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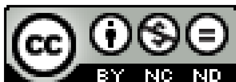

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LOYOLA UNIVERSITY CHICAGO

AGING EFFECTS ON ACUTE LUNG INFLAMMATION AFTER BURN INJURY

A DISSERTATION SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN CANDIDACY FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

PROGRAM IN MOLECULAR AND CELLULAR BIOCHEMISTRY

BY

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I would like to thank my family for persisting with me through the madness of medical school and exasperation of graduate school. Nothing could have grounded me more than them. I would also like to thank Liz for putting up with me and for being my second mom.

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LIST OF ABBREVIATIONS

AP-1	Activator protein-1
ARDS	Acute respiratory distress syndrome
BAL	Bronchoalveolar lavage
CARS	Compensatory anti-inflammatory response syndrome
CD	Cluster of differentiation
ECM	Extracellular matrix
ELISA	Enzyme linked immunosorbent assay
ELR	Glutamic acid-leucine-arginine
ERK	Extracellular signal-regulated kinase
fMLP	N-formyl-methionyl-leucyl-phenylalanine
GAP	GTPase-activating protein
G-CSF	Granulocyte-colony stimulating factor
GDP	Guanine diphosphate
GEF	Guanine nucleotide exchange factor
GM-CSF	Granulocyte monocyte-colony stimulating factor
GPCR	G protein coupled receptor
GRK	G protein-coupled receptor kinase

GRO	Growth related oncogene
GTP	Guanine triphosphate
H&E	Hematoxylin and eosin
HBSS	Hank's buffered saline solution
ICAM-1	Intercellular adhesion molecule-1
Ig	Immunoglobulin
IL	Interleukin
IFN-γ	Interferon-gamma
i.p.	Intraperitoneal
i.t.	Intratracheal
i.v.	Intravascular
Jak	Janus kinase
LOS	Length of stay
LPS	Lipopolysaccharide
LTB_4	Leukotriene B_4
MAPK	Mitogen activated protein kinase
MMP	Matrix metalloproteinase
MIP-2	Macrophage inflammatory protein-2
MOF	Multiple organ failure
NF-κB	Nuclear factor-κB
PECAM-1	Platelet-endothelial cell molecule-1
PI_3K	Phosphatidyl inositol 3 kinase

PIC	Protease inhibitor cocktail
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PLD	Phospholipase D
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SaO_2	Arterial oxygen saturation
SDF-1	Stromal derived factor-1
SEM	Standard error of the mean
SIRS	Systemic inflammatory response syndrome
STAT	Signal transducer and activator of transcription
TBSA	Total body surface area
TGFβ	Transforming growth factor β
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
TREM-1	Triggering receptor expressed on myeloid cell-1
VCAM-1	Vascular cell adhesion molecule-1
VLA	Very late antigen

CHAPTER 1

INTRODUCTION

In the United States, there are 500,000 burn injuries per year that require medical treatment (ABA 2007). About 40,000 of these result in hospitalization and an average of 4,000 result in death (ABA 2007). While only comprising approximately 13% of burn patients, individuals over the age of 65 are at the greatest risk of morbidity and mortality. With advances in treatment strategies over the last few decades, the overall mortality in elderly burn patients has decreased considerably (ABA 2005; Lionelli, Pickus et al. 2005). In the 1970's, burn patients over the age of 65 had an overall mortality of 77% (Linn 1980; Lionelli, Pickus et al. 2005). Today, only 20% of this same population succumbs to burn injury (Lionelli, Pickus et al. 2005). Unfortunately, these patients still tend to require more aggressive treatment strategies and have an increased length of stay in the hospital (Linn 1980; Hammond and Ward 1991). In addition, for aged individuals who survive burn injury, many end up in long-term care facilities, have greater disabilities, and report to have a decreased quality of life than younger patients (Slater and Gaisford 1981; Hammond and Ward 1991). As these outcomes translate into a greater financial and societal burden, improvement in care for elderly burn patients in the acute care setting is warranted.

Regardless of age, the lungs are the most frequent organ to fail as a result of burn (Dancey, Hayes et al. 1999; Fitzwater, Purdue et al. 2003). The most common pulmonary complications are acute respiratory distress syndrome (ARDS) and pneumonia (Teixidor, Novick et al. 1983; Sheridan, Ryan et al. 1998; Fitzwater, Purdue et al. 2003; Davis, Santaniello et al. 2004). Moreover, when an elderly individual sustains a burn, the chances of pulmonary sequelae are significantly greater than younger individuals (Li, Hsu et al. 1990). It is therefore important that better treatment strategies which target the lung are developed to combat this serious issue.

The presence of comorbidities has been shown to be a major factor in predisposing the aged to complications after burn. Others have demonstrated that, even when controlling for comorbidities, the risks of developing serious consequences in the elderly are still considerably higher. This observation indicates that there are underlying defects associated with increased age which need to be considered in order to properly treat the elderly burn patient.

Previous experiments in our laboratory and others suggest that a pre-existing inflammatory state in aged mice prior to injury is likely to be the cause for the increased susceptibility to burn (Ershler 1993; Meyer, Rosenthal et al. 1998; Dinarello 2006; Sarkar and Fisher 2006; Franceschi, Capri et al. 2007; Gomez, Hirano et al. 2007). We propose that burn injury in the aged is actually a “two hit” process, in which increased age primes

the immune system and makes the individual vulnerable to complications. As in other two hit models of systemic inflammation, the occurrence of the second challenge results in an exaggerated response, greater than that which could be achieved by either challenge alone (Davis, Santaniello et al. 2004; Samonte, Goto et al. 2004; Moore, Moore et al. 2005; Maegele, Sauerland et al. 2007). Since these types of situations pose the greatest difficulties in the clinical setting, developing effective treatment strategies to combat them is of great importance. The studies performed herein examine acute lung inflammation in a murine model of scald injury to determine why the aged are at an increased risk for pulmonary complications than a young individual receiving a comparable injury.

Hypothesis: Exaggerated acute pulmonary inflammation after a dorsal scald injury in aged mice parallels increased neutrophil sequestration in the pulmonary vasculature. Blocking the neutrophil chemoattractant receptor, CXCR2, will alleviate neutrophil accumulation in the lungs following burn in the aged.

Aim 1: To determine whether aged mice have greater pulmonary neutrophil sequestration after a remote scald injury than young mice.

Aim 2: To determine whether an elevation in neutrophil chemoattractant cytokines, MIP-2 and KC, parallel the prolonged pulmonary sequestration of neutrophils in aged mice after burn injury. If so, whether blocking the receptor for these chemokines, CXCR2, will reduce this aberrant response of aged mice.

Aim 3: To determine whether the neutrophils from uninjured aged mice have a defective response to KC in *in vitro* chemotaxis assays relative to young and whether this correlates with an alteration in the surface expression of CXCR2 and CD11b as a result of burn injury.

CHAPTER 2

REVIEW OF THE RELATED LITERATURE

Clinical Outcomes after Burn Injury in the Aged

Regardless of age, the main causes of death following burn injury are multiple organ failure (MOF), sepsis, and acute respiratory distress syndrome (ARDS) (Sheridan, Ryan et al. 1998; Fitzwater, Purdue et al. 2003). In other words, mortality from a burn does not necessarily result from the wound alone, but from complications that occur systemically. In a burn patient over the age of 65, morbidity and mortality is significantly increased relative to younger patients (Dancey, Hayes et al. 1999; Fitzwater, Purdue et al. 2003; ABA 2005). For example, a moderate sized burn covering 20% of the total body surface area (TBSA) produces a mortality of only 4% in healthy young adult patients, while elderly patients with the same injury have a mortality of up to 35% (ABA 2005). One explanation for this difference is that elderly patients typically have one or more comorbidities, such as cardiovascular disease or diabetes (Tran, Groeneveld et al. 1990; Lionelli, Pickus et al. 2005). However, even in the absence of clinically detectable disease, persons over the age of 65 still have an increased risk of complications and death after burn (Dancey, Hayes et al. 1999; Fitzwater, Purdue et al. 2003). Unfortunately, the reasons for this have yet to be fully understood.

The Biology of Aging

Since the elderly are at an increased risk for both early and late MOF, as well as other complications following injury, it is important to understand the factors involved with aging and how they could be affecting the body's response to systemic injury. In the most basic sense, aging is simply the process of "getting older." However, if you survey the elderly population, it is quite obvious that everyone "gets older" in their own distinct way. As defined by Mangel in 2001, aging is "a decline in physiologic repair, an increase in probability of death and a decline in fertility with advancing adult age" (Mangel 2001). There is a great deal of evidence suggesting that the vast majority of problems which can manifest over time may be traced back to the changes that occur on a cellular level (Campisi 1996; Faragher and Kipling 1998; Campisi 2005). For decades, it has been known that, when cells are removed from the body and grown in culture, they have a limited ability to replicate, acquire a multitude of phenotypic changes, and undergo cellular senescence (Hayflick 1965; Campisi 2005). Although initially thought of as a purely *in vitro* phenomenon, further studies confirmed that there is a greater number of senescent cells in tissues from aged animals and humans that is not seen in those of the young (Dimri, Lee et al. 1995; Choi, Shendrik et al. 2000; Paradis, Youssef et al. 2001). Since senescent cells do not function normally, many have hypothesized that their accumulation over time is responsible for decreased tissue structure and function seen with age (Campisi 2005).

Cells senesce in order to protect themselves against stressors. These stressors can come either in the form of exogenous agents (ionizing radiation, UV radiation, etc.) or as naturally occurring byproducts of normal cellular activity, such as the reactive oxygen species (ROS) that are generated from oxidative phosphorylation (Wallace 1999). It is because of these stressors that we have developed a system, governed by the aptly named caretakers and gatekeepers, to prevent overt crises from occurring on a daily basis (Kinzler and Vogelstein 1997). The caretakers—including ROS scavengers, DNA repair enzymes, chaperones, and protein degradation pathways—act to quell the effects of the endogenous stressors that are consistently produced (Sierra 2006). If the cellular stress exceeds that which the caretakers can handle, the gatekeepers—mainly p53 and pRb, which control key aspects of the cell cycle and cellular response to DNA damage—control the decision to senesce or to die by apoptosis (Campisi 2005; Sierra 2006).

Although the data are still correlative, there is strong support for the notion that increased DNA damage, leading to the accumulation of senescent cells, is responsible for many of the changes seen with age (Campisi 1996; Faragher and Kipling 1998; Baker, Jeganathan et al. 2004; Lombard, Chua et al. 2005; Vijg and Suh 2005). When DNA is damaged within a cell, response proteins are upregulated and cause a series of downstream events to occur, including increased expression of p53. Generally, p53 is thought to have a main role in cellular protection by initiating either senescence or apoptosis, depending on the degree of damage that is present within a cell (Campisi 2005; Lombard, Chua et al. 2005; Salvioli, Olivieri et al. 2006). When p53 is knocked out in

rodents, the animals exhibit decreased longevity and significant increases in tumor development, as would be expected (Tyner, Venkatachalam et al. 2002). Conversely, when p53 activity is augmented, mice have a striking resistance to tumor development and an increase in longevity. However, when these animals with increased p53 activity are aged to 24 months, they show accelerated development of gross phenotypic changes commonly associated with aging, such as osteoporosis, muscle atrophy, decreased hair growth, and impaired wound healing (Tyner, Venkatachalam et al. 2002). Evolutionarily, these studies may explain why the aging phenotype is so much more prevalent now than in the past. Since increased p53 expression benefits organisms early in life, it is possible that this phenotype has been selected for in the population (Franceschi, Bonafe et al. 2000; Campisi 2005; Kirkwood 2005). This selection would create a high proportion of individuals with longer life-spans but a greater propensity for organ dysfunction with time. The concept that certain genes may be protective early in life, but cause detrimental changes that manifest later in life is called antagonistic pleiotropy (Williams 1957; Kirkwood and Austad 2000; Campisi 2005; Campisi 2005).

It is important to note that the immune system—both the innate and the adaptive—is not exempt from the accumulation of DNA damage and cellular senescence. Although a great deal has been uncovered about the adaptive immune changes that occur with time, age-related defects in components of innate immunity have gained more attention in recent years (Albright and Albright 2003; Plackett, Boehmer et al. 2004; Plowden, Renshaw-Hoelscher et al. 2004; Gomez, Boehmer et al. 2005; Sebastian, Espia

et al. 2005). Commonly observed in the elderly are decreased T cell memory, exhaustion of the naïve T cell population with involution of the thymus, and a chronic inflammatory status that has come to be called, "inflamm-aging" (Franceschi and Bonafe 2003; Meyer 2005; Capri, Monti et al. 2006). As a result, the elderly are more susceptible to viral and bacterial infections, reactivation of latent viruses, autoimmune diseases, and neoplasias compared to young adults (Franceschi, Bonafe et al. 2000; Effros 2003; Pawelec, Akbar et al. 2004; Prelog 2006; Weng 2006).

According to one theory of aging introduced by Harman in the 1950's, the accumulation of free radicals is the main mechanism behind many of the age-related diseases and disorders seen in the population (Harman 1956). However, other sources of cellular stress may also be implicated (Lombard, Chua et al. 2005). Current thought is that the way a person ages is dependent on both the internal and external environment, as well as the genetics which determine the ability for the individual to combat the cellular stresses and DNA damage (Franceschi, Bonafe et al. 2000; Salvioli, Capri et al. 2006). When studying the factors involved with increased longevity, it is evident that the ability to resist cellular stresses—either by decreasing the exposure to them, by having the optimal genetic makeup to oppose them, or both—is the key (Franceschi and Bonafe 2003; Salvioli, Capri et al. 2006).

As stated above, ROS and reactive nitrogen species (RNS) are produced during a variety of normal cellular activities, as well as in numerous pathologic states (Brigham

1990; Lavrovsky, Chatterjee et al. 2000; Balaban, Nemoto et al. 2005; Sarkar and Fisher 2006). The main issue with ROS in a biological system results from the instability of free radicals. If one molecule is converted to a free radical, the reaction can be quickly propagated and potentially turn any molecule nearby into a free radical as well. As oxidized proteins, lipids, and DNA do not function optimally, damage to entire areas of tissue can severely compromise normal function. The direct tissue injury free radicals cause may also stimulate the inflammatory response, generating even more free radicals and creating a feed-forward loop of oxidative damage (Moraes, Zurawska et al. 2006). Free radicals can also stimulate innate immune cells directly by upregulating redox-sensitive transcription factors—mainly nuclear factor-kappaB (NF-κB) and activator protein-1 (AP-1)—that are involved in inflammatory pathways (Lavrovsky, Chatterjee et al. 2000; Li and Verma 2002; Radak, Chung et al. 2004; Moraes, Zurawska et al. 2006).

Since free radicals can be so detrimental to the cell, it is imperative that the body's defense mechanisms that protect against these agents are optimal. Unfortunately, this does not happen with age. Over time, free radicals tend to accumulate, both because of excessive production and because of defects in the machinery that help eliminate them (Lavrovsky, Chatterjee et al. 2000; Salvioli, Olivieri et al. 2006). As a result of the increased cell stress and damage, many of the elderly are in a state of chronic inflammation, marked by an elevation in circulating inflammatory markers, such as IL-6, tumor necrosis factor-α (TNF-α), C-reactive protein, and soluble TNF-α receptor (Franceschi, Bonafe et al. 2000; Gomez, Boehmer et al. 2005; Salvioli, Capri et al. 2006).

In fact most, if not all, problems or diseases associated with aging have some defective inflammatory component to them; these include but are not limited to cardiovascular disease, atherosclerosis, impaired wound healing, cancer, Alzheimer's disease, Parkinson's disease, Type II diabetes, and rheumatoid arthritis (Ashcroft, Horan et al. 1998; Franceschi, Bonafe et al. 2000; Caruso, Lio et al. 2004; Chung, Sung et al. 2006; Salvioli, Capri et al. 2006; Sarkar and Fisher 2006). Thus, to understand aging, it is not only important to determine the mechanisms of cellular damage, but also to understand how the defects in the immune system can lead to even greater problems.

The Inflammatory Response

In order to gain knowledge in the pathogenesis of burn injury in the elderly, it is important to first characterize the steps involved in an appropriate response to an inflammatory challenge. In general, the function of inflammation is to prevent infection, to remove cellular debris, and eventually to promote tissue repair (Henson 2005). To initiate this process, pro-inflammatory cytokines, such as interleukin (IL)-1β and TNF-α, from resident leukocytes and damaged cells must be released at the initial site of injury or infection to recruit more inflammatory cells (Sibille and Reynolds 1990; Garcia-Ramallo, Marques et al. 2002). Typically, the first cells to infiltrate the site of inflammation are neutrophils (Demling, LaLonde et al. 1989; Baskaran, Yarmush et al. 2000; Nathan 2006), closely followed by macrophages (Martin and Leibovich 2005). In the case of a major insult, such as burn, the particular events surrounding this acute phase of inflammation tend to set the stage for consequences that can occur later. Thus, a careful

examination of how these cells are recruited in the early inflammatory response may help to elucidate the mechanisms which lead to detrimental outcomes following burn.

Setting the Stage

Before entering the circulation, granulocyte-monocyte precursors are retained in the bone marrow via the chemokine, stromal cell-derived factor-1 (SDF-1), acting through its receptor, CXCR4 (Levesque, Hendy et al. 2003; Martin, Burdon et al. 2003; Nathan 2006; Christopher and Link 2007). During an inflammatory challenge, the release of either granulocyte-colony stimulating factor (G-CSF) or granulocyte monocyte-colony stimulating factor (GM-CSF) mediates the movement of neutrophils from the bone marrow into circulation by disrupting this SDF-1/CXCR4 axis (Basu, Hodgson et al. 2002; Semerad, Liu et al. 2002; Levesque, Hendy et al. 2003; Nathan 2006; Christopher and Link 2007).

In the absence of a stimulus, circulating neutrophils undergo apoptosis within 8-12 hours of their release from the bone marrow (Savill, Wyllie et al. 1989). As neutrophils play an important role in immune surveillance under resting conditions, a constant neutrophil turnover is therefore required to maintain adequate numbers in the circulation (Weinmann, Scharffetter-Kochanek et al. 2003). Upon insult, GM-CSF, secreted from a number of cellular sources, causes a delay in neutrophil apoptosis. This delay increases the lifespan of neutrophils to 1-2 days, allowing more time for the cells to

infiltrate the source of inflammation (Chilvers, Cadwallader et al. 2000; Mukae, Zamfir et al. 2000).

While macrophages are derived from the same progenitors as neutrophils, their divergent maturation process creates a number of functional differences between them (DeKoter, Walsh et al. 1998; Rosmarin, Yang et al. 2005; Sugimoto, Katayama et al. 2006). For one, macrophages are released from the bone marrow as monocytes, where they remain in circulation for approximately 1-3 days (Martinez, Gordon et al. 2006). Unlike neutrophils which undergo spontaneous apoptosis, the "life" of a monocyte ends when it migrates into tissues and differentiates into a macrophage (Martinez, Gordon et al. 2006).

Neutrophils and Macrophages in the Inflammatory Response

Once recruited to the site of inflammation, activated neutrophils can phagocytose bacteria and cellular debris (Reynolds 1985; Segal 2005), as well as release cytoplasmic granules containing proteases, antimicrobial proteins, and enzymes which generate ROS (Ward 1983; Moraes, Zurawska et al. 2006; Lehrer 2007). However, while the actions of neutrophils are critical in the defense against microbes, they are also relatively nonspecific. As a result, an overproduction or a prolonged release of these products can paradoxically lead to excessive tissue destruction and exacerbation of the inflammatory response (Ward 1983; Simon, DeHart et al. 1986; Brigham 1990; Hewett, Schultze et al. 1992; Jaeschke and Smith 1997; Moraes, Zurawska et al. 2006). Therefore, while

recruitment and activation of neutrophils are important components of inflammation, the ability to turn off this response is just as imperative.

The main role for macrophages is phagocytosis of cellular debris and bacteria to maintain normal tissue function and to prevent infection (Goldstein, Lippert et al. 1974; Reynolds 1985; Savill, Wyllie et al. 1989). As macrophages are sensitive to various cytokines and other inflammatory stimuli, they also play a significant role in initiating and resolving the inflammatory response within tissues (Sibille and Reynolds 1990; Heinrich, Messingham et al. 2003; Martin and Leibovich 2005; Smith, Ochsenbauer-Jambor et al. 2005).

Again, following their release from the bone marrow, the fate of both neutrophils and monocytes is dependent on the particular milieu as the cells circulate through various tissues. When an inflammatory insult is present, a particular series of events must take place in order for the cells to migrate into the injured tissue: chemoattraction, adhesion, and diapedesis (or transendothelial migration) (Muller 2003; Cook-Mills and Deem 2005). While the specific events involved in this process occurs similarly for all leukocytes, this review will only focus on neutrophils from this point forward, as this is the cell type which dominates the acute phase of inflammation after injury.

Neutrophil Chemoattraction

Neutrophils migrate to the site of inflammation following the local release of chemokines. There are four main categories of chemokines, classified by the spacing of conserved cysteine residues near the N-terminal region of the protein: C, CC, CXC, and CXXXC, with each X standing for a nonconserved amino acid (Clark-Lewis, Kim et al. 1995; Baggiolini 1998; Murphy, Baggiolini et al. 2000). The way in which each of these cysteines forms disulfide bonds with other cysteines in the protein determines their three-dimensional structure (Murphy, Baggiolini et al. 2000; Olson and Ley 2002). In general, the CC chemokines attract monocytes, NK cells, and dendritic cells; one CC chemokine also attracts lymphocytes (Clark-Lewis, Kim et al. 1995; Baggiolini 1998). The CXC chemokines can be further classified into two categories, based on the presence of a conserved sequence of glutamic acid-leucine-arginine (or ELR). ELR^+ CXC chemokines specifically attract neutrophils, whereas ELR^- CXC chemokines tend to stimulate lymphocyte migration (Clark-Lewis, Kim et al. 1995; Murphy, Baggiolini et al. 2000; Olson and Ley 2002). Only two C chemokines (lymphotactin α and β, which stimulate T lymphocytes) and one CXXXC chemokine (fractalkine, which can act as both a chemokine and an adhesion molecule) have been discovered (Clark-Lewis, Kim et al. 1995; Murphy, Baggiolini et al. 2000). In mice, KC and macrophage inflammatory protein-2 (MIP-2) are the main ELR^+ CXC neutrophil chemokines (Piccolo, Wang et al. 1999; Reutershan and Ley 2004; Lomas-Neira, Chung et al. 2005). The homologues for these genes in humans are growth-related oncogene alpha (GROα) and beta (GROβ), respectively (Murphy, Baggiolini et al. 2000).

In order to stimulate migration, chemokines must bind to their cognate receptors on leukocytes. Interestingly, nearly all chemokine receptors in the body—including those involved in development, angiogenesis, and neuronal growth—signal through the G_i subclass of G-protein coupled receptors (GPCR) (Baggiolini 1998; Murphy, Baggiolini et al. 2000; Rossi and Zlotnik 2000; Olson and Ley 2002). In general, GPCRs are seven transmembrane receptors that, when activated by ligand binding, a conformational change leads to the activation of a specific guanine nucleotide-binding protein (the G-protein) [reviewed in (Neves, Ram et al. 2002)]. In the inactive state, G-proteins consist of three subunits: α, β, and γ (**Figure 1**). When stimulated, the G_α subunit—which is bound to GDP when active—dissociates from the $G_{\beta\gamma}$ subunits and a guanine nucleotide exchange factor (GEF) replaces the bound GDP with a new GTP molecule. The G_α subunit can then activate other downstream pathways via hydrolysis of GTP to GDP. In some situations, the $G_{\beta\gamma}$ subunit can also act as a signaling molecule. Once GTP hydrolysis occurs, the G_α re-associates with the $G_{\beta\gamma}$ subunit and the signal is terminated. While the G_α subunit has endogenous GTPase activity, another group of regulatory proteins, called GTPase-activating proteins (GAP), can accelerate the hydrolysis and terminate the signal faster. However, if the chemokine receptor is still available at the surface of the neutrophils, the signal can continually become activated if chemokines are present. Therefore, distinct mechanisms for signal termination must be in place to prevent over-stimulation via this pathway.

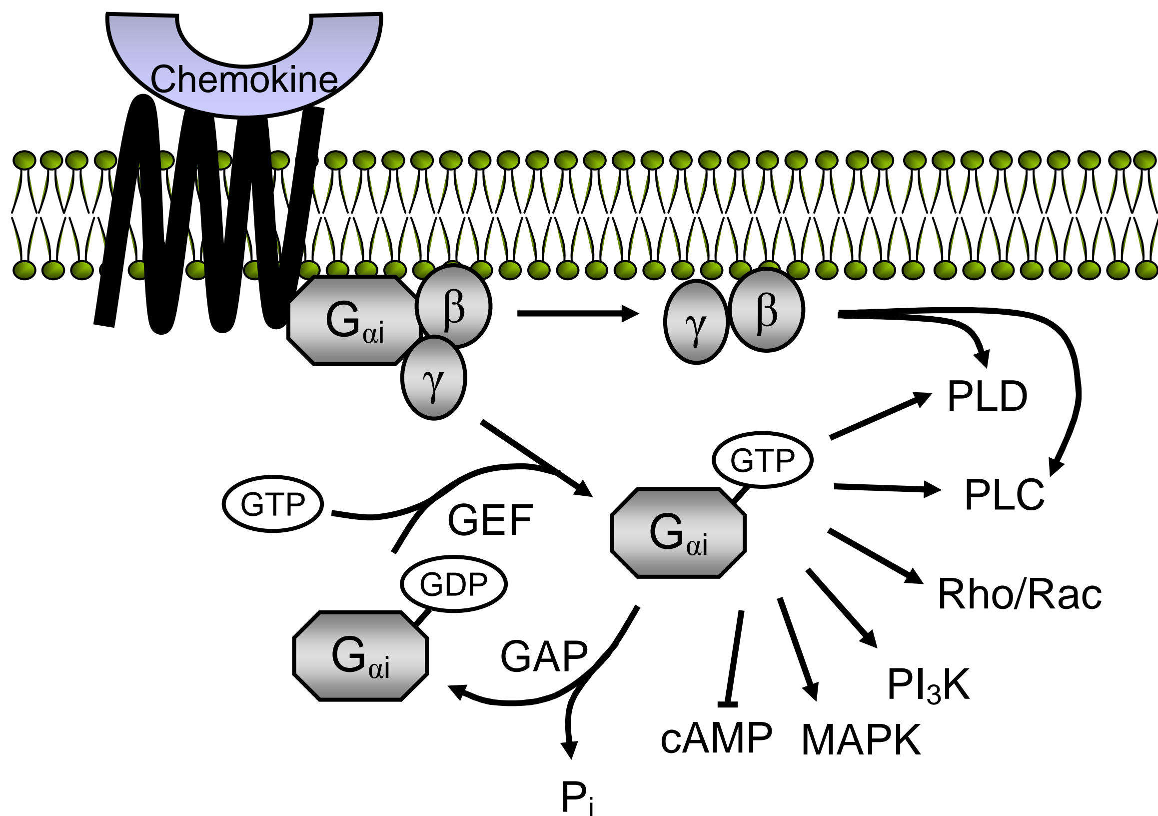


Figure 1: Mechanisms of chemokine receptor activation via $G_{\alpha i}$. Abbreviations: GEF: guanine nucleotide exchange factor; GAP: GTPase activating protein; GDP: guanine diphosphate; GTP guanine triphosphate; P_i: inorganic phosphate; PLD: phospholipase D; PLC: phospholipase C; PI_3K: phosphatidyl inositol 3 kinase; MAPK: mitogen activated protein kinase.

Both KC and MIP-2 signal through the receptor, CXCR2, which leads to the activation of phospholipase A_2, C, and D, Src-related kinases, and small GTPases (**Figure 1**) (Bokoch 1995; Olson and Ley 2002). Signaling through CXCR2 on neutrophils thus stimulates a number of neutrophil functions. Neutrophil chemotaxis, in

particular, involves the small GTPase pathways of Rac and Rho. These pathways culminate in actin cytoskeleton rearrangement to allow movement of cells towards the chemoattractants released at the site of inflammation (Reutershan and Ley 2004). Other neutrophil chemokines that are not part of the CXC classification, but which signal through the same intracellular pathways, include the complement protein, C5a, and the bacterial peptide, N-formyl-methionyl-leucyl-phenylalanine (fMLP) (Hammond, Lapointe et al. 1995; Terashima, English et al. 1998; Piccolo, Wang et al. 1999; Burns, Smith et al. 2003; Reutershan and Ley 2004).

Neutrophil Rolling

Once neutrophils arrive at the site of inflammation, they must move out of central circulation and into the marginating zone of the vasculature before they can infiltrate the tissue. This process is termed, “rolling adhesion” (Tedder, Steeber et al. 1995; Guo and Ward 2002; Beck-Schimmer, Schimmer et al. 2004). Here, P-selectin and E-selectin become upregulated on the surface of activated endothelial cells and weakly bind to sialyl Lewis groups on neutrophils passing through the blood (Tedder, Steeber et al. 1995; Carraway, Welty-Wolf et al. 1998; Guo and Ward 2002). Another molecule, L-selectin, is constitutively expressed on most leukocytes, but it mostly aids in the homing of cells to secondary lymphoid tissues, such as the spleen and lymph nodes (Tedder, Steeber et al. 1995).

This process of rolling adhesion is only temporary, but it allows for neutrophils to integrate and process the signals being produced from the surrounding tissue. The main signals that the neutrophils respond to during this phase are the concentration of chemokines being secreted from the site of inflammation and the degree of expression of adhesion molecules on the endothelium (Luu, Rainger et al. 2000; Zhang, Liu et al. 2001; Ley 2002). If a certain threshold of these stimuli is reached, neutrophils then enter the "tight adhesion" phase and the selectins are shed from the endothelium (Furie and Randolph 1995; Tedder, Steeber et al. 1995). If this threshold is not achieved, leukocytes will become untethered from the endothelium and will continue through the circulation (Ley 2002).

Neutrophil Adhesion

In the tight adhesion phase, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are upregulated on endothelial cells and act to strengthen the interaction with neutrophils (**Figure 2**) (Elangbam, Qualls et al. 1997; Cook-Mills and Deem 2005). The expression of cell adhesion molecules on the endothelium is highly dependent on stimulation by IL-1β and TNF-α (Lo, Everitt et al. 1992; Mulligan, Vaporciyan et al. 1993; Elangbam, Qualls et al. 1997). Neutrophils interact with these adhesion molecules mainly through the α integrins, CD11a and CD11b, for ICAM-1 and the β_1 integrins of the very late antigen (VLA) family for VCAM-1 (Tonnesen 1989; Arnaout 1990; Mulligan, Till et al. 1994; Elangbam, Qualls et al. 1997; Piccolo, Wang et al. 1999). The CD11 integrins can also associate with the β_2

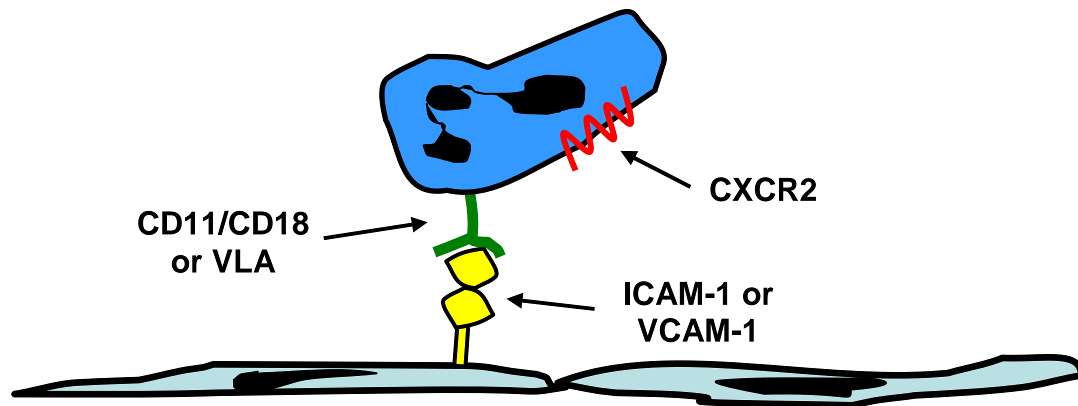


Figure 2: Minimum requirements for neutrophil diapedesis.
Not shown: actin cytoskeletal rearrangements causing endothelial cell retraction and neutrophil migration. Abbreviations: CD: cluster of differentiation; VLA: very late antigen; ICAM-1: intercellular adhesion molecule-1; VCAM-1: vascular cell adhesion molecule-1.

integrin, CD18, for increased migratory capacity (Tonnesen 1989; Hellewell, Young et al. 1994; Walzog, Seifert et al. 1994; Reutershan and Ley 2004). In addition, integrins (mostly β_1) bind to extracellular matrix (ECM) components, via a consensus sequence, RGD (arginine-glycine-aspartate), present in many ECM proteins, such as fibronectin, laminin, and collagen (Arnaout 1990; Elangbam, Qualls et al. 1997; Burns, Smith et al. 2003). In general, it is thought that the β_2 integrins function more in cell-cell interactions and mediate the initial phases of adhesion to the vasculature, while the β_1 integrins participate more in cell-matrix interactions to guide the cell through the tissue once migration begins to occur (Arnaout 1990; Elangbam, Qualls et al. 1997; Burns, Smith et al. 2003).

While CD11 is constitutively present on leukocytes, the relative levels of the a, b, and c isoforms differ according to their maturation state (Arnaout 1990; Mazzone and Ricevuti 1995). CD11a is found mostly on granulocyte-monocyte precursors in the bone marrow; here, CD11b and c are not detectable (Arnaout 1990; Mazzone and Ricevuti 1995). Upon mobilization from the bone marrow, CD11a significantly decreases and CD11b and c begin to appear on the surface of neutrophils (Arnaout 1990; Burdon, Martin et al. 2005). In resting states, most of the CD11b and c is contained within intracellular storage granules of mature neutrophils. Upon activation with stimulants, such as C5a, CXC chemokines, and TNF-α, these integrins are upregulated on the surface of neutrophils to aid in the migration into tissues (Tonnesen 1989; Arnaout 1990; Mazzone and Ricevuti 1995).

Interestingly, there are many other roles for the CD11/CD18 complex on leukocytes, including the binding of the complement component, C3bi, phagocytosis, and antibody dependent cell-mediated cytotoxicity (Arnaout 1990; Mazzone and Ricevuti 1995). On lymphocytes, CD11/CD18 is also involved with proliferation and cytotoxicity (Arnaout 1990; Mazzone and Ricevuti 1995). Given that CD11/CD18 is involved in most leukocyte functions, it is understandable why disease states in which some component of this system is disrupted can lead to a fatal infection (Arnaout 1990; Tedder, Steeber et al. 1995; Elangbam, Qualls et al. 1997).

Neutrophil Diapedesis

While the mechanisms involved in the transition from neutrophil adhesion to migration through the endothelium are not completely understood, it is generally accepted that there are minimum requirements for both the endothelium and neutrophils in this process. The necessary components on the endothelium are the upregulation of cell adhesion molecules (Luu, Rainger et al. 2000; Zhang, Liu et al. 2001) and the activation of actin cytoskeletal rearrangement pathways, leading to cellular retraction and increased permeability (Dudek and Garcia 2001; Murphy and Duffy 2003; Tinsley, Teasdale et al. 2004). The essential components for neutrophils are the increased surface expression of the relevant integrin ligands and actin cytoskeletal reorganization to allow for cell movement (**Figure 2**) (Luu, Rainger et al. 2000; Rosseau, Selhorst et al. 2000; Reutershan and Ley 2004).

When all factors are present and functioning normally, neutrophils can then extravasate into the tissue with the help of platelet-endothelial cell adhesion molecule-1 (PECAM-1), a constitutively expressed molecule on the lateral surfaces of endothelial cells and on neutrophils (Guo and Ward 2002; Beck-Schimmer, Schimmer et al. 2004; Cook-Mills and Deem 2005). To aid in this process, proteins contained in junctional complexes—mainly occludin for tight junctions and β-catenins and cadherins for adhesion junctions (Saitou, Ando-Akatsuka et al. 1997; Dudek and Garcia 2001; Schneeberger and Lynch 2004)—become disrupted and vascular permeability is increased (Dudek and Garcia 2001; Reutershan and Ley 2004; Tinsley, Teasdale et al.

2004). In addition, neutrophils can secrete matrix metalloproteinases (MMP) to digest the intercellular junctional proteins, which further aids in migration through the tissue (Alexander and Elrod 2002; Chakrabarti and Patel 2005). MMPs can be released by neutrophils in response to CXCR2-mediated pathways (Chakrabarti and Patel 2005) and by NADPH-dependent ROS production (Cook-Mills and Deem 2005). With this change in vascular permeability, fluid also leaks out of the vasculature, leading to edema formation, which significantly contributes to the pathology seen in various tissues after injury (Lo, Everitt et al. 1992; Kowal-Vern, Walenga et al. 1997; Dudek and Garcia 2001; Turnage, Nwariaku et al. 2002; Tinsley, Teasdale et al. 2004).

Inflammatory Resolution

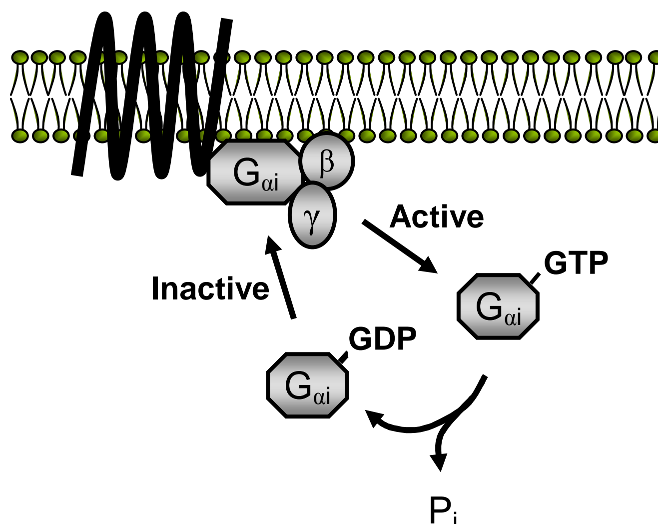
Again, as the consequences of inflammatory cell activation are relatively nonspecific, a prolonged response can eventually lead to tissue destruction. Therefore, to consider an inflammatory response “appropriate”, the resolution may be just as important as the initiation (Henson 2005; Martin and Leibovich 2005; Serhan, Brain et al. 2007). Perhaps the most obvious way to end an inflammatory reaction is to remove the source—whether it is a microbe, a foreign body, or cellular debris. This, of course, is the function of the inflammatory response in the first place, which requires the activation of inflammatory cells, the release of reactive oxygen species and proteolytic enzymes, and phagocytosis (Ayala, Chung et al. 2003; Henson 2005).

In certain scenarios, however, the source or extent of the inflammatory stimulus may persist despite all efforts, such as in chronic inflammatory diseases (Ayala, Chung et al. 2003; Henson 2005). Mechanisms to remove the inflammatory cells from the system, such as apoptosis, therefore exist as another level of regulation. Again, the lifespan of a neutrophil exiting the bone marrow is limited, even when activated (Ayala, Chung et al. 2003; Henson 2005; Serhan, Brain et al. 2007). While the need for constant neutrophil turnover may seem uneconomical, especially in resting states, its benefit in resolving inflammation may ultimately outweigh the costs. Furthermore, it has been shown that macrophages which have ingested apoptotic neutrophils secrete anti-inflammatory mediators, creating another pathway for limiting the response (Savill, Wyllie et al. 1989; Fadok, Bratton et al. 1998; Henson 2005).

Yet another level of regulation for the inflammatory response is the inhibition or downregulation of pro-inflammatory mediators. In general, cell signaling by pro-inflammatory cytokines can be terminated by either removing the extracellular ligands which induce the reaction or by inhibiting the ability for the cell to respond. The release of anti-inflammatory mediators from a number of cell types—including parenchymal cells, macrophages, natural killer cells, and natural killer T cells—is one of the main mechanisms to combat the actions of pro-inflammatory cytokines. One group of anti-inflammatory mediators includes those which target the pro-inflammatory cytokines directly, such as the soluble TNF-α receptor or IL-1 receptor antagonist (Ayala, Chung et al. 2003); these agents have been exploited clinically in the treatment of a number of

inflammatory conditions (Abraham and Allbee 1994; Dinarello 2000; Remick, Call et al. 2001). Another group—which includes anti-inflammatory cytokines, such as IL-10, IL-4, and transforming growth factor β (TGFβ) (Ayala, Chung et al. 2003; Henson 2005), and certain acute phase proteins (Tilg, Dinarello et al. 1997; Hochepied, Berger et al. 2003; Arredouani, Kasran et al. 2005)—acts indirectly on the cells to turn off the production of pro-inflammatory mediators. While modulation of this second group of secreted products may seem to be clinically efficacious, results from *in vivo* studies have been highly variable (Dinarello 2000; Barnes 2001; Ayala, Chung et al. 2003; Marklund, Keck et al. 2005).

While anti-inflammatory mediators can effectively shut down the release of pro-inflammatory cytokines, there are two key processes involved in termination of the chemokine response: spontaneous GTP hydrolysis via endogenous G_α GTPase activity and receptor desensitization (**Figure 3**) (Pitcher, Freedman et al. 1998; Ferguson 2001; Olson and Ley 2002; Ishii and Kurachi 2003; Vroon, Heijnen et al. 2006). As described above, even when $G_{\alpha i}$ is hydrolyzed to its inactive (GDP-bound) form, if the chemokine receptor is still available at the cell surface, the presence of any remaining ligand can trigger re-activation. To completely render a cell unresponsive to chemoattractant stimulation, the receptor is downregulated through the process of desensitization (**Figure 3**). Current thought is that G protein-coupled receptor kinases (GRKs), which phosphorylate the intracellular portion of chemokine receptors, are the main modulators of receptor desensitization (Prado, Suzuki et al. 1996; Mueller, White et al. 1997; Nasser,



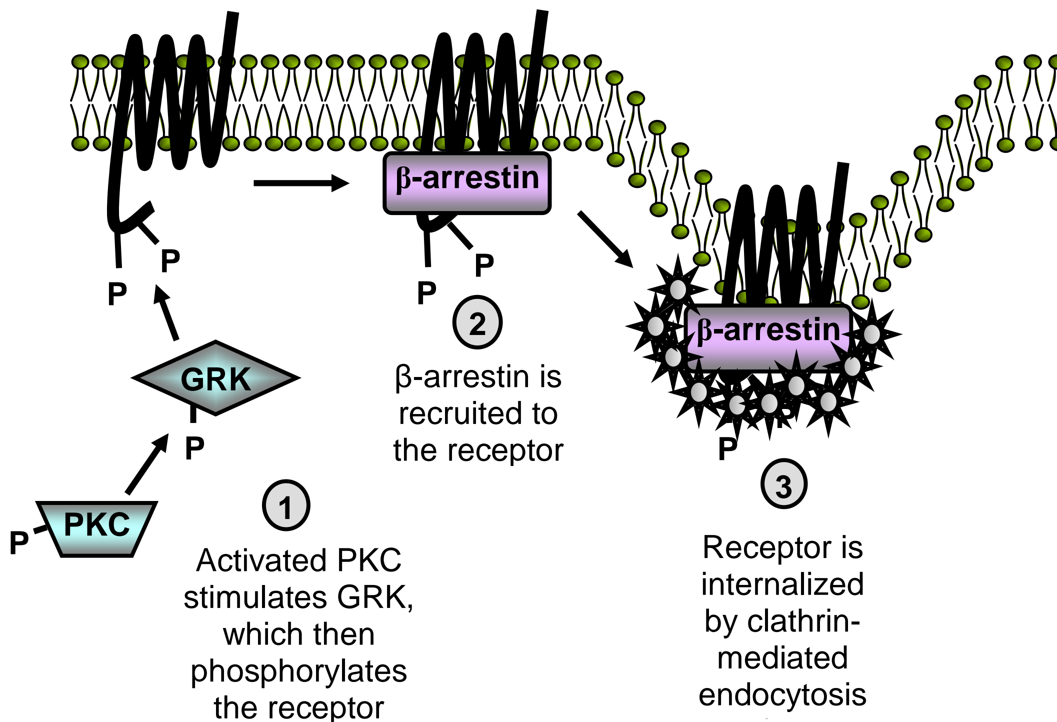


Figure 3: Mechanisms of terminating chemokine signaling pathways. Abbreviations: GDP: guanine dinucleotide phosphate; GTP: guanine trinucleotide phosphate; P_i: inorganic phosphate; PKC: protein kinase C; GRK: G-protein coupled receptor kinase.

Raghuwanshi et al. 2007). GRKs are activated mainly by the protein kinase A (PKA) or C (PKC) pathways—both of which become active upon signaling by the chemokine receptor (Pitcher, Freedman et al. 1998; Ferguson 2001; Olson and Ley 2002). As a result, phosphorylated receptors are unable to signal, even in the presence of extracellular ligand (Luu, Rainger et al. 2000). Moreover, receptor phosphorylation leads to β-arrestin recruitment and endocytosis of the receptor via the formation of clathrin-coated vesicles (Mueller, White et al. 1997; Pitcher, Freedman et al. 1998; Ferguson 2001; Vroon, Heijnen et al. 2006; Nasser, Raghuwanshi et al. 2007). Upon receptor internalization, the potential for chemokine signaling is abolished. Interestingly, as all chemokines signal through the same intracellular pathways, the activation of one chemokine receptor, including those for C5a and fMLP, has the potential to desensitize all other chemokine receptors (Pitcher, Freedman et al. 1998; Ali, Richardson et al. 1999; Le, Li et al. 2000; Luu, Rainger et al. 2000; Ferguson 2001; Vroon, Heijnen et al. 2006). This process, termed heterologous desensitization, therefore allows for absolute termination of receptor signaling, rendering the neutrophil incapable of migration towards any type of stimulant.

The Systemic Response to Burn Injury

Overall, the process of neutrophil recruitment to an inflamed tissue is quite complex. Therefore, careful regulation of the steps involved in the inflammatory response, including those which terminate it, are critical to prevent infection and to initiate tissue repair. For a small wound, the process described above is limited to the injury site, where cytokine secretion and endothelial cell activation occurs locally. In the

case of a moderate to severe burn injury, however, a great degree of cellular and tissue damage is generated over a significant portion of the body. As a result of this large surface area of damage, a local elevation of pro-inflammatory mediators progresses to a systemic circulation of these factors (Davis, Moore et al. 1987; Botha, Moore et al. 1996; Piccolo, Wang et al. 1999; Mimasaka, Hashiyada et al. 2001). In this situation, as pro-inflammatory cytokines travel through the blood and activate endothelial cells, even more cytokines are released into circulation, creating a positive feedback loop of inflammation (Ware and Matthay 2000). Clinically, this can lead to what is called the systemic inflammatory response syndrome (SIRS), marked by hyper or hypothermia, increased heart and respiratory rate, and leukocytosis or leukopenia (Rangel-Frausto, Pittet et al. 1995; Bone 1996).

Again, since it is important that the inflammatory process is countered by anti-inflammatory mechanisms, the reaction to SIRS manifests as a compensatory anti-inflammatory response syndrome (CARS) (Robertson and Coopersmith 2006). This is essentially an exaggerated, global immunosuppressive phase, marked by decreased interferon-gamma (IFN-γ) and IL-10, as well as increased IL-4 (Kovacs, Duffner et al. 2004; Schneider, Schwacha et al. 2004). The clinical outcomes of burn injury, as described above, are therefore determined by which particular phase of the inflammatory response the patient is in (**Figure 4**). In the initial pro-inflammatory phase, the patient is at a significant risk for early MOF as a result of inflammatory-mediated destruction of vital organs, such as the heart and lungs (Demling, LaLonde et al. 1989; Moore, Sauaia et

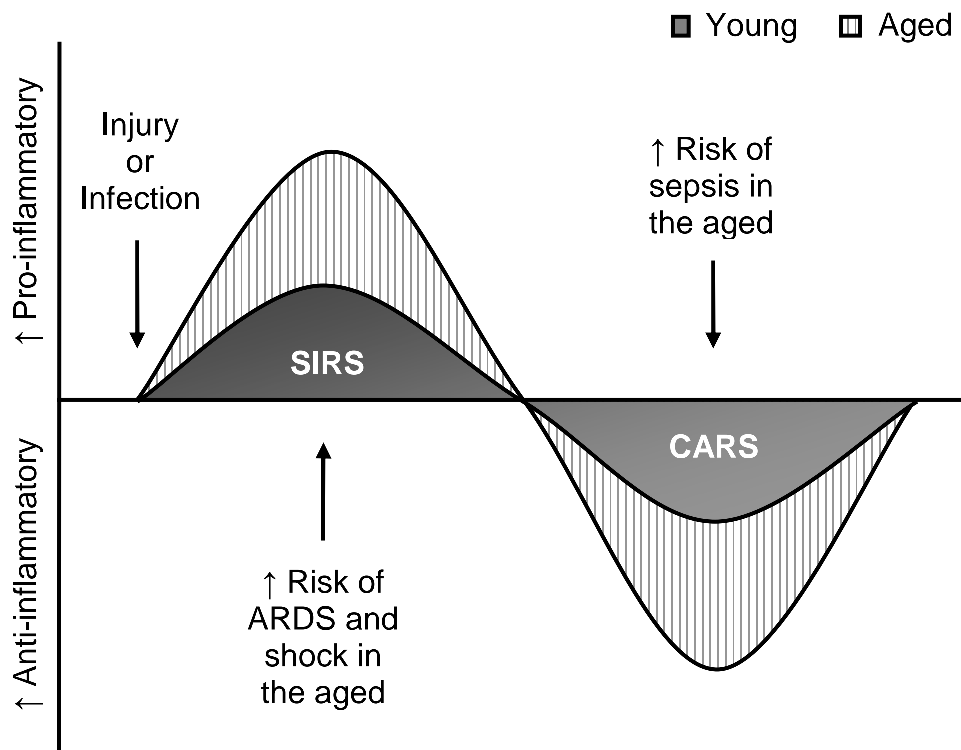


Figure 4: Effects of aging on the evolution of the systemic inflammatory response. Abbreviations: SIRS: systemic inflammatory response syndrome; CARS: compensatory anti-inflammatory response syndrome; ARDS: acute respiratory distress syndrome; LOS: length of stay.

al. 1996), and shock, whereby hypoperfusion renders tissues incapable of sustaining aerobic metabolism (Gutierrez, Reines et al. 2004). In the later phase, where immunosuppression dominates, the risk of MOF is more likely to result from infection and sepsis (Moore, Sauaia et al. 1996; Fitzwater, Purdue et al. 2003; Davis, Santaniello et al. 2004; Tarlowe, Duffy et al. 2005).

It has long been recognized that one of the most serious threats to the burn patient is the development of respiratory complications, such as pneumonia and the development of acute lung injury, leading to ARDS (Shook, MacMillan et al. 1968; Achauer, Allyn et al. 1973; Teixidor, Novick et al. 1983; Hollingsed, Saffle et al. 1993; Dancey, Hayes et al. 1999). Moreover, when pulmonary dysfunction develops in elderly patients, the risk of death is even higher than that of younger burn patients (**Figure 4**) (Le, Zamboni et al. 1986; Clayton, Solem et al. 1995; Ely, Wheeler et al. 2002). Whether from an infection or as a result of systemic inflammation, approximately 45% of burn patients show some degree of damage to the lungs, which is markedly higher than any other organ system, next to the skin (Fitzwater, Purdue et al. 2003). Although the inciting injury is not in the lung, the accompanying tissue damage and edema formation can significantly compromise normal function (Lund, Onarheim et al. 1992; Iliopoulou, Markaki et al. 1993). One reason is that all the blood returning from the site of injury, containing cytokines and activated leukocytes, must eventually circulate through the lung to become reoxygenated. Other characteristics of the lung that make it one of the most susceptible organs to damage following injury include its delicate alveolar architecture and the presence of numerous alveolar macrophages, which can independently respond to systemic cytokines (Williams, Bankey et al. 1994; Arbak, Ercan et al. 1999).

While methods to block pro-inflammatory cytokines would seem advantageous to burn patients, a clinical efficacy for this mode of treatment has not been demonstrated. The timing and dose of administration may be the critical reasons for why this strategy

has not been successful. Chemokines, on the other hand, are also involved in neutrophil exit from the bone marrow, recruitment to inflammatory sites, integrin activation, tissue migration, and effector functions. As their release occurs hours after key cytokines, such as IL-1β, it is possible that targeting the actions of chemokines following burn injury would be promising (Piccolo, Wang et al. 1999; Toth, Alexander et al. 2004).

Neutrophil Defects with Aging

Again, since the neutrophil response dominates the acute stages of inflammation, understanding the defects that occur in neutrophils with age may help to reveal some of the reasons why the elderly are more susceptible to complications after burn. With increased age, there tends to be a decreased number of granulocyte precursors in the bone marrow of both mice and humans (Izumi-Hisha, Ito et al. 1990; Ogawa, Kitagawa et al. 2000). While the numbers in circulation are typically not different from younger individuals, the production of mature neutrophils under inflammatory conditions in aged individuals can be significantly compromised (Chatta and Dale 1996; Schroder and Rink 2003). This defect may have important implications for why the elderly are at an increased risk for infection (Schroder and Rink 2003; De Martinis, Modesti et al. 2004).

While the average lifespan of neutrophils from an aged individual is typically not different from that of neutrophils from the young, the G-CSF mediated delay in apoptosis is not present in neutrophils from old mice and humans (Fulop, Fouquet et al. 1997; Tortorella, Piazzolla et al. 2001; Fortin, Larbi et al. 2007). This is thought to be a result

of defective signaling through the Janus kinase (Jak)/signal transducer and activator of transcription (STAT) pathway (Fortin, Larbi et al. 2007). It can be speculated that this defect in protecting neutrophil apoptosis may lead to a decrease in the ability for the elderly to effectively clear infections.

Once neutrophils are released into circulation, a number of functional defects have been observed in the aged. A few laboratories have reported that phagocytosis is significantly compromised in neutrophils from both humans and animals of increased age (Wenisch, Patruta et al. 2000; Butcher, Chahal et al. 2001; Lord, Butcher et al. 2001). However, there is controversy over whether the ability for neutrophils from the aged to generate ROS is dysfunctional. Some have observed a decrease in superoxide production following stimulation with latex particles or fMLP (Nagel, Pyle et al. 1982; Biasi, Carletto et al. 1996; Fulop, Larbi et al. 2004), while others showed an increase following fMLP stimulation *in vitro* (Ito, Kajkenova et al. 1998; Butcher, Chahal et al. 2001). These differing results are likely a function of the experimental conditions by which they were performed. Nonetheless, the dysfunction in neutrophil phagocytosis and ROS generation contribute to the decreased microbicidal activity of neutrophils from the aged. It has been speculated that these changes may be attributed to decreased intracellular Ca^{2+} release (Fulop, Fouquet et al. 1997; Wenisch, Patruta et al. 2000) and a reduction in actin polymerization following stimulation (Rao 1986; Piazzolla, Tortorella et al. 1998). Both of these processes are integral to the activation of both phagocytosis and NADPH oxidase-dependent ROS production.

In fact, a number of intracellular signaling pathways have been shown to be defective in neutrophils from the aged, such as extracellular signal-regulated kinase (ERK), p38, phospholipase C, and cyclic AMP activation (Lipschitz, Udupa et al. 1991; Fulop, Larbi et al. 2004; Fortin, Larbi et al. 2007). To explain this, it has been found that advanced age is associated with increased membrane fluidity, leading to the alteration in toll-like receptor (TLR) (Fulop, Larbi et al. 2004) and triggering receptor expressed on myeloid cell-1 (TREM-1) signaling (Fortin, Lesur et al. 2007). As both of these pathways are involved in a number of neutrophil functions (Medzhitov and Janeway 2000; Radsak, Salih et al. 2004), these results imply that changes in membrane fluidity may significantly affect overall neutrophil functions in the aged.

In vivo studies show that aging is associated with an exaggerated neutrophil accumulation following a number of different insults, suggesting that there is an increased chemotactic response (Ashcroft, Horan et al. 1998; Corsini, Di Paola et al. 2005; Gomez, Hirano et al. 2007; Ito, Betsuyaku et al. 2007). However, *in vitro* studies show conflicting results. Chemotaxis of neutrophils from aged subjects in response to fMLP or GM-CSF is reported as either decreased or unchanged compared to those of the young (Niwa, Kasama et al. 1989; Biasi, Carletto et al. 1996; Wenisch, Patruta et al. 2000; Fulop, Larbi et al. 2004). The inconsistencies in these results may be a function of the particular stimulant or experimental conditions employed. To note, there are no publications characterizing the response to the CXCR2 chemokines, KC and MIP-2, in neutrophils from the aged. However, levels of GRK2—involved in desensitization of a

number of receptors including CXCR2—have been found to be upregulated in aortas from aged animals. Therefore, receptor desensitization may play a role in the defective chemotaxis seen in neutrophils from the aged (Gros, Chorazyczewski et al. 2000; Schutzer, Reed et al. 2001).

Data indicate that the adhesion molecule profile is altered in aged individuals, which could potentially explain the results from *in vivo* models of inflammation (De Martinis, Modesti et al. 2004). Even in the absence of clinically detectable disease, aged subjects had decreased levels of L-selectin on neutrophils, suggesting either decreased activation or increased shedding from overstimulation (Richter, Rassoul et al. 2003; De Martinis, Modesti et al. 2004). In addition, increased levels of the soluble forms of ICAM-1 and VCAM-1 in circulation and membrane forms in the aorta were found in "healthy" aged humans and rats (Miles, Thies et al. 2001; Richter, Rassoul et al. 2003; Zou, Jung et al. 2004; Zou, Yoon et al. 2006). This is consistent with the concept of inflamm-aging described above. Furthermore, the relative levels of soluble VCAM-1 were found to positively correlate with the risk for cardiovascular disease in the elderly (Richter, Rassoul et al. 2003). Interestingly, however, neutrophil adhesion under basal conditions was not different between young and aged humans, which correlated with no change in CD11a and b on the cell surface of neutrophils (Biasi, Carletto et al. 1996; Butcher, Chahal et al. 2001).

Aging and Burn Injury: a Two-Hit Model?

As inflammation is a factor associated with increased age in the absence of clinically detectable disease, it is interesting to speculate whether the inflammatory processes described above may be exacerbated in elderly burn patients. Many of the neutrophil defects associated with aging—including impaired chemotaxis and elevated levels of circulating cytokines—are also seen in young individuals sustaining a systemic injury. Therefore, taking into account the pathogenesis of burn injury, it can be proposed that increased age primes various cells throughout the body, leading to an exaggerated response once the insult occurs. In other words, burn injury in aged individuals can be considered a "two hit" phenomenon. As in other two-hit models of systemic inflammation, such as burn injury complicated by infection, the occurrence of the second challenge results in a response greater than that which could be achieved by either challenge alone (Davis, Santaniello et al. 2004; Samonte, Goto et al. 2004; Moore, Moore et al. 2005; Maegele, Sauerland et al. 2007). Clinically, these situations pose the greatest challenge. Therefore, developing effective treatment strategies to combat them is of great importance. Current standards of care for burn patients are based on the typical response of a young, healthy adult. Unfortunately, effective treatment strategies for elderly burn patients have yet to be realized. Our understanding of the mechanisms involved in increased morbidity and mortality for burn patients over the age of 65 is missing a great deal of substance. As a result, insights about what may be happening clinically are currently unattainable.

CHAPTER 3

PULMONARY INFLAMMATION IS EXACERBATED IN AGED MICE AFTER BURN INJURY

Abstract

Burn patients over the age of 65 are at a greater risk for developing pulmonary complications than younger patients. The mechanisms for this, however, have yet to be elucidated. The objective of this study was to determine whether increased chemoattraction plays a role in the age-related differences in pulmonary inflammation after burn injury. At 6 or 24 hours after receiving sham or 15% TBSA scald injury, lungs from young and aged mice were analyzed for leukocyte content by histological examination and immunostaining. Lungs were then homogenized and levels of neutrophil chemokines, MIP-2 and KC were measured. At 6 hours after burn, the number of neutrophils was 4 times higher in the lungs of both burn groups compared to aged-matched controls ($p<0.05$), but no age difference was evident. At 24 hours, in contrast, neutrophils returned to sham levels in the lungs of young, burn-injured mice ($p<0.05$), but did not change in the lungs of aged, burn-injured mice. Pulmonary levels of the neutrophil chemokine, KC, but not MIP-2, was consistently 3 times higher in aged, burn injured mice compared to young, burn injured mice at both time points analyzed.

Administration with anti-CXCR2 antibody completely abrogated the excessive pulmonary neutrophil content by 24 hours ($p<0.05$) while not affecting the inflammatory response of the wounds. These studies show that CXCR2-mediated chemoattraction is involved in the pulmonary inflammatory response after burn and suggest that aged individuals sustaining a burn injury may benefit from treatment strategies that target neutrophil chemokines.

Introduction

Individuals over the age of 65 are at a greater risk of developing serious complications after burn injury than younger, otherwise healthy, adults (Linn 1980; McGill, Kowal-Vern et al. 2000; ABA 2005). A main reason is that approximately 70% of elderly patients are admitted to the emergency room with one or more pre-existing conditions, such as cardiovascular disease or diabetes (Tran, Groeneveld et al. 1990; Lionelli, Pickus et al. 2005). However, simply being over the age of 65 has been found to be an independent risk factor for the development of multiple organ failure, sepsis, and acute respiratory distress syndrome after traumatic injury (Dancey, Hayes et al. 1999; Fitzwater, Purdue et al. 2003). Improvements in treatment strategies for burn patients have provided great benefit for younger individuals (2002). Unfortunately, the current advancements have made little progress for elderly burn patients (Griffiths and Laing 1981; ABA 2005).

It has long been recognized that one of the most serious threats to the burn patient is the development of respiratory complications (Achauer, Allyn et al. 1973; Hollingsed, Saffle et al. 1993). Moreover, when pulmonary dysfunction develops in elderly patients, the risk of death is even higher than that of a younger burn patient (Clayton, Solem et al. 1995; Ely, Wheeler et al. 2002). It is currently thought that, with a moderate to severe burn injury, an extensive amount of pro-inflammatory mediators enter into circulation, incite a systemic inflammatory response, and affect organs other than the skin (Ward and Till 1990). Since the lungs receive 100% of the cardiac output, their risk of being affected by a systemic inflammatory response is considerable. The reasons why the lungs of aged individuals are even more susceptible to damage following injury than those of younger individuals, however, are not completely understood (Slater and Gaisford 1981; Duchateau 2003; Lionelli, Pickus et al. 2005).

One of the acute markers of remote organ damage after burn injury is the infiltration of neutrophils (Demling, LaLonde et al. 1989; Baskaran, Yarmush et al. 2000). When activated, neutrophils release numerous proteases and reactive oxygen species, which can result in destruction of the surrounding tissue (Hansbrough, Wikstrom et al. 1996; Ravage, Gomez et al. 1998). There are three main mechanisms involved in neutrophil recruitment to the site of inflammation: chemoattraction, endothelial cell adhesion, and vascular permeability (Hillyer, Mordelet et al. 2003; Reutershan and Ley 2004). In this study, we will only focus on chemoattraction. Neutrophil chemokines—mostly KC and MIP-2, which are orthologs of human GROα and β, respectively—are

released from a number of cell types in the lung in response to inflammatory stimuli (Piccolo, Wang et al. 1999; Lomas-Neira, Chung et al. 2005). Upon their release, chemokines bind to their cognate receptors on circulating leukocytes and induce cytoskeletal changes that allow the cells to migrate into the tissue (Lomas-Neira, Chung et al. 2004; Reutershan and Ley 2004). For neutrophils, the main receptor for MIP-2 and KC is CXCR2, which is upregulated in response to various pro-inflammatory mediators (Goodman, Pugin et al. 2003; Reutershan and Ley 2004).

To date, most of the animal models that explore the mechanisms of pulmonary inflammation after injury utilize young adult animals (Stengle, Meyers et al. 1996; Dries, Lorenz et al. 2001; Lomas-Neira, Chung et al. 2005; O'Dea, Young et al. 2005). Although many have shown that pulmonary sequelae have a higher incidence in elderly burn patients and are more detrimental, few have investigated the cause (Linn 1980; Slater and Gaisford 1981; Lionelli, Pickus et al. 2005). The main objective of this study was to examine pathologic differences in the lungs of young and aged animals in a murine model of burn injury and to determine whether the neutrophil chemokines, MIP-2 and KC, play a role in this process. We have employed a murine model in which animals receive only a moderate sized burn injury (15% of the TBSA). In the human population, only about 1% of individuals from 2-60 years old would succumb to this size burn, whereas 15% of those over the age of 60 would die from a similar sized injury (ABA 2005). Here, we show for the first time that a moderate sized burn in aged mice parallels what is observed in humans and that a greater neutrophil accumulation is related to a

protracted expression of KC in the lungs of aged mice. Blocking neutrophil chemoattraction through administration of anti-CXCR2 antibody effectively reduces pulmonary inflammation in the lungs of aged mice in the first 24 hours after burn.

Materials and Methods

Animals

Young (3-6 months) and aged (18-22 months) BALB/c female mice were obtained from the National Institute of Aging colony at Harlan Laboratories (Indianapolis, IN) and maintained on a 12 hour light/dark cycle with standard laboratory rodent chow and water ad libitum. All experimental procedures were performed according to the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals, National Institutes of Health, and approved by the Animal Care and Use Committee at Loyola University Medical Center.

Induction of Burn Injury

Mice were anesthetized with Nembutal (50 mg/kg) intraperitoneally (i.p.), shaved, and placed into a plastic template designed to give a 15% TBSA, full-thickness, dorsal scald injury when immersed in a boiling water bath for 8 seconds, according to a modified protocol of Walker and Mason (Walker and Mason 1968; Faunce, Gregory et al. 1997). As a control, a separate group of mice received a sham injury, which entailed administration of anesthesia and shaving, but a room temperature water bath was used

instead. Immediately following injury, the mice received warm saline resuscitation (1 ml per 20 g body weight) and their cages were placed on heating pads to prevent circulatory collapse and cardiovascular shock. After recovering from anesthesia, this procedure leaves young, healthy mice able to eat, drink, groom, and ambulate at their pre-injury capacity. Aged mice, on the other hand, take longer to recover from anesthesia, show labored breathing, and do not ambulate as well as before the injury. The mice were sacrificed using CO_2 inhalation and cervical dislocation. No other therapeutic intervention was provided, as administration of anti-inflammatory or analgesic medication may introduce confounding factors into the assessment of inflammatory responses. To eliminate the complication of hormones regulated by circadian rhythms, all burn injury procedures were administered between 8 and 10 am. In addition, all mice—including those which died before the time of sacrifice—were examined for visible tumors and, if found, were removed from the study.

Histologic Examination of the Lungs

Lungs were removed and inflated with formalin immediately after sacrifice, as previously described (Patel, Faunce et al. 1999). After overnight fixation, the lungs were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Lung sections were examined by light microscopy for pathologic changes and neutrophil content (Patel, Faunce et al. 1999). The total number of neutrophils in the lungs of each animal was determined in ten 400x fields.

Immunofluorescence of Leukocytes in the Lungs and Skin

The lungs were removed at the time of sacrifice, inflated with 25% O.C.T. freezing medium, and embedded for frozen sectioning. To compare the changes in the lung to the wound itself, the skin from the edge of the burn wound was also removed at the time of sacrifice and embedded in O.C.T freezing medium for sectioning. Skin was not taken from the center of burn wounds, as most of this tissue is necrotic (Faunce, Llanas et al. 1999). The lung and skin sections were fixed in acetone and blocked with normal goat serum. Sections were first incubated with 1 μg/mL of rat anti-Gr-1 antibody (Invitrogen, Carlsbad, CA) followed by 4 μg/ml of goat anti-rat IgG conjugated to Alexa Fluor 488 (Invitrogen). Since Gr-1 can also be found on certain macrophage populations (Vermaelen and Pauwels 2004; Sugimoto, Katayama et al. 2006), the sections were dual-stained with 0.2 μg/mL of biotinylated anti-MOMA-2 antibody (BMA Biomedicals, Augst, Switzerland), a pan-macrophage marker, and detected with 2 μg/ml of Cy3 Streptavidin (Invitrogen). Using fluorescent microscopy, the total number of neutrophils (designated as Gr-1^{+} MOMA-2^{-} cells) were counted across 10 high power fields for each animal in both lungs and skin (Lam, Caterina et al. 2002). The total tissue area across which cells were counted was quantified and determined to be consistent between animals in all treatment groups (data not shown).

KC and MIP-2 Levels in Lung and Skin Homogenates

One lobe per mouse or one 5 mm punch from the edge of the wound was homogenized in protease inhibitor cocktail (Roche Applied Sciences, Indianapolis, IN)

(Faunce, Llanas et al. 1999; Gomez, Hirano et al. 2007). Samples were analyzed for MIP-2 and KC content by enzyme linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN), according to the manufacturer's specifications (Patel, Faunce et al. 1999). Total protein content of the same aliquot of lung homogenate used for ELISA was determined by BioRad protein assay (BioRad Laboratories, Hercules, CA). Final concentrations of each chemokine are in pg/mg protein.

Blocking CXCR2

An initial set of dose response experiments were performed to determine the lowest dose of anti-CXCR2 antibody that would block neutrophil accumulation in lungs of young mice after burn injury without disturbing the inflammatory process in the skin. With these experiments, we determined that an i.p. dose of 20 μg per animal was sufficient to reduce neutrophil content in lungs of young, burn-injured mice to that of sham animals (data not shown). In a separate set of experiments, both young and aged mice receiving a sham or 15% TBSA burn injury were injected i.p. with either 20 μg of control IgG (R&D Systems) or 20 μg of CXCR2 neutralizing antibody (R&D Systems). Mice were then sacrificed at 6 or 24 hours and the lungs and skin were collected for further analysis, as described above.

Statistical Analysis

Data were analyzed using GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA) and are expressed as mean ± SEM. For comparisons of two groups, an

unpaired student's t-test was used. For time course experiments, a three-way analysis of variance was used. Groups were considered significantly different at p values less than 0.05.

Results

Histologic Changes in the Lungs after Burn

To first examine whether pathologic differences exist in the lungs of young and age mice at 6 and 24 hours after burn injury, frozen sections were stained with H&E. As shown by other laboratories (Stengle, Meyers et al. 1996; Arbak, Ercan et al. 1999; Baskaran, Yarmush et al. 2000), the lungs of young mice were found to have a greater accumulation of inflammatory cells, increased edema formation, and thickened alveolar walls at 6 hours after injury compared to young sham controls (**Figures 5A and 5C**). At this time point, similar pathological changes were found in lungs of aged, burn-injured mice, which were not apparent in lungs of aged control animals (**Figures 5B and 5D**). By 24 hours, the inflammatory cell accumulation in the lungs of young, burn-injured animals diminished, making them indistinguishable from sham controls (**Figures 5E and 5G**). In the lungs of aged animals that sustained injury, however, the inflammatory infiltrate did not decrease at 24 hours compared to 6 hours (**Figures 5F and 5H**). To note, the lungs of young and aged sham-injured mice did not appear different from young and aged unmanipulated animals (data not shown).

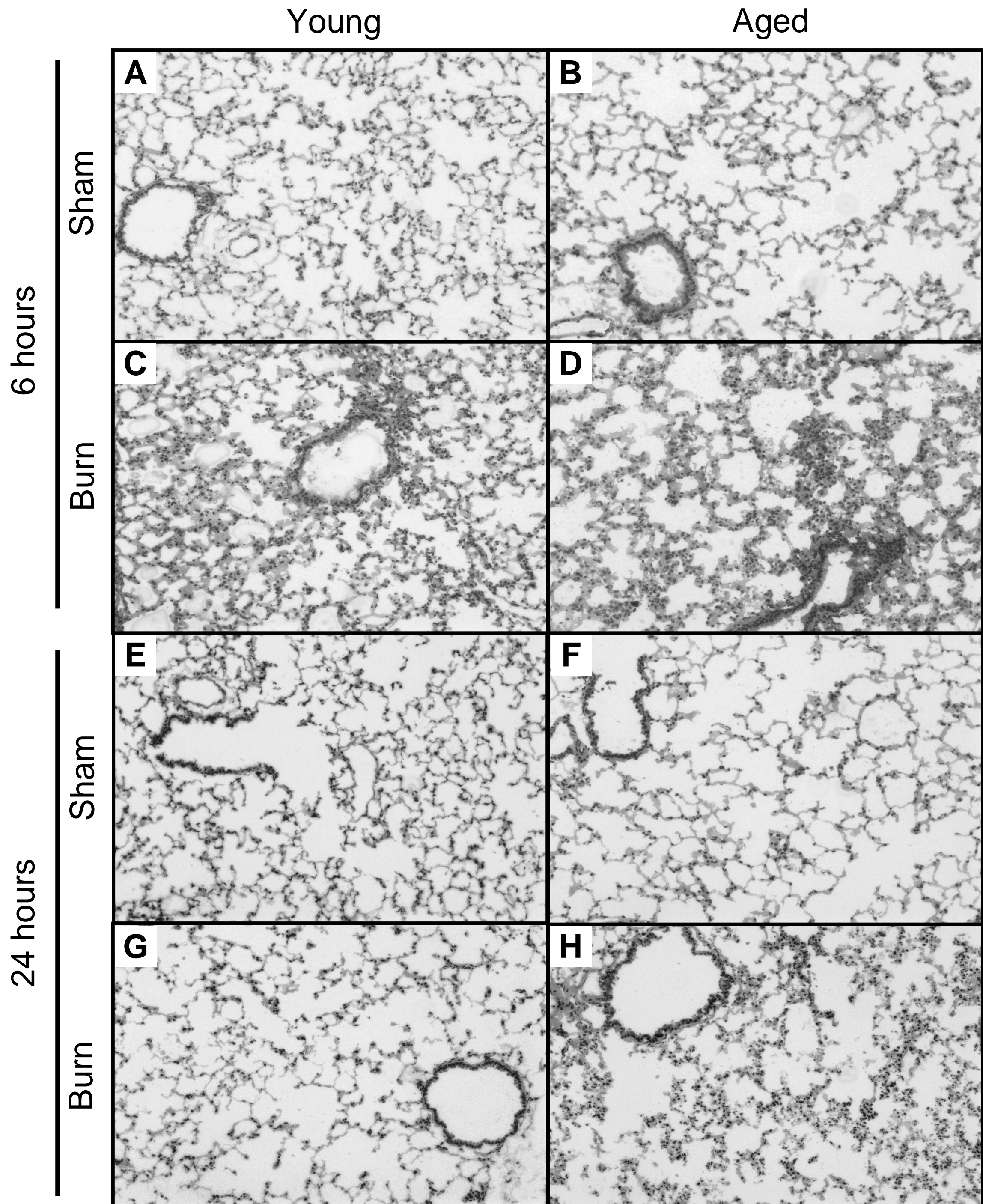


Figure 5. Pathological changes in the lungs of young and aged mice after burn. Representative micrographs of H&E stained lung sections are shown from young (A, C, E, and G) and aged (B, D, F, and H) animals at 6 hours after sham injury (A and B), 6 hours after burn injury (C and D), 24 hours after sham injury (E and F), and 24 hours after burn injury (G and H). All images are at 100x.

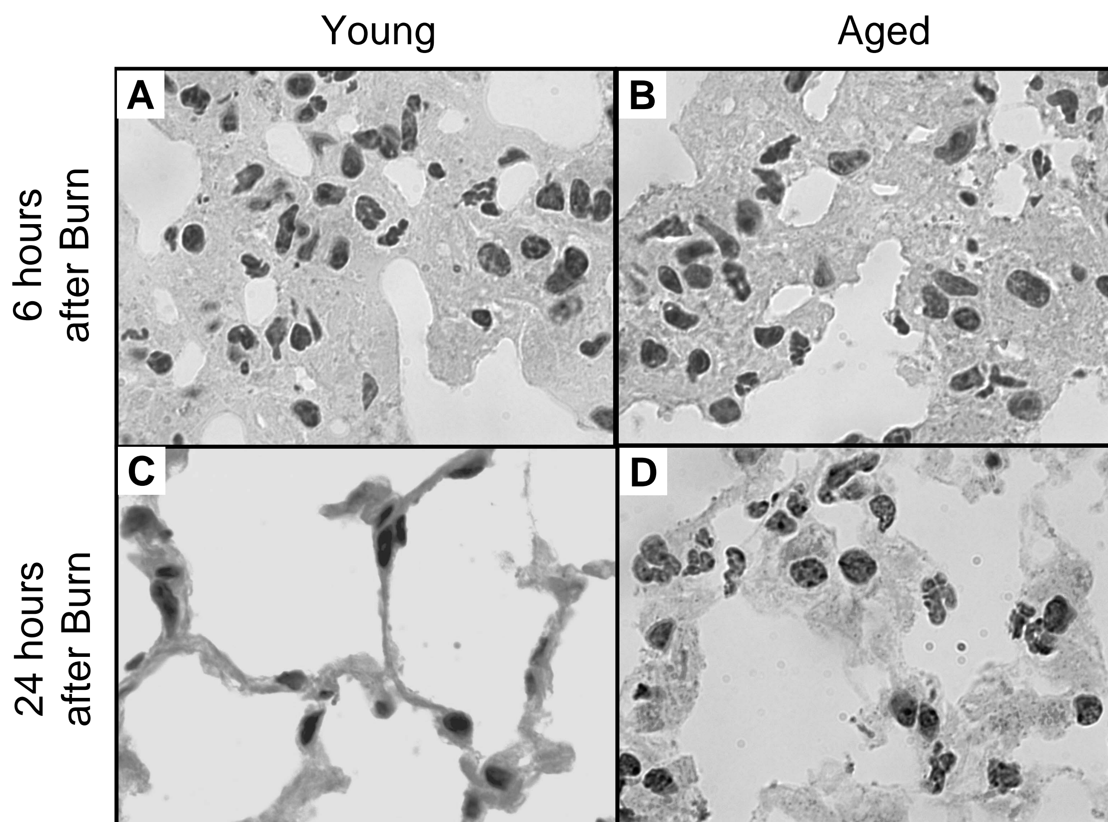


Figure 6. Neutrophil accumulation and alveolar wall thickening in the lungs of young and aged mice after burn. High power view of H&E lung sections illustrating neutrophils within thickened alveolar walls of young and aged burn-injured mice at 6 hours (A and B) and at 24 hours (C and D). High power images of young, burn injured mice at 24 hours did not appear different from those of sham-injured mice (not shown). All images are at 1000x magnification.

Upon closer examination, the vast majority of the inflammatory cells in the lungs after injury were neutrophils. To determine whether these neutrophils migrated into the tissue or remained in the circulation, lung sections from all burn-injured animals were examined at higher power (1000x). High power images of lungs from young mice 24 hours after burn looked identical to those of sham-injured mice; in these groups, the

alveolar walls were thin and not very cellular. In lungs of both young and aged mice at 6 hours after burn, as well as in those of aged mice at 24 hours after burn, the opposite was the case. While many neutrophils were also observed within the vasculature, the majority appeared to have extravasated and localized within the alveolar walls leading to increased wall thickness (**Figure 6**).

Inflammatory Cell Accumulation in Lungs of Aged Mice After Burn

To confirm that the injury-associated inflammatory cells seen in the lungs after burn injury were indeed neutrophils, frozen sections of lung were immunostained with anti-Gr-1 (Sugimoto, Katayama et al. 2006). Since anti-Gr-1 can also detect certain macrophage populations, lungs were simultaneously stained with anti-MOMA-2—a pan-macrophage marker (Vermaelen and Pauwels 2004; Sugimoto, Katayama et al. 2006). Thus, cells that were Gr-1 positive but were negative for MOMA-2 were considered neutrophils. Representative images of immunostained lungs from all treatment groups are shown in **Figure 7** and quantification of neutrophils is shown in **Figure 8**. At 6 hours after injury, the number of neutrophils was more than 4 times higher in lungs of young mice compared to sham-injured controls ($p<0.05$) (shown in **Figure 7A, 7C, and 8**). Similar increases in pulmonary neutrophils were found at 6 hours in the lungs of aged, burn-injured mice (**Figures 7B, 7D, and 8**). By 24 hours after injury, the number of neutrophils in the lungs of young, burn-injured mice decreased to sham levels (**Figures 7E, 7G, and 8).** Parallel to the H&E analysis in **Figures 5 and 6,** the neutrophils remained elevated in the lungs of aged, burn-injured animals at 24 hours compared to

sham controls ($p<0.05$) (**Figures 7F, 7H, and 8**). Neither age nor burn injury affected the number of Gr-1^{+} MOMA-2^{+} cells or Gr-1^{+} MOMA-2^{+} cells observed in the lungs, at either time point analyzed (data not shown). In addition, the number of neutrophils in the lungs of young and aged sham injured mice was not different from those of young and aged unmanipulated mice (data not shown).

Chemokines in Lungs of Aged Mice after Burn Injury

To determine whether increased numbers of neutrophils seen in the lungs of aged mice after injury correlated with enhanced levels of chemokines, lung homogenates obtained from young and aged mice were analyzed for neutrophil chemokines, MIP-2 and KC, by ELISA. At 6 hours after burn injury, both young and aged mice had significantly higher pulmonary KC levels than their sham controls, but levels were 3 times higher in the lungs of aged mice compared to young mice after burn (**Figure 9A**). At 24 hours, KC levels remained elevated in the lungs of both young and aged, burn-injured mice compared to shams, but levels were still 3 times higher in the lungs of aged mice receiving a burn injury. In contrast, although pulmonary levels of MIP-2 were 2-6 times higher than sham levels in all animals receiving a burn, there were no differences between age groups or time points after injury ($p<0.05$) (**Figure 9B**). These data imply that, while both KC and MIP-2 are elevated in the lungs in the acute phases of burn injury, only levels of KC can account for the age-related differences in pulmonary neutrophil recruitment.

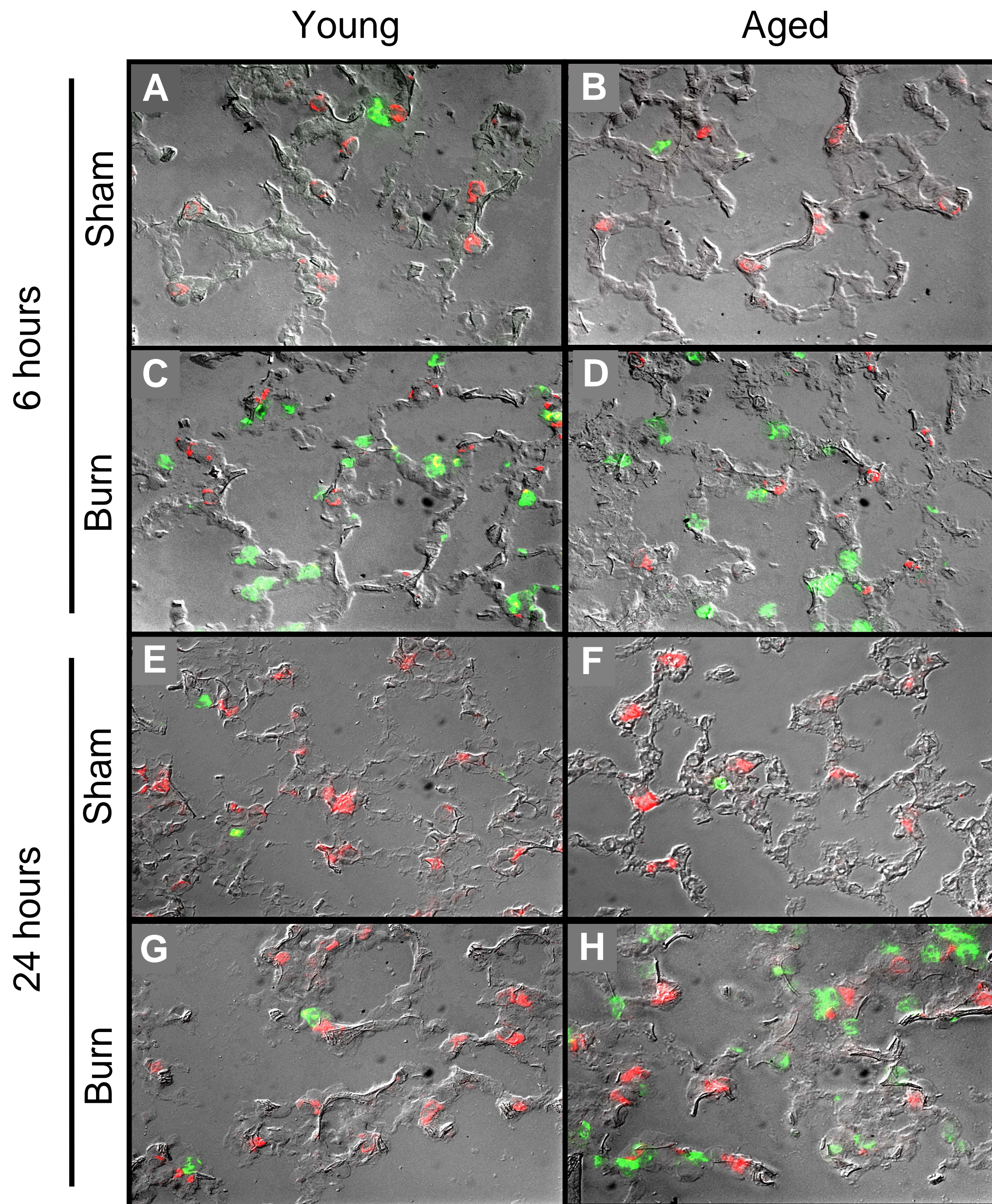


Figure 7. Immunostaining for neutrophils in the lungs after burn. Sections of lungs from young (A, C, E, and G) and aged (B, D, F, and H) mice at 6 and 24 hours after receiving a burn injury were stained with anti-Gr-1 (green) and anti-MOMA-2 (red) antibodies. Representative images are shown with a differential interference contrast (DIC) overlay from animals 6 hours after sham injury (A and B), 6 hours after burn injury (C and D), 24 hours after sham injury (E and F), and 24 hours after burn injury (G and H). All images are at 400x magnification.

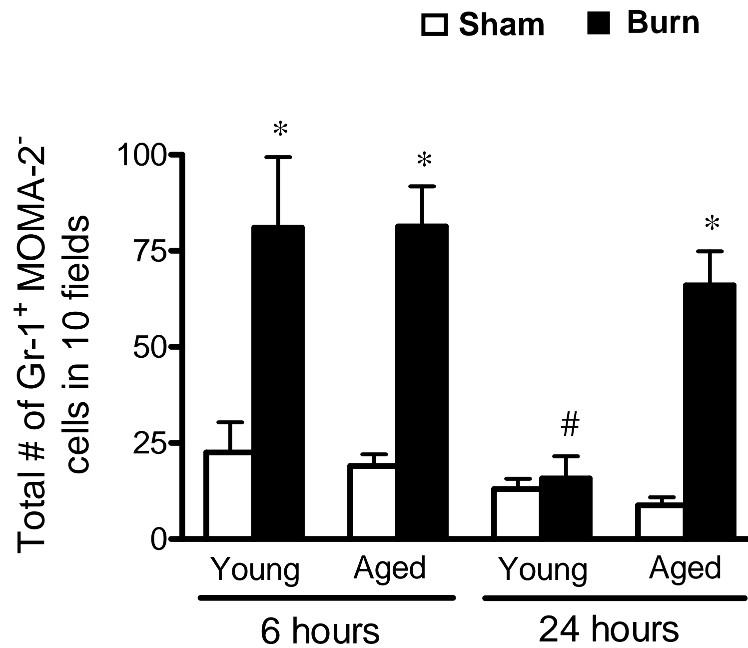


Figure 8. Neutrophil content in the lungs after burn. Total numbers of Gr-1$^+$ MOMA-2$^-$ cells from lungs of young and aged animals at 6 and 24 hours after sham (white bars) or burn (black bars) injury were counted in sections of lung tissue. Data are represented as the average number of cells counted in ten 400x fields for each group ± SEM. N = 4-7 mice per group. *, $p<0.05$ compared to age and time matched sham groups; #, $p<0.05$ compared to young burn group at 6 hours.

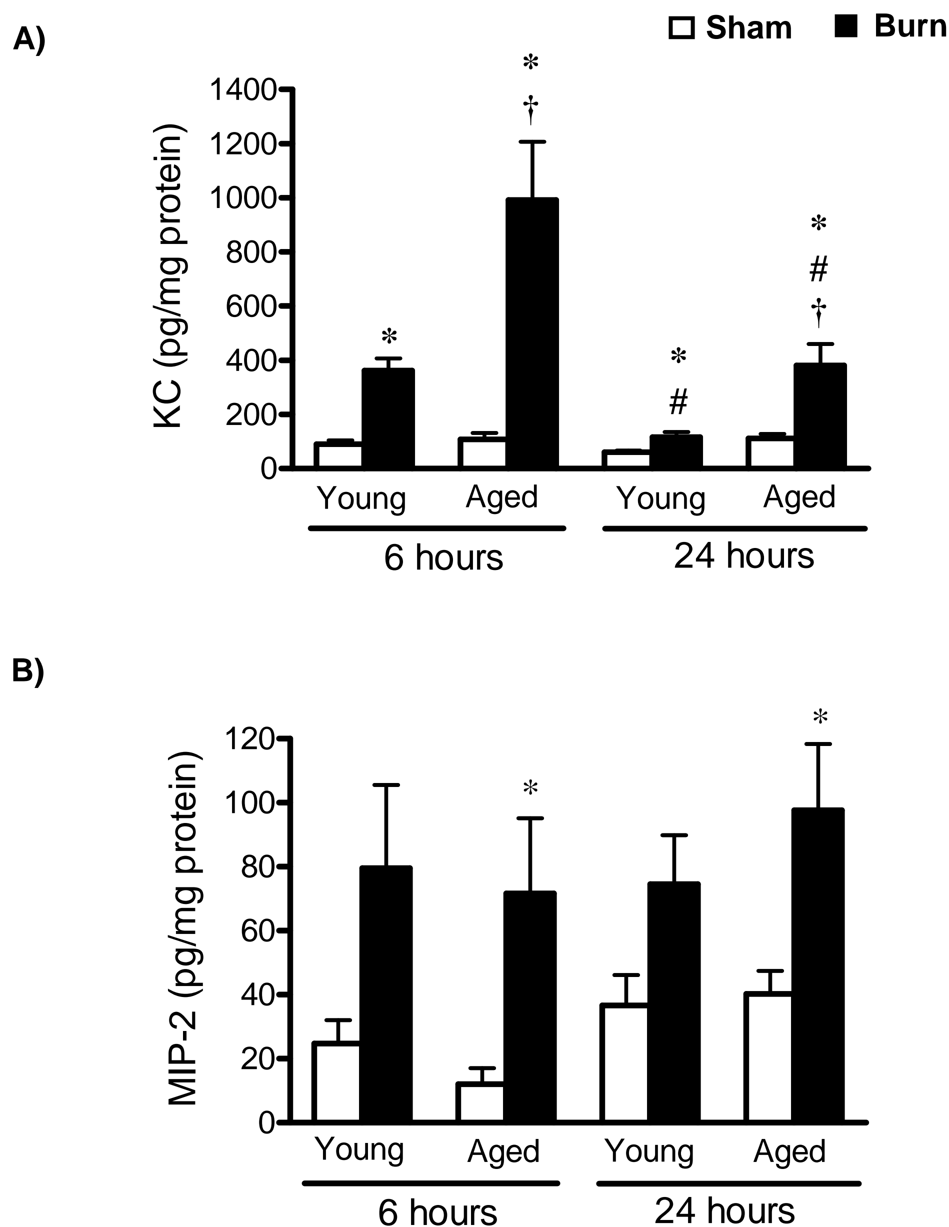


Figure 9. Neutrophil chemokines in the lungs after burn. Levels of A) KC and B) MIP-2 were measured in lung homogenates of young and aged mice at 6 and 24 hours after sham (white bars) or burn (black bars) injury. Data are represented as average concentration in pg/mg protein ± SEM. N = 8-13 mice per group. *, $p<0.05$ compared to age and time matched sham groups; #, $p<0.05$ compared to burn animals at 6 hours; †, $p<0.05$ compared to young burn at the same time point.

Blocking CXCR2

To test the hypothesis that neutrophil chemokines are directly involved in the accumulation of pulmonary neutrophils after burn injury, mice were administered with either control IgG or a neutralizing antibody against CXCR2 i.p. thirty minutes after receiving a burn or a sham injury. Animals were then sacrificed at 6 and 24 hours after injury. As shown above, this time point correlated with neutrophil clearance in the lungs of young mice, while the lungs of aged mice still had a considerable degree of inflammation (**Figures 5-8**). At 6 hours, anti-CXCR2 neutralization only caused about a 50% reduction in the neutrophil count in the lungs of both young and aged mice after burn compared to IgG controls (**Figure 10**). At 24 hours, on the other hand, the exaggerated neutrophil response seen in the lungs of aged, burn injured mice with control IgG was completely inhibited by the anti-CXCR2 antibody (**Figure 10**).

While anti-CXCR2 treatment seemed to be effective in reducing neutrophil counts, it is important to assess the effects it had on the pulmonary pathology following burn. Since the 24 hour time point was most critical for age-related differences, we only examined lungs at this time point. As expected, the lungs of young mice 24 hours after burn did not appear different from the lungs of sham animals following injection of either control IgG or anti-CXCR2 antibody (**Figure 11A-11D, compare to Figure 5**). Administration of the anti-CXCR2 antibody to aged mice at this time point after injury not only blocked the neutrophil content, but it also significantly reduced the pulmonary pathology seen with the injection of control antibody (**Figure 11E-11H**). Interestingly,

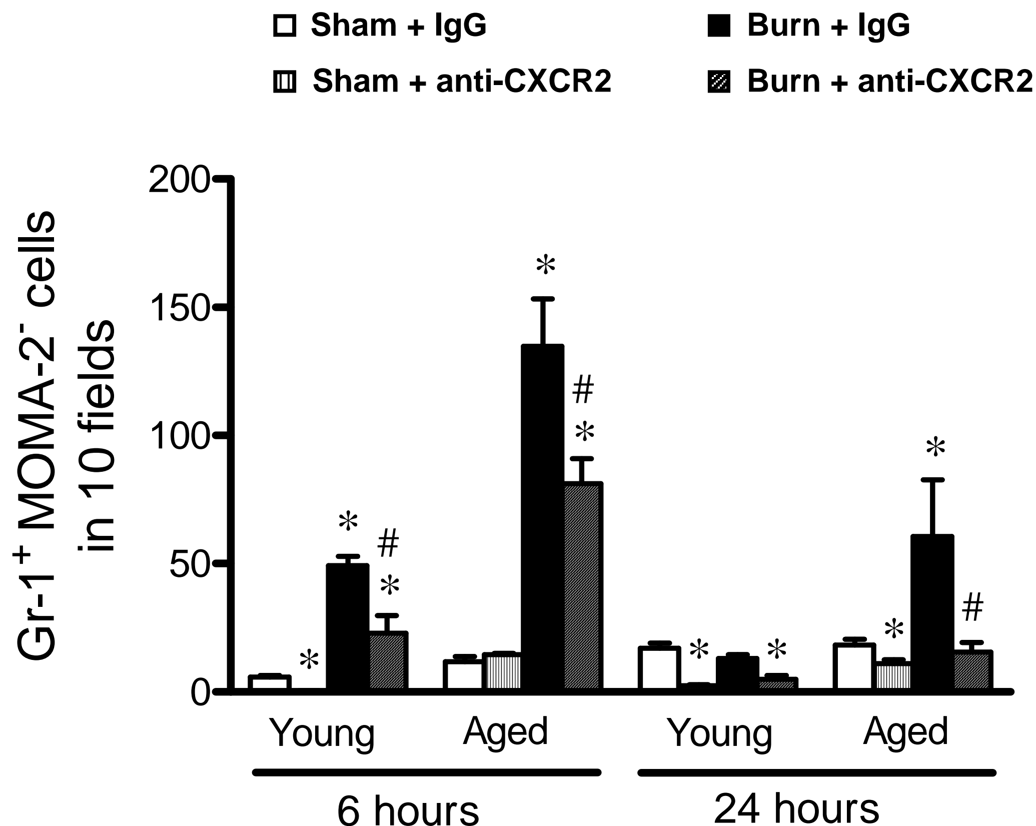


Figure 10. The effects of blocking CXCR2 on neutrophil content in the lungs of young and aged mice after burn. Total numbers of Gr-1+ MOMA-2⁻ cells from lungs of young and aged animals at 6 and 24 hours after sham or burn injury, receiving either 20 µg i.p. of control IgG or 20 µg ip. of anti-CXCR2 antibody were counted in sections of lung tissue as described above. Data are represented as the average number of cells counted in ten 400x fields for each group ± SEM. The average tissue area over which cells were counted did not differ between groups. N = 3-10 mice per group. *, $p<0.05$ compared to sham IgG controls; #, $p<0.05$ compared to IgG controls.

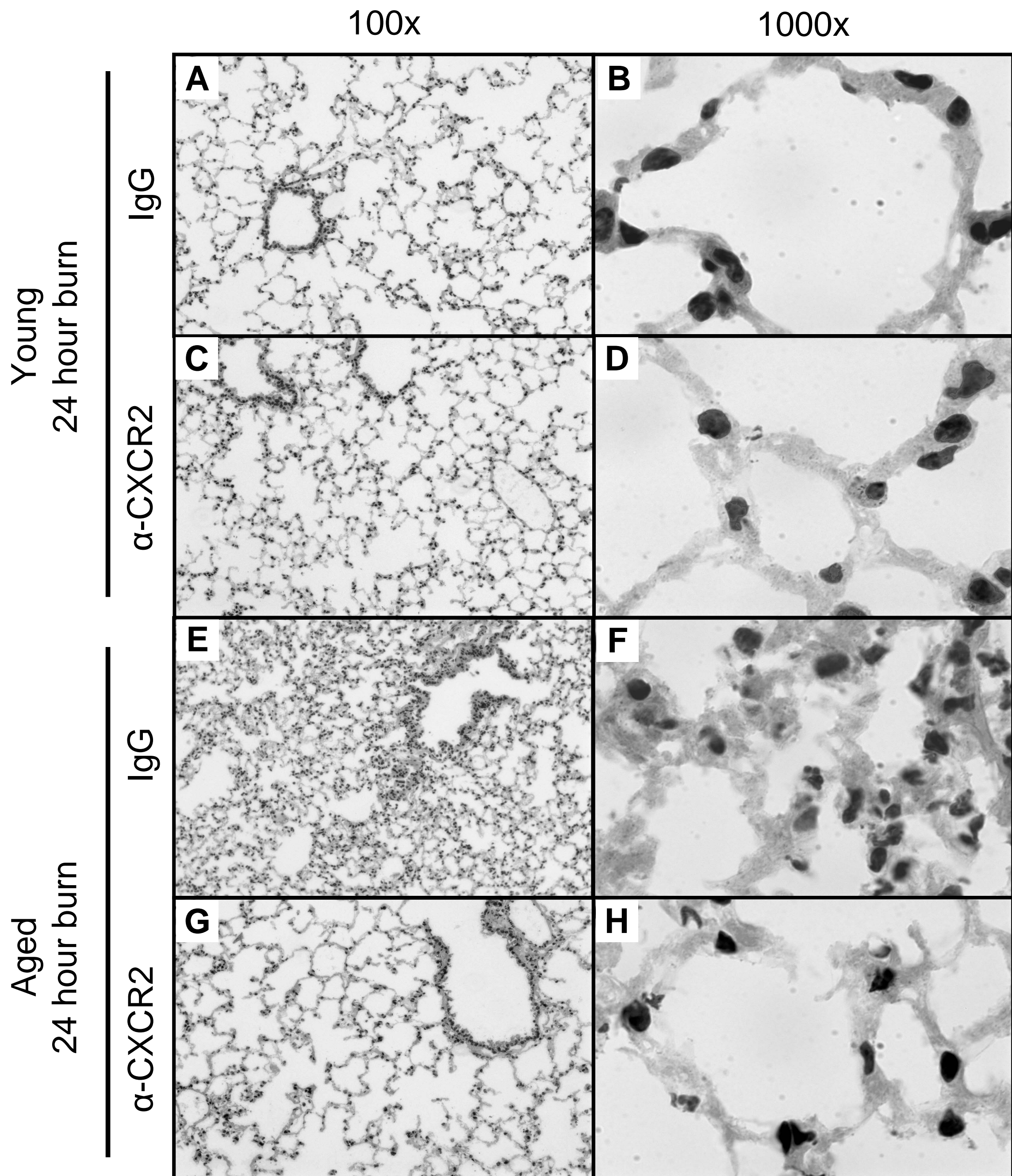


Figure 11. The effects of blocking CXCR2 on the histopathology of the lungs after burn. Representative micrographs of H&E stained lung sections are shown from young (A-D) and aged (E-H) animals receiving either control IgG i.p. (A, B, E, and F) or anti-CXCR2 i.p (C, D, E, and H) at 6 hours after burn injury. Images in the left column are at 100x magnification and images shown on the right are at 1000x magnification.

inhibiting CXCR2 significantly lowered the pulmonary levels of neutrophils in all sham treatment groups as well, except for the aged at 6 hours after injury, suggesting that this receptor also has a role in the normal homeostatic maintenance of neutrophil numbers in the lungs. To note, KC, MIP-2, and IL-1β levels in the lungs were not different between control IgG and anti-CXCR2 administration in any of the treatment groups (data not shown), indicating that antibody treatment did not decrease neutrophil accumulation in the lungs through diminishing the pro-inflammatory response itself.

Wound Analysis

With the observation that chemokines are higher in the lungs of aged mice after burn and lead to greater neutrophil accumulation, we sought to determine whether this held true for the burn wound as well. As shown in **Table 1**, levels of KC were significantly elevated in the wounds of burn injured mice at 24 hours, but age differences in the lungs at this time point were not apparent. MIP-2 levels at this time point were below the minimum level of detection by ELISA in the wounds of all treatment groups (data not shown). Neutrophils in the skin at 24 hours after burn were also elevated compared to shams ($p<0.05$), but were not significantly different between the age groups (**Table 2**). These data suggest that the exaggerated inflammatory response to burn injury in aged mice is specific for the lung.

Table 1. Chemokine levels in wounds at 24 hours after burn

	Sham	Burn
Wound KC (pg/mg protein)		
Young	35.6 ± 144	200.0 ± 28.1 *
Aged	44.7 ± 16.7	210.3 ± 32.2 *

Levels of KC and MIP-2 were measured by ELISA in wound homogenates of young and aged mice at 24 hours after sham or burn injury. Data are represented as average concentration in pg/mg protein ± SEM. N= 8-12 mice per group *, p<0.05 compared to sham controls. MIP-2 was below the minimum detection level for all groups.

Since the anti-CXCR2 antibody was given systemically, there was a concern that neutrophil migration to the wound itself would be compromised, potentially leading to abnormal wound healing or a risk of infection. For this reason, the dose of anti-CXCR2 antibody used (20 μg per mouse) was intentionally kept at a level which would effectively reduce the neutrophil accumulation in the lung while preserving the inflammatory response in the burn wound. The results in **Table 2** show that, in fact, the dose of anti-CXCR2 antibody given did not significantly affect the neutrophil accumulation in the wounds of mice at either age at 24 hours after burn injury. Since anti-CXCR2 antibody treatment did not affect levels of KC or MIP-2 in the lungs, these chemokines were not measured in the wounds of this treatment group.

Table 2. Neutrophil counts in wounds at 24 hours after burn

	IgG (i.p.)		Anti-CXCR2	
	Sham	Burn	Sham	Burn
Young	1.3 ± 0.9	74.0 ± 8.3 *	2.3 ± 1.1	46.8 ± 11.1 *
Aged	9.5 ± 3.1	76.9 ± 9.2 *	7.3 ± 2.8	75.0 ± 7.0 *

Tissue from the edge of burn wounds of young and aged animals at 24 hours after sham or burn injury, receiving either 20 µg i.p. of control IgG or 20 µg ip. of anti-CXCR2 antibody were collected. Total numbers of Gr-1^{+} MOMA-2^{-} cells in paraffin sections were counted as described above for the lungs. Data are represented as the average number of cells counted in ten 400x fields for each group ± SEM. N = 7-14 mice per group. *, $p<0.05$ compared to sham controls.

Discussion

It is widely recognized that advanced age is a significant risk factor for increased pulmonary complications after burn injury (Achauer, Allyn et al. 1973; Clayton, Solem et al. 1995). However, few studies have directly examined the mechanisms that could contribute to this age-associated susceptibility. In studies utilizing young animals, neutrophils are a main mediator of pulmonary inflammation and tissue damage in the acute stages of injury (Stengle, Meyers et al. 1996; Arbak, Ercan et al. 1999; Abraham, Carmody et al. 2000). Here, we have shown that chemokines acting through CXCR2 play a role in causing acute inflammation in the lungs after burn injury. We have also shown that aged mice exhibit an exacerbated pulmonary response to burn as a result of an

increased and sustained level of KC in the lungs. Most importantly, these data indicate that, in this model of burn injury, blocking CXCR2 is an effective way to reduce acute pulmonary inflammation, especially in aged mice.

Many have previously shown that chemokines are instrumental in neutrophil-mediated pulmonary damage after injury in young animals (Mercer-Jones, Shrotri et al. 1999; Piccolo, Wang et al. 1999; Calkins, Bensard et al. 2002; Goodman, Pugin et al. 2003; Lomas-Neira, Chung et al. 2004). Here, we demonstrate that levels of both KC and MIP-2 are elevated in the lungs within the first 24 hours after injury, but that only pulmonary KC levels are affected by age. Experiments aimed at blocking CXCR2 show that inhibiting the neutrophil response to KC completely abrogates pulmonary inflammation at 24 hours after burn in young and aged mice, indicating that chemoattraction is indeed part of this mechanism for both age groups. However, since antibody treatment at 6 hours after burn only reduced the neutrophil content of the lungs by half, perhaps there are temporal differences in mediating neutrophil migration into the lungs. These results are consistent with those from other laboratories studying the effects of inhibiting CXCR2-mediated chemoattraction in various models of systemic inflammation, such as hemorrhagic shock and sepsis, as well as in wound healing (Ness, Hogaboam et al. 2003; Lomas-Neira, Chung et al. 2004; Gordon, Li et al. 2005; Lomas-Neira, Chung et al. 2005). Whether using CXCR2 knockout mice, neutralizing antibody against the receptor, small molecule inhibitors of the receptor, or antibodies against KC and MIP-2 themselves, these studies show that blocking CXCR2-mediated

chemoattraction sufficiently attenuates acute inflammatory responses following a systemic challenge.

As a caveat, *in vitro* experiments conducted by other laboratories have indicated that neutrophils from aged animals actually display decreased chemotaxis in response to various inflammatory stimuli (Niwa, Kasama et al. 1989; Fulop, Larbi et al. 2004; Gomez, Boehmer et al. 2005). The contradiction between these *in vitro* experiments and those conducted in the current study reveal the importance of cellular environment when analyzing defects associated with aging. It is well known that, *in vivo*, aging is associated with increased circulating pro-inflammatory cytokines, such as IL-6, TNF-α , IL-1, and IL-8 (Ershler 1993; Franceschi, Bonafe et al. 2000; Bruunsgaard, Andersen-Ranberg et al. 2003; Sarkar and Fisher 2006). Interestingly, we have also found that IL-1β levels are significantly higher in the lungs of aged mice (V. Nomellini and E.J. Kovacs, unpublished observations). A significant increase in the levels of IL-1β in the lungs of elderly humans in the absence of clinically detectable disease has also been observed (Meyer, Ershler et al. 1996). This increase in pulmonary IL-1β, however, does not correlate with higher levels of KC and MIP-2—both of which can be induced by IL-1β (Calkins, Bensard et al. 2002; Chen, Chang et al. 2007)—in the lungs of uninjured, aged mice (**Figure 9**). After burn injury, on the other hand, KC levels are 3-fold greater in the lungs of aged mice compared to those of young mice at both 6 and 24 hours.

In contrast, IL-1β is not different in the skin of young and aged uninjured mice (V. Nomellini and E.J. Kovacs, unpublished observations). Following this, neither KC nor MIP-2 show age differences in the skin after burn (**Table 1**). Differential levels of IL-1β in the absence of injury may help explain why the lungs of aged mice show an exacerbated response following an inflammatory challenge compared to those of young mice, while the wounds themselves do not. To note, levels of IL-6, another key player in the inflammatory response, are not different in the lungs of young and aged, uninjured mice (data not shown).

With the observation that aging is associated with an increased pro-inflammatory state, the results of the current study are similar to those seen in "two hit" models of injury. In these models, the clinically important situation, whereby two inflammatory challenges occur simultaneously or as subsequent challenges results in an exaggerated response beyond that of either injury alone. Examples of this include hemorrhagic shock plus sepsis or burn injury plus infection (Davis, Santaniello et al. 2004; Perl, Chung et al. 2005). We propose that burn injury in aged individuals parallels these two hit models. Advanced aged acts as the first hit by increasing the inflammatory milieu of the lungs. Once receiving a burn injury (the second hit), aged mice exhibit an augmented response beyond that of young mice receiving a comparable injury. We have shown that blocking this excessive inflammation effectively reduces the pulmonary consequences in the aged mice after burn. In the current studies, though, the anti-CXCR2 treatment did not decrease the mortality rate of the aged mice (data not shown). Regardless of whether

they received anti-CXCR2 antibody, approximately 20% of the aged mice succumb to the burn injury within the first 24 hours, while all of the young mice survive. In other words, a burn size that is normally manageable in young mice leaves aged mice at a greater risk for complications and death; this is very similar to what is seen in the human population (Hammond and Ward 1991; Clayton, Solem et al. 1995; Suchyta, Clemmer et al. 1997). In humans, on the other hand, mortality rates are 4 times higher in elderly patients with pulmonary failure compared to those without (Clayton, Solem et al. 1995). Therefore, while anti-CXCR2 treatment does not affect mortality within the first 24 hours after burn, perhaps it will prove to be a valuable tool to prevent or limit pulmonary failure and death at later time points.

While we have shown that CXCR2 chemokines are mechanistically important in mediating pulmonary neutrophil accumulation after burn, the therapeutic implications of these data are also intriguing. As described above, the intended use of the anti-CXCR2 antibody was to effectively reduce the systemic component to burn injury, while preserving the inflammatory response of the wound itself. Using only 20 μg per mouse, we were able to accomplish this. The reasons for this are unknown, but we believe that it is related to the degree of tissue injury and the number and types of pro-inflammatory mediators involved. At the primary site of injury in the skin, there is a great deal of cellular damage and necrosis that is not seen in the lungs (Faunce, Llanas et al. 1999). The persistence of this necrotic tissue acts as a nidus for a protracted inflammatory response. In addition, chemokine signaling via CXC receptors is not the only mechanism

that contributes to neutrophil migration to the wound. Other candidate chemoattractants not examined in this study include C5a, platelet activating factor, and leukotriene B_4 (LTB_4), all of which are potent mediators of neutrophil migration (Piccolo, Wang et al. 1999; Burns, Smith et al. 2003; Reutershan and Ley 2004). C5a, in particular, has been shown to play a role in burn injury (Schmid, Piccolo et al. 1997; Piccolo, Wang et al. 1999).

Interestingly, these data also show that blocking CXCR2 also decreases the number of neutrophils in the lungs of uninjured animals. It is well known that there is a highly-controlled regulation of neutrophil numbers in the peripheral blood and in tissues. The proposed mechanisms for neutrophil homeostasis are related to the β-integrin, CD18 (Weinmann, Scharffetter-Kochanek et al. 2003), as well as G-CSF and IL-23 (Christopher and Link 2007). Some have also suggested that there is an important interplay between neutrophil responses to the CXCR4/SDF-1 axis and the CXCR2/KC axis (Martin, Burdon et al. 2003). Upon downregulation or cleavage of CXCR4, neutrophils have an increased propensity to leave the bone marrow and migrate to the periphery via CXCR2 ligands. Our results support this hypothesis, indicating that blockage of CXCR2 attenuates neutrophil numbers in the lungs of uninjured mice (**Figure 11**). Although not a primary goal for the current study, these data provide further insight into normal maintenance of neutrophil homeostasis.

From the results of this study alone, it is not possible to predict whether the neutrophil retention in lungs of aged, burn-injured mice was only a result of enhanced recruitment to the tissue or whether increased adhesion to the pulmonary vasculature and/or diminished clearance also plays a role. There has been extensive research on the role of endothelial adhesion molecules on neutrophil accumulation in the lungs after burn injury (Mulligan, Till et al. 1994; Muller, Cronen et al. 2002). In young animals, mRNA expression of ICAM-1 is reportedly elevated in the lungs following burn injury (Jin, Zhu et al. 2003). To date, there are no published reports that assess the cell adhesion molecule profile in lungs of aged animals after injury. However, many have reported an upregulation of membrane and soluble forms of ICAM-1, VCAM-1, and selectins in the serum of aged humans and animals, whether in the absence or in the presence of injury (Forsey, Thompson et al. 2003; Richter, Rassoul et al. 2003; Laudes, Guo et al. 2004; Zou, Jung et al. 2004). In addition, since both aging and burn injury are known to compromise the phagocytic capacity of macrophages, decreased removal of apoptotic neutrophils may provide an alternative explanation for the increased pulmonary inflammation seen in aged mice after burn (Plowden, Renshaw-Hoelscher et al. 2004; Sebastian, Espia et al. 2005). In our hands, histologic examination does not reveal an accumulation of apoptotic bodies. Specific staining for active caspase 3 and similar studies aimed at characterizing the state of neutrophil apoptosis, as well as macrophage phagocytosis, are required to define the role of neutrophil clearance in the lungs of aged animals in response to injury.

In summary, these data show that CXCR2-mediated neutrophil migration is important in the development of pulmonary inflammation in the acute stages of burn injury. In addition, increased age is associated with an exaggerated response in the lungs, but not in the wounds, following burn injury, possibly as a result of differential levels of IL-1β expression before injury. Regardless of age, prolonged neutrophil exposure can lead to excessive tissue destruction as a result of protease release and oxidative stress (Ward and Till 1990; Hansbrough, Wikstrom et al. 1996; Ravage, Gomez et al. 1998). This may explain why the elderly are at an increased risk for pulmonary complications after burn injury. Low doses of CXCR2-neutralizing antibody are effective in attenuating acute pulmonary neutrophil accumulation in aged mice, while maintaining the inflammatory environment of the wound tissue. Importantly, these data imply that the development of more targeted therapies against neutrophil chemokines may be beneficial for preventing or diminishing remote organ damage after injury, especially in the aged population.

CHAPTER 4

INCREASED CXCR2 ACTIVATION PRIMES NEUTROPHILS FROM AGED MICE AND CAUSES PROLONGED PULMONARY INFLAMMATION AFTER INJURY

Abstract

Pulmonary complications are the most frequent and often the most fatal for the burn patient, especially for individuals over the age of 65. The main etiology of lung damage after burn, regardless of age, is the development of a systemic inflammatory response. To determine why advanced age predisposes an individual to exaggerated pulmonary inflammation after burn injury, a murine model using a 15% TBSA scald injury was employed. Using immunofluorescence on lung sections and flow cytometry on whole lung cell suspensions, neutrophils were found to be significantly higher in the lungs of aged mice after burn ($p<0.05$). However, these neutrophils were not localized to the airspaces, as measured by flow cytometry of BAL. To determine whether increased adhesion molecule expression could explain this neutrophil sequestration in the lungs, pulmonary vascular ICAM-1 expression was quantified by immunofluorescence on lung sections by dual staining with PECAM-1. At 6 hours, there were no differences between any of the treatment groups. At 24 hours, in contrast, ICAM-1 and PECAM-1 coexpression was significantly higher in the lungs of aged, burn injured mice ($p<0.05$).

When CD11b, was measured on peripheral blood neutrophils, levels were decreased in aged, sham injured animals ($p<0.05$). At 24 hours after burn, CD11b was found to be elevated only on neutrophils from young mice, although this was not significantly different. Interestingly, neutrophils from aged mice showed increased migratory behavior in the absence of stimulus, but no further response to KC, when chemotaxis assays were employed. This correlated with decreased expression of CXCR2 on pulmonary neutrophils, but not on peripheral blood neutrophils from aged mice in the absence of injury ($p<0.05$). In response to burn, CXCR2 did not decrease further on neutrophils in the lungs of aged mice, but expression levels were significantly attenuated on peripheral blood neutrophils of aged mice ($p<0.05$), with no change on those of young mice. In summary, increased age seems to prime neutrophils by generating an elevated state of activation in the absence of injury. In response to burn injury, neutrophils from the aged appear to accumulate within the pulmonary vasculature in an ICAM-1 dependent manner. Targeting this hyperresponsiveness of the aged may help to attenuate the inflammatory response of the lungs following burn.

Introduction

While the overall mortality of burn patients over the age of 65 has improved over the last few decades (ABA 2005), these patients still have poorer clinical outcomes and an increased length of stay (LOS) in the hospital (Gomberg, Gruen et al. 1999; Roth, Velmahos et al. 2001; Taylor, Tracy et al. 2002; Bergeron, Clement et al. 2006; 2007). As the proportion of elderly individuals is expected to grow considerably in the future,

this issue translates into a greater socioeconomic burden to our society. It is therefore important that new treatment strategies are developed to minimize the effects of age on the response to injury.

Similar to other insults which lead to systemic inflammation, the development of pulmonary complications, such as pneumonia and ARDS, are often the most serious threat to the burn patient, especially in elderly (Shook, MacMillan et al. 1968; Le, Zamboni et al. 1986; Hollingsed, Saffle et al. 1993; Dancey, Hayes et al. 1999; Ely, Wheeler et al. 2002). In contrast to most other organs in the body, the lung has two potential routes for an inflammatory insult to enter: through the airway and through the bloodstream. Interestingly, the pathogenesis of pulmonary inflammation is completely dependent on where the stimulus is localized. When an inflammatory source is located in the airway, a rapid neutrophil recruitment to the alveolar space can be seen (Frevert, Huang et al. 1995; Gupta, Feng et al. 1996; Xing, Gauldie et al. 1998; Czermak, Friedl et al. 1999; Beck-Schimmer, Madjdpour et al. 2002; Sentman, Brannstrom et al. 2002; Quinton, Nelson et al. 2004; Gordon, Li et al. 2005; Reutershan, Basit et al. 2005; Speyer, Rancilio et al. 2005; Basit, Reutershan et al. 2006). However, when the source is systemic (or administered intravascular (i.v.)), neutrophil accumulation within the lung tissue occurs, but cells do not migrate into the alveoli. The neutrophils are said to be "sequestered" in alveolar capillaries (Johnson, Brigham et al. 1991; Standiford, Kunkel et al. 1995; Gupta, Feng et al. 1996; van Eeden, Kitagawa et al. 1997; Carraway, Welty-Wolf et al. 1998; O'Malley, Matesic et al. 1998; Czermak, Friedl et al. 1999; Murphy,

Paterson et al. 2005; Rojas, Woods et al. 2005; Gomez, Hirano et al. 2007). The differences in these results are not simply due to the location of the chemokines, as levels of these mediators can be detected in bronchoalveolar lavage (BAL) fluid following either type of injury (Schmid, Piccolo et al. 1997; Czermak, Friedl et al. 1999).

The localization of neutrophils after an inflammatory challenge has important implications. When activated, neutrophils in the alveoli can generate considerable damage through the release of proteolytic enzymes and ROS, but this response is typically limited to the lung (Brigham 1990; Ward and Till 1990; Hansbrough, Wikstrom et al. 1996; Jaeschke and Smith 1997; Carden, Xiao et al. 1998; Arbak, Ercan et al. 1999; Moraes, Zurawska et al. 2006). On the other hand, activated neutrophils that are sequestered in pulmonary capillaries also cause damage to the endothelium and can exacerbate the systemic inflammatory response to injury (Simon, DeHart et al. 1986; Kowal-Vern, Walenga et al. 1997; Carden, Xiao et al. 1998; Usatyuk and Natarajan 2005).

Many laboratories have shown that the acute inflammatory response in the lungs of young rodents after burn injury peaks around 4-6 hours (Hansbrough, Wikstrom et al. 1996; Stengle, Meyers et al. 1996; Arbak, Ercan et al. 1999; Baskaran, Yarmush et al. 2000; Dries, Lorenz et al. 2001). By 24 hours, this response is completely resolved, as assessed by neutrophil counts and levels of myeloperoxidase activity (a marker of neutrophil activity) (Hansbrough, Wikstrom et al. 1996; Stengle, Meyers et al. 1996;

Baskaran, Yarmush et al. 2000; Dries, Lorenz et al. 2001). We recently published that the pulmonary inflammatory response to burn, in terms of neutrophil counts and KC levels, persisted at 24 hours in the lungs of aged mice receiving the same injury (Nomellini, Faunce et al. 2008). When anti-CXCR2 neutralizing antibody was administered i.p. 30 minutes after burn, the prolonged response in aged mice was prevented (Nomellini, Faunce et al. 2008). This study suggested that neutrophil accumulation in the lungs of the aged is a result of increased chemoattraction towards the CXCR2 chemokines, KC and MIP-2.

In vitro and *in vivo* evidence indicates that chemokine signaling is much more complex than originally thought. Not only do chemokines act to stimulate neutrophil movement towards an inflammatory stimulus, but also to induce firm adhesion to the endothelium and to mediate diapedesis (Luu, Rainger et al. 2000; Cinamon, Grabovsky et al. 2001; Zhang, Liu et al. 2001; Kim, Carman et al. 2004). These studies also imply that the particular order of events in chemokine signaling is required for the appropriate response to occur. First, CD11/CD18 on neutrophils binds loosely to ICAM-1 on the endothelium. Once this interaction is in place, chemokine signaling causes CD11/CD18 to become upregulated and to cluster, leading to firm adhesion (Chatila, Geha et al. 1989; Kim, Carman et al. 2004). Once adherent, neutrophils can then interact with chemokines immobilized on the apical side of endothelial cells and begin the process of diapedesis (Rot, Hub et al. 1996; Luu, Rainger et al. 2000; Cinamon, Grabovsky et al. 2001). Once movement through the endothelium occurs, other adhesion molecules, such as VCAM-1,

coordinate migration through the basement membrane and into the tissue (Burns, Smith et al. 2003).

Decreased CXCR2 expression has been implicated in mediating pulmonary complications after a systemic insult (Cummings, Martin et al. 1999; Adams, Hauser et al. 2001; Tarlowe, Duffy et al. 2005). Receptor desensitization is thought to regulate this effect in order to prevent overstimulation following injury (Cummings, Martin et al. 1999; Adams, Hauser et al. 2001; Arraes, Freitas et al. 2006). This observation seems to contradict the beneficial effect of systemic administration of CXCR2 inhibitors (Ness, Hogaboam et al. 2003; Lomas-Neira, Chung et al. 2004; Gordon, Li et al. 2005; Nomellini, Faunce et al. 2008). The interpretation of these studies to determine a precise mechanism for the beneficial effects of anti-CXCR2 treatment in aged mice after burn injury is still not completely understood. In the current study, we hypothesize that the prolonged pulmonary inflammation in aged mice following burn is a result of continued neutrophil sequestration in the vasculature caused by persistent downregulation of CXCR2.

Materials and Methods

Animals

Young (3-6 months) and aged (18-22 months) BALB/c female mice were obtained from the National Institute of Aging colony at Harlan Laboratories (Indianapolis, IN) and maintained on a 12 hour light/dark cycle with standard laboratory

rodent chow and water ad libitum. All experimental procedures were performed according to the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals, National Institutes of Health, and approved by the Animal Care and Use Committee at Loyola University Medical Center.

Induction of Burn Injury

Mice were anesthetized with Nembutal (50 mg/kg i.p.), shaved, and placed into a plastic template designed to give a 15% TBSA, full-thickness dorsal scald injury when immersed in a boiling water bath for 8 seconds, according to a modified protocol of Walker and Mason (Walker and Mason 1968; Faunce, Gregory et al. 1997). As a control, a separate group of mice received a sham injury, which entailed administration of anesthesia and shaving, but a room temperature water bath was used instead. Immediately following injury, the mice received warm saline resuscitation (1 ml per 20 g body weight) and their cages were placed on heating pads to prevent circulatory collapse and cardiovascular shock. After recovering from anesthesia, this procedure leaves young, healthy mice able to eat, drink, groom, and ambulate to their pre-injury capacity. Aged mice, on the other hand, take longer to recover from anesthesia, show labored breathing, and do not ambulate as well as before the injury. The mice were sacrificed using CO_2 inhalation and cervical dislocation. No other therapeutic intervention was provided, as administration of anti-inflammatory or analgesic medication may introduce confounding factors into the assessment of inflammatory responses. To eliminate the complication of hormones regulated by circadian rhythms, all burn injury procedures were administered

between 8 and 10 am. In addition, all mice—including those which died before the time of sacrifice—were examined for visible tumors and, if found, were removed from the study.

Immunofluorescence

The lungs were removed at the time of sacrifice, inflated with 25% O.C.T. freezing medium, and embedded for frozen sectioning. The lung sections were fixed in acetone and blocked with normal goat serum. To determine neutrophil content in lungs, sections were first incubated with 1 μg/mL of rat anti-Gr-1 antibody (Invitrogen) followed by 4 μg/ml of goat anti-rat IgG conjugated to Alexa Fluor 488 (Invitrogen). Since Gr-1 can also be found on certain macrophage populations (Vermaelen and Pauwels 2004; Sugimoto, Katayama et al. 2006), the sections were dual-stained with 0.2 μg/mL of biotinylated anti-MOMA-2 antibody (BMA Biomedicals), a pan-macrophage marker, and detected with 2 μg/ml of Cy3 Streptavidin (Invitrogen). Using fluorescent microscopy, the total number of neutrophils (designated as Gr-1^+ MOMA-2^- cells) were counted across 10 high power fields for each animal (Lam, Caterina et al. 2002). Data are expressed as mean number of neutrophils counted in ten 400x fields ± SEM. The total tissue area across which cells were counted was quantified and determined to be consistent between animals in all treatment groups (data not shown).

To determine the expression of adhesion molecules in the lung after burn or sham injury, sections were incubated with 0.25 μg/ml of Armenian hamster anti-mouse ICAM-

1 (BD Pharmingen, San Diego, CA), followed by 3 μg/ml of goat anti-Armenian hamster IgG conjugated to Cy3 (Jackson Immunoresearch). Since ICAM-1 is also expressed on lung epithelium, sections were dual stained with 0.16 μg/ml of rat anti-mouse PECAM-1 (BD Pharmingen), followed by 4 μg/ml of goat anti-rat IgG conjugated to Alexa Fluor 488 (Invitrogen). Expression of ICAM-1 on lung endothelium was determined by quantifying the total area of ICAM-1 and PECAM-1 colocalization across ten 400x fields per animal. Data are expressed as mean levels of ICAM-1^{+} PECAM-1^{+} area ± SEM.

To determine the expression of CXCR2 on neutrophils in the lungs, sections were first stained with 1 μg/ml rat anti-Gr-1 (Invitrogen) followed by 4 μg/ml of goat anti-rat IgG conjugated to Alexa Fluor 488 (Invitrogen) to detect neutrophils. Then, sections were stained with 1.25 μg/ml of PE-conjugated rat anti-mouse CXCR2. Expression of CXCR2 on pulmonary neutrophils was determined by quantifying the total area of CXCR2 on Gr-1^{+} cells in each field and dividing by the total number of cells. Ten fields were measured for each animal in all treatment groups. Data are expressed as mean level of CXCR2 expression per Gr-1^{+} cell ± SEM.

Flow Cytometry

Analyses utilizing flow cytometry were performed as previously described (Boehmer, Meehan et al. 2005). Cells were washed with Hank's Buffered Saline Solution (HBSS) and blocked with anti-CD16/32 antibody for 30 minutes. Cells were then stained using anti-mouse antibodies of saturating concentrations at 4°C. After

incubating the cells for 30 minutes, they were washed twice and fixed with 1% paraformaldehyde. Fluorescence was measured by flow cytometry (FACSCanto, BD Biosciences). Anti-mouse antibodies were used at the following concentrations: 2 μg/ml of PE-conjugated rat anti-mouse Gr-1 (Invitrogen), 20 μg/ml of APC-conjugated rat anti-mouse F4/80 (eBioscience, San Diego, CA) 10 μg/ml of FITC-conjugated rat-anti mouse Gr-1 (eBioscience), 12.5 μg/ml of PE-conjugated rat anti-mouse CXCR2 (R&D Systems), 10 μg/ml of PE-conjugated rat-anti mouse CD11a (Invitrogen), 10 μg/ml of PE-conjugated rat anti-mouse CD11b (eBioscience).

Bronchoalveolar Lavage

To determine the cell populations in the alveolar space, BAL was performed on young and aged mice 24 hours after receiving either a burn or a sham injury (Czermak, Breckwoldt et al. 1999). Immediately following sacrifice, the tracheas were exposed and a small incision was made just below the cricothyroid cartilage. Tracheas were then cannulated using 22 gauge needles and 1 ml of cold phosphate buffered saline was repeatedly injected until 5 ml of fluid was recovered for each animal. BAL cells were centrifuged for 5 minutes at 300 g, then stained for Gr-1 and F4/80 using flow cytometry.

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Isolation of Peripheral Blood Neutrophils

Blood was taken via cardiac puncture of the left ventricle. Samples were diluted 1:1 in HBSS and layered on Histopaque 1083 (Sigma, St. Louis, MO), and centrifuged at 400 g for 30 minutes at 20°C without the brake applied. The monocyte and plasma layers

were aspirated, leaving granulocytes and erythrocytes. Samples were resuspended in HBSS and 3% dextran was added to sediment the erythrocytes. After 45 minutes at room temperature, the top layer containing granulocytes was removed and centrifuged at 300 g for 5 minutes. Any remaining erythrocytes were lysed using ACK buffer (Invitrogen).

Chemotaxis Assay

Neutrophils were isolated as described above. Cells were centrifuged at 300 g for 5 minutes and resuspended in 40 μM of Cell Tracker Green (Invitrogen) in media containing HBSS, antibiotics, 25 mM HEPES and 1% bovine serum albumin at 10^6 cells/ml. Cells were incubated in the dark for 45 minutes at 37°C and 5% CO_2. Cells were then washed and resuspended in chemotaxis media at 10^6 cells/ml. The bottom wells of a chemotaxis chamber (NeuroProbe, Gaithersburg, MD) were filled with various doses of recombinant mouse KC (R&D Systems). A separate set of wells were filled with media alone as a negative control or 10^{-7} M fMLP (Sigma) as a positive control. Another set of wells were filled with sample inputs to determine the fluorescence of the starting cell suspension. The filter membrane was then placed over the wells and cell suspensions were added to the upper side of the membrane at 10^6/ml. Samples were incubated for 60 minutes at 37°C and 5% CO_2. Cell suspensions were then aspirated off the top membrane and 20 μM EDTA was added to the upper side of the membrane for 15 minutes to allow any cells adhering to the membrane to detach. The membrane was then removed and the fluorescence of the bottom wells was measured in a fluorescence

spectrophotometer. The percent of cells migrating was determined by comparing the fluorescence of the cells in the sample wells to that of the input wells.

Statistical Analysis

Data were analyzed using GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA) and are expressed as mean ± SEM. For comparisons of two groups, an unpaired student's t-test was used. Groups were considered significantly different at p values less than 0.05.

Results

Localization of Neutrophils in the Lungs of Aged Mice after Burn

As a number of reports indicate, neutrophil accumulation in the lungs of mice after a systemic injury occurs within alveolar walls and capillaries rather than the airways (Johnson, Brigham et al. 1991; Standiford, Kunkel et al. 1995; Gupta, Feng et al. 1996; van Eeden, Kitagawa et al. 1997; Carraway, Welty-Wolf et al. 1998; O'Malley, Matesic et al. 1998; Czermak, Friedl et al. 1999; Murphy, Paterson et al. 2005; Rojas, Woods et al. 2005; Gomez, Hirano et al. 2007). To determine the precise location of neutrophils in the prolonged response in the lungs of aged mice at 24 hours after burn, a number of methods were employed. First, lung sections were immunostained with anti-Gr-1 antibody (Sugimoto, Katayama et al. 2006). Since Gr-1 is also present on certain macrophage populations, lungs were simultaneously stained with anti-MOMA-2—a pan-macrophage marker, shown to adequately detect alveolar macrophages (Vermaelen and

Pauwels 2004; Sugimoto, Katayama et al. 2006). Thus, Gr-1 positive cells that were negative for MOMA-2 were considered neutrophils. Quantification of the number of neutrophils counted in a total of ten fields per animal in each group is shown in **Table 3**. At this time point, neutrophils in the lungs of young mice did not differ from shams. However, neutrophils were 4 times higher than sham controls in the lungs of aged mice ($p<0.05$).

To confirm these studies, flow cytometry on intact cells harvested from whole lung homogenates was performed. However, instead of using anti-MOMA-2 antibody for alveolar macrophages, anti-F4/80 antibody was used, since it also has the ability to detect circulating monocytes (Austyn and Gordon 1981; Hirsch, Austyn et al. 1981; Vermaelen and Pauwels 2004). As shown in **Table 3**, while neutrophils (Gr-1^{+} F4/80^{-} cells) in the lungs of young mice were similar to sham injured animals, those from aged mice were 6 times greater than sham controls ($p<0.05$).

While immunohistochemistry and flow cytometry confirmed that there is increased neutrophil accumulation in the lungs of aged mice 24 hours after burn injury, these methods do not allow for precise localization of the neutrophils. Therefore, lungs were lavaged to determine if the neutrophils migrated into the alveolar space (**Table 3**). As expected, BAL cells of both young and aged mice sham-injured mice were predominantly macrophages (F4/80^{+} Gr-1^{-} cells, not shown). Neutrophils (F4/80^{-} Gr-1^{+} cells) only comprised 4% in the young and 7% in the aged of cells recovered from BAL.

Table 3. Neutrophil localization in lungs at 24 hours after burn

Method	Young		Aged	
	Sham	Burn	Sham	Burn
Immunofluorescence [a] using lung sections (total # in 10 fields)	16.0 ± 2.4	16.1 ± 2.4	12.8 ± 2.5	63.29 ± 11.5 *
Flow cytometry of [b] lung homogenates (% $Gr\text{-}1^{+}$ $F4/80^{-}$ cells)	2.0 ± 0.4	1.8 ± 0.3	2.1 ± 0.2	11.8 ± 3.0 *
Flow cytometry of [c] BAL cells (% $Gr\text{-}1^{+}$ $F4/80^{-}$ cells)	4.1 ± 3.5	0.4 ± 0.1	7.0 ± 2.7	0.5 ± 0.2 *

[a] Total numbers of $Gr\text{-}1^{+}$ $MOMA\text{-}2^{-}$ cells in lungs of young and aged animals at 24 hours after sham or burn injury were counted in sections of lung tissue. Data are represented as the average number of cells counted in ten 400x fields for each group ± SEM. N = 8-14 mice per group. *, $p<0.05$ compared to all other groups.

[b] All five lung lobes were homogenized in HBSS as described above. Cells were stained for Gr-1 and F4/80 as described above and analyzed by flow cytometry. Data are represented as the average percent of $Gr\text{-}1^{+}$ $F4/80^{-}$ cells in the homogenate (after excluding cell debris) ± SEM. N = 6-8 mice per group. *, $p<0.05$ compared to all other groups.

[c] Lungs were lavaged with 1 ml of saline until 5 ml of sample was collected. Cells were spun down and stained with Gr-1 and F4/80 as described above and analyzed by flow cytometry. Data are represented as the average percent of $Gr\text{-}1^{+}$ $F4/80^{-}$ cells in BAL (after excluding cell debris) ± SEM.

At 24 hours after burn, the proportion of macrophages did not change. Interestingly, the proportion of neutrophils in the BAL actually decreased in both young and aged mice, but was only significant in the aged mice receiving injury ($p<0.05$). Altogether, these

data indicate that, while neutrophils continue to accumulate in the lungs of aged mice 24 hours after injury, these cells do not have the capacity to migrate into the alveoli.

The Role of Adhesion Molecules after Burn Injury

Many have shown that adhesion via the interaction of ICAM-1 on the endothelium and its ligand, CD11/CD18, on neutrophils is the main mechanism of neutrophil recruitment in the lungs after injury (Doerschuk 1992; Lo, Everitt et al. 1992; Mulligan, Till et al. 1994; Jin, Zhu et al. 2003; Reutershan and Ley 2004). Expression of these adhesion molecules were therefore measured to test whether an alteration in either or both of them could explain the age-related differences in the lungs after burn. First, lung sections from young and aged mice at 6 and 24 hours following burn or sham injury were immunostained for ICAM-1. Since ICAM-1 is also expressed by pulmonary epithelial cells, sections were also stained for PECAM-1, a constitutively expressed endothelial marker (Eppihimer, Russell et al. 1998). Coexpression indicated ICAM-1 levels on the endothelium. Results in **Figure 12** show that ICAM-1 expression on the pulmonary vasculature did not differ in young and aged mice at 6 hours after injury. In contrast, ICAM-1 expression on the pulmonary endothelium of aged mice at 24 hours after burn was increased greater than 5 fold compared to sham controls ($p<0.05$), while no differences were detected in the lungs of young, burn injured mice.

Although ICAM-1 was found to show age-related differences in expression after burn, the presence of its ligand on neutrophils is required in order for the interaction with

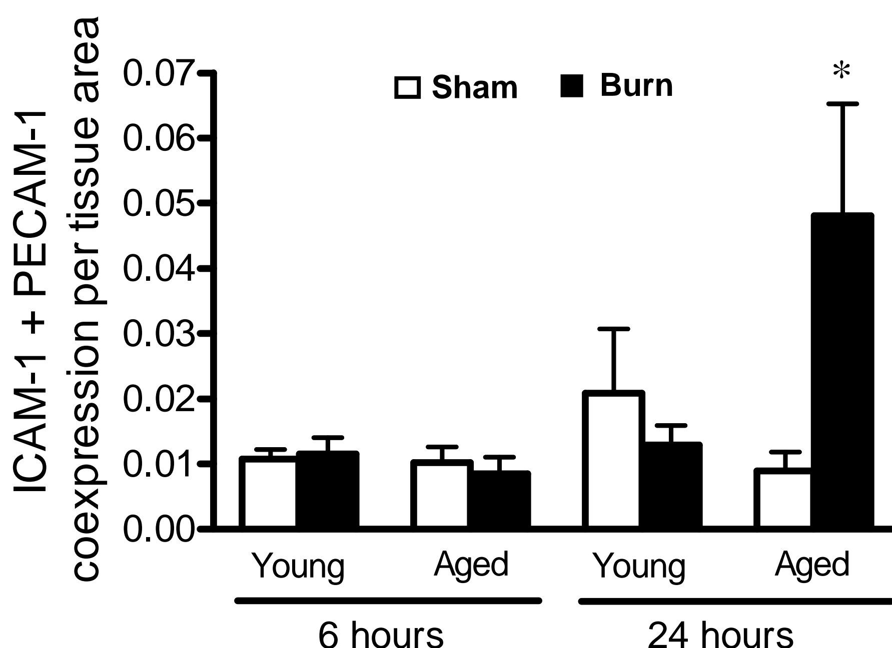
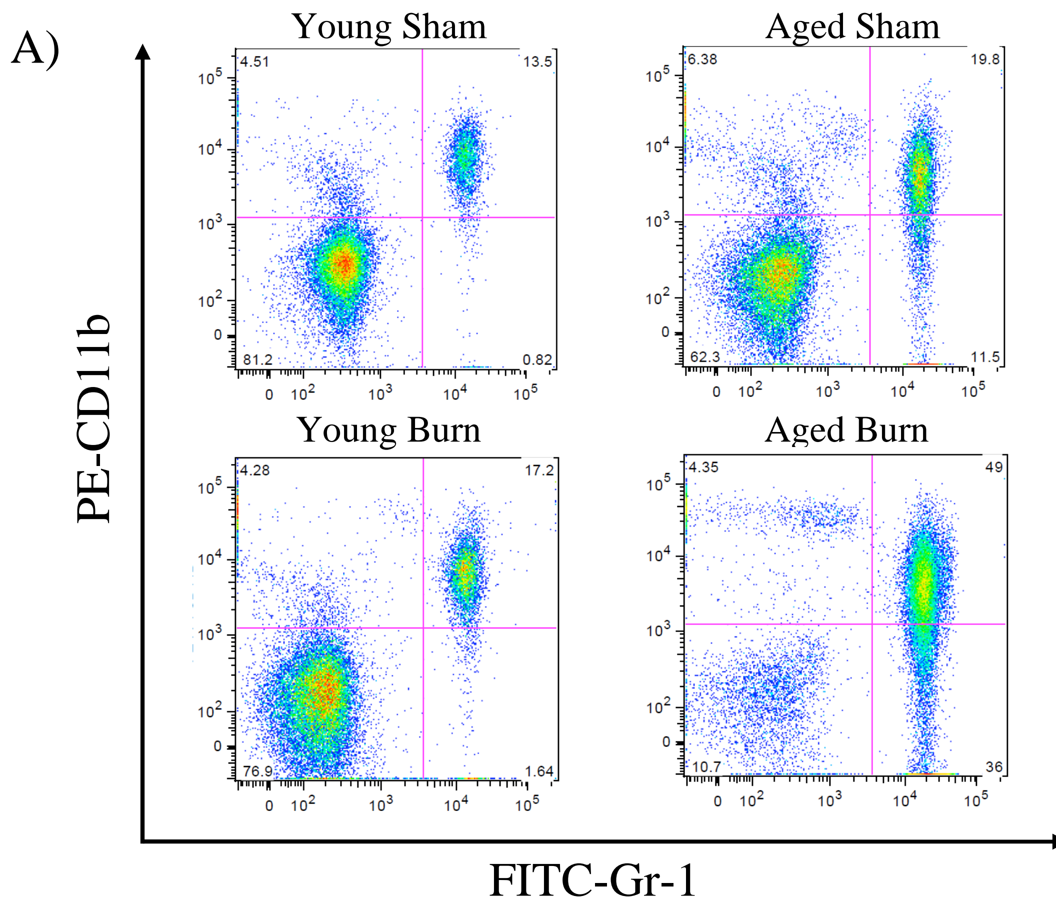


Figure 12. Pulmonary endothelial ICAM-1 expression after burn. Sections of lungs from young and aged mice at 6 and 24 hours after sham (white bars) or burn (black bars) injury were stained with anti-ICAM-1 and anti-PECAM-1 antibodies. Since ICAM-1 is also expressed on alveolar epithelial cells, expression of endothelial ICAM-1 was determined by measuring the total area in which the two markers co-localized (μm^2), normalized to the total tissue area (μm^2). Data are represented as mean ± SEM. N = 4-11 animals per group. *, $p<0.05$ compared to sham control.

the endothelium to occur (Elangbam, Qualls et al. 1997). To determine the surface expression of the ICAM-1 ligand on neutrophils after burn, peripheral blood was stained and analyzed for expression of CD11b by flow cytometry (**Figure 13**). Interestingly, there was a decreased surface expression of CD11b on neutrophils from sham-injured aged compared to sham-injured young mice ($p<0.05$). After burn injury, neutrophils from the young had elevated CD11b expression, although this did not reach statistical

A)



B)

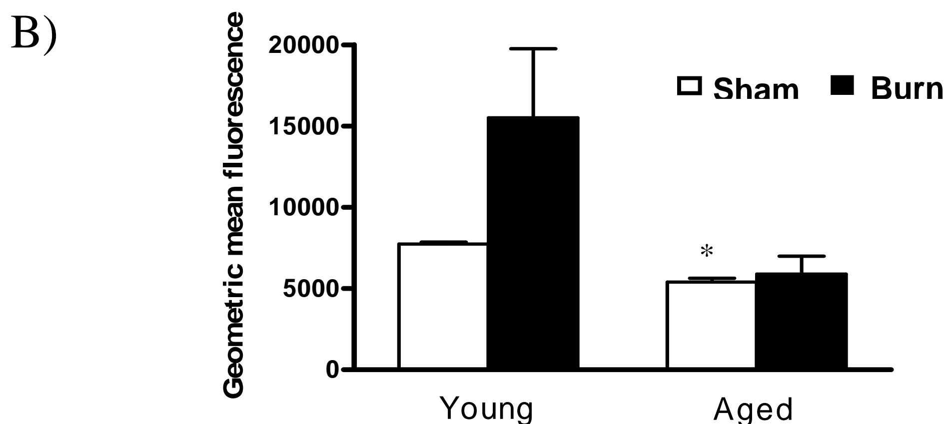


Figure 13. Peripheral blood neutrophil CD11b expression following burn. CD11b expression on peripheral blood cells of young and aged mice 24 hours after sham or burn injury was determined by flow cytometry. A) Representative graphs from each group. B) Quantification of the geometric mean fluorescence of CD11b expression per cell in sham (white bars) versus burn (black bars) injury. Data are represented as mean ± SEM. N = 4-6 mice per group. *, $p<0.05$ compared to young sham. To note, $p = 0.07$ when comparing young and aged burn injured groups.

significance. CD11b levels on neutrophils from aged mice at 24 hours after burn, on the other hand, were not different from sham controls.

CXCR2-mediated Neutrophil Chemotaxis

While these data suggest that increased vascular adhesion may be responsible for neutrophil sequestration in the lungs after burn, the reasons why they are not capable of transmigrating out of the vasculature is still unclear. It was hypothesized that chemotaxis was defective in neutrophils from aged mice, rendering them incapable of transmigration. To test this, cells were isolated from the blood and assayed for their ability to migrate towards KC. This particular chemokine was chosen because our previous results indicated a role for CXCR2 in mediating pulmonary inflammation in aged mice after burn (Nomellini, Faunce et al. 2008). Given that our previous data showed that there were age-related differences in levels of only one neutrophil chemokine in lung homogenates after burn (KC), we performed chemotaxis assays using this chemokine and purified peripheral blood neutrophils from young and aged mice. The results in **Figure 14A** showed that, in the absence of any stimulant, migration of neutrophils from aged mice was significantly higher compared to young mice ($p<0.05$). In the presence of physiologic doses of KC, neutrophils from young mice had a robust response compared to unstimulated controls ($p<0.05$). However, no further increases in migration were found in neutrophils from aged mice in response to the same doses of KC when expressed as percent of cells migrating (fluorescence of cells migrated/fluorescence of input wells x 100). Since baseline migration was different, the results were re-expressed

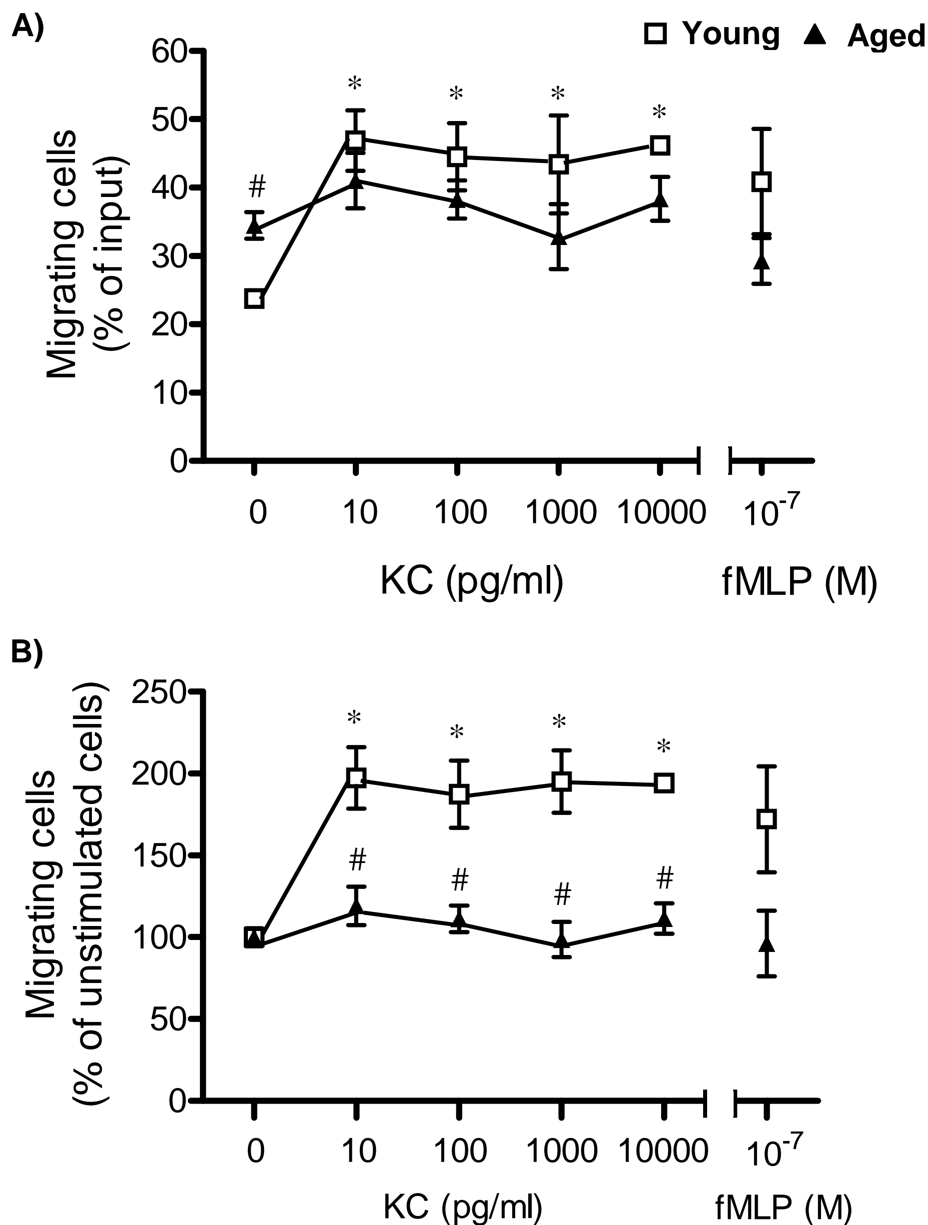


Figure 14. Defects in KC-mediated chemotaxis of neutrophils from aged mice. Peripheral blood neutrophils isolated from young (white squares) and aged (black triangles) mice were tagged with a fluorescent dye and incubated with varying concentrations of KC in a Boyden-like chemotaxis chamber for 1 hour at 37°C. fMLP was used as a positive control. Migrating cells are expressed as A) % of input (fluorescence of migrated cells/fluorescence of input x 100) or B) % of unstimulated cells (% input / % input of unstimulated cells x 100). Data are represented as mean ± SEM. N = 3-6 mice per group. *, $p<0.05$ compared to young unstimulated control; #, $p<0.05$ compared to young at the same chemokine dose.

in terms of percent baseline (percent of cells migrating/percent of cells migrating at baseline x 100), as shown in **Figure 14B**. This allowed the age differences to become more apparent. While neutrophils from young mice showed a robust response at all KC doses tested ($p<0.05$), those from aged mice showed no reaction to the same doses. Similar results were found in response to the positive control, fMLP, but this was not found to be statistically significant. In summary, these data indicate that neutrophils from aged mice are in an elevated activation state, causing increased migratory behavior, but that the specific response to KC is blunted.

CXCR2 Expression

As described above, chemokine receptor desensitization is commonly seen after a systemic insult and is thought to be responsible for many of the resulting defects seen in neutrophil function. Given that neutrophils from the aged were not able to respond to KC, it was hypothesized that the levels of CXCR2 may be altered. To determine cell surface receptor expression, blood was collected from young and aged mice after sham or burn injury and red cells were lysed immediately. The remaining leukocytes were stained with anti-CXCR2, as well as anti-Gr-1 and anti-F4/80, to determine expression on neutrophils by flow cytometry. Baseline levels of CXCR2 expression on neutrophils were not found to be different between the age groups (**Figure 15A**). At 24 hours after burn, there was an increase in the percentage of neutrophils ($p<0.05$), but no change in the relative amount of monocytes in the peripheral blood of aged mice compared to that of young mice (**Figure 15A**). The majority of these circulating neutrophils in the aged

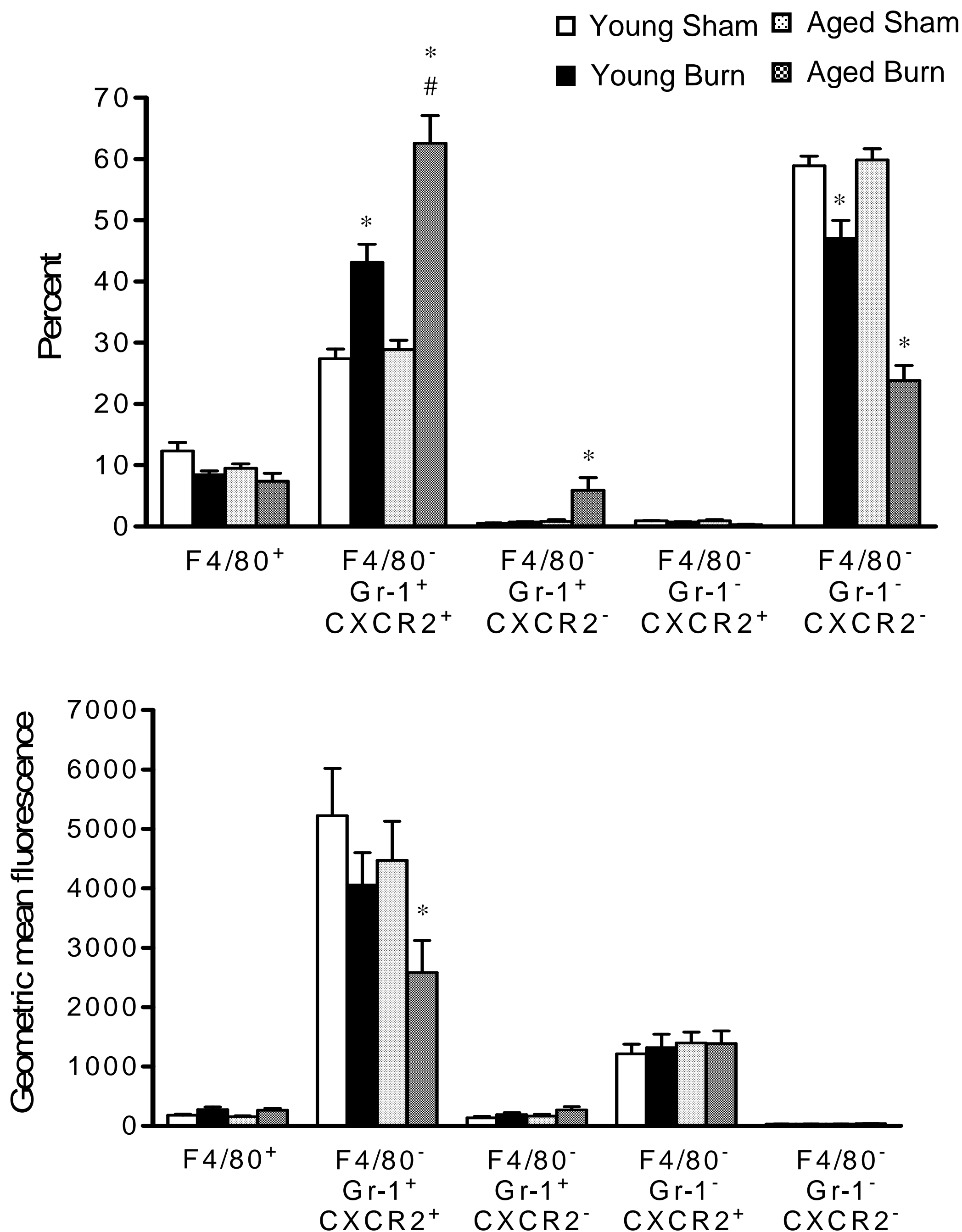


Figure 15. CXCR2 expression on peripheral blood neutrophils following burn. Peripheral blood from young and aged mice at 24 hours after sham or burn injury was stained for F4/80, Gr-1, and CXCR2 and analyzed by flow cytometry. Results are expressed as A) percent of each cell type and B) CXCR2 expression per cell. Data are represented as mean ± SEM. N = 12-15 mice per group. *, $p<0.05$ compared to sham control; #, $p<0.05$ compared to young burn.

were CXCR2$^+$. Interestingly, a small but significant population of neutrophils completely lacking CXCR2 was found only in the aged, burn injured group (**Figure 15A**, $p<0.05$). Moreover, while the amount of CXCR2$^+$ neutrophils was elevated in the blood after burn, the total expression per cell was significantly diminished only in the aged mice (**Figure 15B**, $p<0.05$). While is has been reported that monocytes express CXCR2 (Browning, Diehl et al. 2000; Smith, Galkina et al. 2005), we were unable to detect significant levels on F4/80$^+$ cells. It is thought that other cell types express CXCR2 as well, such as endothelial and epithelial cells (Schraufstatter, Chung et al. 2001; Hillyer, Mordelet et al. 2003). Although F4/80$^-$ Gr-1$^-$ cells comprised less than 1% of the population of cells analyzed, CXCR2 expression was detected. However, levels of CXCR2 in this population did not change with burn injury.

While determining CXCR2 expression on neutrophils in the peripheral blood may be a useful tool to predict pulmonary outcomes, this does not necessarily depict what is happening in the lungs. To determine whether similar receptor changes occur in the lungs of aged mice at 24 hours after burn, sections were immunostained using anti-CXCR2 and anti-Gr-1 antibodies. Although Gr-1 can also be detected on certain monocyte populations, we have shown that these double positive cells comprise less than 5% of the leukocytes in the lungs and do not express CXCR2. As a result, all Gr-1$^+$ CXCR2$^+$ cells were considered to be neutrophils. Surprisingly, CXCR2 expression on neutrophils from aged mice was significantly lower than that of neutrophils from young

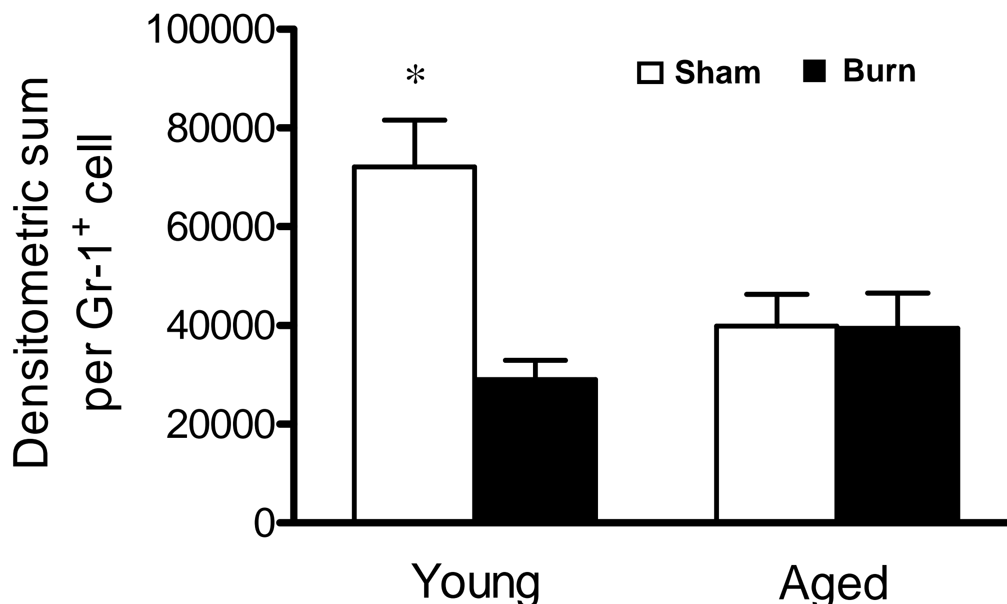


Figure 16. CXCR2 expression on neutrophils in the lungs after burn. Lung sections of young and aged mice at 24 hours after sham or burn injury were stained with anti-Gr-1 and anti-CXCR2 antibodies. The average CXCR2 expression was determined for each cell across ten 40x fields per animal by dividing the densitometric sum of one field by the number of Gr-1 positive cells in that field. Data are represented as mean ± SEM. N = 12-19 mice per group. *, $p<0.05$ compared to all other groups.

mice in the absence of injury (**Figure 16**). At 24 hours after burn, CXCR2 was also downregulated in pulmonary neutrophils from young mice ($p<0.05$), but did not decrease any further in those of aged mice (**Figure 16**). These data indicate that there is tissue-specific regulation of this receptor.

Discussion

In sum, the data presented in this study implicate decreased CXCR2 expression as a mediator of prolonged neutrophil sequestration in the lungs of aged mice after burn injury. We propose that this delayed response is a result of an inherent hyperresponsiveness of neutrophils from aged mice. Consistent with the idea of inflamm-aging (Franceschi, Bonafe et al. 2000), neutrophils seem to have an elevated basal level of activation, measured by increased migratory capacity and decreased CXCR2 expression in the lungs. Upon a challenge such as burn injury, the systemic inflammatory environment of aged mice persists longer that of young animals, as seen by increased ICAM-1 and KC levels in the lungs at 24 hours (Nomellini, Faunce et al. 2008), rendering neutrophils incapable of further activation. Although this concept seems contradictory to many reports showing defective neutrophil function in the aged, most studies tend to focus on the proinflammatory cytokines, fMLP, lipopolysaccharide (LPS), and C5a pathways, which are mainly present only during an infectious or inflammatory challenge (Rao 1986; Biasi, Carletto et al. 1996; Schroder and Rink 2003; Fulop, Larbi et al. 2004; Fortin, Lesur et al. 2007). The CXCR2 pathway, on the other hand, is a constitutively active pathway, required to maintain neutrophil homeostasis and immune surveillance of tissues (Martin, Burdon et al. 2003).

A few studies are consistent with our observations, indicating that advanced age is associated with an elevated activation state under resting conditions, as measured by increased intracellular Ca^{2+} and increased GRK activation (Wenisch, Patruta et al. 2000;

Schutzer, Reed et al. 2001; Fulop, Larbi et al. 2004). Similar to the current study, this increased activity makes the cells incapable of generating a peak response once a secondary stimulus is applied (Wenisch, Patruta et al. 2000; Fulop, Larbi et al. 2004). This dysfunction may explain the increased susceptibility to infection in elderly individuals sustaining an injury (Linn 1980; Nagy, Smith et al. 2000; Bergeron, Lavoie et al. 2003).

Despite reports that adhesion molecules are upregulated in the aged (Miles, Thies et al. 2001; Forsey, Thompson et al. 2003; Kletsas, Pratsinis et al. 2004; Chung, Sung et al. 2006; Zou, Yoon et al. 2006), we did not find pulmonary ICAM-1 to be increased in aged, sham-injured mice in the current study. Only in response to burn injury, were age-related differences found in ICAM-1 expression in the lungs. Alternatively, CD11b was only elevated in the young mice after burn. Contrary to what was expected, CD11b expression on neutrophils from aged mice was not different from sham controls. According to most reports, chemokine signaling increases the affinity of CD11b for ICAM-1 by receptor clustering (Arnaout 1990; Ley 2002; Kim, Carman et al. 2004). Therefore, it is possible that flow cytometry is not the most appropriate method to measure the effects of age on CD11b function. As CD11b is important in pulmonary neutrophil accumulation after burn, other means of analysis are required to fully understand the mechanism of chemokine signaling in neutrophils from aged mice (Mulligan, Till et al. 1994; Jin, Zhu et al. 2003).

As stated above, the precise role for CXCR2 in mediating neutrophil adhesion and transmigration is still not completely understood. The current study suggests that neutrophil accumulation in the lungs occurs only when vascular adhesion molecules are elevated and CXCR2 is downmodulated. In young mice, levels of CXCR2 on peripheral blood neutrophils were similar between injury groups. Baseline levels of CXCR2 on peripheral blood neutrophils from aged mice were not different than those from young mice. At 24 hours after burn, circulating neutrophils from the aged had attenuated CXCR2 levels and a significant number did not have any detectable receptor expression. Thus, expression levels of CXCR2 on circulating neutrophils may help predict pulmonary outcomes after injury, as previously suggested (Adams, Hauser et al. 2001; Tarlowe, Duffy et al. 2005).

As seen in the lung sections from young mice, on the other hand, burn injury induced a significant attenuation of receptor expression on neutrophils. These data suggest that, since pulmonary adhesion molecules were not elevated at this time point after burn, neutrophils were not able to accumulate within the vasculature. Conversely, CXCR2 was found to be significantly reduced on neutrophils in the lungs of aged mice after sham injury. These data may be explained by the increased inflammatory state seen in the lungs of "healthy" aged individuals (Meyer, Rosenthal et al. 1998; Nomellini, Faunce et al. 2008). Again, since adhesion molecules were not present in the lungs of uninjured aged mice, this decreased CXCR2 expression did not translate into pulmonary neutrophil accumulation. Consistent with our hypothesis, reduced CXCR2 on neutrophils

from aged mice at 24 hours after burn resulted in neutrophil accumulation, as elevated pulmonary ICAM-1 was also present. As a limitation to this study, differential methods were used to detect surface expression of the receptor on peripheral blood neutrophils versus on neutrophils in tissue sections. In addition, it is not completely evident whether the decreased CXCR2 expression seen in the lungs was on neutrophils within the vasculature or on those which were able to migrate into the alveoli. Regardless, only a small number of neutrophils comprise the alveolar space and are not likely to affect the results of this study.

Further support for the importance of CXCR2 availability for transmigration can be seen in the BAL analysis. While neutrophils comprise an extremely small proportion of BAL cells under resting conditions, there was still a significant reduction following burn injury in the aged. These data indicate that even the homeostatic regulation of neutrophil content in the lungs is compromised when CXCR2 is not available.

The reasons for prolonged CXCR2 downmodulation in our model of injury have yet to be completely delineated. It is not clear whether this decrease in CXCR2 is a result of prolonged desensitization or simply because of the continued exposure to high levels of chemokines in the lungs of aged mice at 24 hours after burn. There is some evidence that GRK activity is increased in the aged (Schutzer, Reed et al. 2001). While this may explain increased receptor downregulation, the role of GRK in neutrophils in response to

burn is unknown. Again, GRK is activated by PKC, which is also elevated in the aged. Therefore, GRK inhibitors may be another potential target after burn.

One question that arises from this study is that, if CXCR2 availability correlates with increased vascular adhesion following a systemic insult, how do blocking studies alleviate this response? While only speculative, we believe that this relates back to the concept that a sequential order of events is required for the appropriate response to occur. Following a systemic challenge, circulating proinflammatory mediators can activate neutrophils in the blood (Drost, Larsen et al. 1993; Bone 1996; Botha, Moore et al. 1996; Yeh, Lin et al. 1997; Rojas, Woods et al. 2005). As chemokine signaling pathways are likely to be simulated before neutrophils reach the lungs, CD11b may continue to be active, allowing firm adhesion to take place. However, since receptor levels are low, these neutrophils cannot sense the local chemokines produced by the lung and, therefore, cannot be guided through the endothelium (Le, Li et al. 2000; Luu, Rainger et al. 2000). This mechanism is supported by *in vitro* observations utilizing endothelial monolayers and neutrophils (Luu, Rainger et al. 2000). When neutrophils from healthy humans are allowed to flow over an activated endothelial monolayer, they rapidly adhere and transmigrate. Notably, migration through the endothelium occurred only when chemokines were also present in the media; activated endothelium alone did not cause firm adhesion. When neutrophils are preincubated with fMLP, IL-8, C5a, or GROα, the cells adhere but they do not transmigrate, similar to what is observed in a systemic insult. In the case of chemokine receptor neutralizing experiments, anti-CXCR2 antibody was

administered i.p. at 30 minutes after burn or sham injury (Nomellini, Faunce et al. 2008). Here, it is thought that chemokine signaling is blunted immediately upon neutrophil release into circulation, inhibiting firm adhesion altogether. Other laboratories have also shown a beneficial effect of blocking CXCR2 following a systemic response (Lomas-Neira, Chung et al. 2004; Gordon, Li et al. 2005). Further experiments analyzing the affects of neutralizing CXCR2 in the early response to burn are required to better define this mechanism.

This study also raises the question of why only certain intracellular pathways are enhanced in neutrophils from the aged. A common signaling molecule reported to be involved in the hyperresponsiveness of neutrophils from aged mice in this study is PKC. Activated PKC has been shown to mediate chemokine receptor cross-desensitization through the activation of GRKs (Richardson, Ali et al. 1995; Pitcher, Freedman et al. 1998; Ali, Richardson et al. 1999; Le, Li et al. 2000; Ferguson 2001; Heit, Tavener et al. 2002; Arraes, Freitas et al. 2006), CD11/CD18 phosphorylation to increase receptor clustering (Chatila, Geha et al. 1989; Kim, Carman et al. 2004), and increased NADPH oxidase-mediated ROS production (Martins Chaves, Prates Rodrigues et al. 2002; Olson and Ley 2002). Interestingly, an increase in PKC activity and a decrease in the G-proteins involved in chemokine signaling ($G_{\alpha i}$) has been found in aged humans (Busquets, Ventayol et al. 1996). Therefore, increased basal levels of chemokine signaling, leading to elevated PKC activity may play a causal role in mediating the results of the current study. Future studies are required to characterize PKC activity in neutrophils from the aged.

Since a number of differences between humans and rodents exist, the observations we have made in this study may not necessarily translate to the human condition. Most importantly, human neutrophils also express CXCR1, which binds almost exclusively to IL-8—a chemokine that does not have a direct murine ortholog (Baggiolini 1998; Richardson, Pridgen et al. 1998; Murphy, Baggiolini et al. 2000; Adams, Hauser et al. 2001; Nasser, Raghuwanshi et al. 2007). While CXCR1 signals through the same pathway as CXCR2, the kinetics of cell surface expression are very different. Both CXCR1 and CXCR2 are rapidly downregulated after chemokine binding (Chuntharapai and Kim 1995). While CXCR2 stays internalized for a significant amount of time, CXCR1 expression is quickly restored on the surface and is available for further signaling (Cummings, Martin et al. 1999).

Clinical studies have indicated that post-traumatic outcomes depend mostly on the relative levels of CXCR2 expression rather than CXCR1 (Adams, Hauser et al. 2001; Tarlowe, Duffy et al. 2005). Decreased CXCR2 responses correlated with increased susceptibility to pneumonia (Adams, Hauser et al. 2001; Tarlowe, Duffy et al. 2005). Fitting this observation with the current study, if CXCR2 is not available, neutrophils cannot migrate into the alveoli and protect against offending organisms. On the other hand, when neutrophils from trauma patients expressed increased levels of CXCR2, the development of ARDS was more likely (Adams, Hauser et al. 2001). Presumably, if CXCR2 activation is enhanced, migration into the alveoli will be increased, leading to the

airway congestion seen in ARDS. In summary, despite the presence of CXCR1, these results can still be explained by the mechanism proposed in the current study.

Overall, these data indicate that an increased basal activity of neutrophils from the aged in the absence of injury acts to "prime" the cells for a protracted response following burn. In particular, it seems as though defects in CXCR2 pathways are a main mediator in this process, causing increased vascular adhesion and congestion of the pulmonary vasculature. Therefore, CXCR2 may be an effective target of therapy.

CHAPTER 5

SUMMARY AND DISCUSSION

In our murine model of a 15% TBSA scald injury, pulmonary sequelae parallel that which is seen clinically (Teixidor, Novick et al. 1983; Dancey, Hayes et al. 1999; George, Gupta et al. 2003). In young animals, neutrophil infiltration into the lungs begins around 4 hours, but recedes by 24 hours (Stengle, Meyers et al. 1996). When the same type of injury is administered to aged mice, neutrophils begin to enter the lungs at a similar time as in young mice, but are still present at 24 hours (Nomellini, Faunce et al. 2008). While the data are still correlative, there is strong evidence that neutrophils persist in the lungs of aged mice after burn because of increased adhesion and congestion within the pulmonary vasculature.

The Established Model of Neutrophil Infiltration into Inflamed Tissues

Normally, neutrophils entering the circulation of an inflamed tissue undergo margination in the post-capillary venules by the tethering of selectins (**Figure 17**) (Muller 2003). Neutrophils then undergo loose adhesion with ICAM-1 (Luu, Rainger et al. 2000). This interaction is stabilized via signaling through CXCR2 to cause CD11b clustering and firm adhesion (Muller 2003; Kim, Carman et al. 2004). The continued

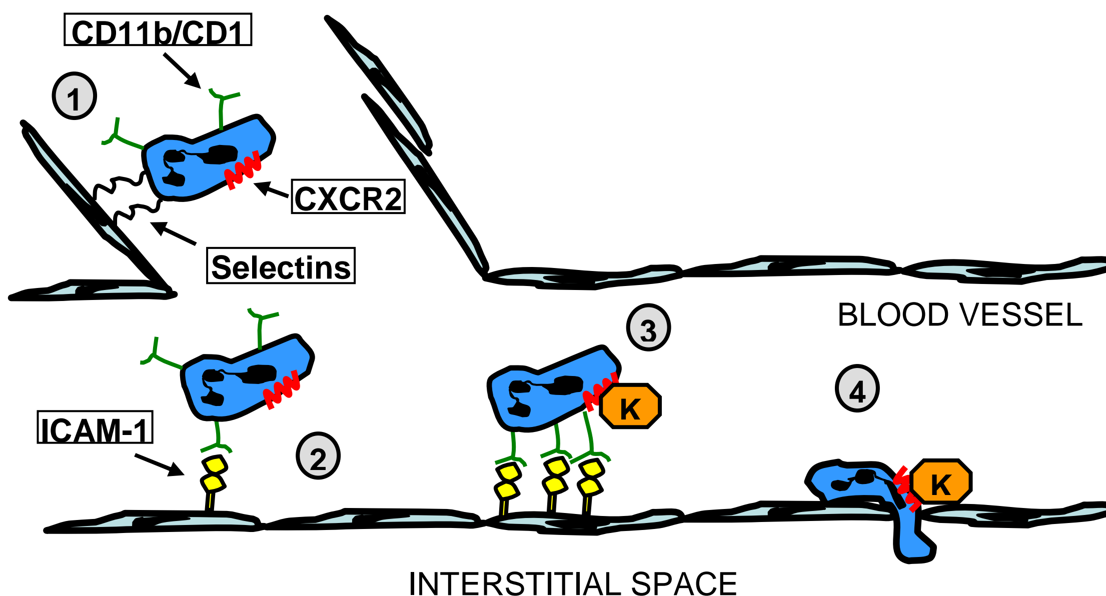


Figure 17. Established model of neutrophil infiltration into the interstitium of inflamed tissues. According to reports published by other laboratories, this mechanism requires a series of events occurring in sequential order. In most tissues of the body, this takes place in post-capillary venules. Neutrophils passing through inflamed tissue are initially slowed by the tethering of selectins **(1)**. The initial interaction between ICAM-1 and CD11b is then established for loose adhesion **(2)**. Signaling through CXCR2 mediates CD11/CD18 clustering and firm adhesion to ICAM-1 **(3)**. Further stimulation through CXCR2 mediates neutrophil migration over the endothelium and, upon reaching an intercellular junction between endothelial cells, diapedesis into the interstitial space **(4)**.

availability of CXCR2 to guide the cell towards endothelial junctions is crucial for the final step of transmigration into the interstitial space (Luu, Rainger et al. 2000).

<u>The proposed model for prolonged inflammation in the lungs of aged mice after burn</u>

As neither CXCR2 nor CD11b was measured at 6 hours after burn on neutrophils from mice of either age, the exact mechanism of neutrophil accumulation at this time

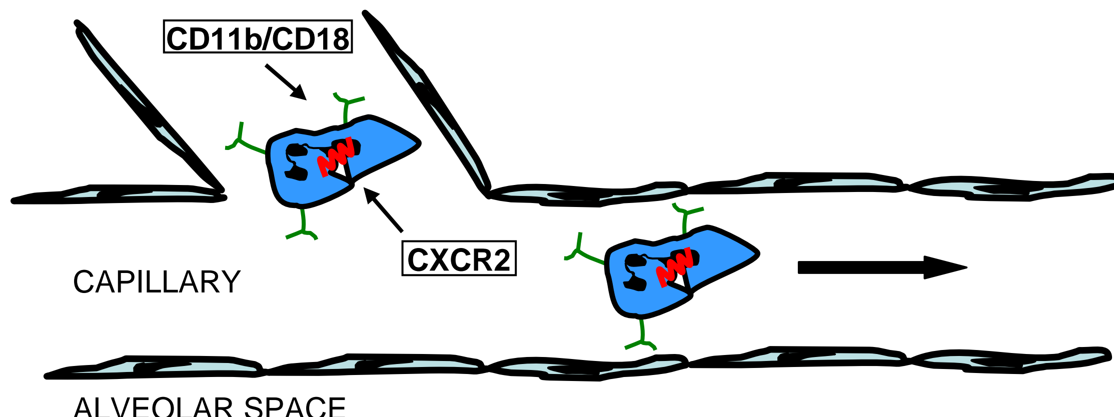
point is still unclear. However, since ICAM-1 was not upregulated, it is thought that the cells are sequestered in the lungs at this early phase via ICAM-1 and CD11b independent pathways, as previously suggested (Doerschuk 1992; Hellewell, Young et al. 1994)

By 24 hours, it is thought that neutrophil transmigration requires ICAM-1 and CD11b dependent pathways (Doerschuk 1992). Since ICAM-1 was still not increased in the lungs of young mice at this later time point, we propose that the neutrophils were not able to undergo loose adhesion and thus continued on in circulation (**Figure 18A**). The lungs of aged mice at 24 hours after burn, on the other hand, exhibited increased ICAM-1 on endothelium and KC in tissue homogenates (**Figures 12** and **9**, respectively). While these conditions normally lead to neutrophil transmigration, CXCR2 surface expression was observed to be decreased in the lung of aged mice ate this time point. We therefore propose that prolonged KC expression in the lungs of aged mice after burn causes CXCR2 to be saturated. As such, neutrophils may undergo adhesion, but the lack of ability to further respond to KC and MIP-2 in the tissue correlates with decreased migration through the endothelium and increased sequestration within the vasculature.

Passive Neutrophil Accumulation in the Lungs after Burn

Since many pathways of neutrophil activation stimulate actin remodeling (Piazzolla, Tortorella et al. 1998; Dinauer 2003; Hannigan, Huang et al. 2004; Reutershan and Ley 2004), some laboratories have posited that cells become more “stiff” and cannot migrate through the intricate pulmonary capillary network (Worthen, Schwab et al. 1989;

A) The Lungs of Young Mice 24 Hours Post-Burn



B) The Lungs of Aged Mice at 24 Hours Post-Burn

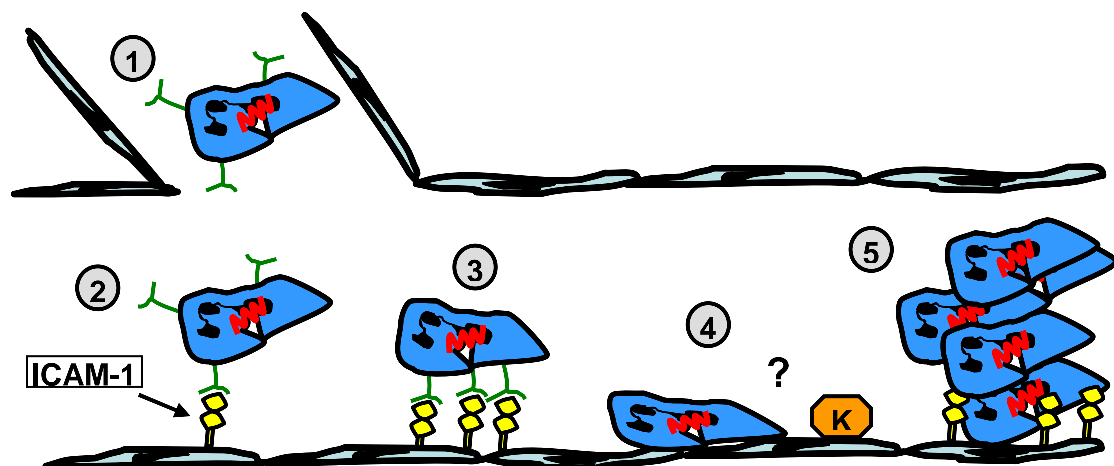


Figure 18. Proposed mechanism of neutrophil sequestration in the lungs of young versus aged mice at 24 hours after burn. A) Since ICAM-1 is not upregulated in the lungs of young mice at 24 hours after burn, vascular adhesion does not occur and cells may continue in circulation. B) Neutrophils enter pulmonary circulation with levels of CD11b and CXCR2 that do not differ from those of young mice at this time point **(1)**. Since ICAM-1 is upregulated, however, neutrophils loosely adhere **(2)** and are potentially able to undergo firm adhesion **(3)**. Since CXCR2 expression is significantly low, neutrophils cannot be guided to migrate through the endothelium via further activation of the receptor **(4)**. As a result, neutrophils sequestered in the vasculature and continue to accumulate **(5).**

Skoutelis, Kaleridis et al. 2000; Suwa, Hogg et al. 2001; Yoshida, Kondo et al. 2006). These studies have concluded that the inability for neutrophils to alter their cell shape is the main cause of pulmonary vascular congestion. While not measured in this study, the prolonged stimulation of neutrophils from the aged mice may cause increased actin polymerization and cell stiffness. It is thus possible that part of the mechanism of neutrophil accumulation seen in the lungs of aged mice at 24 hours also involves decreased deformability.

The Role of ICAM-1 Dependent and Independent Adhesion in the Lungs

Despite proposals that actin polymerization is the only mechanism of neutrophil sequestration in the lungs after burn, passive accumulation is not likely the only cause. Data indicate that ICAM-1 is also important in mediating vascular adhesion in the lungs after burn (Mulligan, Till et al. 1994; Jin, Zhu et al. 2003). While other adhesion molecules are known to be involved in the inflammatory response to burn, such as VCAM-1, VLA, and PECAM-1, studies aimed at blocking these factors do not show an effect (Mulligan, Till et al. 1994; Reutershan and Ley 2004). Since ICAM-1 inhibition significantly attenuates pulmonary consequences after injury (Mulligan, Till et al. 1994), this particular adhesion molecule was chosen for our study.

Interestingly, at 6 hours, levels of ICAM-1 on the pulmonary endothelium were not different between any of the treatment groups (**Figure 12**). The concept that neutrophils utilize ICAM-1 independent pathways to migrate into the lung has been

recognized by others (Doerschuk 1992; Hellewell, Young et al. 1994; Issekutz, Chuluyan et al. 1995; Burns, Smith et al. 2003). For nearly all tissues in the body, including the skin, leukocytes undergo diapedesis at post-capillary venules (Burns, Smith et al. 2003; Muller 2003; Reutershan and Ley 2004). In the lungs, however, leukocyte transmigration, in general, occurs at the capillaries (Burns, Smith et al. 2003; Laudes, Guo et al. 2004; Reutershan and Ley 2004). Since post-capillary venules have a diameter that is much wider than that of leukocytes, flow through these vessels is much faster than in capillaries (Jung, Norman et al. 1998; Burns, Smith et al. 2003). As such, to ensure that a leukocyte enters an inflamed tissue, margination, rolling, and firm adhesion in post-capillary venules are required to give cells enough time to sense and to respond to the locally produced chemokines. The diameter of a capillary, on the other hand, is typically the same or smaller than leukocytes (Burns, Smith et al. 2003). Therefore, the rate of flow is much slower through these vessels. The increased transit time offered by capillary circulation seems to be adequate for leukocytes to recognize any local inflammation (Lien, Henson et al. 1991). This is thought to be the reason why cells can migrate via ICAM-1 and CD11/CD18-independent mechanisms in the lungs, which is predominantly composed of capillaries (Doerschuk 1992; Hellewell, Young et al. 1994).

The Required Components for Neutrophil Adhesion and Migration

Chemokine signaling, particularly via CXCR2, seems to be an important factor in determining where neutrophils accumulate within the lung tissue. Again, the role of CXCR2 is not only for cell movement in general, but also for upregulating and clustering

CD11b, and transmigrating through the endothelium (Chatila, Geha et al. 1989; Arnaout 1990; Frevert, Huang et al. 1995; Ley 2002). Other laboratories have indicated that, when CXCR2 is functional in an inflamed tissue, CD11b on neutrophils binds ICAM-1 and the endothelial-bound KC helps to guide the cell out of the vasculature (Tonnesen 1989). If any one of these components is absent, cells do not transmigrate (Luu, Rainger et al. 2000; Zhang, Liu et al. 2001).

Although others have shown that CXCR2 signaling upregulates CD11b on neutrophils, this observation was not found after burn injury (**Figure 13**) (Frevert, Huang et al. 1995; L'Heureux, Bourgoin et al. 1995). However, the particular method of analysis chosen may not have been appropriate for a few reasons. First, the levels of CD11b were measured on peripheral blood neutrophils. As seen with the CXCR2 analysis (**Figure 15**), expression may depend on the tissue analyzed. Therefore, the effects of CXCR2 signaling on CD11b may be better understood by determining expression within the lung tissue itself. In addition, it has been shown that CXCR2 activation not only stimulates CD11b upregulation, but also clustering on the cell surface (Arnaout 1990; Ley 2002; Kim, Carman et al. 2004). Since flow cytometry can only measure the average expression per cell, the clustering effect may have been obscured. Moreover, it has been suggested that the ability for chemokine signaling to induce CD11b expression requires cells to first be bound to ICAM-1 (Luu, Rainger et al. 2000; Kim, Carman et al. 2004). In other words, CXCR2 signaling may not be necessary for the

initial CD11b/ICAM-1 interaction to take place, but for the formation of a more stable interaction between them.

The need for loose adhesion prior to CXCR2-mediated firm adhesion of the CD11b and ICAM-1 interaction is exemplified by *in vitro* experiments from Luu et al (Luu, Rainger et al. 2000). If neutrophils were allowed to flow over activated endothelium, many of the cells were able to migrate through. When various chemokines were added to the flow, neutrophils immediately stopped rolling, adhered firmly to the endothelium, and changed shape, but only migrated over and not through the endothelium. This lack of transmigration was presumably because there was no chemotactic gradient on the other side of the monolayer to guide the cells through. If neutrophils were preincubated with these chemokines, however, cells were only able to undergo rolling and not firm adhesion or migration, presumably because the chemokine receptors were already downregulated and not available for transmigration. Moreover, this study showed that the ability for neutrophils to adhere and to migrate was a result of CXCR2 signaling and the upregulation of CD11b.

The Effects of Age on Pulmonary Neutrophil Accumulation after Burn

From the results of our study, it seems that neutrophils from aged mice have an elevated activation status, making them predisposed to aberrant responses following a secondary challenge. Evidence for this hypothesis is increased chemotaxis and low CXCR2 expression on pulmonary neutrophils in the absence of injury (**Figures 14** and

16). As described above, most of the studies analyzing neutrophil defects with age focus on pathways that are only relevant during inflammation (Rao 1986; Biasi, Carletto et al. 1996; Schroder and Rink 2003; Fulop, Larbi et al. 2004; Fortin, Lesur et al. 2007). Conclusions from these analyses implicate that the aging environment "primes" neutrophils, making them unable to respond as robustly upon an inflammatory challenge. By assessing the effects of age on a constitutively active pathway (CXCR2) we have demonstrated that these neutrophils may, in fact, be in an increased activation state under resting conditions. Using the example of CXCR2, our data showed that, while expression levels may not necessarily be different on peripheral blood neutrophils isolated from aged mice, chemotactic behavior was increased (**Figure 14**). Upon further stimulation with KC, these cells were no longer able to migrate. After burn injury, this overstimulation translated into prolonged neutrophil accumulation in the lungs of aged mice.

One particular signaling pathway that may be involved in the observed increase in activation of neutrophils from aged mice is protein kinase C (PKC). As described above, activated PKC has been shown to activate G-protein related kinases (GRKs) to induce chemokine receptor desensitization (Richardson, Ali et al. 1995; Pitcher, Freedman et al. 1998; Ali, Richardson et al. 1999; Le, Li et al. 2000; Ferguson 2001; Heit, Tavener et al. 2002; Arraes, Freitas et al. 2006) and to increase CD11/CD18 clustering (Chatila, Geha et al. 1989; Kim, Carman et al. 2004). Since some laboratories have reported increased PKC activity in the aged (Busquets, Ventayol et al. 1996; Martins Chaves, Prates

Rodrigues et al. 2002), it is possible that this pathway may play a role in priming neutrophils from the aged in the absence of injury.

It is important to note that the effects of age on all cells of the body need to be considered in order to fully explain the pulmonary consequences of burn. Saturation of chemokine pathways may explain the decreased expression of CXCR2 observed in the current study. However, chemokine signaling is not known to trigger ICAM-1 expression. It can therefore be proposed that aging also primes endothelial cells in the lungs. Our laboratory and others have reported that aged individuals express elevated levels of IL-1β—the main mediator of ICAM-1 upregulation—in the lungs of "healthy" aged mice and humans (Meyer, Rosenthal et al. 1998; Nomellini, Faunce et al. 2008). As a caveat, we did not find increases in pulmonary endothelial ICAM-1 until 24 hours after burn (**Figure 12**). Therefore, we hypothesize that the ICAM-1 becomes expressed in aged mice receiving a burn only as a result of prolonged accumulation of pulmonary neutrophils leading to excessive tissue damage. In the lungs of young mice, the neutrophil accumulation is only transient and therefore may not induce the same degree of tissue injury (**Figures 5-8**). It is interesting to note that we did not find elevated IL-1β in the skin of uninjured aged mice (Nomellini, Faunce et al. 2008). In addition, no age-related differences in neutrophil accumulation or KC levels were found in the wounds after burn injury (**Tables 1 and 2**). Therefore, the effects of age on the response to burn injury are tissue specific and may be dependent on the pre-injury environment.

Since pulmonary endothelial ICAM-1 does not appear until 24 hours after burn in aged mice, we believe that the mechanism we proposed for pulmonary neutrophil accumulation is most relevant at this later time point. Support for this hypothesis is exemplified by CXCR2 blocking studies (**Figures 10** and **11**). Anti-CXCR2 neutralizing antibody was only able to reduce neutrophil counts in the lungs of both young and aged mice at 6 hours after burn by about 50%. This means that neither CXCR2 nor ICAM-1 is the only mediator of neutrophil adhesion in the lungs at this time point. Here, we suggest that neutrophil accumulation occurs in the lungs of both young and aged mice via both ICAM-1/CD11b dependent and independent pathways, as described above.

At 24 hours after burn, in contrast, anti-CXCR2 was able to completely inhibit pulmonary neutrophil accumulation in aged mice. One explanation is that ICAM-1/CD11b dependent adhesion dominates at this later time point. Studies by Doerschuk et al also suggest that early pulmonary neutrophil accumulation after a systemic insult is mainly CD11/CD18 independent, while later time points require CD11/CD18 dependent adhesion (Doerschuk 1992). Since the precise mechanism of ICAM-1 and CD11b independent adhesion is not understood, however, further exploration into this mechanism is necessary. In addition, future experiments are required to determine the precise mechanism of the benefit of anti-CXCR2 therapy.

Overall, it seems that burn injury in elderly individuals is an example of a two hit injury, as suggested above. Before an insult, aging may affect a number of different cells

throughout the body—including endothelial cells and neutrophils. Once the injury occurs, the response becomes exaggerated beyond that which is seen with burn alone (*i.e.* in young individuals). As two hit injuries are more clinically challenging, an awareness of the effects of age is important in order for better treatment strategies to be developed.

Fitting Our Model to Other Animal Models of Pulmonary Inflammation

This proposed mechanism of neutrophil migration shown in **Figure 18** can be applied to a number of conditions which lead to pulmonary inflammation. It has been demonstrated that, when an inflammatory stimulus is located within the airway, neutrophil content in the BAL is significantly increased (Frevert, Huang et al. 1995; Gupta, Feng et al. 1996; Xing, Gauldie et al. 1998; Czermak, Friedl et al. 1999; Beck-Schimmer, Madjdpour et al. 2002; Sentman, Brannstrom et al. 2002; Quinton, Nelson et al. 2004; Gordon, Li et al. 2005; Reutershan, Basit et al. 2005; Speyer, Rancilio et al. 2005; Basit, Reutershan et al. 2006). In a study by Frevert, et al, when recombinant KC was instilled intratracheally in rats, a dose-dependent infiltration of neutrophils into the alveolar space was observed (Frevert, Huang et al. 1995). In this same study, neutrophil content in BAL was significantly attenuated when animals were given anti-KC or anti-MIP-2 antibody i.p. immediately before administration of LPS into the airway. As explained by our proposed model, neutrophils were able to migrate into the airway only when increased CXCR2 signaling within the lungs was possible.

When the inflammatory stimulus is systemic, neutrophils accumulate within the lungs, but do not migrate into the airspace (Johnson, Brigham et al. 1991; Standiford, Kunkel et al. 1995; Gupta, Feng et al. 1996; van Eeden, Kitagawa et al. 1997; Carraway, Welty-Wolf et al. 1998; O'Malley, Matesic et al. 1998; Czermak, Friedl et al. 1999; Murphy, Paterson et al. 2005; Rojas, Woods et al. 2005; Gomez, Hirano et al. 2007). Wagner et al showed that, while LPS instillation into the airway caused a significant accumulation of neutrophils in the BAL, this response was completely ablated when LPS was given i.v. at 1.5, 3, or 6 hours beforehand (Wagner, Harkema et al. 2002). In accordance with our model, if LPS was first given systemically, CXCR2 expression on the cell surface was likely to be reduced. This effect was likely due to the systemic release of proinflammatory mediators rather than the LPS itself, as signaling through the LPS receptor (TLR4) has actually been shown to augment CXCR2 activity (Fan and Malik 2003). Therefore, by the time these neutrophils reached the lung, they could no longer respond to the locally produced chemokines and could not migrate into the airway. Interestingly, this same study showed that i.v. LPS given after intratracheal (i.t.) instillation can also inhibit neutrophil influx into the airspaces, only if it is given within the first 1.5 hours of i.t administration (Wagner, Harkema et al. 2002). If LPS was injected i.v. at 2 hours after i.t. LPS, neutrophils were still able to migrate into the airspace. These results imply that the effects of the systemic inflammatory mediators cause immediate CXCR2 desensitization. If functional neutrophils are allowed enough time to enter the alveoli (after 2 hours, according to this report), LPS administered i.v. no longer has an effect.

Fitting Our Model to Clinical Data

The results from this study and others may explain why patients suffering from systemic inflammation have a significantly increased risk of pneumonia (Teixidor, Novick et al. 1983; Shirani, Pruitt et al. 1987; Hollingsed, Saffle et al. 1993; Adams, Hauser et al. 2001). It has been shown that trauma patients with decreased CXCR2 expression on peripheral blood neutrophils are more predisposed to pulmonary infection (Adams, Hauser et al. 2001; Tarlowe, Duffy et al. 2005). As a caveat, some neutrophils from humans show increased CXCR2 activity. Interestingly, these patients tend to be predisposed to acute respiratory distress syndrome (ARDS) with increased neutrophil congestion of the alveoli (Adams, Hauser et al. 2001). Currently, there are no studies which examine CXCR2 expression in neutrophils from elderly trauma or burn patients. However, since burn patients over the age of 65 have a significantly greater risk of pneumonia (Martin, Mannino et al. 2006), it is interesting to speculate whether these individuals will be more likely to have decreased CXCR2 expression on neutrophils.

The Clinical Use of Anti-CXCR2 Therapy in Burns

While our model minimally requires three components, a number of factors are involved in the inflammatory response. Theoretically, targeting any one of these components may be effective in preventing pulmonary inflammation after a systemic insult. We believe that targeting CXCR2 will prove to be the most effective strategy for a number of reasons.

Inhibition of cytokines upregulated early in the inflammatory response, such as IL-1β or TNF-α, have shown to be beneficial in animal studies of systemic insults, but have yet to be efficacious in the clinical setting (Dinarello 2000; Calkins, Bensard et al. 2002). It is thought that the timing of administration, as well as the futility of trying to block only one cytokine is a cause for this discrepancy (Dinarello 2000; Remick, Call et al. 2001). Blocking selectins has also been performed in animal models, but the results are highly dependent on the particular tissue being targeted and the modality of injury (Mulligan, Till et al. 1994; Carraway, Welty-Wolf et al. 1998; Chandra, Katahira et al. 2003). Inhibiting other adhesion molecules, such as VCAM-1, VLA, and PECAM-1, generally do not show a benefit in animal models of a systemic insult (Mulligan, Till et al. 1994; Reutershan and Ley 2004). In contrast, inhibition of the ICAM-1/CD11b interaction has been demonstrated in animals (Mulligan, Till et al. 1994; Cotran and Mayadas-Norton 1998; Jin, Zhu et al. 2003). Because both of these molecules have a number of roles on a wide variety of cell types, the severe side effects that may precipitate from anti-ICAM-1 or anti-CD11b therapy may not be a desired mode of treatment for burn patients.

As CXCR2 is fairly specific for neutrophils, targeting this receptor in patients with burn injury may show the most benefit. As described above, however, CXCR2 expression in human trauma patients can be either increased or decreased (Adams, Hauser et al. 2001; Tarlowe, Duffy et al. 2005). If CXCR2 levels are high, methods applied in the current study may be effective. Alternatively, if CXCR2 levels are low,

methods to increase the receptor via GRK inhibitors—which would decrease receptor phosphorylation and desensitization—may be more appropriate, as enhanced activity of this pathway has been shown to correlate with neutrophil defects following a systemic insult (Arraes, Freitas et al. 2006). Standards of care when targeting CXCR2 may therefore require quick and effective tests to determine the receptor status of the patient before a particular treatment option is chosen.

APPENDIX A

ADDITIONAL FIGURES

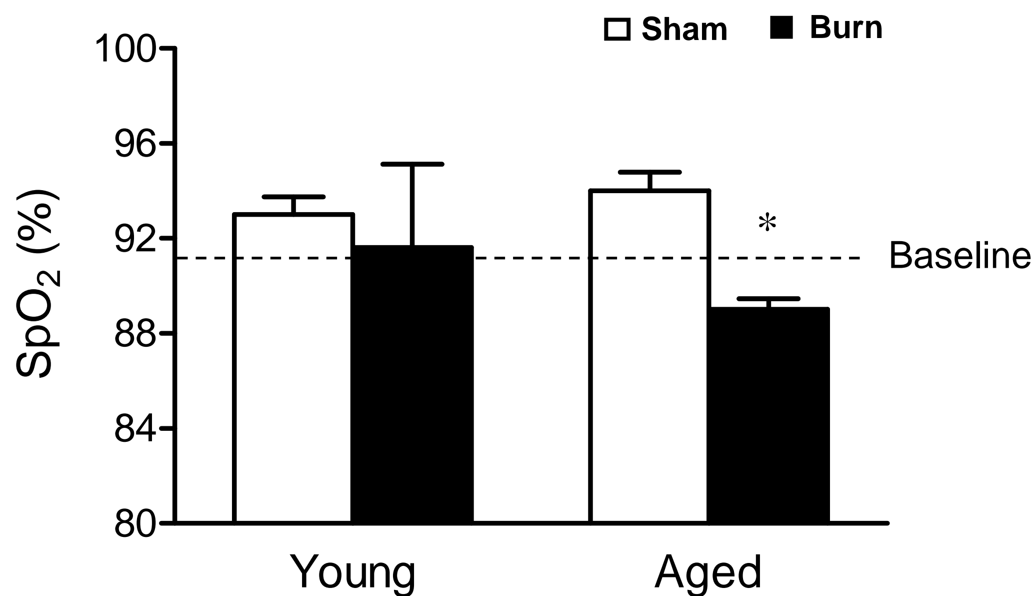


Figure 19. Oxygen saturation in young and aged mice at 24 hours after burn. Oxygen saturation was measured at 24 hours after sham (white bars) or burn (black bars) injury in young and aged mice. Animals with tumors were not included. Data are represented as mean ± SEM. N = 3-4 mice per group. *, $p<0.05$ compared to all other groups.

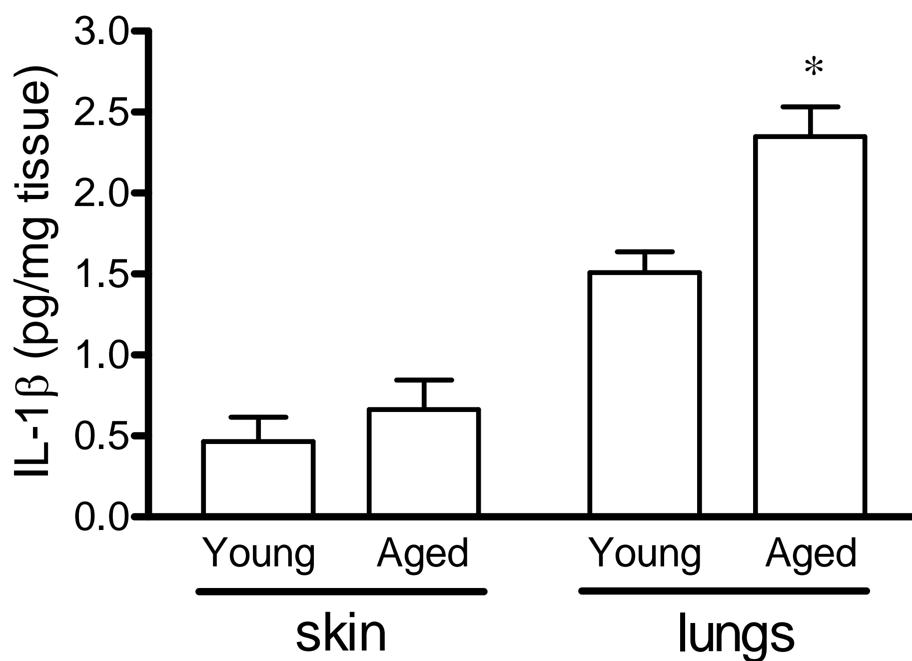


Figure 20. Differential IL-1β expression in the skin and lungs of young and aged uninjured mice. Levels of IL-1β were measured in lung and skin homogenates of young and aged uninjured mice. Data are represented as average concentration in pg/mg protein ± SEM. N = 13-19 mice per group. *, $p<0.05$ compared to young lungs.

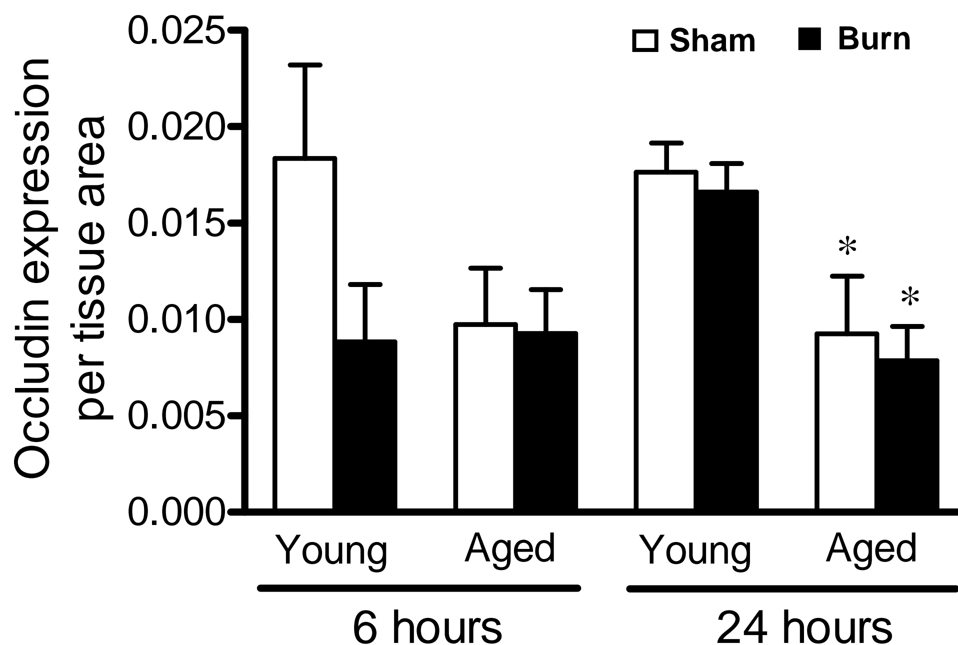


Figure 21. Pulmonary occludin expression after burn. Sections of lungs from young and aged mice at 6 and 24 hours after sham (white bars) or burn (black bars) were stained with anti-occludin antibody. Expression of pulmonary occludin was determined by measuring the total area of positive staining (μm^2), normalized to the total tissue area (μm^2). Data are represented as mean ± SEM. N = 3-7 mice per group. *, $p<0.05$ compared young receiving the same injury at the same time point.

APPENDIX B

SPECIFIC METHODS

Induction of Burn Injury

Materials

-CO_2 tank and chamber
-scale
-Nembutal (50 mg/kg)
-Normal saline (0.9%)
-1 ml syringes with 27 g needle
-3 ml syringes with 27 g needle
-Heating plate
-2 H_2O basins: one with room temperature H_2O and one with boiling H_2O
-Electric shavers
-Burn templates (to give 15% TBSA burn)
-Warming pads

Procedure

1. Tailmark and weigh animals. Be sure to replace dirty cage bottoms with clean ones.
2. Fill 1 ml syringes with Nembutal to give 50 mg/kg to each animal.
3. Fill 3 ml syringes with normal saline.
4. Inject animals with Nembutal.
5. When they are asleep, shave their backs.
6. Administer 15% TBSA sham or burn injury.
7. Resuscitate with normal saline (give 1 ml per 20 g)
8. Place animal cages on top of warming pads. Keep room temperature warm.
9. Monitor animals until they awaken. Record any deaths.

Lung Inflation for Immunohistochemistry

Materials

-27 gauge needle
-3 ml syringe
-25% O.C.T. in PBS
-100% O.C.T
-Weigh boats
-Pennies or embedding boats
-Dry ice

Procedure

1. Fill a 3 ml syringe with diluted O.C.T. and attach a 27 gauge needle.
2. Remove lung lobe of interest from hilar structures.
3. Place lung lobe on the side of a weigh boat, dorsal side up.
4. Insert needle into the dorsal side of the lung lobe nearly parallel with the lobe surface and inject diluted O.C.T. until the lobe is fully inflated.
5. Place one drop of 100% O.C.T onto a penny or in embedding boats.
6. Immerse the inflated lung lobe in the O.C.T.
7. Cover any exposed lung with the 100% O.C.T.
8. Allow embedded lobe to freeze using dry ice.

Indirect Immunofluorescence

Materials

-Frozen tissue sections, cut at a maximum of 5 µm thick, on Superfrost Plus slides
-Copland jar
-Acetone
-PAP pen
-PBS, pH 7.2-7.4
-Humidified chamber
-Normal serum (from same species as the secondary antibody was made in)
-Primary antibody against mouse antigen to detect
-Fluorochrome-conjugated secondary antibody against the IgG of the species that the primary antibody was made in
-Coverslips
-Mounting medium (Crystal Mount, Aqua Polymount, or other anti-fading medium)

Procedure

1. Remove slides from -20°C freezer, dry at room temp until condensation completely evaporates (minimum 30 mins, maximum 24 hrs).
2. Fix in ice-cold acetone for 10 min at -20°C.
3. Let slides air dry.
4. Encircle tissue samples with PAP pen.
5. Rehydrate in PBS for 5 min.
6. Tap off excess PBS and apply normal serum (diluted 1:10 in PBS) to block.
7. Incubate 20-30 min in humidified chamber.
8. Pour off serum, DO NOT RINSE.
9. Dilute primary antibody in PBS containing 2% normal serum.
   *Dilution will need to be determined for each antibody beforehand.
10. Apply primary antibody to each slide.
11. Incubate 30-60 minutes in humidified chamber.
12. Rinse slides in PBS for 5 min.
13. Dilute secondary antibody in PBS containing 2% normal serum.
   *Dilution will need to be determined for each antibody beforehand.
14. Apply secondary antibody to each slide.
15. Incubate 30 min in humidified chamber.
16. Rinse in PBS for 5 min.
17. Quick rinse in tap H_2O and allow slides to dry.
18. Apply mounting medium and cover slip.
19. Let dry overnight at 4°C.

Hematoxylin and Eosin Staining for Frozen Sections

Materials
-Slide holders and staining wells
-EtOH (80%, 95%, 100%)
-Formalin
-Harris Hematoxylin
-0.5% Acid Alcohol (250 ml of 80% EtOH + 1.2 ml HCL)
-Ammonia H_2O (250 ml of tap water + 9-10 drops of ammonia)
-Eosin
-Xylene
-Cover slips
-Mounting medium

Procedure
1. 50% alcohol – 5 min
2. Tap H_2O – 20 dips
3. 10% formalin – 2 min
4. Tap H_2O – 20 dips
5. Hematoxylin – 8 min
6. Running tap H_2O – until clear
7. 0.5% acid alcohol – 3 dips
8. Running tap H_2O – until clear
9. Ammonia H_2O – 10 dips
10. Running tap H_2O – 3 min
11. 95% EtOH – 10 dips
12. Eosin – 5 dips
13. 3x 95% EtOH – 20 dips each
14. 2x 100% EtOH – 20 dips each
15. Xylene – minimum 5 min
16. Coverslip (keep the rest of the slides in xylene).

Lung Homogenization

Materials
-Lung lobes, flash frozen
-Liquid nitrogen
-Scale
-Wet and dry ice
-2 containers for washes: 1 for ddH_2O and 1 for EtOH
-Small beaker of ice H_2O
-14 ml polypropylene tubes, 17mm x 100 mm
-Protease inhibitor cocktail (PIC)
-Homogenizer
-Sonicator
-1 ml syringes with 25 gauge needles
-1.2 µm syringe filters
-1.5 ml tubes (2x sample number)

Procedure
1. Weigh lung samples. Keep samples on dry ice, flash freeze in liquid nitrogen after weighing.
2. Fill 12 x 100 mm plastic tubes with 0.9 ml PIC. Keep on ice.
3. Wash homogenizer, first with EtOH (minimum10 sec), then with ddH_2O (minimum10 sec).
4. Place frozen lung sample in tube with PIC.
5. Place tube in beaker of ice H_2O.
6. Homogenize sample. Start at speed 4, moving tube slowly up and down 5 times. Turn off for 10 sec, then turn to speed 7 and repeat.
7. Be sure that all tissue is homogenized.
8. Wash homogenizer between samples, first with EtOH then with ddH_2O, making sure to check for any pieces of tissue that may be stuck to the blade. Dry with kimwipes.
9. Sonicate samples at 30% output for 30 sec each. Keep samples on ice.
10. Wash sonicator between samples, first with EtOH then with ddH_2O, and dry probe with kimwipes.
11. Centrifuge samples at about 800 g (2000 rpm in large Beckman centrifuge) for 2 minutes at 4°C to pellet debris.
12. Transfer supernatant to 1.5 ml tubes.
13. Spin in tabletop centrifuge at 14000 rpm for 15 minutes at 4°C.
14. Remove supernatants using 1 ml syringes with 25 gauge needles.
15. Detach needle and filter contents of the syringe through 1.2 µm syringe filters into new 1.5 ml tubes.
16. Aliquot filtered samples.
17. Store samples at -80°C.

Preparation for anti-CXCR2 Injection

Materials
-Anti-mouse CXCR2 antibody (R&D Systems, Catalog #: MAB2164, Lot KPM016071)
-IgG_{2A} Isotype Control (R&D Systems, Catalog #: MAB006, Lot CAO076061)
-Sterile PBS
-1 ml syringes with 27 gauge needles

Procedure
1. To stock vial of anti-CXCR2, add 1 ml sterile PBS (= 500 µg/ml)
2. Dilute anti-CXCR2 to 200 µg/ml, estimate about 200 µl per animal
   -Example: for 3 animals, need 600 µl. Make 1000 µl total according to the calculation:
   (500 µg/ml)(x)=(200 µg/ml)(1000 µl)
   x = 400 µl of stock antibody + 600 µl of PBS
3. To stock vial of IgG control, add 1 ml sterile PBS (=500 µg/ml)
4. Dilute IgG to 200 µg/ml, again estimate about 200 µl per animal
5. Fill needles with 100 µl of either anti-CXCR2 or control IgG for a total of 20 µg per mouse.
6. Administer sham or burn injury
7. Inject animals i.p. with either control IgG or anti-CXCR2 30 minutes after injury.
8. Sacrifice at desired time point.

Peripheral Blood Neutrophil Isolation

Materials

-1 ml syringes with 27 gauge needles
-Heparin
-HBSS
-Histopaque 1083 (Sigma, Catalog #: 18031)
-3% Dextran in PBS
-ACK lysis buffer
-15 ml conical tubes (3x number of samples)
-Ice bucket

Procedure

1. Fill conical tubes with 3 ml Histopaque. Let sit at room temperature while collecting samples.
2. Coat 1 ml syringes with heparin (fill syringe to 1.0ml and decant, leaving some heparin in the hub).
3. Collect blood from animals via cardiac puncture of the right ventricle.
4. Decant into 15 ml conical tubes. Keep samples on ice.
5. Dilute blood 1:1 with HBSS.
6. Carefully add diluted blood on top of Histopaque. Do not disturb the interface between the 2 fluids.
7. Centrifuge at 400 g for 30 min at 20°C. TURN OFF BRAKE.
8. Aspirate off top layer and save the pellet containing erythrocytes and neutrophils.
9. Add 2 ml of HBSS and resuspend.
10. Add 5 ml of 3% dextran to each sample.
11. Mix by inverting 3x slowly.
12. Incubate at room temperature for 45-60 min.
13. Remove top layer and place into new 15 ml tubes. Discard the pellet.
14. Add 5 ml HBSS to each sample.
15. Centrifuge at 300 g for 5 min.
16. Aspirate supernatant.
17. Add 2.5 ml ACK lysis buffer. Immediately vortex or pipette samples up and down.
18. Incubate for 2 min.
19. Stop the reaction by adding 10 ml ice cold HBSS.
20. Centrifuge at 300 g for 5 min.
21. Aspirate supernatant.
22. Repeat lysis if necessary.
23. Resuspend cells in 0.5 ml HBSS.
24. Count cells at 1:1 dilution with trypan blue (20 µl of sample + 20 µl trypan blue).

Chemotaxis Assay

Materials

-See protocol for “Peripheral Blood Neutrophil Isolation”
-Fluorescent cell dye (ex; Cell Tracker Green: Invitrogen, Catalog #: C2925)
-Chemotaxis media (HBSS, Penicillin/Streptomycin/Glutamate, 25 mM HEPES, 1%BSA)
-Recombinant mouse CXCL1/KC, Carrier Free (R&D Systems, Catalog #: 453-KC/CF)
-N-formyl-methionyl-leucyl-phenylalanine (Sigma, Catalog #: F3506)
-Chemotaxis chamber (NeuroProbe, Catalog #: 101-8)

Procedure

1. Isolate neutrophils from peripheral blood.
2. Count cells in 0.5 ml HBSS.
3. Centrifuge at 300 g for 5 min.
4. Dilute Cell Tracker Green to 40 µM in chemotaxis media.
5. Aspirate the supernatant and resuspend to10^6 cells/ml in diluted Cell Tracker Green.
6. Incubate samples for 45 min in the dark at 37°C and 5% CO_2.
7. Wash at least twice in HBSS.
8. Resuspend in chemotaxis media to 10^6 cells/ml.
9. Remove 100 µl of each sample for input wells.
10. Centrifuge input samples at 300 g for 5 min.
11. Prepare dilutions of rmKC in chemotaxis medium.
12. Add 29 µl of each chemokine or Prepare a separate set of wells for negative controls (media alone) and positive controls (fMLP (10^{-7} M)).
13. Resuspend sample inputs in 58 µl of chemotaxis media.
14. Plate 29 µl of sample inputs in a separate set of wells in duplicate.
15. Place filter membrane over wells.
16. Pipette 50 µl of each sample onto upper side of membrane in duplicate.
17. Incubate 60 min in incubator (37°C and 5% CO_2).
18. Aspirate cells from upper side of membrane.
19. Pipette 50 µl of 20 µM EDTA in PBS to upper side of membrane to detach adherent cells.
20. Incubate 15 min at room temperature.
21. Read plate in fluorescence spectrophotometer at an excititation of 492 nm and an emission of 517 nm to measure Cell Tracker Green.

Flow Cytometry

Materials

-Cells for control staining (unstained cells + single stain control for each antibody)
-1.5 ml tubes
-Flow buffer: 1% BSA, 0.1% NaN_3 in PBS, 0.2 μm filtered
-Blocking solution: 1:20 dilution (0.5 mg/ml) of anti-CD16/32 FcRδ II/III (clone 2.4G2, BD Pharmingen, Catalog #553142)
-Fluorochrome-conjugated antibodies of choice (determine dilutions beforehand)
-1% paraformaldehyde in PBS – make new each time

Procedure

1. Wash and count cells.
2. Centrifuge at 300 g for 5 min.
3. Aspirate supernatant and add 50 μl of blocking buffer to each tube.
4. Mix samples and incubate 20 min at 4°C.
6. Add antibodies of choice to each sample tube and to control tubes.
7. Incubate for 30 min at 4°C protected from light.
8. Centrifuge at 300 g for 5 min.
9. Wash 3x in flow buffer.
10. Aspirate supernatant and resuspend in 300 μl of 1% paraformaldehyde.
11. Cover tubes with foil and store at 4°C until analysis.
12. Wash 3x before running on FACS machine to remove paraformaldehyde.

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