high power field. Error bars indicate SEM.

The inhibitory activity of lactoferrin is specific to granulocyte migration

To determine whether the migration-inhibitory effects of lactoferrin were specific to neutrophils among professional phagocytes, its effects on monocyte and macrophage migration *in vitro* was analysed. As shown in **Figures 25A and 25B**, C5a-induced chemotaxis of mononuclear phagocytes was unimpaired by lactoferrin. However, in terms of eosinophil migration, lactoferrin exerted a significant inhibitory effect on eosinophil chemotaxis.

In detail, the migration of eosinophils towards milk-derived purified or towards neutrophil-derived lactoferrin was first determined and it was found that both milk-derived and neutrophil-derived lactoferrin exerted inhibitory effects on eotaxin-induced eosinophil migration (mean number of migrated eosinophils: 21.7 ± 1.07 towards neutrophil-derived lactoferrin; 27.6 ± 1.65 towards milk-derived lactoferrin in the presence of eotaxin (100 nM) vs. 50.8 ± 2.06 towards eotaxin control) **(Figure 26A)**. Furthermore, lactoferrin purified from human milk displayed a concentration-dependent inhibitory activity toward eosinophil migration in response to eotaxin as well as towards other chemoattractants such as fMLP, C5a and LTB_4 to similar levels **(Figures 26B and 27A)**. Moreover, as

in neutrophils, lactoferrin exerted no toxic effects on eosinophils, as assessed by Anx/PI staining of control and lactoferrin-treated eosinophils. In addition, lactoferrin (10 μg ml^{-1}) did not promote neutrophil or eosinophil apoptosis when the cells were cultured over a 24- or 48-h period **(Figure 28)**. Whether lactoferrin acted by inhibiting eosinophil migration directly or by promoting eosinophil repulsion was next investigated. In chemotaxis assays, in which lactoferrin was added to the upper chamber along with eosinophils, an inhibition of eosinophil migration towards eotaxin and control medium was observed, suggesting that lactoferrin, similar to neutrophils, exerts a direct effect on eosinophils by inhibiting their migratory ability and not by forcing them to migrate in all directions away from the chemoattractant **(Figure 27B)**.

Furthermore, subsequent chemotaxis assays were performed in which the inhibitory activities of iron-depleted (apo-form), partially iron-saturated or fully iron-saturated lactoferrin (holo-form) were compared. It was found that the levels of iron saturation were not related to the levels of inhibition in eosinophil migration **(Figure 29A)**. Eosinophils were also induced to migrate towards eotaxin in the presence of transferrin and it was clearly demonstrated that transferrin, in stark contrast to lactoferrin, displayed no inhibitory effect on eosinophil chemotaxis, providing in this way strong evidence that the observed inhibition is lactoferrin-specific and does not require iron-chelating activity [mean number of migrated

eosinophils: 31.3 ± 1.8 towards lactoferrin in the presence of eotaxin (100 nM); 48.2 ± 1.9 towards transferrin vs. 50.3 ± 2.3 mean number of migrated eosinophils towards eotaxin control **(Figure 29B)**.

In summary, it was found that lactoferrin specifically exerts an inhibitory effect on granulocyte migration. This effect was irrespective of the source of lactoferrin (milk- or neutrophil-derived), as both types presented a significant inhibition in neutrophil migration. Also, it was found to be irrespective of the iron-binding properties or the iron-saturation status of lactoferrin.

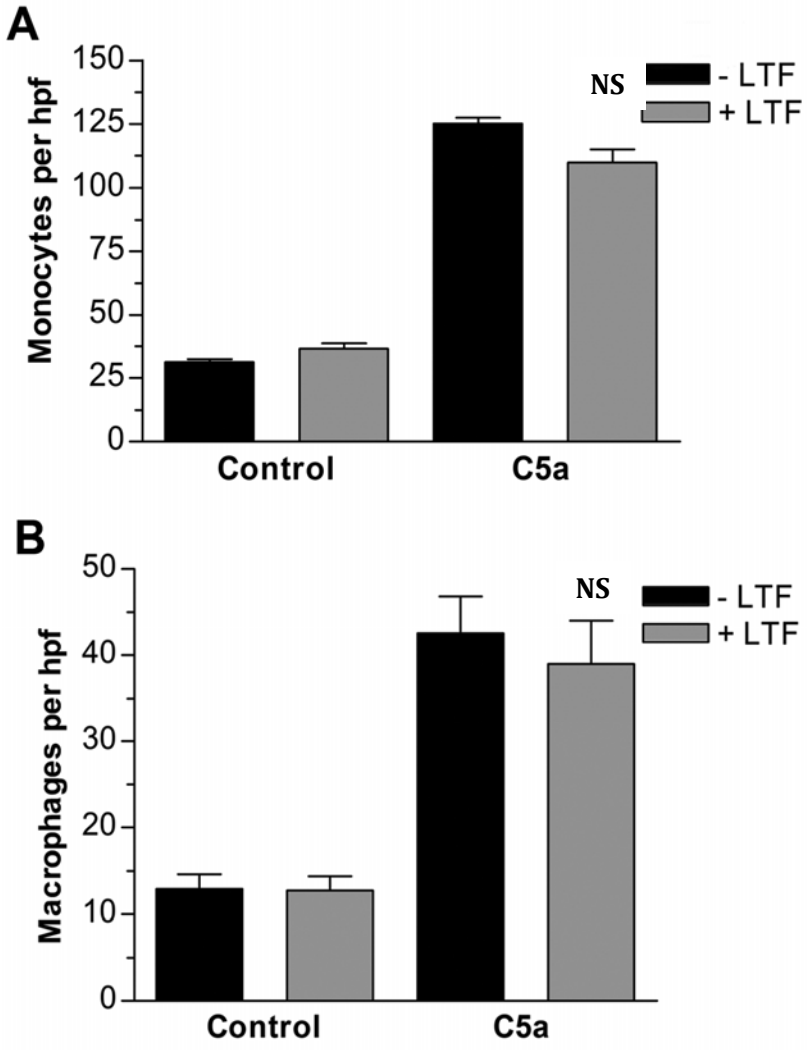


FIGURE 25: Lactoferrin had no effect on monocyte and/or macrophage chemotaxis .

Chemotaxis assays to determine the migration of **(A)** monocytes; n=3; NS=non-significant vs. C5a control or **(B)** macrophages towards C5a (6.25 ng ml^{-1}) in the presence of lactoferrin (10 µg ml^{-1}); n=3; NS=non-significant vs. C5a control. Results showed that lactoferrin exerted no inhibitory effect on the chemotaxis of these cell types. ; C5a, complement 5a; LTF, lactoferrin; hpf, high power field. Error bars indicate SEM.

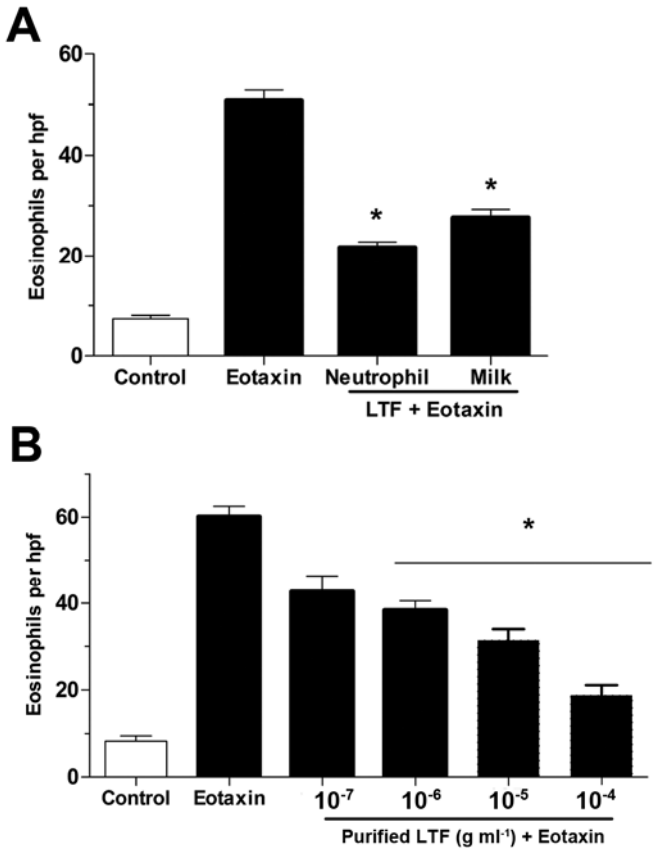


FIGURE 26: Lactoferrin inhibited eosinophil chemotaxis.

(A) Chemotaxis assay to determine eosinophil migration towards milk-derived or neutrophil-derived lactoferrin (10 µg ml^{-1}) in the presence of eotaxin (100 nM) n=4, *p<0.05 vs. eotaxin control **(B)** Eosinophil chemotaxis towards eotaxin (100 nM) in the presence of varying concentrations of purified human lactoferrin; n=3; *p<0.05 vs. eotaxin control. Results showed an inhibition in the eotaxin-induced migration of eosinophils towards lactoferrin. LTF,

lactoferrin; hpf, high power field. Error bars indicate SEM.

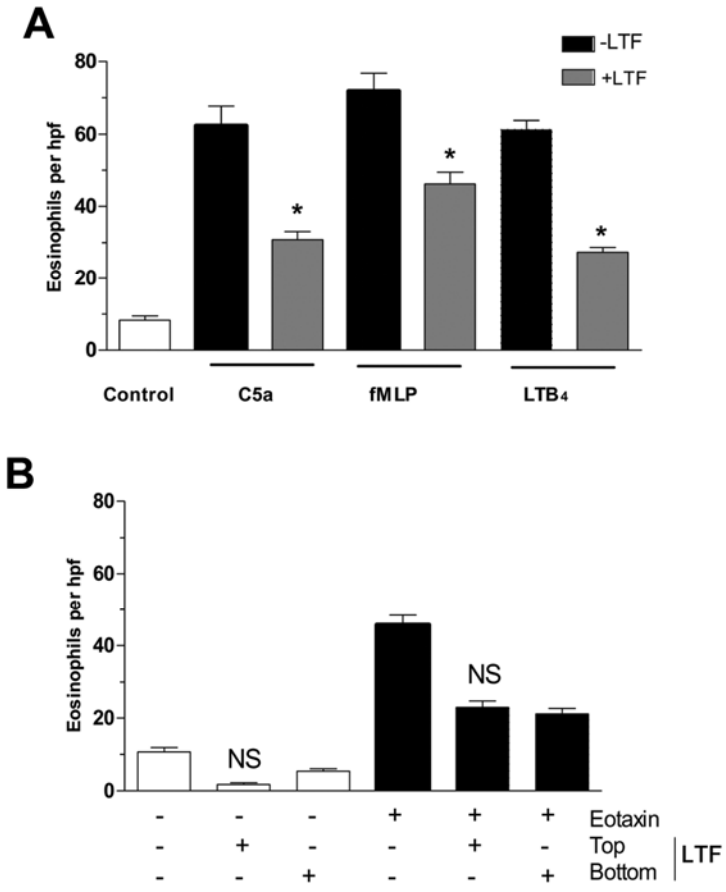


FIGURE 27: Lactoferrin inhibited eosinophil migration irrespective of the chemoattractant used.

(A) Chemotaxis assay to determine eosinophil migration towards the chemoattractants fMLP (100 nM), C5a (726 nM) and LTB_4 (50 nM) in the presence of milk-purified lactoferrin (10 µg ml^{-1}) n=3; *p<0.05 vs. corresponding positive control. **(B)** Eotaxin-induced chemotaxis assay in the presence of lactoferrin (10 µg ml^{-1}) in the top or bottom compartment of the Transwell insert n=3; NS=non-significant vs. corresponding lactoferrin (bottom +) controls; fMLP, formyl-methionyl-leucyl-phenylalanine;

C5a, complement 5a; LTB_4, leukotriene B_4; LTF, lactoferrin; hpf, high power field. Error bars indicate SEM.

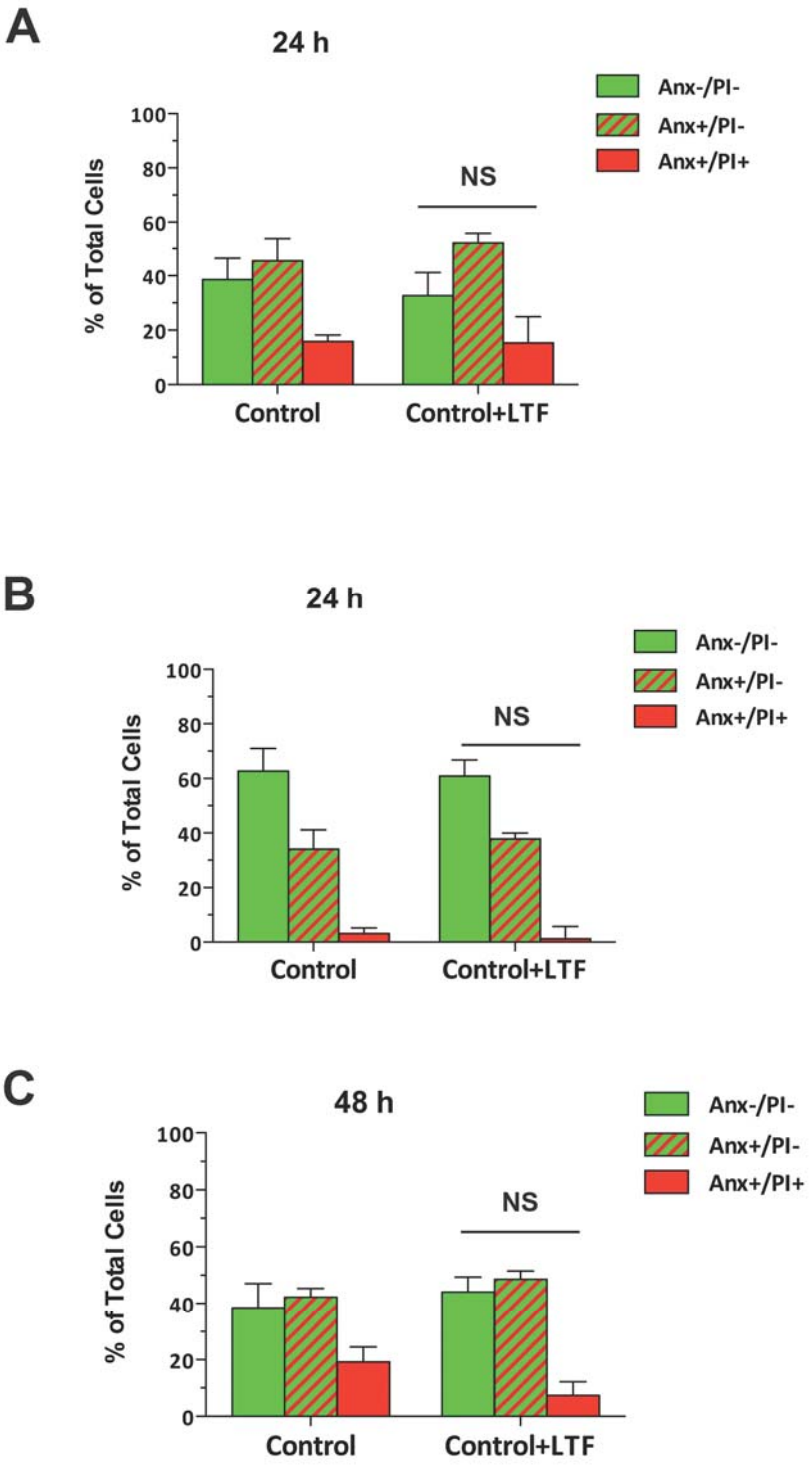

A
24 h
Anx-/PI-
Anx+/PI-
Anx+/PI+
% of Total Cells
100
80
60
40
20
0
NS
Control
Control+LTF
B
24 h
Anx-/PI-
Anx+/PI-
Anx+/PI+
% of Total Cells
100
80
60
40
20
0
NS
Control
Control+LTF
C
48 h
Anx-/PI-
Anx+/PI-
Anx+/PI+
% of Total Cells
100
80
60
40
20
0
NS
Control
Control+LTF

Figure 28: Lactoferrin did not promote neutrophil and/or eosinophil apoptosis.

Measurement of apoptosis levels in neutrophils after 24 h **(A)** or eosinophils after 24 h **(B)** and after 48 h cultured in the presence (Control + LTF) or absence (Control) of purified human lactoferrin (10 µg ml^{-1}), as assessed by Anx/PI staining; n=3 for A, B and C; NS=non-significant compared to control cells (no lactoferrin); LTF, lactoferrin. Error bars indicate SEM.

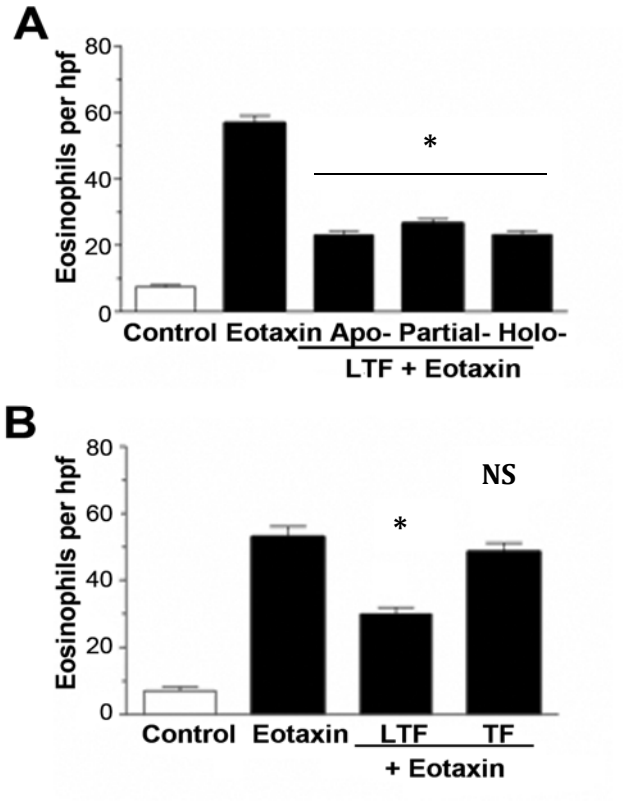


FIGURE 29: The effect of lactoferrin on eotaxin-induced chemotaxis occurred irrespective of its iron-saturation status.

Chemotaxis assay to determine **(A)** eosinophil migration towards eotaxin (100 nM) in the presence of recombinant iron-depleted (Apo-), partially iron saturated and fully iron-saturated (Holo-) recombinant lactoferrin (10 µg ml^{-1}); n=3 *$p<0.05$ vs. eotaxin control and **(B)** eosinophil migration towards eotaxin (100 nM) in the presence of purified human lactoferrin (10 µg ml^{-1}) or purified human transferrin (TF, 10 µg ml^{-1}); n=3 *$p<0.05$ and NS, non-significant vs. eotaxin control. Results showed that the inhibitory effect exerted by lactoferrin on eosinophil migration was irrespective of the iron-saturation status or the iron-binding properties of the protein. LTF,

lactoferrin; hpf, high power field. Error bars indicate SEM.

Lactoferrin impairs neutrophil activation morphology

Neutrophil migration involves activation, adhesion and extravasation; processes that are accompanied by gross changes in cell morphology (Servant *et al.*, 2000). While non-activated neutrophils are rounded, activated neutrophils acquire a polarised morphology with spreading and adhesion to the available substratum. In order to initially assess the effects of lactoferrin on the acquisition of neutrophil activation a time-lapse video microscopy of neutrophils was performed and recorded directly their activation morphology, cell spreading and locomotion. During a 1-h time course, lactoferrin pre-treated neutrophil populations (10 µg ml^{-1}; 40 min at 37°C) stimulated with fMLP (1 µM) displayed a greater proportion of non-adherent cells with a rounded, non-activated morphology as compared with neutrophils treated with fMLP alone **(Figure 30A)**. These quantitative differences between lactoferrin-treated and untreated neutrophils stimulated with fMLP were reflected in the locomotion of the cells around the substratum, with lactoferrin-treated cells displaying markedly reduced movement (for example, at 30 min: about 25.0% non-polarised neutrophils after lactoferrin pre-treatment, 49.0% non-polarised neutrophils without lactoferrin pre-treatment; -at 60 min: 36.0% non-polarised neutrophils after lactoferrin pre-treatment, 68.0% non-polarised neutrophils without lactoferrin pre-treatment) **(Figure 30B)**.

Changes in cell morphology following stimulation with fMLP or other neutrophil agonists are characterised by a rapid increase in intracellular cytoplasmic calcium levels through mobilisation of calcium from ER stores and activation of calcium influx channels of the plasma membrane, mediated by the inositol triphosphate (IP_3) and diacylglycerol/phospholipase C (DAG/PLC) pathways (Lew *et al.*, 1984; Nigam *et al.*, 1992; Pozzan *et al.*, 1983). In order to further determine whether the observed cell shape alterations following lactoferrin treatment are related to changes in intracellular calcium concentrations ($[Ca^{2+}]_i$), the levels of $[Ca^{2+}]_i$ in control and lactoferrin-treated cells were measured in response to fMLP stimulation (1 nM or 10 nM). No changes were observed in the fMLP-mediated $[Ca^{2+}]_i$ response between control and lactoferrin-treated neutrophils, as in both cases, a maximum $[Ca^{2+}]_i$ increase of 300 nM at approximately 55 s was noted after stimulation with 10 nM fMLP. These findings suggest that lactoferrin acts downstream or independently of the mechanisms involved in intracellular calcium flux **(Figure 31)**. In general, it was found that the inhibitory effect of lactoferrin on neutrophil migration was linked to the impaired activation morphology of neutrophils, providing in this way initial insights on its mode of activity.

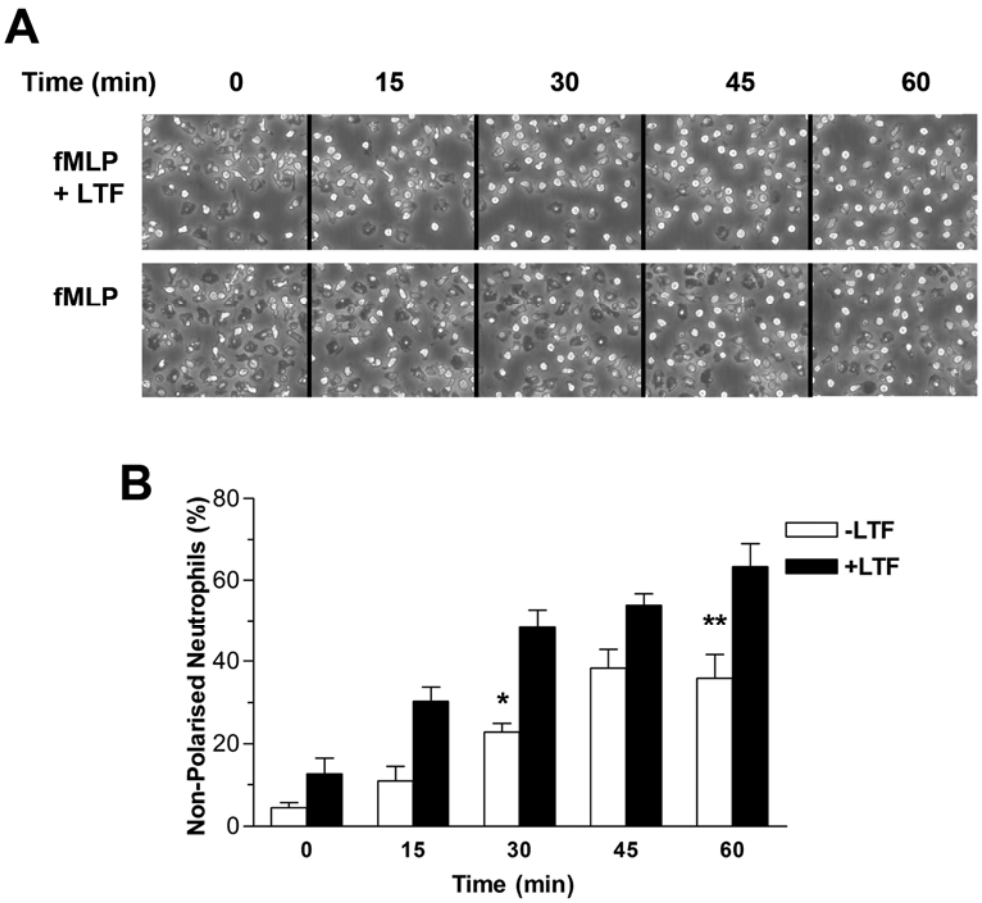


FIGURE 30: Effect of lactoferrin on neutrophil polarisation morphology and spreading.

(A) Time-lapse video microscopy frames of control or lactoferrin pre-treated neutrophils (10 µg ml^{-1}; 40 min at 37°C) stimulated with 1 µM fMLP over a 1-h incubation time course. n=2; Original magnification ×400. **(B)** Quantification of neutrophils (non-polarised) counted from five different fields n=2; *p < 0.05, **p< 0.01 vs. corresponding +LTF control. Results showed that lactoferrin acted by impairing the acquisition of the activation morphology of neutrophils. fMLP, formyl-

methionyl-leucyl-phenylalanine; LTF, lactoferrin. Error bars indicate SEM.

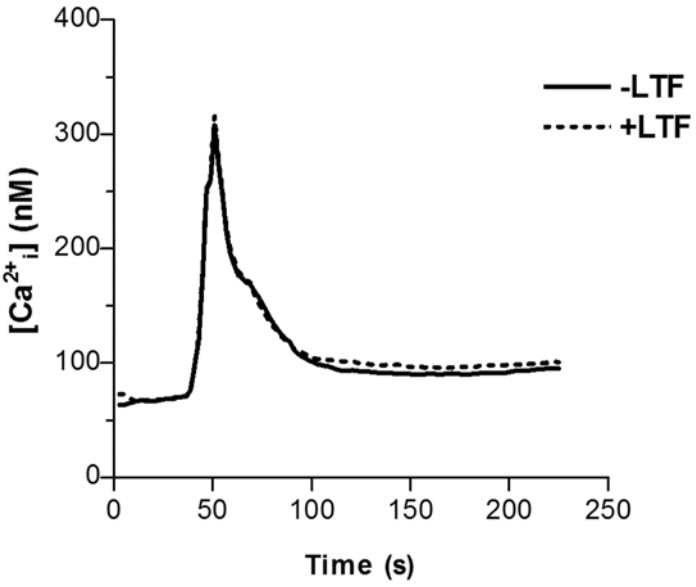


FIGURE 31: Lactoferrin does not impair intracellular calcium levels in neutrophils following activation.

Representative plot (n=3) showing measurement of $[Ca^{2+}]_i$ levels in neutrophils incubated in the presence or absence of lactoferrin (10 μg ml^{-1}; 30 min at 37°C) followed by stimulation with fMLP (10 nM). Results revealed that lactoferrin did not alter the $[Ca^{2+}]_i$ levels in neutrophils activated by fMLP.

Lactoferrin impairs the activation status of neutrophils and affects downstream signalling cascades involved in their activation

As lactoferrin was demonstrated to prevent neutrophil migration and the acquisition of polarised activation morphology, it was next explored whether lactoferrin could also affect the neutrophil activation state. To this end, the expression of two known neutrophil activation-associated markers, CD62L (L-selectin) and CD11b, was measured using two-colour flow cytometry. Upon activation, CD62L is cleaved from the neutrophil surface, whereas CD11b expression is upregulated following translocation from cytoplasmic granules to the cell membrane. Freshly isolated neutrophils were pre-treated with lactoferrin and then exposed to the activation stimuli fMLP (100 nM), TNF-α (1 ng ml^{-1}) and PMA (100 nM). As shown in **Figures 32 and 33**, it was found that, in lactoferrin-treated neutrophils compared with control cells, CD62L expression was significantly higher, whereas CD11b levels were lower. These effects were common to all activation stimuli used. Transferrin-treated neutrophils were also included but showed no significant differences compared with control cells. It is noteworthy that the lactoferrin effect was also evident when PMA, a specific PKC activator, was used as an agonist, indicating that lactoferrin acts downstream of PKC and not on pathways involved in PKC activation and $[Ca^{2+}]_i$ responses, such as the IP_3 and DAG/PLC pathways.

This finding prompted to investigate putative downstream targets of PKC involved in the late signalling cascades following neutrophil activation that also regulate cell motility and actin reorganisation. Such cascades involve the activation of MAP family kinases (Szczur *et al.*, 2006) and the phosphorylation status of p44/42 (ERK1 and ERK2) MAPKs was therefore examined. Whereas in untreated neutrophils, ERK1 and ERK2 were phosphorylated following fMLP stimulation (100 nM), lower levels of phosphorylated ERK1/2 were observed in neutrophils that had been pre-treated with lactoferrin prior to stimulation with fMLP **(Figure 34A).** Collectively, these data suggest that lactoferrin has a clear impact on neutrophil activation, including impairment of neutrophil degranulation, inhibition of expression of β_2 integrins and reduction of activation of intracellular kinases with profound effects on cell migration and motility.

Lactoferrin has been shown previously to bind to receptors on mononuclear phagocytes and to inhibit proinflammatory responses via NF-κB (Birgens *et al.*, 1983; Haversen *et al.*, 2002; Van Snick and Masson, 1976). However, the involvement of the NF-κB pathway in the inhibition of neutrophil migration seems unlikely, since lactoferrin also exerted an inhibitory effect on the chemotaxis of neutrophils treated with gliotoxin (2 ng ml^{-1}), a specific NF-κB inhibitor **(Figure 34B).** In addition, no changes in the levels of IκB, an inhibitor of NF-κB that is degraded following NF-κB activation, were observed in Western blot

analysis of fMLP-stimulated neutrophils treated with or without lactoferrin **(Figure 34C).** In general terms, it was found that the inhibitory effect of lactoferrin on neutrophil migration was linked to the impairments in the activation status of neutrophils and in particular, in downstream signalling molecules involved in neutrophil activation, such as the phosphorylation of MAP kinases.

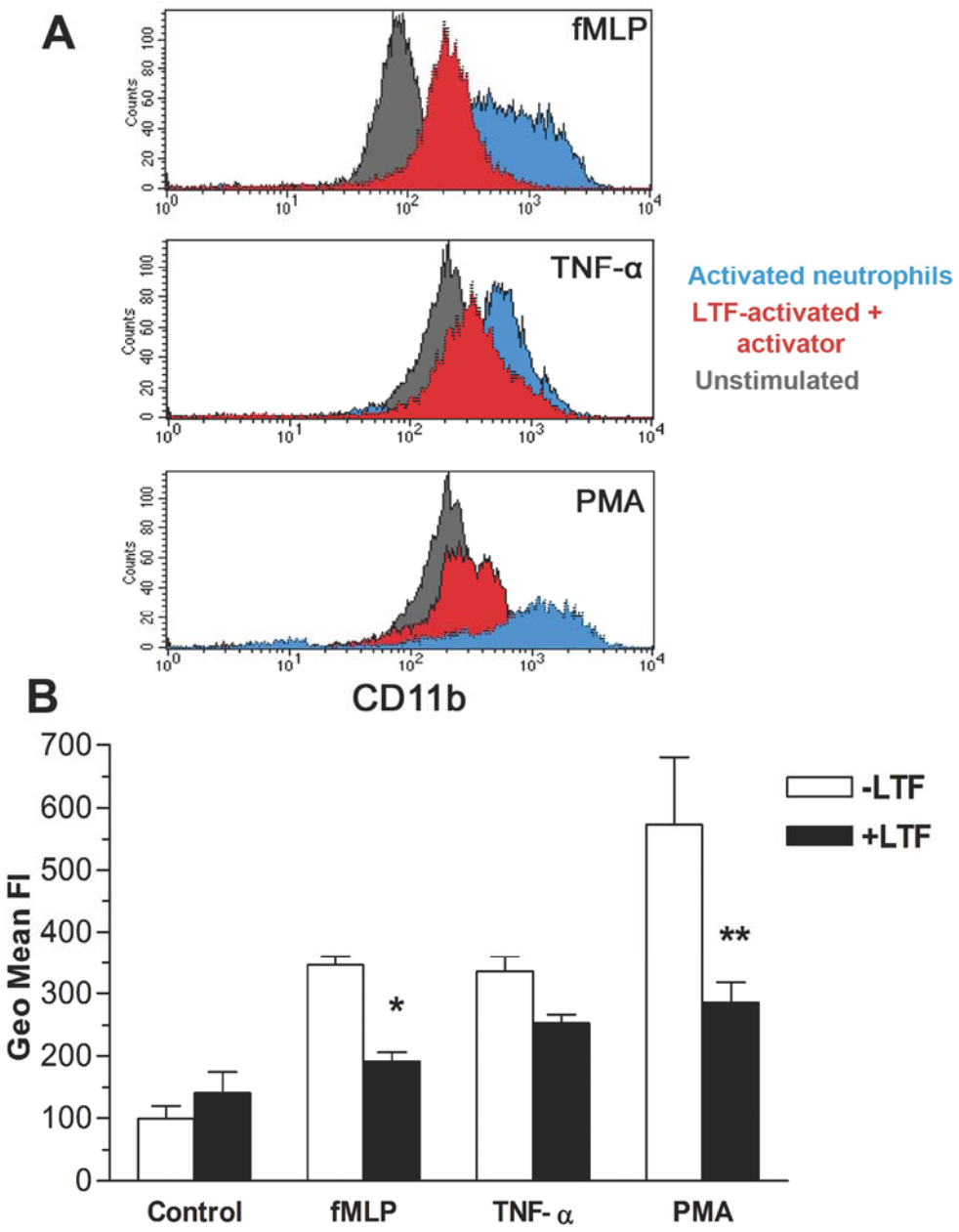
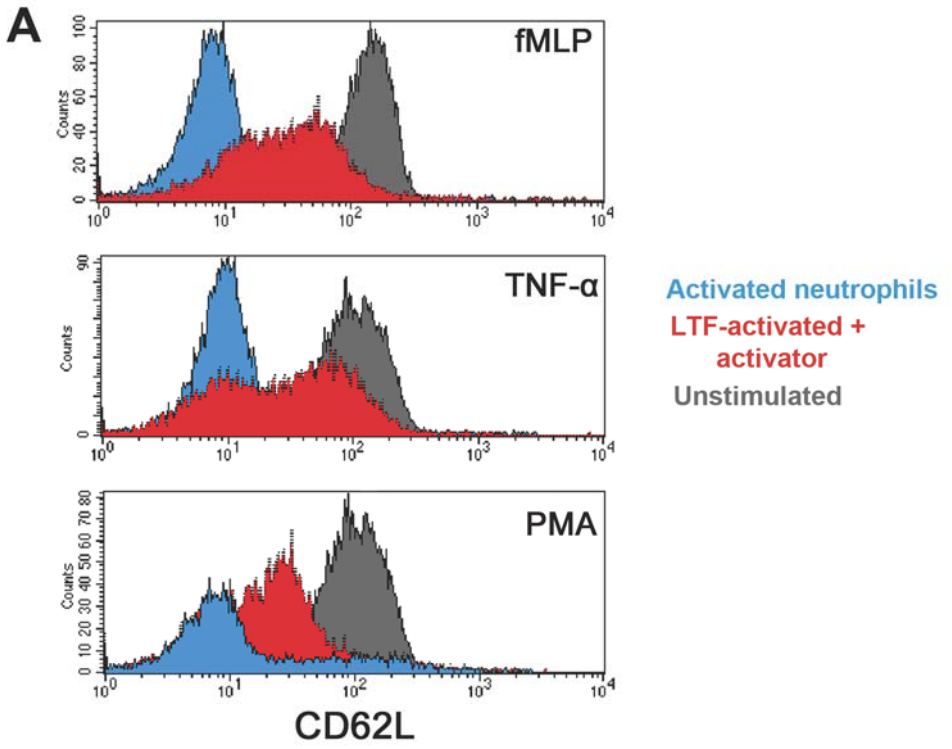

A
fMLP
TNF-α
PMA
Counts
Activated neutrophils
LTF-activated + activator
Unstimulated
CD11b
B
Geo Mean FI
-LTF
+LTF
Control
fMLP
TNF- α
PMA
*
**

FIGURE 32: Effect of lactoferrin on neutrophil activation status.

The expression of CD11b was assessed in fMLP- (100 nM), TNF-α- (1 ng ml^{-1}), or PMA-stimulated (100 nM) neutrophils (30 min at 37°C) that were pre-incubated (40 min at 37°C) in the presence or absence of lactoferrin (10 μg ml^{-1}) n=3. **(A)** Representative flow cytometry overlays of CD11b expression in control (gray) and stimulated neutrophils (lactoferrin-treated: red; untreated: blue). n = 3. **(B)** Flow cytometry results showed lower levels of CD11b expressed i.e. lower activation status, in neutrophils pre-incubated with lactoferrin followed by agonist stimulation, compared to control activated neutrophils n = 3; *p < 0.05, **p < 0.01. fMLP, formyl-methionyl-leucyl-phenylalanine; TNF-α, tumour necrosis factor-α, PMA, phorbol myristate acetate; LTF, lactoferrin. Error bars indicate SEM.


A
fMLP
TNF-α
PMA
Counts
CD62L
Activated neutrophils
LTF-activated + activator
Unstimulated

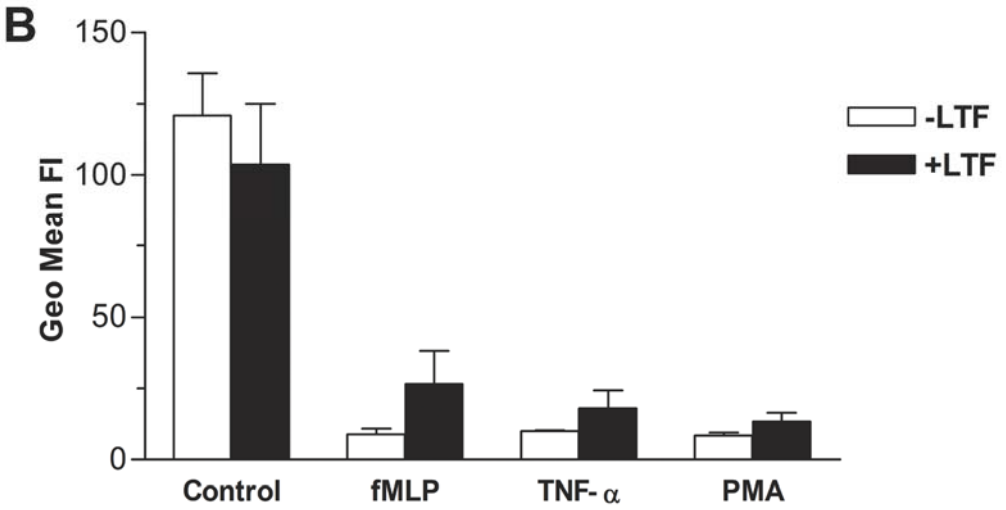

B
Geo Mean FI
150
100
50
0
Control
fMLP
TNF-α
PMA
-LTF
+LTF

FIGURE 33: Effect of lactoferrin on neutrophil activation status.

The expression of CD62L was assessed in fMLP- (100 nM), TNF-α– (1 ng ml^{-1}) or PMA-stimulated (100 nM) neutrophils (30 min at 37°C) that were pre-incubated (40 min at 37°C) in the presence or absence of lactoferrin (10 μg ml^{-1}). **(A)** Representative flow cytometry overlays of CD62L expression in control (gray) and stimulated neutrophils (lactoferrin-treated: red; untreated: blue). n = 3. **(B)** Flow cytometry results showed higher levels of CD62L expressed i.e. lower activation status in neutrophils pre-incubated with lactoferrin followed by agonist stimulation, compared to control activated neutrophils n=3. fMLP, formyl-methionyl-leucyl-phenylalanine; TNF-α, tumour necrosis factor-α, PMA, phorbol myristate acetate; LTF, lactoferrin. Error bars indicate SEM.

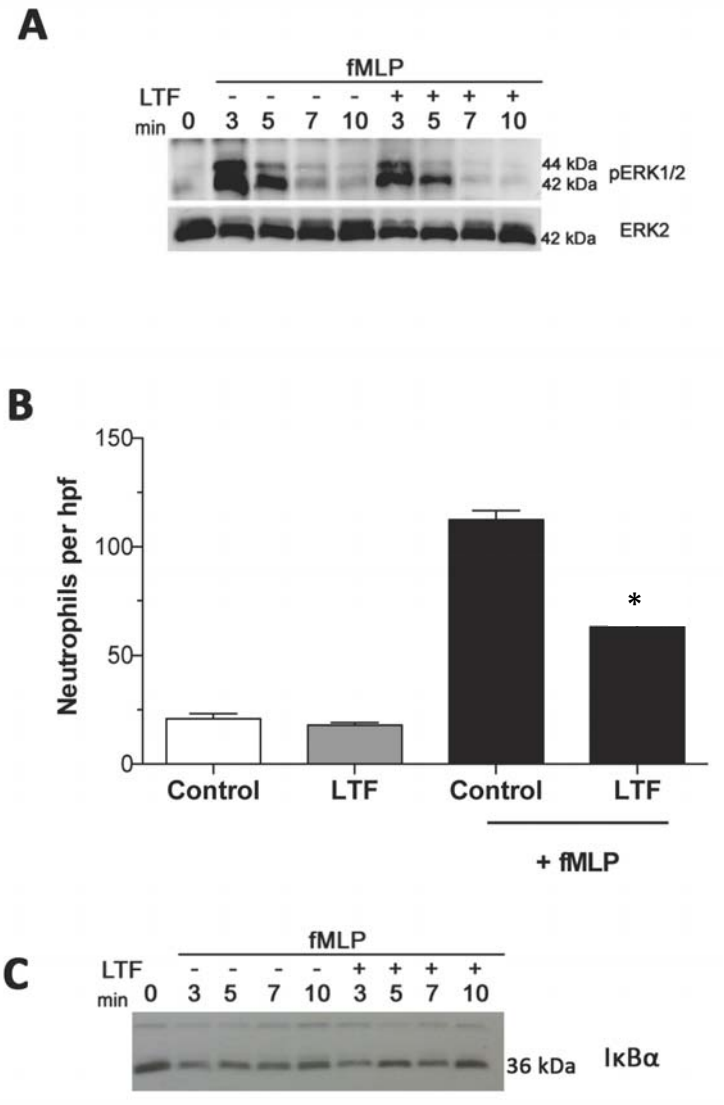

A
fMLP
LTF - - - - + + + +
min 0 3 5 7 10 3 5 7 10
44 kDa
42 kDa
pERK1/2
42 kDa
ERK2
B
Neutrophils per hpf
150
100
50
0
*
Control
LTF
Control
LTF
+ fMLP
C
fMLP
LTF - - - - + + + +
min 0 3 5 7 10 3 5 7 10
36 kDa
IκBα

FIGURE 34: Lactoferrin reduced ERK1/2 phosphorylation status in activated neutrophils.

Immunoblot analysis to determine levels of **(A)** ERK1/2 phosphorylation and of **(C)** IκBα. Neutrophils were incubated with lactoferrin (10 μg ml^{-1}; 40 min at 37°C), followed by stimulation with fMLP (100 nM) for the indicated times. Membrane was stripped and reprobed for total ERK2 and IκBα. Results are representative of three independent experiments. Results showed that neutrophils treated with lactoferrin presented a reduced ERK1/2 phosphorylation status following fMLP stimulation; however no effect on IκBα levels was evident. Results are representative of three (for ERK1/2) or two (for IκBα) independent experiments. **(B)** Chemotaxis assay to determine the effect of gliotoxin (2 ng ml^{-1}) on neutrophil migration towards lactoferrin. Chemotaxis of neutrophils was determined after being pre-incubated in the presence of gliotoxin (black columns; 40 min; 37°C) and washed with PBS prior to use in the assay. Gliotoxin pre-treatment was found to have no effect on the inhibitory activity of lactoferrin on neutrophil migration; n=2 *$p<0.05$ vs. fMLP. Error bars indicate SEM.

Lactoferrin is actively expressed and released upon apoptosis induction in diverse cell types

Pursuing the early observations that the inhibition of neutrophil migration by BL cells was correlated with BL cell apoptosis **(Figure 4C)**, lactoferrin expression was assessed following induction of apoptosis in a panel of cells of diverse lineages. By transcriptional analysis using RT-PCR, lactoferrin was found to be expressed, as reported previously, by MCF7-C3 breast cancer epithelial cells in their viable state but not by the T leukemic cell line Jurkat, the epithelial lung cancer A549 cells or BL2 cells. Upon apoptosis induction, lactoferrin expression was shown to be upregulated in MCF7-C3 cells and expressed *de novo* in Jurkat, BL2, and A549 cells **(Figure 35)**. More specifically, lactoferrin was transcribed *de novo* early after induction of apoptosis in A549 cells by either 100 nM etoposide or 1 μM staurosporine and its expression levels were relevant to the levels of apoptosis **(Figure 36A)**. The RT-PCR results also revealed that the levels of lactoferrin induced by etoposide were reduced in A549 cells treated in the presence of the broad-spectrum caspase inhibitor zVAD-fmk (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone), which prevented apoptosis induction **(Figure 36B)**. The link between lactoferrin expression and apoptosis induction was further supported by the effects of the apoptosis inhibitor, Bcl-2. BL cells expressing exogenous Bcl-2 that provided protection from apoptosis expressed lower levels of

lactoferrin upon exposure to staurosporine than did their parental counterparts **(Figures 9C and 35)**.

Not only was apoptosis-related lactoferrin expression demonstrated at the transcriptional level, lactoferrin protein was also recovered from supernatants of BL2 and A549 cells undergoing apoptosis. Similarly, an analogous effect was also evident from supernatants obtained from primary lymphocytes induced to become apoptotic in the presence of 1 μM staurosporine, showing in this way that the release of lactoferrin extends to all cells undergoing apoptosis used in this study **(Figure 37A)**. Moreover, as shown in **Figure 37B**, treatment of A549 cells with brefeldin A (1 μg ml^{-1}), which interferes with intracellular transport of newly synthesised proteins, resulted in inhibition of apoptosis-induced lactoferrin release, providing further evidence for the *de novo* synthesis and secretion of lactoferrin by cells undergoing apoptosis. Finally, the release of lactoferrin is not linked to necrosis but lactoferrin is expressed and actively released from cells as a consequence of activation of their apoptosis program, as evidenced by immunoblotting analyses and chemotaxis assays using supernatants of BL cells undergoing primary necrosis ($1x10^{6}$ ml^{-1} BL cells stimulated to become necrotic by incubation at 56°C for 1 h) **(Figures 38A and 38B)**. In summary, it was evident that lactoferrin was actively expressed and released upon apoptosis induction in diverse cell types. The release of lactoferrin is a characteristic of

apoptosis, enabling in this way, the anti-inflammatory nature of apoptosis as a physiological process.

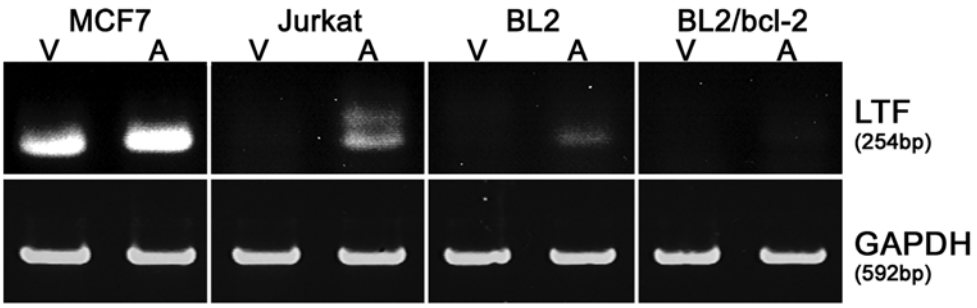


FIGURE 35: Induction of apoptosis upregulated lactoferrin expression.

RT-PCR analysis in cell lines stimulated to undergo apoptosis (A) and unstimulated controls (V). The cell lines included MCF7 cells transfected with caspase-3 (25.4% apoptosis; 100 μM etoposide, 20 h), Jurkat (18.4% apoptosis; 1 μM staurosporine, 3 h), BL2 (12.5% apoptosis), and BL2/bcl-2 (7.4% apoptosis; 1 μM staurosporine, 1 h). Representative image of three independent experiments.

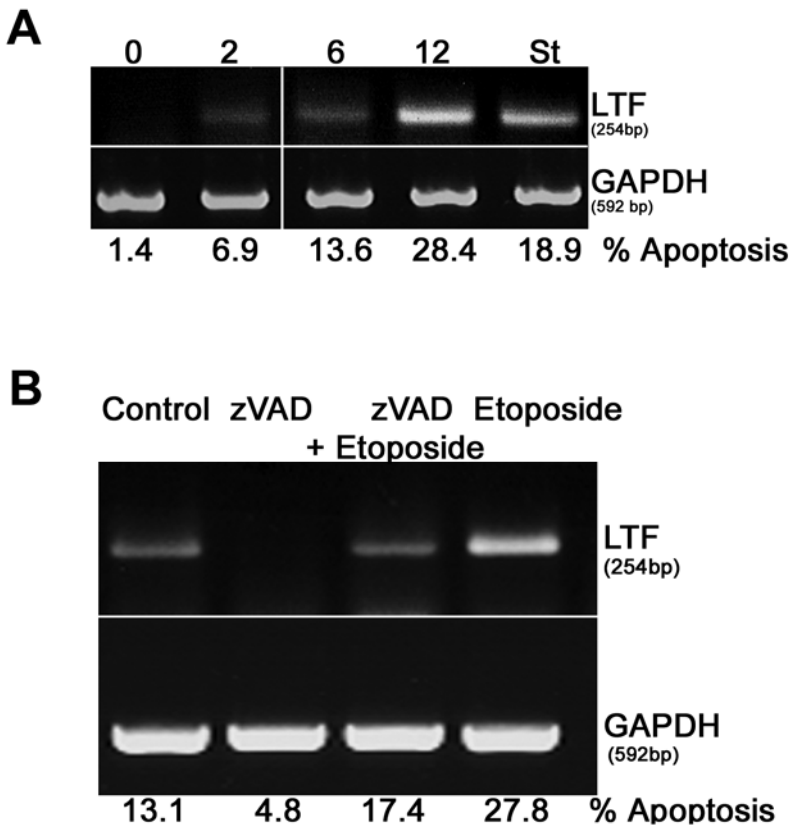


FIGURE 36: Induction of apoptosis upregulated lactoferrin expression.

(A) Lactoferrin expression in A549 cells at defined time points (h) following stimulation with 100 μM etoposide or 1 μM staurosporine. **(B)** Addition of pan-caspase inhibitor zVAD-fmk (100 μg ml^{-1}) for 12 h in order to prevent etoposide-induced apoptosis in A549 cells. It was revealed that lactoferrin expression was linked to and upregulated by the apoptosis levels of the cells. Results (A and B) are representative of three independent experiments.

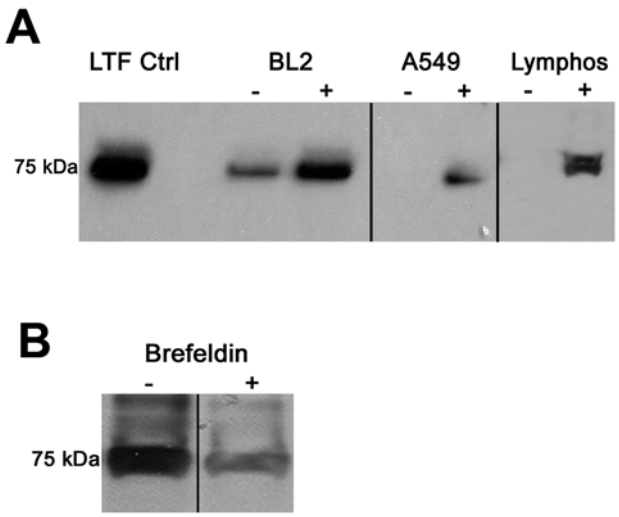


FIGURE 37: Increased release of lactoferrin following apoptosis induction.

(A) Immunoblot analysis of cell supernatants from: BL2 and primary lymphocytes in the presence (+) or absence (–) of staurosporine (1 μM) in serum-free conditions for 1 h. A549 cells were stimulated with (+) or without (–) 100 μM etoposide for 5 h. **(B)** A549 cells were induced to become apoptotic (100 μM etoposide; 20 h) in the presence or absence of brefeldin A (1 μg ml^{-1}), a protein release inhibitor. Treatment with brefeldin A reduced the amount of lactoferrin released from apoptotic A549 cells. Results (A and B) are representative of three independent experiments. Proteins run on the same gel for each experiment.

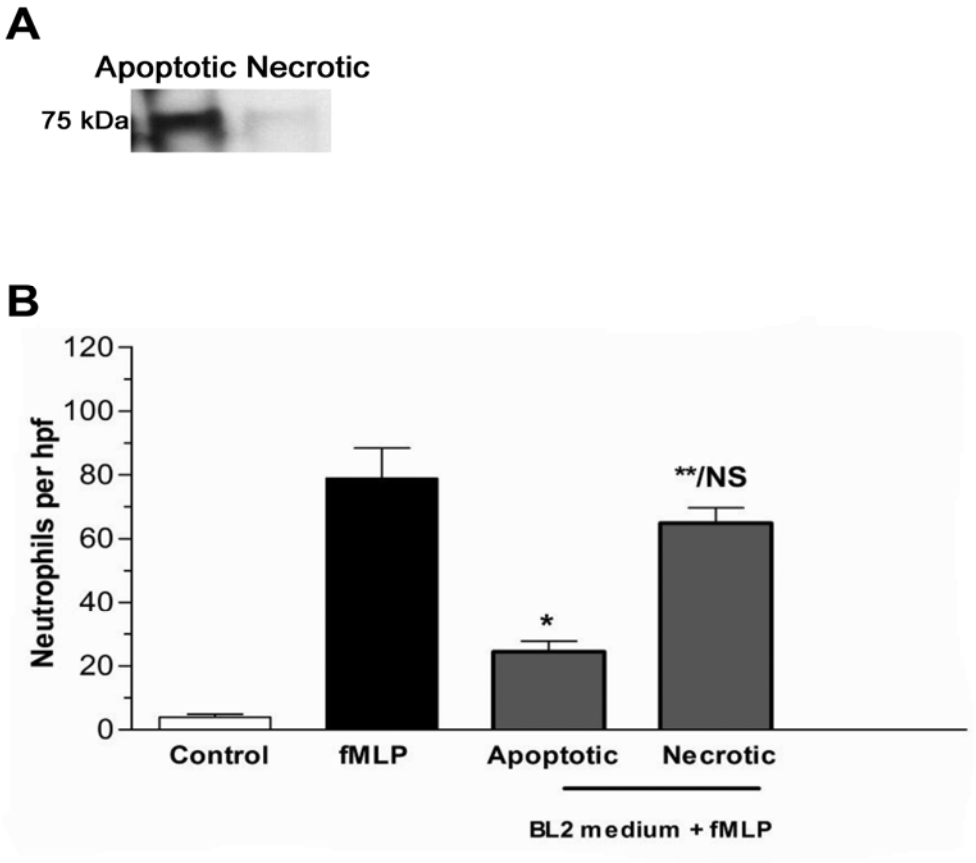


FIGURE 38: Necrotic BL cells did not produce lactoferrin or other mediators that inhibit neutrophil migration.

(A) Immunoblot analysis of conditioned media from apoptotic and/or necrotic BL2 cells ($1x10^6$ cells ml^{-1}) to detect lactoferrin. Image is representative of three independent experiments; proteins run on the same gel. **(B)** Chemotaxis assay to determine neutrophil migration towards conditioned media from BL2 cells ($2x10^6$ cells ml^{-1}) stimulated to undergo apoptosis (1 µM staurosporine; 1 h; 37°C) or primary necrosis (56°C; 1 h) in serum-free conditions n=3; *p<0.001 compared to fMLP control; **p<0.05 vs. apoptotic control;

NS= non-significant vs. fMLP control; hpf, high power field. Error bars indicate SEM.

Lactoferrin promotes BL cell proliferation

These studies indicate that lactoferrin is expressed by a great diversity of cell lineages and it is upregulated upon apoptosis as a feedback mechanism to prevent neutrophil infiltration and stimulate a variety of anti-inflammatory immune effector activities. The next goal was to identify what is the role of lactoferrin in a proliferating tumour cell line. In other words, examine whether the expressed lactoferrin also plays a role in tumour establishment and growth.

Using BL as a model, the EBV+ (Mutu, Wan) and EBV- (BL2, L3055) BL cell lines were cultured at a starting number of $0.3x10^{6}$ cells in X-vivo culture medium (lactoferrin-free). Their cell number was then determined in the presence of monoclonal anti-human lactoferrin antibody (10 µg ml^{-1}) or isotype control and in all cases, it was noted that depletion of lactoferrin significantly inhibited BL cell proliferation over a 48-h time course, suggesting in this way that lactoferrin is an important determinant of cell growth. Indeed, the addition of lactoferrin antibody (10 µg ml^{-1}) resulted in a reduction of the proliferation rate of all the cell lines examined **(Figures 39 and 40)**. In detail, after a 48-h incubation, the number of BL2 cells in control conditions was $9.14x10^{5}$ ± 0.7 cells, whereas in the presence of lactoferrin antibody or isotype control, it was $5.61x10^{5}$ ± 0.7 and $8.14x10^{5}$ ± 0.4 BL2 cells respectively. In terms of Mutu cell proliferation after 48 h culture in X-vivo, in control conditions, $7.69x10^{5}$ ± 0.8

Mutu cells were counted, while $5.32x10^5$ ± 0.5 and $8.51x10^5$ ± 0.6 cells in the presence of anti-lactoferrin antibody or isotype control, respectively. Similarly, in Wan and L3055 culture in control conditions, $8.53x10^5$ ± 0.9 Wan cells and $7.56x10^5$ ± 1.2 L3055 cells were counted in control conditions at the 48 h time point. This number was reduced to $7.20x10^5$ ± 0.5 and $5.20x10^5$ ± 0.3 respectively after the addition of anti-lactoferrin antibody. The isotype control numbers were $8.49x10^5$ ± 1.8 Wan and $7.85x10^5$ ± 0.8 L3055 cells. This result supports earlier studies that have reported the presence of lactoferrin in BL tissue scans as well as studies that proposed the role of endogenously expressed lactoferrin as a growth factor. It should be noted that addition of polyclonal anti-lactoferrin antibody to the BL cells did not stimulate such an effect **(Figure 41).**

Additional experiments were carried out, in which the proliferation of BL2 cells and BL2 cells transfected with shRNA vector for lactoferrin was examined. As a control, shRNA BL2 cells transfected with a negative control vector were included. Although the starting number of all three cell types was $0.3x10^6$ cells, the lactoferrin shRNA-transfected cells were proliferating at a slower rate than their respective control cells over a 48-h time course **(Figure 42)**. In detail, after 48 h, the number of parental control BL2 cells was $8.20x10^5$ ± 0.9 cells, whereas that of the lactoferrin shRNA-transfectant ones was $5.10x10^5$ ± 1.2 cells and of the negative shRNA control BL2 cells was $9.30x10^5$ ± 1.1 cells. Moreover, despite the fact that

purified lactoferrin when added to cells at a concentration of 10 µg ml^{-1} had no effect, it promoted the proliferation of lactoferrin shRNA-transfected cells at analogous levels to their negative control counterparts when added at concentrations of 100 pg ml^{-1} or 1 ng ml^{-1}; therefore increasing cell number to $7.60x10^{5}$ ± 0.6 and $8.10x10^{5}$ ± 0.8 cells, respectively. In general terms, these results revealed that lactoferrin promotes BL proliferation, based on proliferation experiments, in which lactoferrin was depleted via the use of an anti-lactoferrin antibody or via an shRNA-mediated knockdown approach. These findings provide a first line of evidence that lactoferrin is a determinant of BL cell proliferation and further experiments should be carried out to examine this effect in other tumour cell lines.

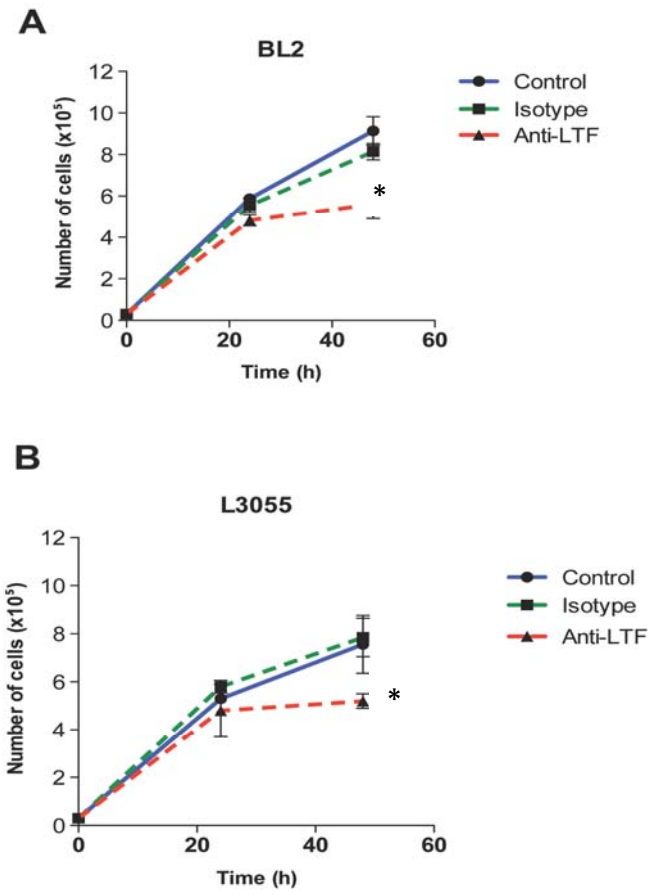


FIGURE 39: Depletion of endogenous lactoferrin reduced the rate of BL (EBV-) cell proliferation.

Proliferation assay to determine the proliferation rate of the EBV- BL cell lines **(A)** BL2 and **(B)** L3055 under conditions, in which lactoferrin was depleted via the use of mouse monoclonal anti-human lactoferrin antibody (clone LF-2B8; 10 µg ml^{-1}). Cells were plated at an initial concentration of $0.3x10^{6}$ cells and their proliferation (relative number) was recorded for 48 h. An isotype control (mouse monoclonal IgG1 isotype control –MOPC21) was also included (10 µg ml^{-1}; n=6 for each cell line;

*p<0.05 vs. isotype control). Error bars indicate SEM.

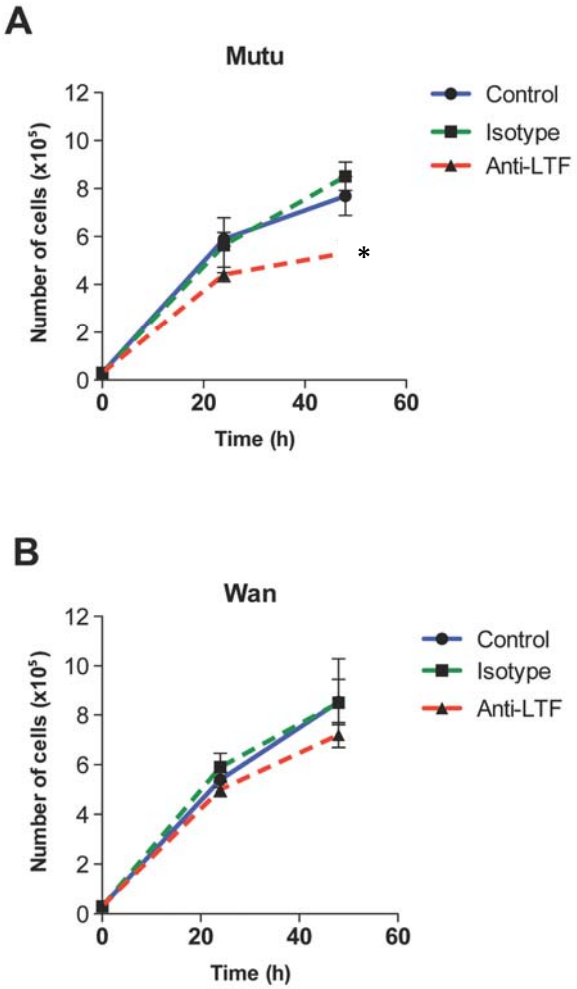


FIGURE 40: Depletion of endogenous lactoferrin reduced the rate of BL (EBV+) cell proliferation.

Proliferation assay to determine the proliferation rate of the EBV+ BL cell lines **(A)** Mutu and **(B)** Wan under conditions, in which lactoferrin was depleted via the use of mouse monoclonal anti-human lactoferrin antibody (clone LF-2B8; 10 µg ml^{-1}). Cells were plated at an initial concentration of $0.3x10^{6}$ cells and their proliferation (relative number) was recorded for 48 h. An isotype control (mouse monoclonal IgG1 isotype control –MOPC21)

was also included (10 µg ml^{-1}; n=6 for each cell line; *p<0.05 vs. isotype control). Error bars indicate SEM.

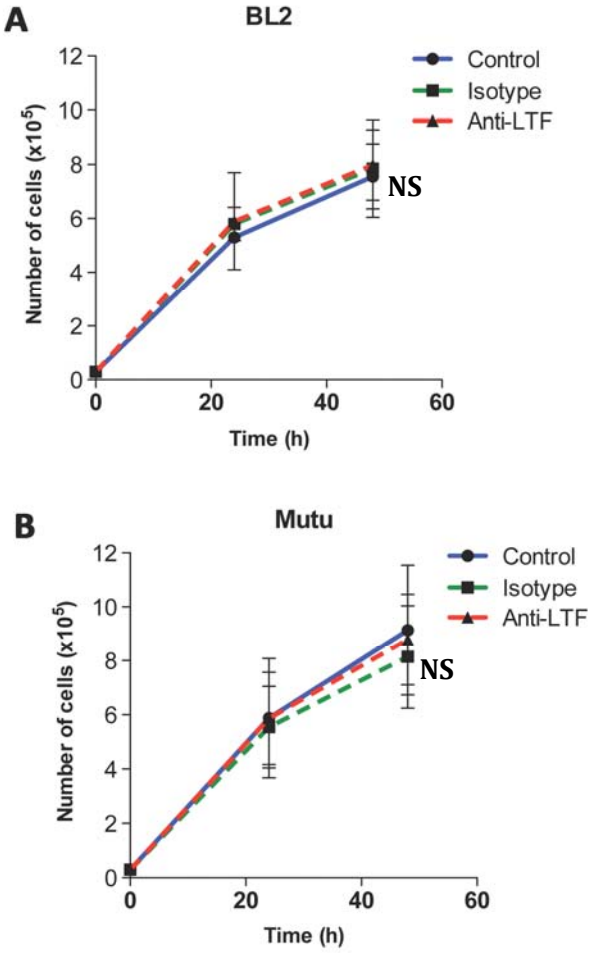


FIGURE 41: No effect on the rate of BL cell proliferation upon depletion of endogenous lactoferrin using a polyclonal antibody.

Determination of the proliferation rate of **(A)** BL2 and **(B)** Mutu under conditions, in which lactoferrin was depleted via the use of polyclonal anti-human lactoferrin IgG antibody. Cells were plated at an initial concentration of $0.3x10^6$ cells and their proliferation (relative number) was recorded for 48 h. An isotype control (rabbit polyclonal IgG negative control) was also included (10 µg ml^{-1}; n=3; NS vs. isotype control). No difference was evident in the

proliferation rate of the cells. Error bars indicate SEM.

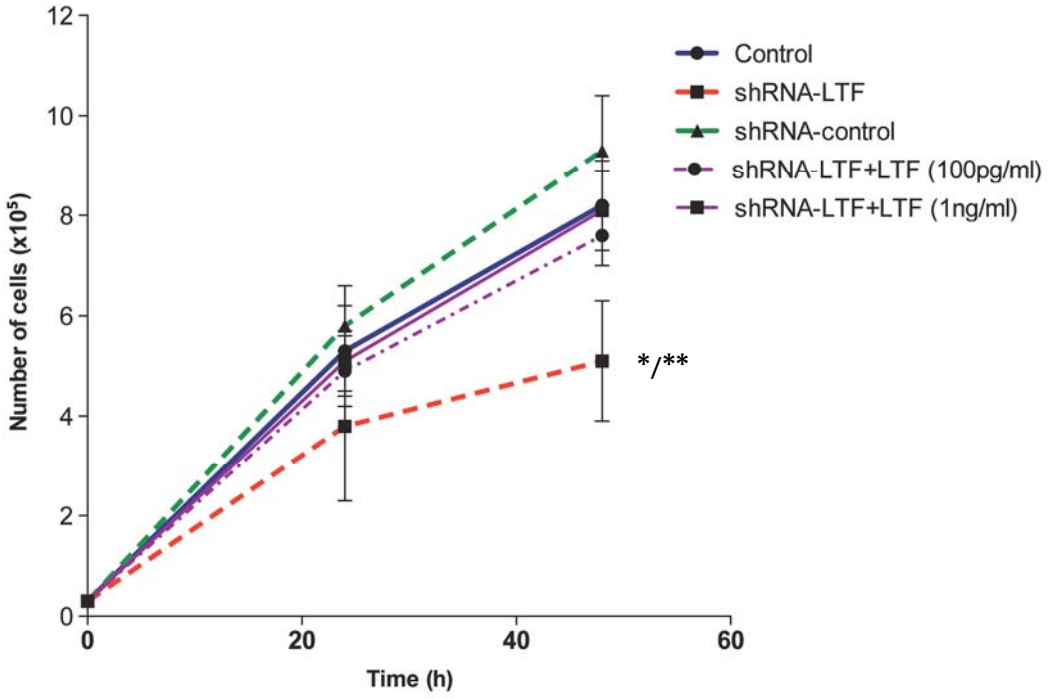


FIGURE 42: shRNA-mediated depletion of endogenous lactoferrin specifically reduced BL cell proliferation.

Proliferation assay to determine the proliferation of the BL2 parental cell line, of lactoferrin shRNA-transfected BL2 cell line and of shRNA-negative control BL2 cell line. As a control, purified lactoferrin (100 pg ml^{-1} and 1 ng ml^{-1}) was added to lactoferrin shRNA-transfected BL2 cells. All cells were plated at an initial concentration of $0.3x10^{6}$ cells and their proliferation (relative number) was recorded for 48 h n=5; *p<0.05 vs. negative shRNA control, **p<0.05 vs. lactoferrin shRNA +purified lactoferrin, NS=

non-significant vs. shRNA negative control. Error bars indicate SEM.

Depletion of lactoferrin induces BL cells to undergo apoptosis

There are many studies that support that exogenous administration of lactoferrin has potent apoptosis-inducing and anti-tumoural effects. However, such studies had not reported possible limitations concerning the origin and quantity of the administered lactoferrin; something that might possibly trigger toxic effects. In this study, it was shown for the first time, that lactoferrin is endogenously expressed and its expression is linked to BL cell proliferation. As Burkitt's lymphoma displays a high apoptosis rate as a tumour, whether lactoferrin expression is related to the rate of cell apoptosis was further examined.

Indeed, by carrying out analogous proliferation experiments in BL2 cells, the addition of the monoclonal anti-lactoferrin antibody was found to promote the induction of apoptosis, as over a 48-h time course, addition of anti-lactoferrin antibody resulted in an increase in the number of necrotic (Anx^+/PI^+) BL cells **(Figure 43)**. Also, addition of purified lactoferrin resulted in a lower proportion of cells undergoing apoptosis.

This effect on apoptosis rates was also prominent in the measurement of the cell cycle status of these cells. Cells incubated in the presence of the anti-lactoferrin antibody presented a classical cell cycle profile of cells undergoing apoptosis that was marked by the inefficiency

of cells to enter the G2 phase **(Figure 44)**. As an analogous effect was not evident in shRNA-transfected BL2 cells **(Figure 45)**, it could be proposed that the addition of the anti-lactoferrin antibody to BL cells induces cellular death via an antibody-dependent cytotoxicity. Moreover, the possibility that the use of antibody causes a rapid deprivation of lactoferrin in these cells, promoting their cellular death shall not be excluded, as the shRNA transfected ones were used to survive in a lactoferrin-deficient environment. Also, in shRNA-transfected cells, lactoferrin was knocked down and not completely neutralised, and therefore, low levels of lactoferrin could have been present.

To better elucidate the effect of lactoferrin on apoptosis, BL cells were cultured in X-vivo medium (serum-free) at a concentration of $1x10^{6}$ cells ml^{-1} over a 24 h time course in the presence or absence of 1 ng ml^{-1}, 100 ng ml^{-1}, 1 µg ml^{-1} and 10 µg ml^{-1} of purified lactoferrin. We observed that lactoferrin did not stimulate BL cells to become apoptotic at all concentrations. Also, when BL2 cells were induced to become apoptotic following staurosporine treatment (1 µM; 1 h at 37°C) or in serum-free conditions (24 h), the addition of purified lactoferrin (1 µg ml^{-1}) did not promote or inhibit apoptosis induction **(Figure 46)**.

In summary, it has been clearly shown that lactoferrin is an endogenous and tumour cell autonomous component of Burkitt's lymphoma and acts both by regulating the type of

immune cells infiltrating the tumour stroma as well as the proliferation of the tumour cells. Distortion of the expression of lactoferrin –if not genetically redundant- might possibly elicit devastating anti-tumour effects. In support to this, very promising preliminary findings on the *in vivo* growth of lactoferrin shRNA-BL cell lines in SCID xenografts showed a reduction in BL tumour establishment and growth compared to shRNA negative BL2 control xenografts **(Figure 47)**. However, due to technical problems and time constraints, the number of mice in each group is not comparable and analogous experiments cannot unfortunately be carried out in the context of this project.

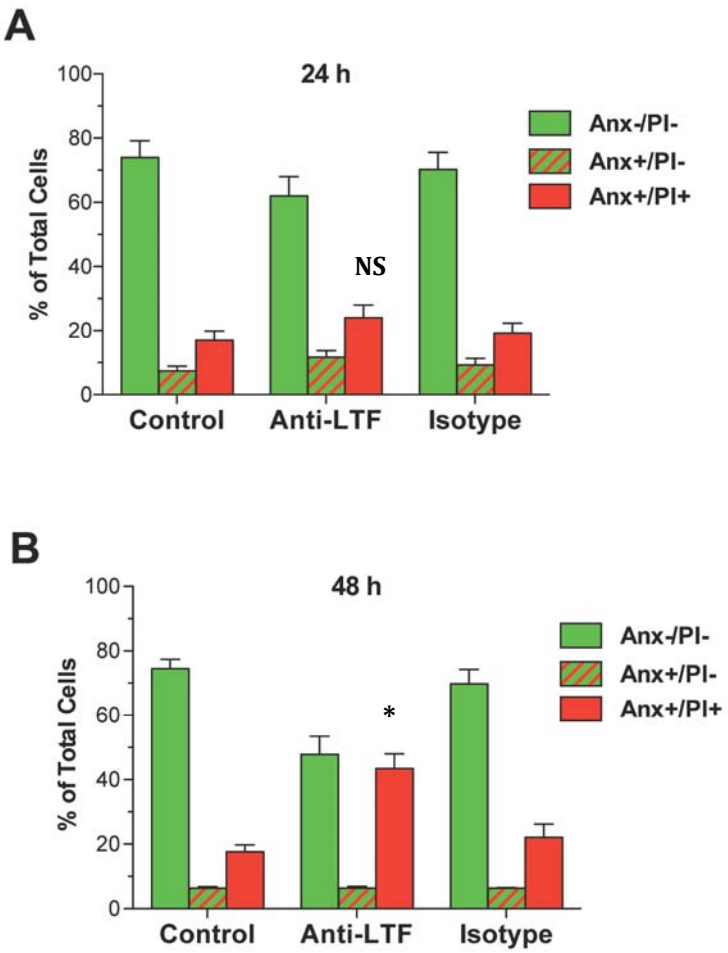


FIGURE 43: Effect of lactoferrin depletion to the rate of BL cell apoptosis.

Measurement of apoptosis levels in BL2 cells cultured in the presence or absence of mouse monoclonal anti-human lactoferrin antibody (LF-2B8; 10 μg ml^{-1}), as assessed by Anx/PI staining. An isotype control (mouse monoclonal IgG1 isotype control –MOPC21) was also included (10 μg ml^{-1}). It was evident that the antibody-mediated depletion of lactoferrin resulted to an increase in the percentage of Anx^{+}/PI^{+} cells. n=6 for 24 h and 48 h; *p<0.05 or NS=non-

significant vs. respective control. Error bars indicate SEM.

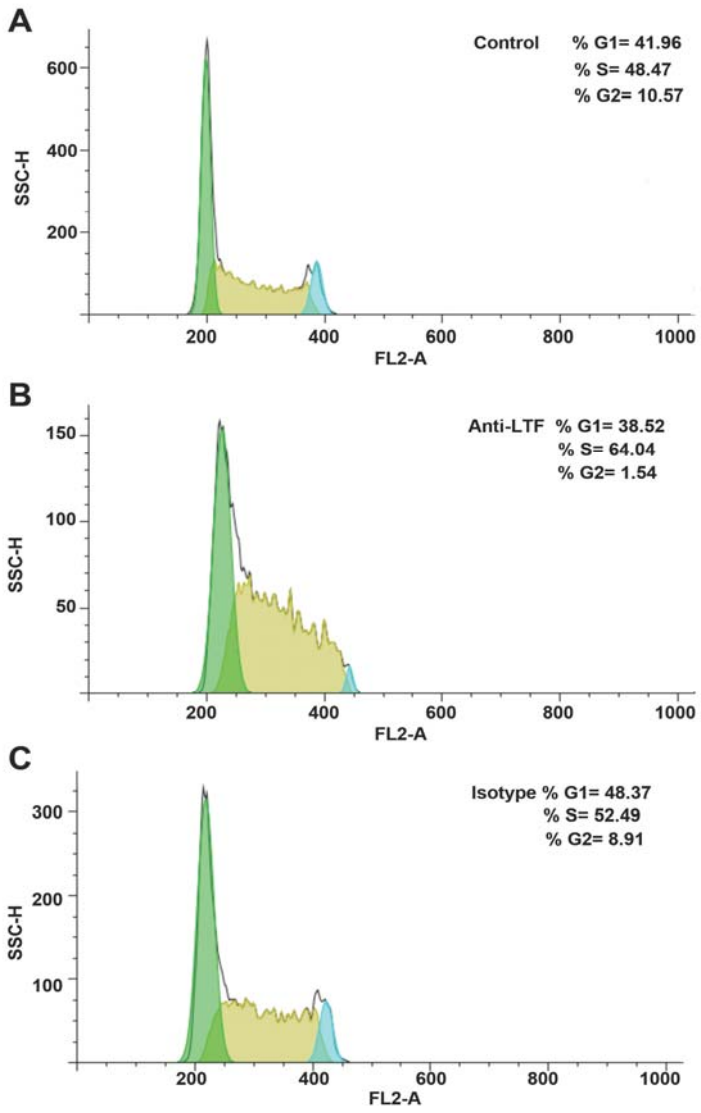


FIGURE 44: Effect of lactoferrin depletion to the cell cycle of BL cells.

Analysis of the cell cycle profile of BL2 cells cultured in the **(B)** presence or **(A)** absence of mouse monoclonal anti-human lactoferrin antibody (LF-2B8; 10 µg ml^{-1}). **(C)** An isotype control (mouse monoclonal IgG1 isotype control -MOPC21) was also included (10 µg ml^{-1}). Results are

representative of six (n=6) independent experiments; *p<0.05 vs. respective control. Results indicated that BL2 cells incubated in the presence of the anti-lactoferrin antibody presented an inefficiency to enter the G2 phase.

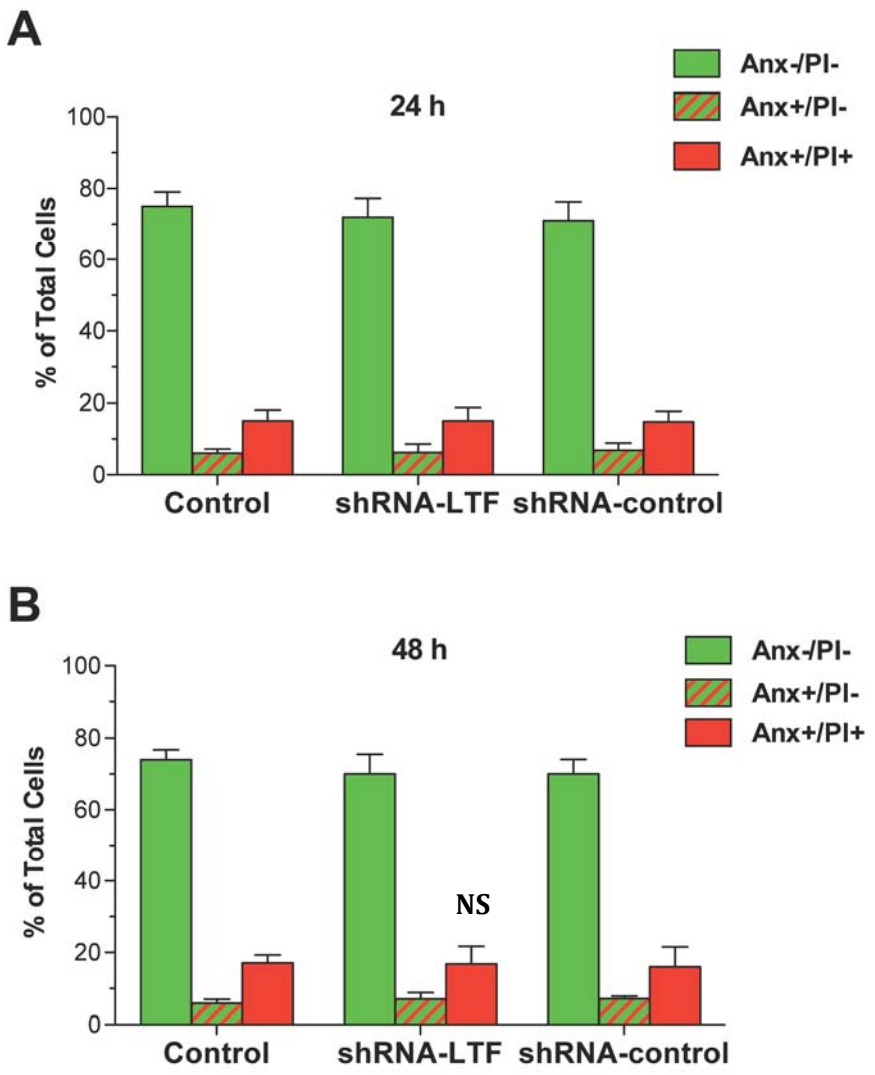


FIGURE 45: Effect of lactoferrin depletion to the rate of BL cell apoptosis.

Determination of apoptosis levels in lactoferrin shRNA-transfected BL2 cells and their control counterparts, as assessed by Anx/PI staining. As a transfection control, a negative shRNA-transfected BL2 cell line was included. No difference was evident in the apoptosis levels of all

three cell lines. n=6 for 24 h and 48 h; NS=non-significant vs. respective control. Error bars indicate SEM.

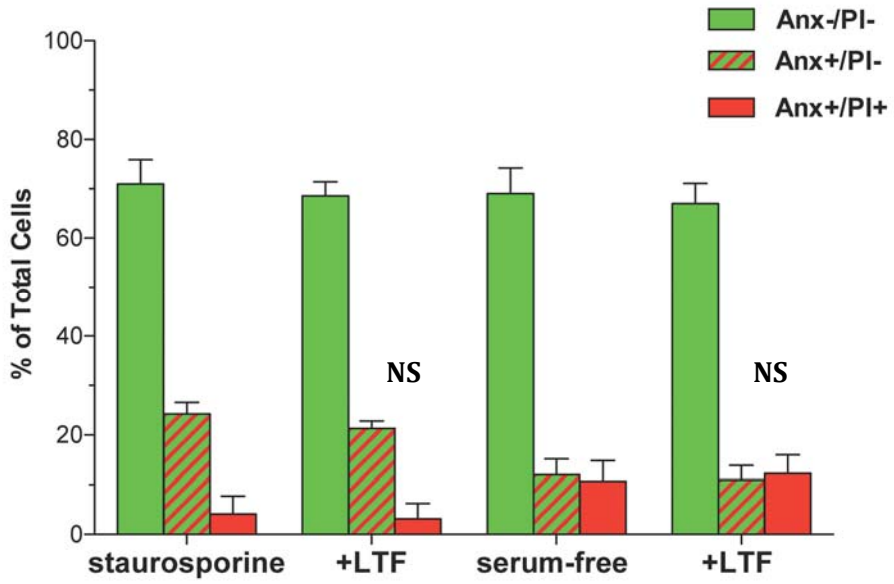


FIGURE 46: Lactoferrin did not promote the induction of apoptosis.

Determination of apoptosis levels in BL2 cells that were induced to become apoptotic following staurosporine treatment (1 µM; 1 h at 37°C) or in serum-free conditions (24 h) in the presence or absence of purified lactoferrin (1 µg ml^{-1}), as assessed by Anx/PI staining. It was revealed that lactoferrin neither stimulated BL2 cells to become apoptotic nor promoted or inhibited apoptosis induction. n =3; NS=non-significant vs. respective control.

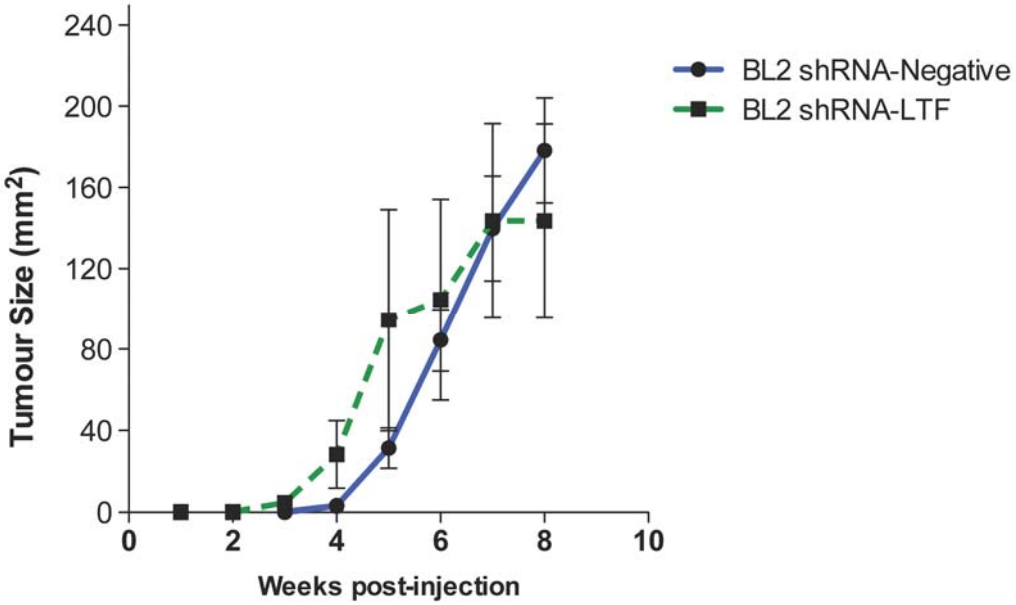


FIGURE 47: Determination of BL tumour growth in vivo.

Lactoferrin depletion seemed to result to decreased xenograft tumour growth. The BL2 LTF-shRNA and the BL2-shRNA negative control xenograft tumours were grown for 8 weeks and tumour size was determined after the appearance of visible tumours. The rate of tumour growth was determined by averaging the tumour size in each cell line (shRNA-LTF n=3; shRNA negative control n=6). Error bars indicate SEM.

DISCUSSION

Apoptosis is an intrinsically non-phlogistic process that regulates many physiological, homeostatic processes of an organism, such as organ morphogenesis, limb development and immune responses (Kerr *et al.*, 1972; Majno and Joris, 1995). Unlike necrosis, which has proinflammatory consequences, apoptosis is an anti-inflammatory process (Kerr *et al.*, 1994; Leist and Nicotera, 1997). For this reason, defining the properties of apoptotic cells that contribute to the non-inflammatory or anti-inflammatory nature of the apoptosis program is critical to the understanding of this fundamental biological process (Savill *et al.*, 1993; Vaux, 1993). Failure to control apoptosis, can result to detrimental effects to the host such as inflammation and autoimmunity (Ekert and Vaux, 1997). In this study, lactoferrin was identified as a cell-autonomous, anti-inflammatory mediator that inhibits granulocyte migration. Lactoferrin is a pleiotropic protein widely known mainly for its iron-binding properties and is a member of the transferrin family of proteins (Baker and Baker, 2005; Ward and Conneely, 2004). It is mainly found in the specific granules of neutrophils and mucosal secretions and is expressed by the epithelial cells of the digestive and respiratory tracts (Bennett and Kokocinski, 1978; Ward *et al.*, 2005). Apart from the bacteriostatic properties of lactoferrin that are attributable mostly due to its iron-binding ability, the anti-inflammatory activities of lactoferrin are ever

increasing (Legrand *et al.*, 2005). The ability of lactoferrin to inhibit granulocyte migration, as described here, is an additional anti-inflammatory feature of this molecule, independent of its iron-chelating activity.

Although its exact involvement in the apoptotic process and its relations to other immune cell types has not been clearly determined yet, in this study, lactoferrin was found to inhibit neutrophil chemotaxis both *in vitro* and *in vivo* with no consequences on neutrophil survival, pose it as a key regulatory molecule in inflammation. In detail, in this study, Burkitt's lymphoma (BL) cells, like all other apoptotic cell types, were demonstrated to actively release lactoferrin so as to inhibit neutrophil recruitment. This effect of BL cells on neutrophils is a possible homeostatic mechanism so as to prevent the cytostatic effects arising from neutrophil infiltration. Indeed, as evidenced from preliminary experiments, when neutrophils were co-cultured with BL cells at different ratios, a decrease in the total viable cell population was noted (I. Bournazou, unpublished data).

The identification of lactoferrin as an inhibitor of neutrophil migration released from BL cells was validated by neutralisation experiments using both monoclonal and polyclonal antibodies specific for human lactoferrin. Moreover, in subsequent chemotaxis experiments, it was demonstrated that purified human lactoferrin inhibited neutrophil migration in a bell-shaped mode. It should be noted that lactoferrin prevented neutrophil recruitment

with no toxic effects on neutrophil survival, as assessed by Anx/PI staining. This inhibitory effect was irrespective of its source (neutrophil-derived or milk-derived), showing in this way that differences in the glycosylation profile of lactoferrin do not seem to account for its described anti-inflammatory activity on neutrophils. In fact, although there are no differences reported until now in terms of their biological activity, the only difference between milk and neutrophil-derived lactoferrin is that lactoferrin from neutrophils lacks terminal fucose residues in the glycan chain that are required for binding to macrophages (Martins *et al.*, 1995); however, this difference has no effect in terms of its activity on neutrophils, as both these types of lactoferrin were shown to inhibit neutrophil chemotaxis at similar levels.

The observed *in vitro* effect on neutrophil chemotaxis was supported by *in vivo* findings, which demonstrated that lactoferrin inhibited neutrophil recruitment in a thioglycollate-induced mouse model. In that model, it was also examined whether transferrin inhibits neutrophil recruitment or the described inhibitory effect was specifically exerted by lactoferrin. Although transferrin is another member of the transferrin family of proteins and highly homologous to lactoferrin (Harrington, 1992; Masson and Heremans, 1971; Metz-Boutigue *et al.*, 1984), it failed to exert an analogous inhibitory effect on neutrophil chemotaxis, showing in this way that lactoferrin specifically inhibits neutrophil chemotaxis

independent of its iron-binding properties. This provided a first clue in attempts to identify the region of lactoferrin that is responsible for this inhibitory activity. Although more detailed investigation for the identification of this specific region is required, mainly employing techniques such as site-directed mutagenesis, it can be suggested that the region of lactoferrin that specifically inhibits neutrophil migration is not its iron-binding site. However, it can be speculated that this region is possibly found in the N lobe of lactoferrin, as this lobe has been demonstrated to account for all the described anti-inflammatory effects of lactoferrin (Baveye *et al.*, 1999; Brock, 2002; Velliyagounder *et al.*, 2003). The fact that iron plays no role in the effect of lactoferrin on neutrophil chemotaxis, as described in this study, was further supported by the finding that lactoferrin exerts the same level of inhibition in neutrophil migration irrespective of its iron saturation status. Indeed, although iron and iron-associated molecules have been previously shown to play an important role in many immunomodulatory functions, when three different iron-saturation variants of lactoferrin (apo, partial and fully-iron saturated) were used in this study, no difference was observed in terms of their efficiency to inhibit neutrophil migration. This result is in contrast to other studies, in which the level of iron saturation plays a key role in the anti-inflammatory response. For example, purified iron-saturated lactoferrin was shown to suppress IL-1 release by monocytes, whereas an inhibition

of GM-CSF activity production by monocytes and macrophages was found to be correlated with the iron saturation status of lactoferrin (Broxmeyer *et al.*, 1978; Broxmeyer *et al.*, 1986; Zucali *et al.*, 1989).

In this study, it was also found that lactoferrin, apart from neutrophils, exerted an inhibitory effect on eosinophil migration. However, unlike other chemorepellent stimulants (Jutila *et al.*, 1991; Tharp *et al.*, 2006), it had no chemorepulsive activities on either neutrophils or eosinophils. This result complemented earlier findings that described the ability of lactoferrin to reduce allergic airway inflammation in *in vivo* models (Elrod *et al.*, 1997; Kruzel *et al.*, 2006) and the inability of soluble, contrary to immobilised, lactoferrin to activate eosinophils (Thomas *et al.*, 2002). It should be emphasised that the inhibitory effect of lactoferrin was restricted to granulocyte migration, as subsequent chemotaxis experiments to determine monocyte or macrophage chemotaxis displayed no difference in the C5a-induced chemotaxis of these cells, despite the presence of lactoferrin. However, it cannot be excluded that lactoferrin exerts multiple effects on the resident monocytes or macrophages by regulating their activation status and promoting functional activities such as phagocytosis. Indeed, it has been reported that lactoferrin has a suppressive effect on the synthesis of proinflammatory cytokines e.g TNF-α, IL-1 and IL-6 by resident, activated monocytes or macrophages following LPS stimulation (Crouch *et al.*, 1992; Mattsby-Baltzer *et al.*, 1996). Other reported properties of

lactoferrin involve NK cell activation, activation of monocytes, activation of GM-CSF, stimulation of antibody-dependent cell cytotoxicity and macrophage cytotoxicity as well as maturation of splenic B cells (Baveye *et al.*, 1999; Legrand *et al.*, 2005).

This diversity in the anti-inflammatory activity of lactoferrin on immune and endothelial cells is evidenced by the presence of lactoferrin-specific receptors on the cell surface (Suzuki and Lonnerdal, 2002; Suzuki *et al.*, 2005). It should be noted that the cationic nature of lactoferrin in combination with its high binding capacity as a protein, make lactoferrin receptors very difficult to identify. Also, the involvement of pseudo-receptors shall not be excluded. For all these reasons, the detailed characterisation of lactoferrin receptors from various cell types had partial success and many candidate receptors have been proposed for various cell types (Suzuki and Lonnerdal, 2002). The lactoferrin receptors that have been characterised till now are expanded to the whole organism and include intestinal lactoferrin receptors, liver lactoferrin receptors such as LRP (LDL receptor-related protein) as well as lactoferrin receptors in the brain and the bronchial epithelial cells (Davidson and Lonnerdal, 1988; Ghio *et al.*, 1999; Herz and Strickland, 2001; Kawakami *et al.*, 1990; Kawakami and Lonnerdal, 1991). Other cell types that display lactoferrin receptors are lymphocytes, megakaryocytes and monocytes (Nillesse *et al.*, 1994; Suzuki *et al.*, 2005). In neutrophils, no distinct receptors for lactoferrin have

been characterised so far. Scatchard binding analysis of ^{125}I-labelled apo-lactoferrin indicated that lactoferrin bound to neutrophils via two classes of receptors that differ in the affinity and the number of binding sites per cell (Bournazou *et al.*, 2009). The higher affinity receptors were determined to be expressed at a density of 9,100 ± 2,500 binding sites per cell with an affinity of 350 ± 65 nM and the lower affinity receptors to be expressed at a density of $2.50x10^{6}$ ± $0.7x10^{6}$ per cell with an affinity of 20 ± 10 μM. This finding was supported by previously published literature on the identification of two types of lactoferrin receptors on neutrophils (Maneva *et al.*, 1983; Spik *et al.*, 1994). Moreover, published evidence on eosinophils, presented two classes of lactoferrin receptors using ^{125}I-labelled lactoferrin with dissociation constants of 47 nM and 260 nM (Thomas *et al.*, 2002). However, in all cases, the detailed characterisation of the relative receptors and the molecular mechanisms underlying neutrophil and eosinophil inhibition by lactoferrin require extensive further work.

Another point that should be mentioned in terms of the recognition of receptors on neutrophil surface by lactoferrin is the finding that lactoferrin does not directly associate with chemoattractants and chemoattractant receptors. The absence of association with chemoattractants is evident since the efficiency of the chemoattractant molecules remained intact following treatment and antibody-based removal of lactoferrin in lactoferrin absorption experiments. Also, lactoferrin

reduced the activation status of neutrophils treated with PMA, an intracellular PKC agonist, showing in this way that its inhibitory effect does not arise due to its interactions with chemoattractant receptors on neutrophil surface. It should also be mentioned that the observed effect of lactoferrin was not due to any association with LPS, as the lactoferrin used was endotoxin-free and the chemotaxis assays were carried out in serum-free conditions.

Proposed Model of Lactoferrin Inhibitory Effect on Neutrophils

Till now, the effect of lactoferrin on neutrophils had not been unravelled, apart from early observations, in which orally administered bovine lactoferrin in influenza virus infected mice reduced the number of infiltrating leukocytes in bronchoalveolar lavage fluid (Yamauchi *et al.*, 2006). Also, the administration of recombinant human lactoferrin prevented injury of non-steroidal anti-inflammatory drugs in the intestine of rats; an effect linked to attenuation of neutrophil migration (Dial *et al.*, 2005).

The results of this study support these findings and further suggest that lactoferrin is one of the few till now identified molecules, along with lipoxins and annexin-1, which are endogenously expressed to inhibit granulocyte migration (Lim and Pervaiz, 2007; Perretti and D'Acquisto, 2009; Schwab *et al.*, 2007). Furthermore, according to results from time-lapse video microscopy of neutrophils, it was demonstrated that lactoferrin prevents neutrophils from attaining classical polarisation morphology. This was confirmed by subsequent findings that lactoferrin prevents neutrophil migration by affecting the acquisition of particular neutrophil activation states, as assessed by the levels of two known activation markers, CD62L and CD11b in response to the activation stimuli of fMLP, TNF-α and PMA. It is noteworthy that the lactoferrin effect was also evident when PMA, a specific PKC activator, was used

as an agonist, also indicating that lactoferrin acts downstream of PKC and not on pathways involved in PKC activation and $[Ca^{2+}]_i$ responses, such as the IP_3 and DAG/PLC pathways. This was validated by the fact that lactoferrin did not influence intracellular calcium levels $[Ca^{2+}]_i$. Following lactoferrin treatment, no differences were noted in intracellular calcium concentrations ($[Ca^{2+}]_i$) in response to fMLP stimulation. Based on these findings, putative downstream targets of PKC were examined that were involved in the late signalling cascades following neutrophil activation and also regulate cell motility and actin reorganisation (Szczur *et al.*, 2006). Such cascades involve the activation of MAP family kinases and therefore, the phosphorylation of p44/42 (ERK1 and ERK2) MAPKs was next examined. Indeed, following fMLP stimulation, lower levels of phosphorylated ERK1/2 were observed in neutrophils that had been pre-treated with lactoferrin.

The arising data clearly suggest that the observed lactoferrin-mediated inhibitory effects on neutrophil chemotaxis could be attributed to modulation of molecules other than those involved in the $[Ca^{2+}]_i$ response following agonist stimulation. In addition, analysis of the expression of early activation markers such as CD62L and CD11b following stimulation with diverse neutrophil agonists, including PMA, a specific synthetic intracellular activator of PKC, further suggested that lactoferrin acts downstream or independently of the PKC pathway, affecting signalling cascade components of the

later phases of neutrophil activation. This notion was strengthened by the observation that lactoferrin inhibited the phosphorylation of p44/42 (ERK1 and ERK2) MAPKs, indicating that its action is mediated, at least in part, via the MAPK pathway, a key pathway downstream of PKC with a crucial role in the regulation of cytoskeletal rearrangement and cell adhesion (Thompson *et al.*, 1994; Thompson *et al.*, 1993; Van Lint *et al.*, 1993).

Although the exact molecular mechanism of lactoferrin concerning neutrophil chemotaxis requires further more detailed investigation, a model of lactoferrin activity can be proposed **(Figure 48)**. According to this model, it can be speculated that the previously reported specific interaction of lactoferrin with the calcium-calmodulin complex (Ca^{2+}/CaM) might also be a potential intracellular signalling target of lactoferrin (de Lillo *et al.*, 1992). The Ca^{2+}/CaM complex is formed following the increase in $[Ca^{2+}]_i$ and PKC activation, and regulates the activity of a number of pathways involved in cell adhesion and migration including the ERK1/2 kinases (Gaines *et al.*, 2008; Schmitt *et al.*, 2004; Verploegen *et al.*, 2005; Verploegen *et al.*, 2002). Indeed, the use of specific inhibitors of calmodulin activity has been demonstrated to mimic the effect of lactoferrin, i.e. inhibition of neutrophil migration as reported in this study (Downey *et al.*, 1996; Lian *et al.*, 2001; Naccache *et al.*, 1980; Verploegen *et al.*, 2005).

At a molecular level, it was further investigated whether the described effect of lactoferrin on neutrophils is NF-κB-associated. Indeed, it has been previously shown that lactoferrin binds to receptors on mononuclear phagocytes and inhibits proinflammatory responses via NF-κB (Birgens *et al.*, 1983; Haversen *et al.*, 2002; Van Snick and Masson, 1976). Also, on a rat model of trinitrobenzensulfonic acid (TNBS)-induced colitis, administration of lactoferrin has resulted in a significant reduction in the proinflammatory cytokines, TNF-α and IL-1β, a downregulation of the NF-κB pathway and an upregulation of the anti-inflammatory IL-4 and IL-10 (Togawa *et al.*, 2002a; Togawa *et al.*, 2002b). However, the involvement of the NF-κB pathway in the inhibition of neutrophil migration seems unlikely, since the lactoferrin-mediated inhibition of chemotaxis of neutrophils treated with gliotoxin, a specific NF-κB inhibitor, was found to be identical to that of untreated cells. In addition, no changes in the levels of IκB, an inhibitor of NF-κB that is degraded following NF-κB activation, were observed in Western blot analysis of fMLP-stimulated neutrophils treated with or without lactoferrin.

In general, the identification of lactoferrin is of particular importance, as it is one of the few physiological mediators that have been characterised so far to negatively regulate neutrophil migration. In this way, insights on the way that the other negative regulators act can prove beneficial so as to identify the

signalling pathway or the molecules affected by lactoferrin. For instance, lipoxins, mainly lipoxin A_4 (LXA_4), have been reported to inhibit neutrophil recruitment by inhibiting integrin clustering and mobility and by inhibiting TNF-α secretion via a block in ERK activation (Filep *et al.*, 1999; Serhan, 1997). Also, it stimulates the uptake of apoptotic neutrophils by macrophages (Godson *et al.*, 2000). Recently, it was found that LXA_4 associates with polyisoprenyl phosphate (PIPP) signalling pathway leading to accumulation of presqualene disphosphate (PSDP), a potent negative intracellular signal in neutrophils that acts by inhibiting superoxide generation and recombinant PLP (Jozsef *et al.*, 2002; Levy *et al.*, 1999; Maderna *et al.*, 2002; Ohira *et al.*, 2004). LXA_4 intracellular activity has also been demonstrated to involve a block in the phosphorylation and activation of leukocyte-specific protein-1 and of several other components of the p38 MAPK pathway. In terms of the recognition receptor for LXA_4, it has been shown that LXA_4 acts via an identified lipoxin A_4 receptor (ALX), which is a seven-transmembrane G-protein-coupled receptor (Chiang *et al.*, 2006). Annexin-1, which is also another negative regulator of neutrophil migration, acts via the same receptor (ALX), proposing in this way a similar mode of activity (Babbin *et al.*, 2006; Chiang *et al.*, 2006; Perretti, 2003). Moreover, ALX was found to share a conserved homology to ChemR23 that is a receptor for resolvin E1 (RvE1) (Arita *et al.*, 2005; Flower and Perretti, 2005). Whether lactoferrin binds or associates

with ALX is something that needs to be investigated. Also, whether lactoferrin modulates the expression of cytokines or related adhesion molecules on neutrophils so as to elicit its inhibitory effect is till now not known.

It should also be highlighted that lactoferrin was reported to be linked to an upregulation in IL-10 levels (Hwang *et al.*, 2007). This is a common characteristic with annexin-1 activity, as exposure of macrophages to annexin-1-derived peptides was demonstrated to induce IL-10 secretion; an effect also mimicking glucocorticoid activity (Clark, 2007; Ferlazzo *et al.*, 2003; Parente and Solito, 2004; Perretti *et al.*, 2002; Perretti and D'Acquisto, 2009; Souza *et al.*, 2007). In this way, comparison of annexin-1 or glucocorticoid effects with the anti-inflammatory effects of lactoferrin would be interesting to examine.

At this stage, a useful tool to study the molecular effects of lactoferrin would be the generation of lactoferrin knock-out mice ($LTF^{-/-}$). Unfortunately, $LTF^{-/-}$ mice are not commercially available. However, one study has reported the generation of such a knock-out strain, but there is controversy as to whether this model is successful, as these mice were presented to be viable and fertile and displayed a normal development. Also, the iron status of these mice was normal (Ward *et al.*, 2003). This is quite unexpected, based on the crucial role of lactoferrin in development, reproduction and as an iron-binding protein. Other reported evidence of a $LTF^{-/-}$ mouse

model presented a different phenotype, confirming the assumption that the generation of such a knock-out model is not feasible. Indeed, that $LTF^{-/-}$ model was lethal at the pre-implantation stage of the embryo (Spik, 1998). The embryo appeared normal until 8-cell stage, but failed to reach the 16-cell stage. The validity of this model was confirmed when the embryos were rescued following addition of recombinant mouse lactoferrin to the culture medium, showing in this way, that lactoferrin is involved in fetal haemopoiesis as well.

Therefore, based on these reported studies, several attempts should be made in order to generate a successful $LTF^{-/-}$ model. If such a model is made experimentally possible, then many insights would be gained in terms of the molecular effect of lactoferrin on neutrophil chemotaxis and the identification of the underlying factors and mechanisms. Also, using this $LTF^{-/-}$ model, the role of lactoferrin on the expression of cytokines could be easily determined. The factors that promote or regulate lactoferrin expression will be therefore better characterised, as till now there is restricted evidence as the ERE (estrogen response element) region is the only till now characterised regulatory molecule located in the 5' flanking region of the gene that controls the expression of the lactoferrin gene (Liu and Teng, 1992). The location and expression of ERE overlaps the binding site of the COUP transcription factor that acts as a negative regulator of oestrogen. Lactoferrin gene activity was also found to be dependent on prolactin expression

within a species' mammary gland (Green and Pastewka, 1978), whereas the uterine lactoferrin gene expression was highly dependent on 17β-oestradiol treatment (Pentecost and Teng, 1987; Teng *et al.*, 1989). In this way, the identification of the exact pattern of the molecular activity of lactoferrin will prove to be beneficial in several aspects of reproductive biology and inflammation.

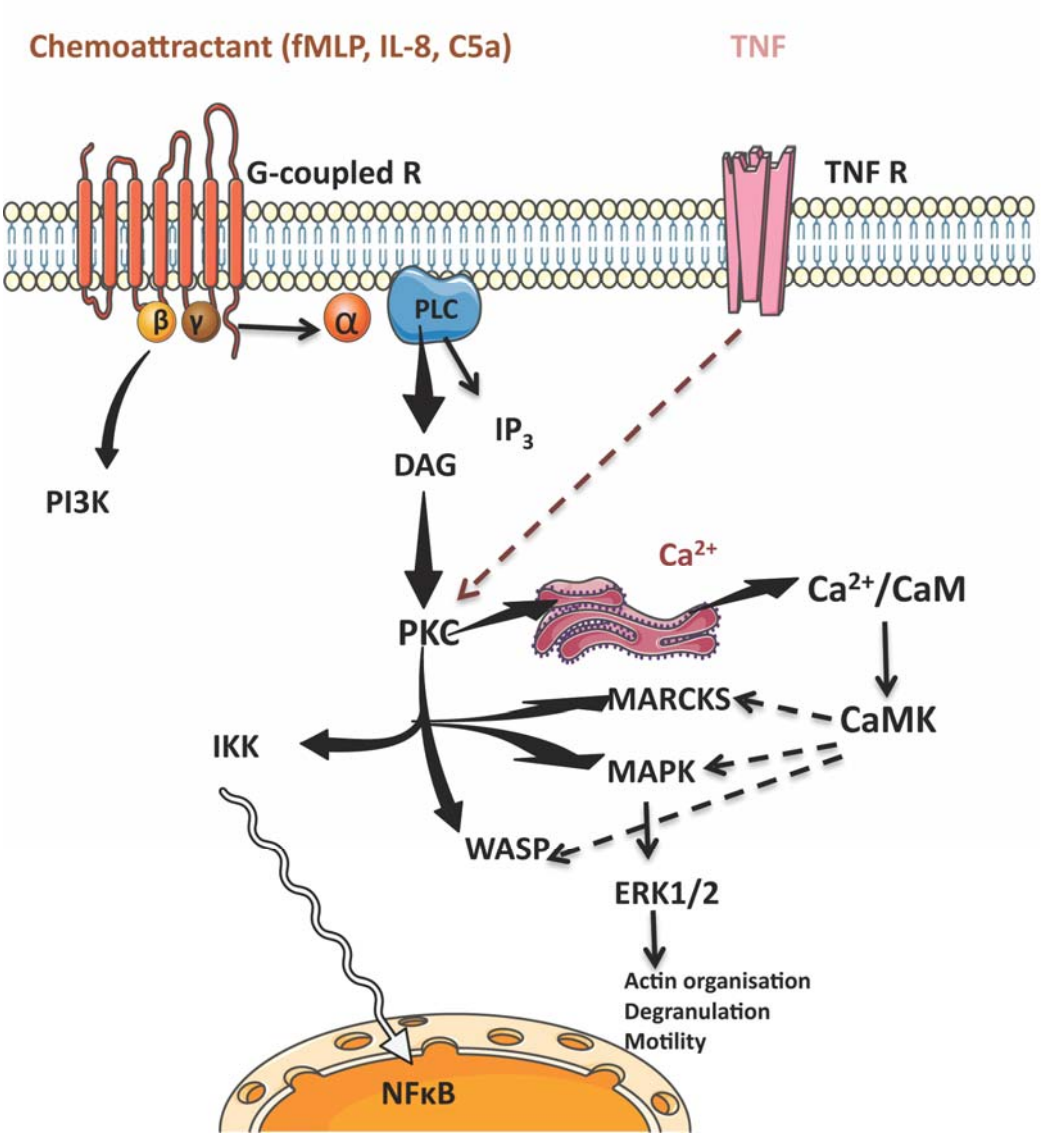


FIGURE 48: Proposed model of lactoferrin inhibitory activity on neutrophil chemotaxis

It is speculated that lactoferrin acts intracellularly and more specifically, it interacts and inhibits the activity of calcium-calmodulin (Ca^{2+}/CaM) complex. The Ca^{2+}/CaM complex is formed following PKC activation and $[Ca^{2+}]_i$ increase and regulates the activity of cell adhesion and migration pathways including ERK1/2 phosphorylation. Inhibitors of calmodulin activity mimic the inhibitory effect of lactoferrin on neutrophil migration.

The Role of Lactoferrin in The Resolution of Inflammation

The identification of lactoferrin, apart from its anti-microbial properties, bears strong implications in the therapeutic potential of many inflammatory disorders that are marked by aberrant neutrophil infiltration. Such disorders include glomerulonephritis, interstitial lung disease, rheumatoid arthritis etc. (Weiss, 1989). This is even supported by the finding that the presence of significant numbers of neutrophils found in the pulmonary fluid from infants with hyaline membrane disease (HMD) and bronchopulmonary dysplasia (BPD) are linked to lactoferrin and lysozyme deficiency in the airway secretion of the affected individuals (Revenis and Kaliner, 1992). Thus, although lactoferrin represents a major protein in respiratory epithelial lining fluid, its deficiency may lead to predisposition of altered host defense towards lung injuries and result to neutrophil accumulation. Moreover, the identification of the anti-inflammatory properties of lactoferrin enabled to gain better insights on its role in disorders that are marked by the presence of anti-lactoferrin antibodies, especially ulcerative colitis, primary sclerosing cholangitis, Crohn's disease and SLE (Audrain *et al.*, 1996; Caccavo *et al.*, 2005; Roozendaal *et al.*, 1998).

Due to its broad role in inflammation, many attempts have been carried out to obtain large amounts of the protein. In detail, a recombinant form has been available and was established using various expression systems such as

filaments of *Aspergillus* fungus (Ward *et al.*, 1992), yeast and other bacteria (Kim *et al.*, 2006; Tian *et al.*, 2007). Also recently, transgenic *Bombyx mori* insects were created, each expressing 205 µg of lactoferrin per infected pupa (Liu *et al.*, 2005b). This was followed by the generation of transgenic animals that produce lactoferrin in goats, mice, rabbits (levels up to 2 g of human lactoferrin per litre of milk) (Nuijens *et al.*, 1997; Zhang *et al.*, 2008). The development of lactoferrin peptides with highly efficient bacteriostatic, anti-microbial and apoptotic properties has also yielded tremendous applications (Elass *et al.*, 2002; Roy *et al.*, 2002; Ward and Conneely, 2004). The surprising fact is that the physical and biological characteristics of the recombinant protein are indistinguishable from the native one; however, in both cases, the possibility of LPS contamination shall not be excluded that might account for experimental variations among groups. Despite this limitation, it should be emphasised that lactoferrin is a commercially attractive molecule that is a natural component of the immune system with an ever-increasing list of properties. It can be readily supplied in large quantities and is one of the few physiological, endogenous expressed molecules characterised so far that negatively regulate neutrophil infiltration.

The inhibitory effect of lactoferrin on eosinophil migration should also be highlighted, as it can be therapeutically beneficial in allergic inflammatory conditions, including allergic rhinitis and asthma, which

are characterised by excessive eosinophil infiltration. Many modes of lactoferrin administration have been characterised and involve oral administration, subcutaneous or inhaled (Ward *et al.*, 2005). Another described mode involves the use of liposomes as carriers for lactoferrin (Chen *et al.*, 2010a; Roseanu *et al.*, 2010). Liposomes are prepared from biodegradable, non-toxic lipids and are used for the local delivery of therapeutic agents. A study by Trif *et al.* (2001), has introduced the application of human lactoferrin entrapped in positively charged liposomes in the inflamed joint in a model of collagen-induced arthritis. Therefore, the use of lactoferrin-entrapped liposomes can be a good candidate in inflammatory conditions to prevent their exacerbation. Also, the effect of lactoferrin on the activity of disease-specific anti-inflammatory drugs or whether the application of lactoferrin could enable the resolution and eradication of the inflammatory responses are two points that would be interesting to examine.

Despite its therapeutic potential, the identification of lactoferrin also reveals novel insights on the processes that take place during the resolution of an inflammatory response. What could therefore be proposed is that, based on the findings of this study, in inflammatory conditions, recruited, activated neutrophils arrive at the site of inflammation and release lactoferrin. Secreted lactoferrin creates a concentration gradient that stops neutrophil movement, while inhibiting the chemotaxis of other neutrophils that arrive from circulation **(Figure 49)**. In

fact, it is the same protein that is released by neutrophils, as a means of their effector activities, while it prevents their further recruitment. This response triggers the creation of an anti-inflammatory environment that marks the later phases of a response, which in parallel is also a homeostatic mechanism that promotes the initiation of the resolution phase of inflammation. Indeed, the local concentration of lactoferrin at sites of inflammation or infection was found to be up to 200 $\mu g\, ml^{-1}$ maximum; a concentration that was demonstrated in this study to be able to inhibit neutrophil migration *in vitro*.

Furthermore, in sites where homeostatic apoptosis rates are high, which are characterised by an anti-inflammatory environment, it can be suggested that the constitutive production of lactoferrin accounts for the absence of neutrophils from such sites and therefore, it contributes to the non-inflammatory nature of apoptosis. In general terms, the anti-inflammatory nature of the apoptotic program is stimulated mainly by the release of anti-inflammatory cytokines, such as IL-10, TGF-β by macrophages or epithelial cells as well as by possible till now unidentified molecular mechanisms that prevent neutrophil recruitment. Also, in professional phagocytes, sensing mechanisms that are not yet well defined allow these cells to navigate to apoptotic cells through chemotactic processes involving lipid signalling molecules, such as lipoxins and classical chemokine mechanisms. It is noteworthy that of the two categories of

professional phagocytes, mononuclear and polymorphonuclear cells, apoptotic cells attract mononuclear phagocytes selectively (Kerr *et al.*, 1972; Ravichandran and Lorenz, 2007). Indeed, the lack of recruitment of granulocytes to apoptotic zones is a hallmark of the non-phlogistic apoptosis program, distinguishing normal areas of cell death from sites of accidental or pathological tissue damage or infection.

Recent evidence suggests that certain recombinant preparations of lactoferrin have the potential to chemoattract monocytes (de la Rosa *et al.*, 2008). Although monocyte attraction to milk-derived lactoferrin failed to be observed in this study, these results indicate that lactoferrin has the potential to function in the selective attraction of monocytes by excluding granulocytes from sites where homeostatic apoptosis rates are high. Furthermore, a point that should be examined is whether lactoferrin interacts or regulates the expression of "find-me" molecules that are exposed on the surface of apoptotic cells or macrophages to promote cell clearance. It can be speculated that lactoferrin, secreted by neutrophils or by cells at the early stages of their apoptosis, as demonstrated in this study, promotes the expression of macrophage chemoattractants or recognition molecules, such as thrombospondin, PS exposure and nucleotides that will enable phagocytic clearance.

In summary, it can be proposed from the results of this study that lactoferrin arising from the secondary granules

of neutrophils and from apoptotic cells constitutes a negative feedback component that limits the influx of neutrophils. On this basis, it could be speculated that neutrophils undergoing apoptosis *en route* to resolution of inflammation would release lactoferrin as do other apoptotic cells but would be unlikely to be required to synthesise the protein *de novo*. Also, this could be a mechanism by which granulocytes are retained at the inflammatory site, as lactoferrin prevents them from moving. Lactoferrin is therefore a constitutive anti-inflammatory component of apoptotic cells that, in addition to its known anti-inflammatory properties, militates against the proinflammatory recruitment of granulocytes to sites where homeostatic apoptosis rates are high.

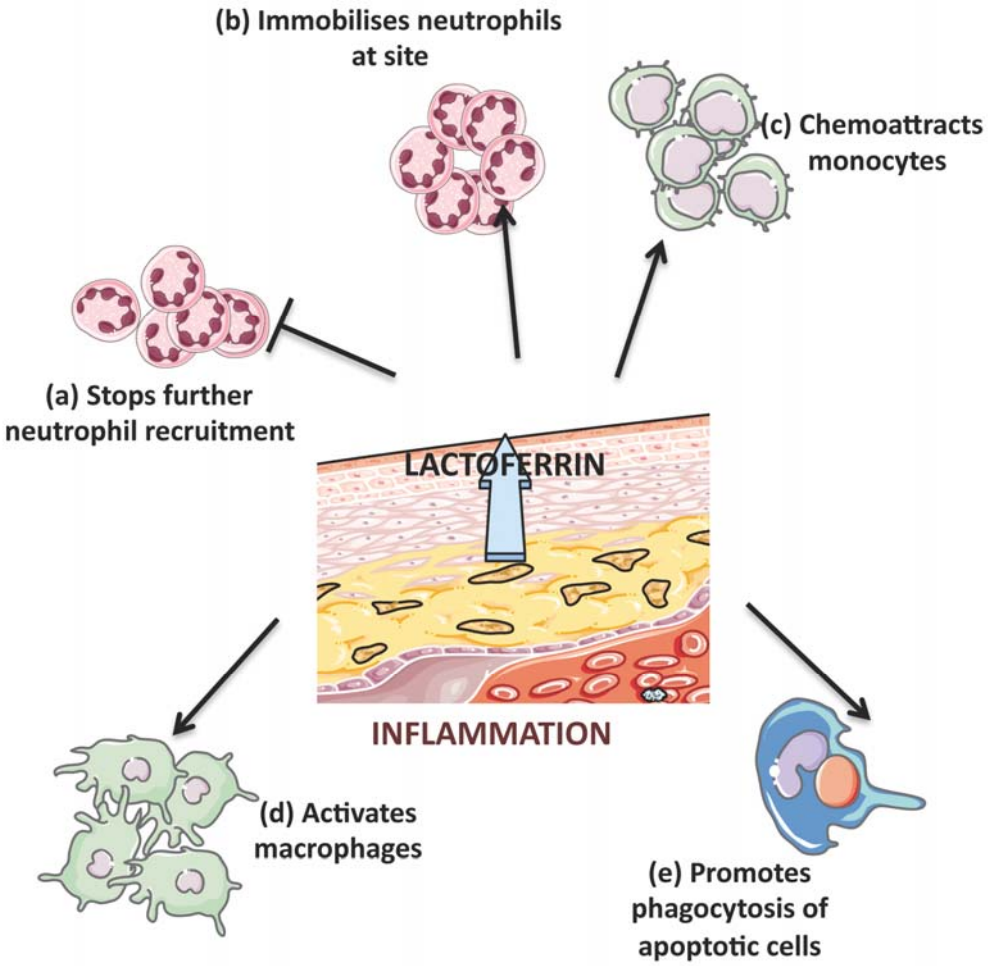


FIGURE 49: Biological contribution of lactoferrin in the resolution of an inflammatory response

Based on the results of this study, in an inflammatory site, lactoferrin is secreted by the recruited neutrophils and by resident apoptotic cells. Secreted lactoferrin creates a concentration gradient that (a) stops further neutrophil recruitment, (b) immobilises resident neutrophils allowing them to exert their effector functions, (c) chemoattracts monocytes (based on de la Rosa *et al.*, 2008), (d) activates resident macrophages

(based on Curran *et al.*, 2006) and (e) promotes phagocytosis (based on Lima and Kierszenbaum, 1985).

Lactoferrin, Apoptosis and Tumour Proliferation

The first published evidence on the role of apoptosis and lactoferrin secretion arose from studies on lactation and the involuting mammary tissue (Monks *et al.*, 2002; Wang *et al.*, 2005; Wilde *et al.*, 1997). During lactation, there is a strong expression of lactoferrin mRNA expression on day 1 of lactation, whereas a cDNA microarray analysis on the expression of genes during the involution of bovine mammary gland further revealed a decreased mRNA expression of the milk protein genes of β-, κ-casein, α-lactalbumin and β-lactoglobulin and a dramatic increase of the expression of lactoferrin 192 h post-milking (Singh *et al.*, 2008; Wang *et al.*, 2005). Therefore, as the involution of the mammary tissue is dominantly characterised by elevated levels of apoptosis, the associated increase in the levels of lactoferrin provided initial insights that link lactoferrin to apoptosis.

In this study, it was revealed that lactoferrin is endogenously expressed and released by a variety of tumour types upon induction of apoptosis. More specifically, when BL2 cells were staurosporine-stimulated, an upregulation in lactoferrin expression was noted parallel to the increase in apoptosis levels. In contrast, BL2 cells transfected with the anti-apoptotic *BCL2*, failed to express lactoferrin to similar levels due to the lower

apoptosis rates. Lactoferrin expression was also found to be upregulated in MCF-7 cells and expressed *de novo* in Jurkat, BL2 and A549 cells, irrespective of the apoptosis-inducing agent. Not only was apoptosis-related lactoferrin expression demonstrated at the transcriptional level, lactoferrin protein was also recovered from supernatants of cells undergoing apoptosis. This finding demonstrated that lactoferrin is actively expressed by several tumour types and highlighted the importance of apoptosis in tumour progression. In this way, lactoferrin can be used as a marker to histologically quantify apoptosis or to assess the progression rate of tumours.

Indeed, several studies supported that in conditions like tumours that are characterised by distortions in the regulation of cell population, apoptosis plays a key role by regulating tumour progression and prognosis. For example, in breast carcinomas, high-grade tumours display more apoptosis (Lipponen *et al.*, 1994; Mustonen *et al.*, 1997). Also, high-grade malignant non-Hodgkin's lymphomas have a higher apoptotic index than low-grade lymphomas and this correlates to Bcl-2 expression, which is increased in low-grade lymphomas (Kiberu *et al.*, 1996; Soini *et al.*, 1998a). In parallel, other studies have supported the role of lactoferrin as a growth factor. For example, lactoferrin induces the development of osteoblast cells by promoting their proliferation via low density lipoprotein receptor related protein-1 (LRP-1) signalling (Cornish *et al.*, 2004; Grey *et al.*, 2004). Also, i*n vivo*, in mice in which lactoferrin (4 mg) had been administered

subcutaneously for 5 days, a marked fourfold increase in bone mass was noted (Cornish *et al.*, 2004). In mammary cells, lactoferrin has been reported to interfere with retinoid signalling (Baumrucker *et al.*, 2006b). The mitogenic effect of lactoferrin also extends to enterocyte formation, B and T lymphocyte expansion as well as macrophage development (Grey *et al.*, 2006). In general, lactoferrin was suggested to be an important contributor in terms of organ formation and bone development, based on its high levels of expression in a foetus as well as in several tumours. Therefore, based on this study and previous published evidence, it can be speculated that the role of lactoferrin in tumours is at least two-fold, as it (i) prevents neutrophil infiltration and thus any arising cytostatic effects and (ii) promotes tumour cell proliferation. The latter is supported by results of this study, which indicated that blocking the activity of lactoferrin or knocking down its expression, also decreases the proliferation rate of the tumour cells in Burkitt's lymphoma irrespective of whether they associate to EBV infection; however, the exact mode of lactoferrin recognition by proliferating cells is something till now not known.

This effect was prominent by assessing the proliferation of cells cultured in the presence of monoclonal antibodies against lactoferrin as well as of cells that were shRNA-transfected to knockdown lactoferrin expression. Such an effect on cell proliferation was accompanied by the observation that BL cells, in which lactoferrin has been

blocked via monoclonal antibodies, underwent apoptosis and an arrest at G1/S cell cycle checkpoint at a significantly higher rate than their control counterparts. Such a negative effect on cell cycle progression was also reported in other studies, in which lactoferrin was demonstrated to interfere with the cell cycle and induce a cell cycle arrest at the G1/S checkpoint by affecting cell cycle regulatory proteins such as cyclin E, p21 and p27 Cdk inhibitory proteins that are associated with the Akt and MAPK pathways (Damiens *et al.*, 1999; Xiao *et al.*, 2004; Zhou *et al.*, 2008). It should be mentioned that the observed effect of lactoferrin on the cell cycle or on the levels of apoptosis was not evident when the proliferation of lactoferrin shRNA-transfected BL2 cells was assessed. Therefore, it could be proposed that the addition of the anti-lactoferrin antibody to BL cells induces cellular death via an antibody-dependent cytotoxicity. Moreover, the possibility that the use of antibody causes a rapid deprivation of lactoferrin in these cells, promoting their cellular death, shall not be excluded as the shRNA transfected ones were used to survive in a lactoferrin-deficient environment. Also, in shRNA-transfected cells, lactoferrin was knocked down and not completely neutralised, and therefore, low levels of lactoferrin could have been present.

Although depletion of endogenous lactoferrin by monoclonal antibodies resulted in an increase in the apoptosis levels of BL cells, lactoferrin *per se* was not found to induce or stimulate apoptosis. Indeed, BL cells that were cultured

in serum-free medium in the presence of lactoferrin underwent apoptosis at a similar rate as those cultured in the absence of lactoferrin. Moreover, staurosporine-induced apoptotic BL cells displayed a similar level of apoptosis in the presence or absence of lactoferrin, showing in this way that lactoferrin does not promote the apoptosis program in Burkitt's lymphoma. This finding comes in contrast to published evidence that reported that lactoferrin induced apoptosis in hepatic epithelial cells via an iron-dependent lysosomal death pathway in a process necessary for caspase-3 activation (Gorria *et al.*, 2008). Recent evidence also suggested that the protease activity of lactoferrin may be instrumental in activating the caspase cascade, as endogenous lactoferrin has been reported to induce apoptosis through activation of caspase-3 (Gorria *et al.*, 2008; Katunuma *et al.*, 2006). Additionally, lactoferrin was demonstrated to promote apoptotic cell death in oral cancer cells by inducing cleavage of caspase-3 and poly (ADP-ribose) polymerase, phosphorylation of ERK1/2 and phosphorylation of cJun N-terminal kinase/stress activated protein kinase (JNK/SAPK) (Sakai *et al.*, 2005). Indeed, it was found that pre-treatment of cells with a JNK/SAPK inhibitor reduced lactoferrin-induced apoptosis. This was also confirmed in subsequent studies in Jurkat T leukemias, to which lactoferrin- induced apoptosis via the regulation of c-Jun-N-terminal kinase (JNK) activity and via the activation of caspase-9 and caspase-3 (Lee *et al.*, 2009). However, how lactoferrin could sustain proliferation and

how its depletion can promote apoptosis could involve distinct signalling pathways -other than those reported so far- that could be caspase-dependent or independent differing among tumour types. For this reason, further investigation is required on the underlying molecular mechanisms.

A possible mechanism of activity could be via the PPAR (peroxisome proliferator activated receptor) pathway, as PPARα was found to be a transcription factor for lactoferrin, while PPARβ/δ was demonstrated to inhibit leukocyte recruitment, expression of cell adhesion molecule (CAM) and chemokine release (Hasmall *et al.*, 2002; Piqueras *et al.*, 2009). Therefore, it can be speculated that a PPAR-mediated mechanism seems able to link and regulate both activities of lactoferrin as a negative regulator of neutrophil migration and as a growth factor; however, it needs to be experimentally verified. Also, in the context of lactoferrin activity as a growth factor, it should be investigated whether the iron binding domain of lactoferrin or the iron saturation of lactoferrin regulate its growth factor activities, as iron and lactoferrin were previously reported to be essential for cell growth (Baumrucker *et al.*, 2006a; Burnham, 1963).

Additionally, in this study, initial insights were gained in terms of the mode of lactoferrin secretion by cells. So far, there is little published evidence on the manner that lactoferrin is released by neutrophils or mammary cells during lactation. In this study, it was demonstrated that

treatment of A549 cells with brefeldin A, which interferes with intracellular transport of newly synthesised proteins, resulted in inhibition of apoptosis-induced lactoferrin release, providing further evidence for the *de novo* synthesis and secretion of lactoferrin. Moreover, although it is clear that the secretory events inhibited by brefeldin A treatment (transport from the endoplasmic reticulum (ER) and Golgi network) are important for the release of lactoferrin from apoptotic cells, further work will be required to elucidate the details of this secretory process, including whether lactoferrin is transported directly from Golgi membranes for release from the cell surface. All these findings support the possibility of exocytosis as a mode of lactoferrin release.

It can be suggested that other insights on the mode of lactoferrin release could be gained from the mode of annexin-1 release. Annexin-1 is also an inhibitor of neutrophil recruitment that is stored in gelatinase granules (Lominadze *et al.*, 2005; Perretti and Flower, 2004). Though the molecular mechanisms of annexin-1 release are cell-specific, in macrophages, the ATP-binding cassette (ABC) transporter system was found to be responsible for annexin-1 release, while in neutrophils, annexin-1 becomes mobilised and relocated to the outer leaflet of the plasma membrane upon activation by chemoattractants (Perretti *et al.*, 2000; Vong *et al.*, 2007; Wein *et al.*, 2004). In pituary cells, phosphorylation at Ser27 of annexin-1 is enough to promote

its secretion (John *et al.*, 2002; Porte *et al.*, 1996; Solito *et al.*, 2006). In this way, in the case of lactoferrin, the mode of its secretion might differ between neutrophils, where it is stored in granules, and apoptotic cells, by which it is *de novo* expressed. Also, lactoferrin release might occur as a stress-response; providing in this way an explanation why it is released at the early stages of apoptosis and thus rendering the involvement of the JNK/SAPK pathway more possible. Although this study failed to detect lactoferrin in apoptotic cells by immunohistochemical or immunofluorescent staining, as these approaches were not sensitive enough in detecting the low amount of expressed lactoferrin, it is anticipated that novel approaches or optimisation of the existing ones would allow more insights to be gained in terms of the mode of lactoferrin secretion.

In general terms, the observation that lactoferrin released into the supernatants of apoptotic cells is able to inhibit the migration of neutrophils, demonstrates not only that physiological concentrations of lactoferrin are functional in mediating this effect but also that apoptotic cells are active in producing a factor that can potently suppress migration of neutrophils, the major subset of professional phagocytes among circulating leukocytes, to sites where homeostatic apoptosis rates are high. The anti-inflammatory role of lactoferrin has also been supported by the fact that the release of lactoferrin was found not to be linked to necrosis, which is

characterised by a highly inflammatory environment, but that it is expressed and actively released from cells as a consequence of activation of their apoptosis program.

In summary, the results of this study have indicated that the overall apoptotic microenvironment of a tumour triggers the synthesis and release of lactoferrin. In addition, despite the fact that lactoferrin has been reported in other studies to exert apoptotic effects on cells, such as hepatocytes, mammary cells and others, something like that was not evident in this study using Burkitt's lymphoma cells. Therefore, lactoferrin may have multiple properties in apoptosis: (a) regulating the initiation of the program, (b) influencing repair in the tissue microenvironment and (c) promoting the non-phlogistic nature of the process; showing in this way that the endogenous production of lactoferrin is linked to the initiation of the apoptosis program, to its anti-inflammatory features as well as to tumour progression.

Lactoferrin as a Molecule of Tumour Immunosurveillance

The involvement of lactoferrin in the regulation of neutrophil recruitment and BL cell proliferation enabled its characterisation as a molecule of the tumour immunosurveillance mechanism **(Figure 50)**. Under physiological conditions, lactoferrin expression extends to nearly all tissues such as breast, stomach, pancreas, salivary gland, endometrium, spleen, prostate liver, blood and others (Siebert and Huang, 1997). Its expression becomes upregulated under specific normal conditions such as organ morphogenesis, lactation and involution of the mammary gland (Monks *et al.*, 2002; Wilde *et al.*, 1997). Based on the results of this study, the absence of neutrophils from these tissues could be attributed to the presence of lactoferrin.

In the case of tumours, studies have reported enhanced lactoferrin expression in neoplastic bone tissues, in human sporadic renal cell carcinomas, in human astrocytomas, in colon carcinomas, in human breast cancer, in Hodgkin's disease and Burkitt's lymphoma (Brock *et al.*, 1994; Campbell *et al.*, 1992; Hoffer *et al.*, 1979; Ieni *et al.*, 2009; Tuccari *et al.*, 1999) and during the neoplastic transformation of the endometrium (Walmer *et al.*, 1995) and of the endocervix (Giuffre *et al.*, 2006). Especially, in the case of endocervix neoplasms, neoplastic formation correlates with downregulation of lactoferrin expression (Farley *et al.*, 1997). Lactoferrin mRNA was also shown by RT-PCR analysis to be expressed by the promyelocytic

leukemia HL-60 cell line, T-lymphoblastic leukemia Jurkat cell line, breast tumour T41D cell line, Burkitt's lymphoma Raji, colorectal adenocarcinoma SW490 and erythroleukemia K562 (Hoffer *et al.*, 1979; Siebert and Huang, 1997). However, the role or how lactoferrin expression becomes regulated in tumour tissues requires further investigation. Moreover, based on the identification of the differential expression of the lactoferrin isoform, delta lactoferrin in tumour tissues, it cannot be excluded that inside a tumour cell, lactoferrin can be cleaved or alternatively spliced (Breton *et al.*, 2004; Siebert and Huang, 1997). Another point that requires investigation is whether the glycosylation profile of lactoferrin or its iron saturation status might promote tumour progression (van Berkel *et al.*, 1996). Also, whether there are polymorphic sites in the lactoferrin gene that account for these properties of lactoferrin shall also be examined.

In general terms, although the exact activities of lactoferrin in relation to tumour biology are currently unclear, based on this study, it can be therefore suggested that due to its anti-inflammatory properties, lactoferrin is a protein that is manipulated by the tumour itself to regulate any possible anti-tumour immune responses by the host. For instance, lactoferrin expression might be altered or upregulated by tumour-derived cytokines that are released to render hypofunctional the immunogenicity of the host organism. One example is IL-10, which is expressed by tumours, like

Burkitt's lymphoma in this case, and was presented to be upregulated following administration of lactoferrin (Hwang *et al.*, 2007). Lactoferrin could also account for B cell maturation by potentially upregulating factors such as B cell-activating factor (BAFF/BLyS)(Ogden *et al.*, 2005), whereas the tight association of lactoferrin with the tumour regulatory protein IL-18 could also support the involvement of lactoferrin in tumour-promoting responses (Iigo *et al.*, 2009; Iigo *et al.*, 2004). Other point that could be examined is the association between lactoferrin and Bcl-2 expression, as alterations in Bcl-2 expression are linked to tumour establishment and progression. Also how lactoferrin expression can influence or be modulated by the hypoxic environment of some tumour regions is a point not known yet.

Despite the fact that lactoferrin exerts no effect on monocyte migration, it might possibly induce TAM differentiation to M2 phenotype, affect their activation status as well as the rate of phagocytosis of engulfed apoptotic tumour cells. Indeed, the phagocytosis rate and the presence of apoptotic bodies in tumours, especially BL, are high and the presence of lactoferrin is very possible to affect these processes. Also, whether recruited monocytes or TAMs express lactoferrin is something that has not been examined yet. On a similar basis, the production of lactoferrin could account for the absence of neutrophils from the Burkitt's lymphoma site. It can thus be predicted that *in situ* production of lactoferrin acts as a homeostatic mechanism to prevent the

cytostatic effects that the migrated neutrophils would exert. This prediction is supported by the observation that Burkitt's lymphoma, in which high rate of apoptosis occurs, has been demonstrated to display *in situ* production of lactoferrin (Hoffer *et al.*, 1979).

Other functions that are still under investigation involve the efficiency of lactoferrin as a growth factor in association to its anti-tumour ability and the arising controversy on how its activities are interrelated. Indeed, there are studies that support lactoferrin as an anti-apoptotic molecule, as oral administration of lactoferrin in a mouse intestinal mucosa was demonstrated to inhibit carcinogenesis and metastasis (Iigo *et al.*, 2004), diminished head and neck squamous cell carcinomas *in vivo* (McKeown *et al.*, 2006; Wolf *et al.*, 2007; Xiao *et al.*, 2004), prevented lung colonisation and experimental metastasis in a B16-F10 melanoma model (Bezault *et al.*, 1994) as well as azoxymethane (AOM)-induced rat colon tumour formation and aberrant crypt foci development (Sekine *et al.*, 1997a; Sekine *et al.*, 1997b; Tsuda *et al.*, 1998; Tsuda *et al.*, 2000). In other words, since lactoferrin is actively synthesised and involved in tumour proliferation, a question that arises is how lactoferrin can exert an anti-tumour effect. This is a point that does not reject the results of this study, but instead it prompts particular attention in terms of the dose and the source of the administered lactoferrin. There are many variations in the mode of administration of lactoferrin, its purity, species' specificity as well as differences in

the time of experimentation or even non-specific immune responses that are elicited by the glycan moieties of the protein. Another determinant is also differences in the production of native and recombinant lactoferrin. Purified lactoferrin usually arises following metal ion affinity chromatography or concanavalin A affinity chromatography and is in the iron-saturated form (Nuijens *et al.*, 1996). However, unlike previous published literature that is based on the exogenous administration of lactoferrin, this study is the first to report that lactoferrin is an endogenously expressed factor by tumours that plays a role in their proliferation.

In this way, based on the endogenous expression of lactoferrin, attempts shall be made in order to beneficially employ this molecule in tumour immunotherapy. Future experiments shall be focused on the knock-down of lactoferrin in tumour models *in vivo* and on the examination of whether this affects the recruitment of neutrophils in order to exert any cytostatic effects against tumour. Also, the level and type of tumour-secreted cytokines are crucial on how tumour responds in the absence of lactoferrin and the type of mechanisms that are employed by the tumour to survive regression should be investigated. Additionally, the rate of proliferation and tumour apoptosis are two parameters that shall be examined. A useful tool to address all these questions would be the establishment of a valid lactoferrin knockout mouse model, which, in parallel, will provide useful

insights on the underlying molecular mechanism of lactoferrin activity.

Therefore, based on the findings of this study, further research is required to unravel the exact molecular mechanisms that enable neutrophil anti-tumoural activity as well as the elaboration of molecules that tumour cells employ in order to prevent neutrophil recruitment. It is evident that the manipulation of mechanisms that induce neutrophil migration and their cytostatic responses can be very successful. Hopefully, lactoferrin is one of the molecules that can be therapeutically beneficial in this context. Although the existence of a tumour and the mechanisms that promote tumour immunosurveillance are too complex to prevent tumour regression, it is anticipated that lactoferrin, in combination with the existing anti-tumour therapies would lead to a massive infiltration of neutrophils to the tumour stroma, decrease the rate of tumour proliferation and finally succeed in the total eradication of the tumour itself.

In summary, given the multifunctional abilities of lactoferrin, the results of this study highlight the influence of apoptotic cells on multiple physiological processes, including cell growth, differentiation, innate and adaptive immune responses, as well as the pathological process of inflammatory and malignant diseases. Since lactoferrin has potent ability to inhibit neutrophil migration, it seems likely that in tumours in which neutrophils are absent, like Burkitt’s lymphoma, this

might act as an evading mechanism by the tumour in order to escape neutrophil infiltration and therefore, their cytotoxic effects. In this way, induction of neutrophil infiltration through inhibition of lactoferrin may lead to tumour destruction. On the contrary, in tumours in which neutrophils play a supportive role, such as gliomas, limitation of neutrophil infiltration through lactoferrin administration could be therapeutically beneficial.

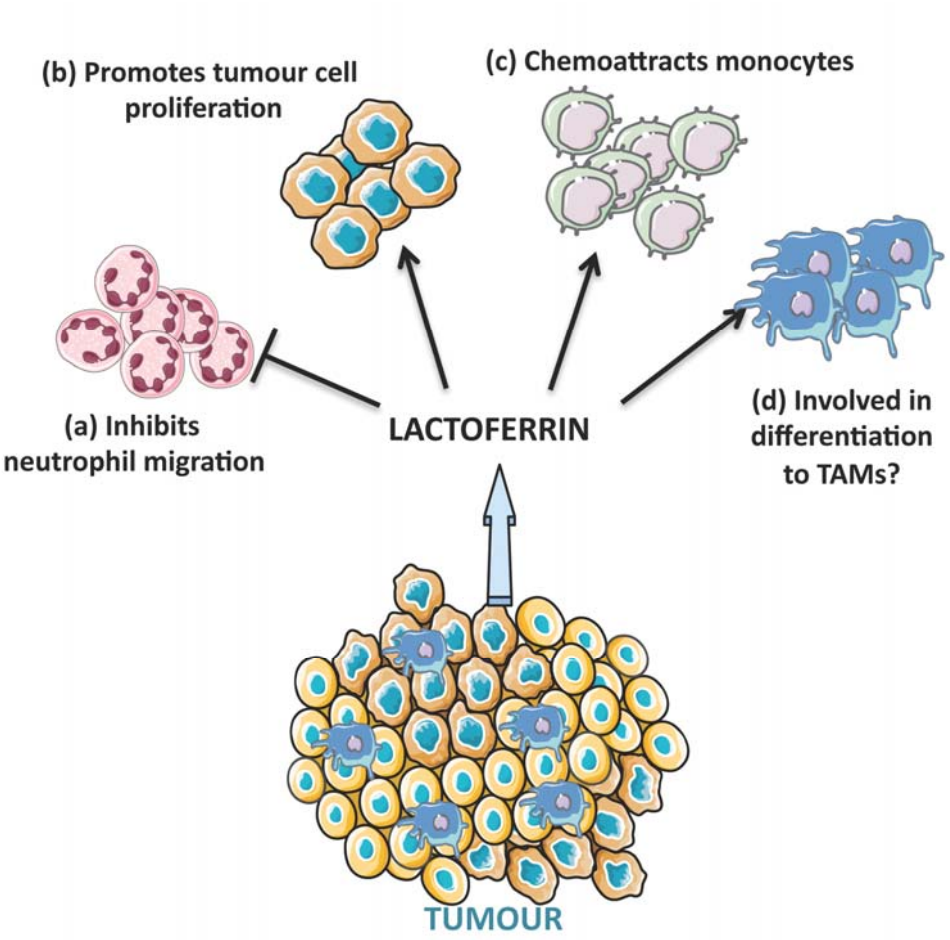


FIGURE 50: Biological contribution of lactoferrin in tumour establishment and progression

Based on the results of this study, in a tumour site, endogenous lactoferrin (a) promotes BL cell proliferation, (b) is released from viable (?) and/or apoptotic tumour cells to inhibit neutrophil migration as a homeostatic mechanism to avoid the cytostatic effects of neutrophils. Also, based on de la Rosa *et al.* (2008), secreted lactoferrin chemoattracts monocytes that could later become differentiated to tumour-associated macrophages (TAMs), which have a symbiotic relationship with the tumour, promoting its growth.

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to my supervisors Profs. *Christopher Gregory* and *Adriano Rossi* for the help, guidance, encouragement and support during all the stages of my PhD project. It is my honour that they trusted me for this project and shared their research enthusiasm with me.

Special thanks to Dr *Simon Brown* for the helpful discussions and for providing me with the Scatchard analysis' results of lactoferrin binding to neutrophils. Prof. *Ian Dransfield* and Dr *Simon Hart* are two persons I particularly wish to thank for the helpful discussions and advice. I would also like to thank Dr *Rodger Duffin* for his help on the *in vivo* peritonitis experiments, *Lynsey Melville* for establishing the xenograft models, measuring tumour growth, preparing histology sections and advice on immunohistochemistry, Dr *Jason King* for helpful advice on shRNA-mediated gene knockdown and for providing the lentiviral packaging vectors, *Marta Pasikowska* for providing the BL2-shRNA negative control cell line and the Sequencing Service (University of Dundee) for performing DNA sequencing.

I am also grateful to all members of the *Inflammation and Cancer group* for their encouragement and the *Medical Research Council* for the financial support.

This thesis wouldn’t have been possible without the constant love, support, encouragement and care from my family and to them I dedicate this thesis.

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ABBREVIATIONS

List of Abbreviations	
ABC	ATP-Binding Cassette
ACTH	Adrenocorticotrophic Hormone
ADCC	Antibody-Dependent Cell-mediated Cytotoxicity
AIDS	Acquired Immune Deficiency Syndrome
AIF	Apoptosis-Inducing Factor
ANOVA	One-way Analysis of Variance
ANXA1	Annexin A1
Anx	Annexin V
AOM	Azoxymethane
AP-1	Transcription factor Activator Protein-1
Apaf-1	Apoptosis Activating Factor-1
APC	Allophycocyanin
Apo2	Apoptosis antigen 2
Apo3	Apoptosis antigen-3
Arp2/3	Actin-Related Proteins 2/3
ATLs	Aspirin-Triggered Lipoxins
BAFF	B cell Activating Factor
BBMVs	Brush Border Membrane Vesicles
BCG	Bacille Calmette-Gurein
BE-LfR	Bronchial Epithelium Lactoferrin Receptors
β2-GPI	β2-glycoprotein I
β-ME	β-mercaptoethanol
BL	Burkitt's lymphoma
BLyS	B Lymphocyte Stimulator
BPD	Bronchopulmonary Dysplasia
BSA	Bovine Serum Albumin
C5a	Complement factor 5a
CAD	Caspase-Activated DNase
CAM	Cell Adhesion Molecule
Cdk	Cyclin-dependent kinases
c-FLIP	FLICE-like Inhibitory Protein
CGD	Chronic Granulomatous Disease
cJNK	c-Jun-N-terminal kinase
CLLs	Chronic Lymphocytic Leukemias
COUP-TF	Chicken Ovalbumin Upstream Promoter-Transcription Factor
COX-2	Cyclooxygenase-2
CRP	C-Reactive Protein
CSF-1	Colony Stimulating Factor-1
CXCR	Chemokine Receptor
DAG	Diacylglycerol
DCR	Decoy Receptor

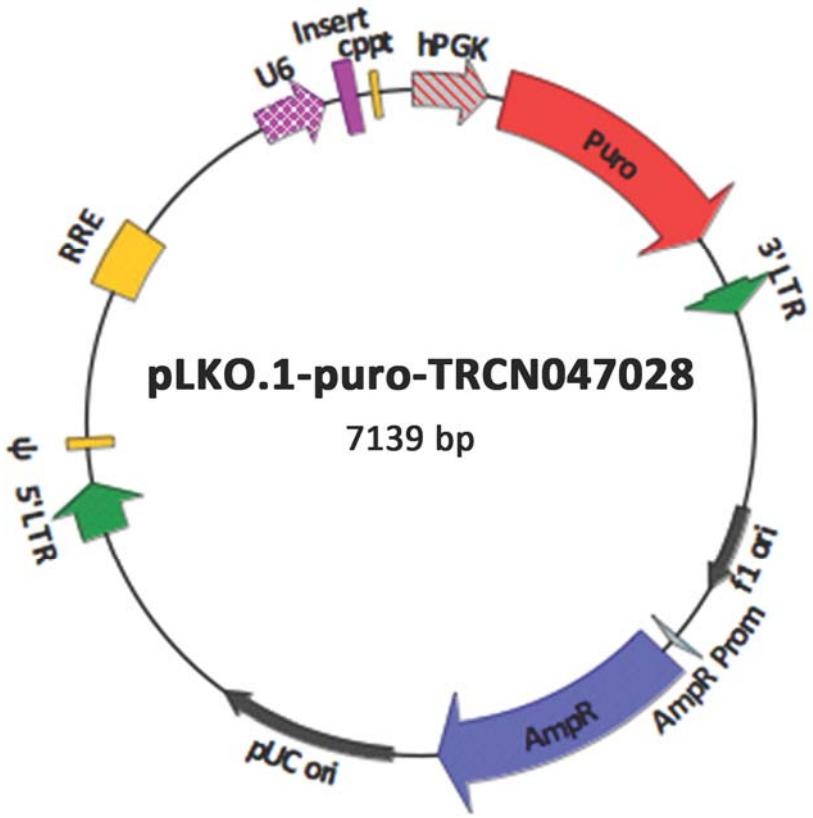
DCs	Dendritic Cells
DHA	Docosahexaenoic acid
DISC	Death-Inducing Signalling Complex
DMBA	Dimethylbenzantracene
DMEM	Dulbecco's Modified Eagle Medium
DR3	Death Domain Receptor 3
DR4	Death Domain Receptor 4
DR5	Death Domain Receptor 5
EBV	Epstein-Barr Virus
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EGTA	Ethylene Glycol Tetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EPA	Eicosapentaenoic acid
ER	Estrogen Receptor or Endoplasmic Reticulum
ERE	Estrogen Response Element
ERK	Extracellular-signal-regulated kinases
FADD	Fas-Associated Death Domain
FasL	Fas Ligand
FBS	Foetal Bovine Serum
FHF	Familial Hibernian Fever
FISH	Fluorescence *In situ* Hybridisation
FITC	Fluorescein isothiocyanate
FMF	Familial Mediterranean Fever
fMLP	formyl-Methionyl-Leucyl-Phenylalanine
FPR	Formyl-Peptide Receptor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAP	GTPase Activating Protein
Gas-6	Growth Arrest-Specific 6
GATA-1	GATA-binding factor 1
G-CSF	Granulocyte Colony-Stimulating Factor
GCT	Giant Cell Tumour
GDIs	Guanosine Dissociation Inhibitors
GDP	Guanosine Diphosphate
GEF	Guanosine Exchange Factor
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GRO/KC	Growth-Related Oncogene
GTP	Guanosine Triphosphate
HBSS	Hanks' Buffered Salt Solution
HDM-2	Human Double Minute-2
HIV	Human Immunodeficiency Virus
HMD	Hyaline Membrane Disease
HOCl	Hypochlorous acid
HRP	Horseradish
HS	Heparin Sulfate

hTERC27	human Telomerase Reverse transcriptase
HUVEC	Human Umbilical Vein Endothelial Cell
IAP	Inhibitor of Apoptosis Protein
ICAD	Inhibitor of CAD
ICAM-1	Inter-Cellular Adhesion Molecule-1
IFN	Interferon
IL-1Ra	IL-1 Receptor antagonist
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
IP3	Inositol triphosphate
i.p.	Intraperitoneal
IκBα	Nuclear factor of κB Inhibitor, α
K5	Plasminogen Kringle 5
LAK	Lymphokine-Associated Killer
LARD	Lymphocyte-Associated Receptor of Death
LB	Lysogeny Broth
LC-LfR	Lymphocyte Lactoferrin Receptor
LEC	Liver Expressed Chemokine
LFA-1	Lymphocyte Function-associated Antigen-1
Lfcin	Lactoferricin
liver LfR1	liver Lactoferrin Receptors
LPC	Lysophosphatidylcholine
LRP	LDL receptor-Related Protein
LRP-1	Low density lipoprotein Receptor related protein-1
LSP-1	Lymphocyte-Specific Protein-1
LT	Lymphotoxin
LTB4	Leukotriene B4
LTF	Lactoferrin
LXA_4	Lipoxin A4
LXB_4	Lipoxin B4
MAC-1	Macrophage-1
MALDI-TOF	Matrix-Assisted Laser Desorption Ionisation–Time Of Flight
MAP kinase	Mitogen-Activated Protein kinase
MBL	Mannan-Binding Lectin
MC-LfR	Monocyte Lactoferrin Receptors
MCP-1	Monocyte Chemotactic Protein-1
M-CSF	Macrophage Colony-Stimulating Factor
MFG-E8	Milk Fat Globule EGF-Factor 8
MHC	Major Histocompatibility Complex
MIP-1	Macrophage-Inflammatory Protein-1
MPO	Myeloperoxidase
MW	Molecular Weight
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NETs	Neutrophil Extracellular Traps
NF-κB	Nuclear Factor -κB
NK cell	Natural Killer Cell

NKT cell	Natural Killer T Cell
NO	Nitric Oxide
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
NSCLC	Non-Small Cell Lung Carcinoma
N-WASP	Wiskott Aldrich Syndrom Protein
PAF	Platelet-Activating Factor
PARP	Poly-ADP-Ribose-Polymerase
PBS	Phosphate Buffered Saline
PD_1	Protectin D_1
PDGF	Platelet-Derived Growth Factor
PE	Phycoerythrin
PGD_2	Prostaglandin D_2
PI	Propidium Iodide
PI(3)P	Phosphatidylinositol-3-phosphate
$PI(3,4)P_2$	Phosphatidylinositol-3,4-bisphosphate
$PI(3,4,5)P_3$	Phosphatidylinositol-3,4,5-trisphosphate
$PI(4,5)P_2$	Phosphatidylinositol-4,5-bisphosphate
PI3 kinase	Phosphoinositide 3-kinase
PIPP signalling	Polyisoprenyl Phosphate signalling
Pit1	Pituitary-specific transcription factor
PKC	Protein Kinase C
PLC	Phospholipase C
PLP	Pyridoxal-phosphate
PMA	Phorbol Myristate Acetate
PMN	Polymorphonuclear
PPAR	Peroxisome Proliferator Activated Receptor
PS	Phosphatidyl Serine
PSDP	Presqualene Disphosphate
PT pore	Permeability Transition pore
ROI	Reactive Oxygen Intermediates
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RvE_1	Resolvin E_1
SAP	Serum Amyloid Protein
SARS	Severe Acute Respiratory Syndrome
SCID	Severe Combined Immunodeficient
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
shRNA	short hairpin RNA
SI-LfR	Intestinal Lactoferrin Receptor
SIRCAMS	Scottish Instrumentation and Resource Centre for Advanced Mass Spectrometry
SLE	Systemic Lupus Erythematosus
TAM	Tumour-Associated Macrophage
TAN	Tumour-Associated Neutrophil

TBS	Tris Buffered Saline
TCA	Trichloroacetic acid
TF	Transferrin or Tissue Factor
TGF-β	Transforming Growth Factor-β
TMB	Tetramethylbenzidine
TNFR1	Tumour Necrosis Factor Receptor-1
TNF-α	Tumour Necrosis Factor-α
TPA	Tetradecanoylphorbol-Acetate
TRADD	TNFR-Associated Death Domain
TRAIL-R1	Tumour Necrosis Factor-Related Apoptosis-Inducing Ligand Receptor 1
TRAIL-R2	Tumour Necrosis Factor-Related Apoptosis-Inducing Ligand Receptor 2
TRAMP	TNF Receptor-related Apoptosis-Mediating Protein
TRICK2	TRAIL Receptor Inducer of Cell Killing 2
TUNEL	Terminal deoxytransferase-mediated dUTP Nick-End Labelling
uPA	urokinase type Plasminogen Activator
UV	Ultaviolet
VEGF	Vascular Endothelial Growth Factor
VPF	Vascular Permeability Factor
VSV	Vesicular Stomatitis Virus
WHO	World Health Organisation
XIAP	X-linked IAP

APPENDIX



U6: U6 promoter; **insert**: shRNA-encoding insert sequence; **cppt**: central polypurine tract; **hPGK**: human phosphoglycerate kinase eukaryotic promoter; **Puro**: puromycin resistance gene for mammalian selection; **3' LTR**: 3' self inactivating long terminal repeat; **f1 ori**: *E.coli* f1 origin of replication; **AmpR Prom**: bacterial constitutive promoter for the ampicillin resistance gene; **AmpR**: ampicillin resistance gene for bacterial selection; **pUC ori**: pUC-based *E.coli* origin of replication; **5' LTR**: L' long terminal repeat; **ψ**: ψ sequence - RNA packaging signal; **RRE**: Rev response element.

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 241 GCCGCACGTCTCACTAGTACCCTCGCAGACGGACAGCGCCAGGGAGCAATGGCAGCGCGC
                                                     M  A  A  R
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      D  R  I  T  D  L  S  P  Q  G  D  P  P  E  L  T  M  T  E  Y
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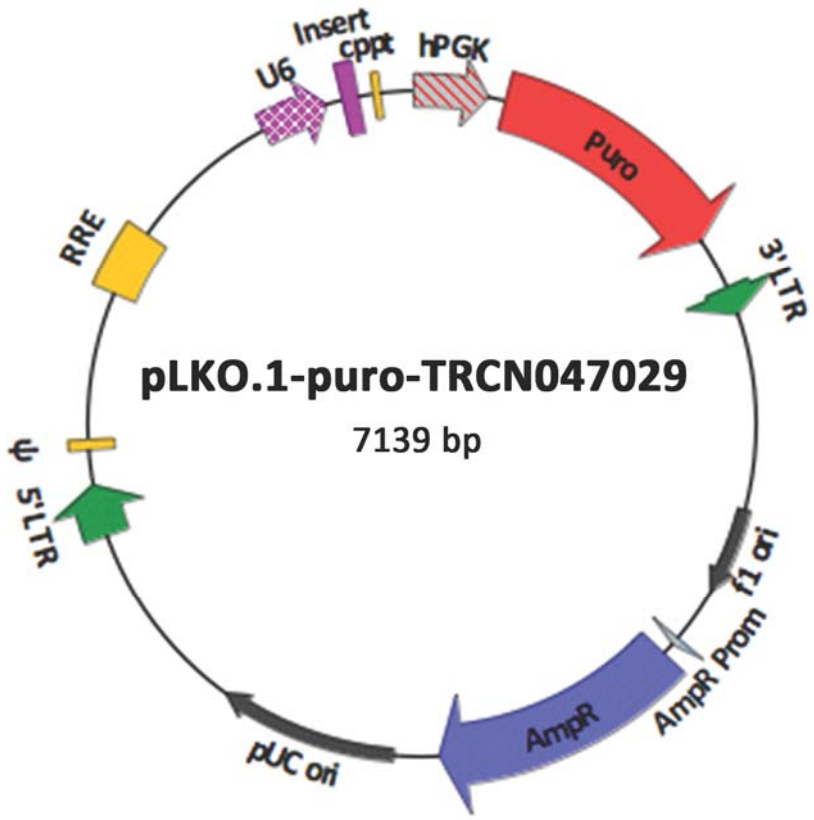

U6: U6 promoter; **insert**: shRNA-encoding insert sequence; **cppt**: central polypurine tract; **hPGK**: human phosphoglycerate kinase eukaryotic promoter; **Puro**: puromycin resistance gene for mammalian selection; **3' LTR**: 3' self inactivating long terminal repeat; **f1 ori**: *E.coli* f1 origin of replication; **AmpR Prom**: bacterial constitutive promoter for the ampicillin resistance gene; **AmpR**: ampicillin resistance gene for bacterial selection; **pUC ori**: pUC-based *E.coli* origin of

replication; **5' LTR**: L' long terminal repeat; **ψ**: ψ sequence - RNA packaging signal; **RRE**: Rev response element.

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 241 GCCGCACGTCTCACTAGTACCCTCGCAGACGGACAGCGCCAGGGAGCAATGGCAGCGCGC
                                                     M   A   A   R
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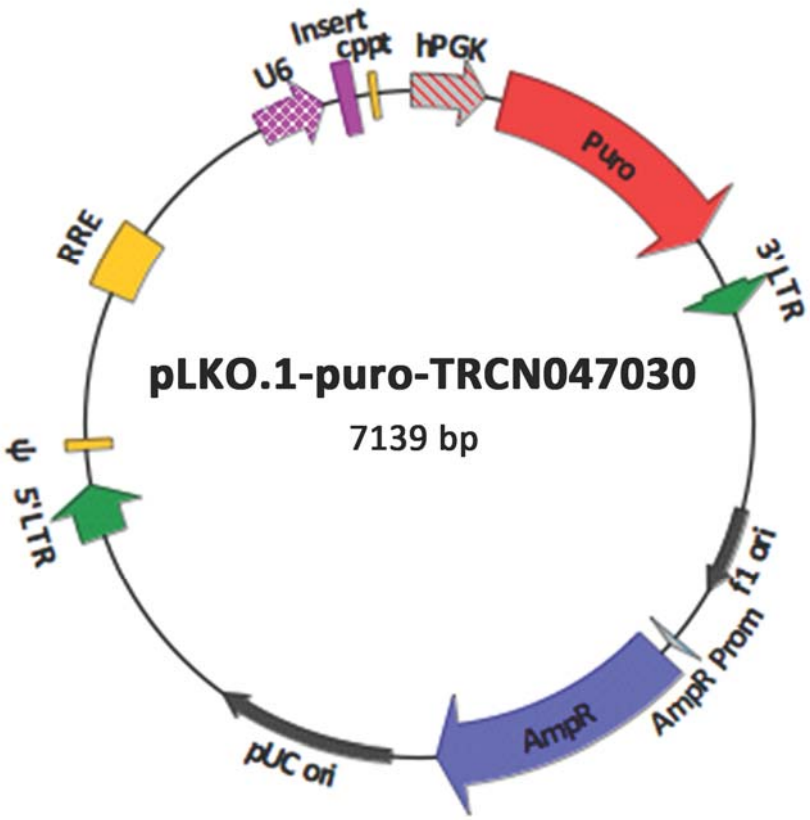

U6: U6 promoter; **insert**: shRNA-encoding insert sequence; **cppt**: central polypurine tract; **hPGK**: human phosphoglycerate kinase eukaryotic promoter; **Puro**: puromycin resistance gene for mammalian selection; **3' LTR**: 3' self inactivating long terminal repeat; **f1 ori**: *E.coli* f1 origin of replication; **AmpR Prom**: bacterial constitutive promoter for the ampicillin resistance gene; **AmpR**: ampicillin resistance gene for bacterial selection; **pUC ori**: pUC-based *E.coli* origin of

replication; **5' LTR**: L' long terminal repeat; **ψ**: ψ sequence - RNA packaging signal; **RRE**: Rev response element.

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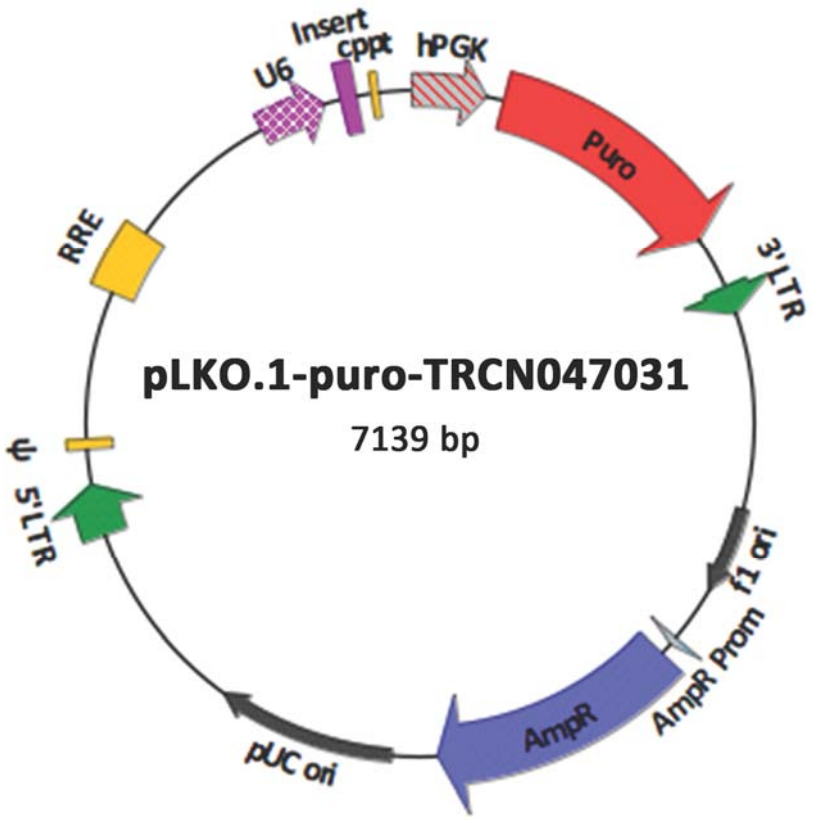

U6: U6 promoter; **insert**: shRNA-encoding insert sequence; **cppt**: central polypurine tract; **hPGK**: human phosphoglycerate kinase eukaryotic promoter; **Puro**: puromycin resistance gene for mammalian selection; **3' LTR**: 3' self inactivating long terminal repeat; **f1 ori**: *E.coli* f1 origin of replication; **AmpR Prom**: bacterial constitutive promoter for the ampicillin resistance gene; **AmpR**: ampicillin resistance gene for bacterial selection; **pUC ori**: pUC-based *E.coli* origin of replication; **5' LTR**: L' long terminal repeat; **ψ**: ψ sequence - RNA packaging signal; **RRE**: Rev response element.

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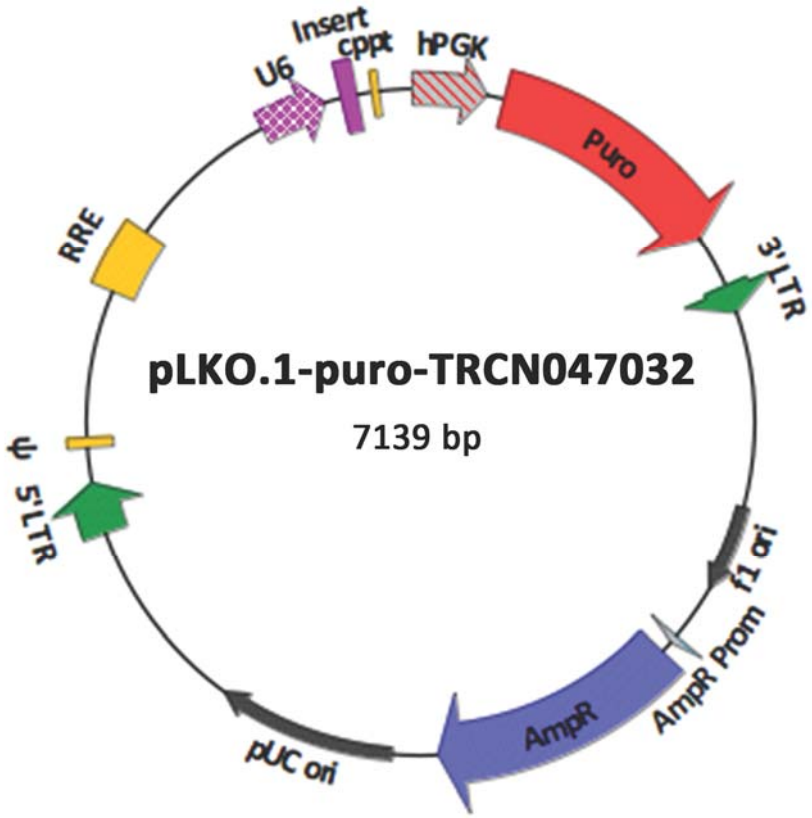

U6: U6 promoter; **insert**: shRNA-encoding insert sequence; **cppt**: central polypurine tract; **hPGK**: human phosphoglycerate kinase eukaryotic promoter; **Puro**: puromycin resistance gene for mammalian selection; **3' LTR**: 3' self inactivating long terminal repeat; **f1 ori**: *E.coli* f1 origin of replication; **AmpR Prom**: bacterial constitutive promoter for the ampicillin resistance gene; **AmpR**: ampicillin resistance gene for bacterial selection; **pUC ori**: pUC-based *E.coli* origin of

replication; **5' LTR**: L' long terminal repeat; **ψ**: ψ sequence - RNA packaging signal; **RRE**: Rev response element.

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 241 GCCGCACGTCTCACTAGTACCCTCGCAGACGGACAGCGCCAGGGAGCAATGGCAGCGCGC
                                                     M   A   A   R
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 361 CGGGAAGGGGCGGTGCGGGAGGCGGGGTGTGGGGCGGTAGTGTGGGCCCTGTTCCTGCCC
      R   E   G   A   V   R   E   A   G   C   G   A   V   V   W   A   L   F   L   P
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