Evidence for a self-enforcing inflammation in neutrophil-mediated chronic diseases

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Evidence for a self-enforcing inflammation in neutrophil-mediated chronic diseases

Nieuwe bevindingen voor een zelfversterkende rol van de neutrofiel
in neutrofiel gerelateerde chronische ziekte
(met een samenvatting in het Nederlands)

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Chapter 1

General introduction

General introduction

Chronic diseases are characterized by a slow progression leading up to a prolonged disease. Chronic diseases, such as chronic respiratory diseases and inflammatory bowel diseases, are the most important cause of mortality in the world, representing 60% of all deaths globally [1]. Since chronic diseases are an enormous health burden [1] and there are no curative therapies, major efforts have been directed towards understanding the pathophysiology of these complicated diseases. This thesis focuses on the neutrophilic inflammation in the diseases chronic obstructive pulmonary disease (COPD) and Crohn's disease (CD) and discusses the self-enforcement of the inflammatory state in the pathogenesis of these chronic diseases.

Pathogenesis of COPD

COPD is one of the leading causes of chronic morbidity and mortality worldwide [2, 3]. It refers to emphysema and chronic obstructive bronchitis with fibrosis and according to the World Health Organization (WHO), COPD is expected to rank fifth in 2020 in burden of disease caused worldwide [2]. This disease has been described by the Global Initiative for Chronic Obstructive Lung Disease as *"a preventable and treatable disease … characterized by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lung to noxious particles or gases"* [2]. The progressive loss of lung function is caused by emphysema due to the destruction of alveolar attachments in lung parenchyma and by narrowing of small airways as a result of inflammatory obstruction of the airway and luminal obstruction with mucus [4].

It is generally accepted that cigarette smoking is the main risk factor for the development of COPD [2, 5]. The WHO has estimated that 73% of COPD mortality is related to smoking in high income countries [6]. However, an estimated 25-45% of patients with COPD have never smoked. Since half the worldwide population is exposed to biomass smoke, it has been suggested that this exposure might be the biggest risk factor for COPD globally [7].

Although the exact etiology of COPD remains unknown, it has been established that COPD pathology is characterized by three mechanisms; an abnormal persistent inflammatory response to stimuli, oxidative stress and the protease-antiprotease imbalance [2, 8, 9]. The effects of smoke in the lungs is strongly linked to the pathogenesis of COPD; smoke and other irritants activate resident macrophages and airway epithelial cells to release chemoattractants, such as CXCL8, which lead to the recruitment of neutrophils. Both neutrophils and macrophages release proteases that break down connective tissue in the lung parenchyma, which ultimately results in the airway remodeling seen in emphysema.

Moreover, this mediator release also stimulates mucus hypersecretion, which is characteristic for chronic bronchitis [8-11].

In addition to the recruitment and regulation of inflammatory cells to the lung, there also is an induction of oxidative stress [9]. Oxidative stress results from an oxidant-antioxidant imbalance; there is an excess of oxidants and/or a depletion of antioxidants [12]. The increase in reactive oxygen species (ROS) from either cigarette smoke or inflammatory cells have several effects on the lung tissue, such as activation of NF-κB which leads to secretion of CXCL8 and TNF-α, inactivation of antiproteases, increased mucus secretion and bronchoconstriction, all of which result in lung parenchymal destruction and airway remodeling [2, 8, 9, 13].

There is also convincing evidence for an imbalance between proteases that break down connective tissue components and antiproteases, which are responsible for the protection against tissue breakdown. The balance tips over in favor of increased proteolysis because of either an increase in proteases (matrix metalloproteinases (MMPs) or neutrophil elastase) or a deficiency in antiproteases (α_1-antitrypsin; α_1-AT) and tissue inhibitors of MMPs (TIMPs) [8-10].

Pathogenesis of CD

CD is one of the major inflammatory bowel diseases (IBD) which is characterized by transmural inflammation involving any part of the gastrointestinal tract [14, 15]. CD is an incurable disease that generally begins in young adulthood and lasts throughout life. CD has the highest prevalence in Western countries [16], and the highest incidences of CD have been reported in North America [17, 18], Northern Europe [19] and the United Kingdom [20, 21]. However, IBD has also recently emerged in countries in which reports were historically uncommon, including China [22], Japan [23] and India [24].

Although the precise mechanism underlying the development of CD remains unknown, it has been postulated that it results from a complex interaction of genetic, environmental and immunologic factors [25, 26]. The general hypothesis is that damage to the bowel mucosa is a result of a deregulated adaptive and innate immune response to mucosal antigens comprised within the constituents of the normal intestinal microbial flora [25].

Alterations in intestinal barrier function may play an essential role in the pathogenesis [14, 25, 27]; the epithelial barrier is leaky in people with CD [28, 29]. Moreover, the epithelial cell layer expresses a different pattern-recognition receptor (PRR) pattern. PRRs are expressed by a broad range of immune and other cells. They are able to recognize pathogen-associated molecular patterns (PAMPs), molecules which are unique to bacteria, fungi, parasites, and viruses; and their stimulation leads to the initiation of immune defense mechanisms. Toll-like receptors (TLRs) and nucleotide-binding oligomerization domains

containing proteins (NODs) are two important groups of PRRs. *NOD2* is the first gene that has been linked directly to IBD development. Decreased NOD2 activity was shown to be positively associated with IBD development [30]. With regard to TLRs, healthy epithelial cell express TLR3 and TLR5, whereas in CD patients TLR3 is significantly downregulated and TLR4 is strongly upregulated on the epithelial cell layer [31]. In addition, TLR9 is also expressed by the epithelial cell layer, enabling the epithelial cells to respond directly to bacterial DNA, which results in a secretion of CXCL8 [32]. In response to this chemoattractant, neutrophils transmigrate from the blood into the intestinal (sub)mucosal and serosal tissue were they release nonspecific inflammatory mediators, which ultimately can lead to tissue damage [25, 27, 33, 34]. Moreover, an impaired active inflammation leading to insufficient neutrophil recruitment in CD has been demonstrated previously and it is hypothesized that this results in a primary immunodeficiency (for a review see [35]).
Other malfunctions of the immune system in CD are the disturbance in antigen recognition and processing by antigen-presenting cells (APCs), the activation of effector T cells by atypical APCs such as epithelial cells, the disturbed clearance of overreactive or autoreactive T cell populations and the trigger of the inflammatory cascade through neuroimmunological interaction by psychosocial stress [14], all of which result in extensive damage to the tissue and disease progression.

Neutrophil functions and actions

Although neutrophils are necessary for healthy lungs and gut, they play an important role in the destructive processes that characterize COPD and CD. In these chronic diseases, neutrophils can be responsible for significant damage when they accumulate at sites of inflammation and are harmful to healthy tissue [25, 36]. Understanding the role of the neutrophil in these chronic diseases can lead to an improvement in knowledge of the pathogenesis, which may lead to new therapeutic strategies.

Neutrophils are produced in the bone marrow from stem cells that proliferate and differentiate to mature neutrophils. They are the most abundant leukocytes in the blood and the first line of defense in innate immunity against invading pathogens [37]. The following section focuses on the main functions and activities of neutrophils in inflammation.

Neutrophilic chemotaxis

Neutrophilic chemotaxis is a process where neutrophils direct their movement by following a chemokine concentration gradient toward the site of an inflammation [38, 39]. The chemokines are homologous 8-10 kD proteins that are subdivided into families on the basis of the relative position of the cysteine residues in the mature protein [39, 40]. The recruitment of neutrophils to the side of infection is mainly directed by the ELR^+ CXC-chemokine subfamily via binding to CXCR1 and CXCR2 [41-43]. In these chemokines the first two cysteine residues are separated by a single amino acid. Moreover, they contain the sequence glutamic acid-leucine-arginine (ELR) preceding the CXC sequence [39, 40]. CXCL8 binds with high affinity to CXCR1 and CXCR2. CXCL6 is also known to bind CXCR1 and CXCR2, although with lower affinity than CXCL8. CXCR2 is a highly promiscuous receptor and can bind CXCL1, CXCL2, CXCL3, CXCL5 and CXCL7 in addition to CXCL6 and CXCL8 [44].

CXCR1 and CXCR2 are G protein-coupled receptors (GPCRs), which are seven-transmembrane-spanning proteins coupled to a heterotrimeric G protein. CXC-chemokine binding to CXC-chemokine receptors leads to a dissociation of $G\alpha_1$ and $G\beta\gamma$ subunits of the heterotrimeric G proteins and subsequent calcium influx and activation of the phosphatidylinositol 3-kinase (PI3K) and the small Rho GTPases signaling pathway [43-45], leading to neutrophilic chemotaxis and migration and integrin adhesiveness [45]. However, also other signaling pathways, such as the activation of the MAP kinase cascade, are involved in the stimulation of CXCR1 and CXCR2 [45]. See figure 1 for the multiple signaling pathways induced by CXCR1/2.

See chapter 2 for the description of other chemokines and chemokine receptors expressed by the neutrophil and other cells.

Neutrophilic chemoattractant N-acetyl Proline-Glycine-Proline

It has long been known that fragments of the extracellular matrix, such as collagen fragments, have chemotactic properties [46, 47]. One of these fragments is N-acetyl Proline-Glycine-Proline (N-ac-PGP). This tripeptide was first identified by Pfister and colleagues in a rabbit model in which it was demonstrated that alkali degradation of whole cornea generated a tripeptide, N-ac-PGP [48]. Injecting N-ac-PGP in normal rabbit corneas resulted in a rapid and severe neutrophil invasion leading to corneal ulceration and perforation, resembling the neutrophil infiltration in the alkali-injured eye [49].

N-ac-PGP and its non-acetylated form, PGP, are active on the neutrophil and act via the CXCR1 and CXCR2 [50]. The basis for N-ac-PGP effects probably lies in its structural homology with the GP motif present in all ELR^+ CXC chemokines, such as CXCL8. This GP

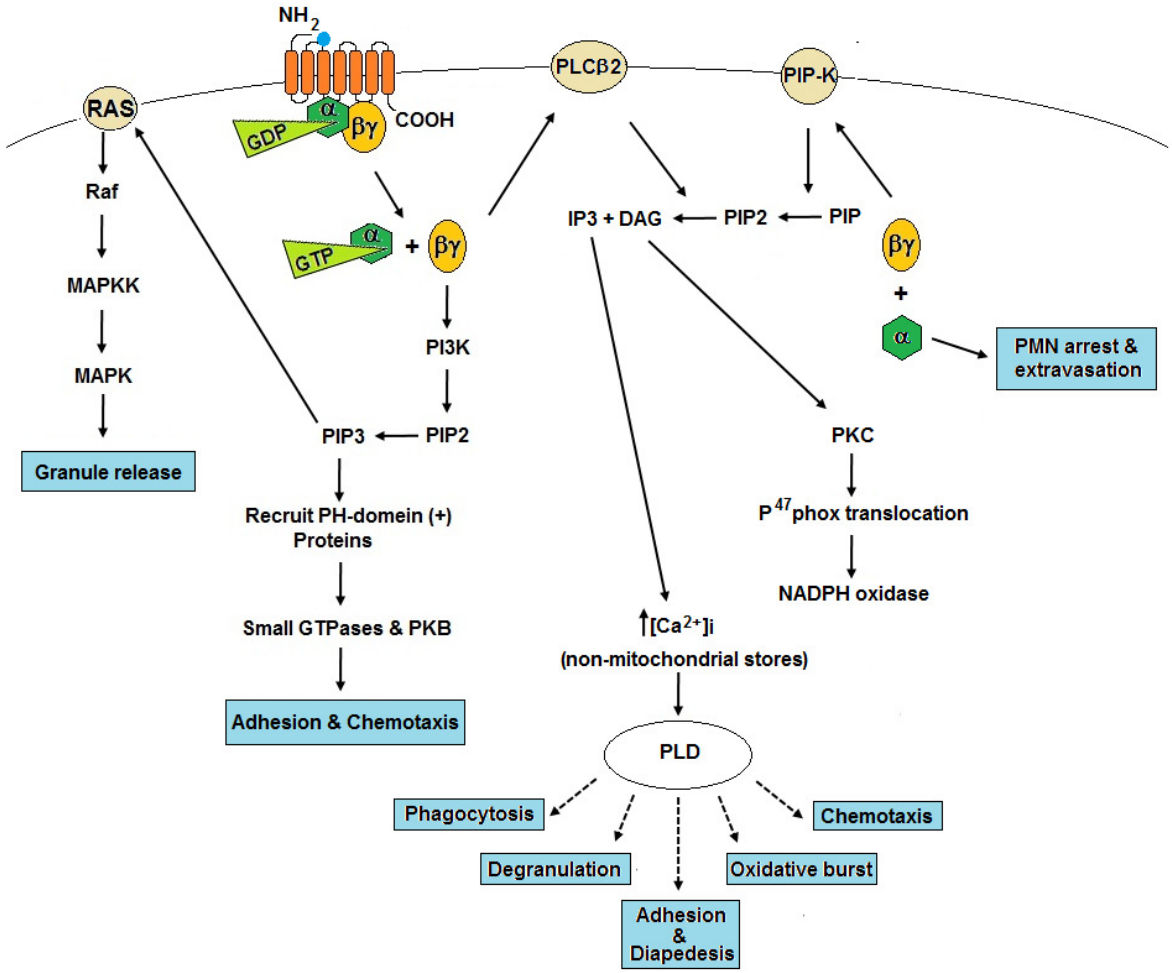

Figure 1. Multiple signaling pathways induced by CXCR1/2.

Chemokine receptors are seven-transmembrane molecules coupled to heterotrimeric G proteins. The β- and γ-subunits are assembled into βγ dimers that act as functional units. The α-subunits bind guanine nucleotides, being active when GTP is bound. The activation of α and βγ sububits leads to the induction of different signaling pathways. PIP-K, Phosphatidylinositol 4-phosphate kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; IP3, inositol 1,4,5-trisphosphate; PKC, protein kinase C; [Ca^{2+}]i, intracellular calcium concentration; PLC, phospholipase C; PI3K, phosphatidylinositol 3-kinase; PH, pleckstrin homology; MAPKK, MAPK kinase. Adapted from [44].

motif is essential for cell activation and ligand binding to CXCR1/2 receptors on neutrophils [50, 51]. The importance of N-ac-PGP in inflammatory diseases, such as COPD, is reinforced by several studies. Clinical data demonstrated that N-ac-PGP can be detected in the bronchoalveolar lavage (BAL) fluid, sputum and serum of COPD patients [50, 52], whereas N-ac-PGP was undetectable in healthy individuals and asthmatics [52]. Moreover, chronic airway exposure to N-ac-PGP causes neutrophil infiltration and lung emphysema in mice [50, 53].

Gaggar *et al.* described the proteolytic cascade that generates the tripeptide PGP from collagen in cystic fibrosis (CF). Using sputum from CF patients, it was shown that MMP8, MMP9 and prolyl endopeptidase (PE) are involved in this multistep pathway [54]. Very recent data from Koelink *et al.* strongly suggests a role for PGP and N-ac-PGP in IBD, since the proteolytic cascade is also present in IBD [55]. Moreover, neutralization of PGP by using RTR (arginine-threonine-arginine) and an anti-PGP antibody significantly reduces intestinal inflammation in a murine IBD model [55].

Neutrophil adherence and transmigration

Neutrophils circulate in the blood as dormant cells [37, 56]. During inflammation, CXC-chemokines are secreted by resident tissue cells, recruited leukocytes, and cytokine-activated endothelial cells. These CXC-chemokines, such as CXCL8 and CXCL1, are locally retained on matrix and cell-surface heparin-like structures, establishing a chemokine concentration gradient surrounding the inflammatory stimulus, as well as on the surface of the overlying endothelium [39]. Neutrophils use a specific set of adhesion and chemokine receptors to migrate from the blood stream to the lung [57-59]. This multistep process of adhesive and migratory events includes selectin-mediated rolling, chemokine-induced activation of integrins and integrin-dependent firm adhesion, leading to transendothelial migration [57]. Figure 2 depicts these four steps in migration. During rolling, neutrophils interact with the endothelial cell surface via selectins binding weakly to mucin-like structures bearing specific carbohydrate moieties [57-59]. These rolling interactions allow neutrophils to sense the CXC-chemokines which are bound to the endothelial cells via heparin-like structures. These chemokines activate the neutrophils via GPCRs, ultimately leading to firm adhesion. Neutrophil firm adhesion to endothelial cells is mediated via interaction between integrins, such as β_2-integrins Lymphocyte Function-associated Antigen 1 (LFA-1; CD11a/CD18; $\alpha_L\beta_2$) and Macrophage 1 Antigen (Mac-1; CD11b/CD18; $\alpha_M\beta_2$) on neutrophils and members of the immunoglobulin superfamily, such as Inter-Cellular Adhesion Molecule (ICAM)-1 and ICAM-2 present on endothelial cells [57-60].

After their firm adhesion, neutrophils crawl over the endothelial cell surface to the nearest junction using their integrins Mac-1 and LFA-1 in a process called locomotion [57], which subsequently leads to neutrophil transmigration. Once in the tissue, neutrophils get activated and migrate towards the pathogen by following the chemokine concentration gradient, after which they kill the pathogen by phagocytosis and/or by releasing cytotoxic proteins from granules. Moreover, neutrophils can extrude strands of DNA with attaching bactericidal proteins that act as extracellular traps for pathogens [37].

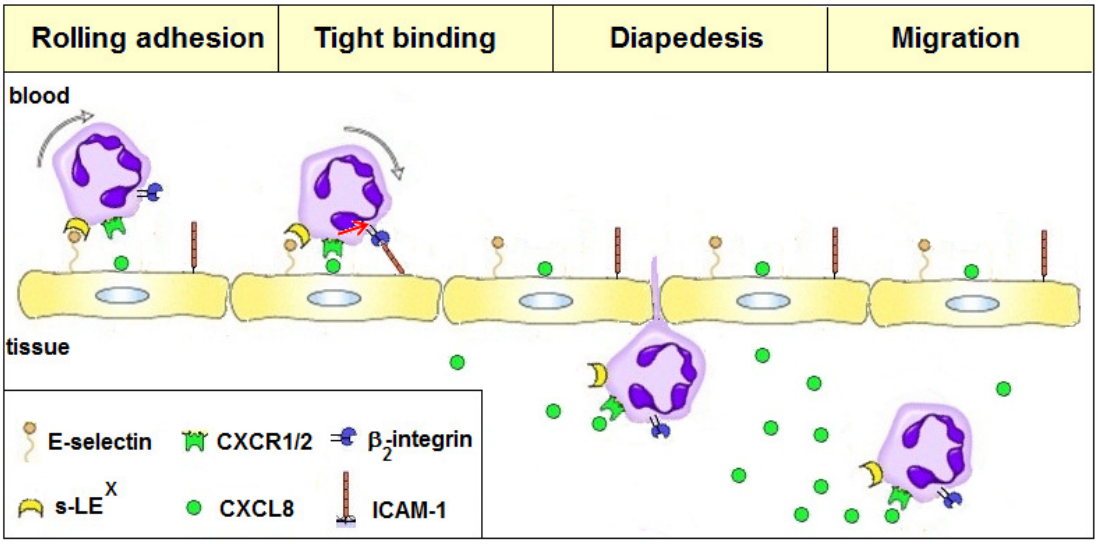

Figure 2. The four steps in neutrophil transmigration.

Neutrophils leave the blood and migrate to sites of infection in a multistep process mediated through adhesive interactions that are regulated by macrophage-derived cytokines and chemokines. Neutrophils reversibly bind to vascular endothelium through interactions between selectins on the endothelium and carbohydrate ligands on the neutrophil (shown here for E-selectin and its ligand the sialyl-Lewis^x moiety (s-Le^x)). This interaction is weak, which allows the neutrophil to roll along the endothelium. However, the neutrophil is arrested on the endothelium after binding ICAM-1 on the endothelium with a CXCL8-activated β_2-integrin (such as Mac-1 or LFA-1). After this tight binding process, the neutrophil squeezes between the endothelial cells forming the wall of the blood vessel. In the tissue, the neutrophil migrates along a concentration gradient of chemokines (here shown as CXCL8) secreted by cells at the site of infection. Adapted from [61].

Phagocytosis, degranulation and synthesis of inflammatory mediators

Neutrophils have different defense mechanisms to eradicate pathogens from the body. One of the defense mechanisms is phagocytosis (engulfment of solid particles by the cell membrane to form a phagosome) and the release of cytotoxic granule proteins [62]. Neutrophilic granules store proteins which are important for the neutrophil to move from the blood stream into the tissue and to kill micro-organisms. There are three types of granules; primary or azurophilic, secondary or specific and tertiary or gelatinase granules. The primary granules confine their release of proteolytic enzymes to the phagosome, whereas the secondary granules secrete antibiotic proteins both into the phagosome and the surroundings [63, 64]. The tertiary granules release MMPs that break down the major components of the basal membrane [63, 65]. Besides granules, neutrophils also contain secretory vesicles. These vesicles quickly provide the membrane proteins needed by the neutrophil for firm adhesion to the activated endothelium and migration into the tissues. One of the membrane proteins is Mac-1 [63], which is important in firm adhesion [57].

A third defense mechanism is the production of ROS. During neutrophil activation, cytoplasmic and membrane components assemble to form a functional multi-component electron-transfer system to catalyze the reduction of molecular O_2 by NAPDH oxygenation. ROS are associated with antimicrobial activity but can also damage host tissue [62].
A fourth defense mechanism is the production of lipid mediators. In response to inflammatory stimuli, activated neutrophils synthesize leukotrienes by cleaving arachidonic acid (AA) from phospholipids by phospholipases. Subsequently, AA is converted to the unstable leukotriene A_4 (LTA_4) and further processing of LTA_4 leads to the production of the potent chemoattractant leukotriene B_4. This chemoattractant released by neutrophils can amplify the immune response by the recruitment of phagocytic cells in an autocrine or paracrine manner and by the stimulation of the production of proinflammatory cytokines [62, 66].
A fifth defense mechanism is the release of neutrophil extracellular traps (NETs). Activated neutrophils can release contents such as chromatin, leading to the formation of a scaffold for the extracellular exposure of antimicrobial proteins and histones [67].
The last mechanism is the synthesis of proteins. After extravasation, neutrophils initiate a second transcriptional burst resulting in the production of various cytokines [68], chemokines, such as CXCL8 [69], and growth factors [70, 71].

The role of neutrophils in COPD and CD

COPD and CD are complex diseases that involve several types of inflammatory cells and inflammatory mediators, such as cytokines, chemokines and proteases [10, 14]. The next section focuses on the neutrophilic component of the chronic diseases. Other inflammatory cells and mediators that play a role in COPD and CD are addressed in chapter 2.

Neutrophilic inflammation in COPD

Neutrophils play a key role in all states of disease; neutrophils are not only abundant in the stable state of COPD, but also during progression or exacerbation of disease increased neutrophil numbers are associated with disease severity [36].
COPD is characterized by an inflammatory response in the lungs as a reaction to inhaled irritants such as cigarette smoke [5]. As early as in the eighties, studies described that smokers have increased neutrophil numbers in BAL fluid in comparison to non-smokers [72, 73]. In COPD, the sputum and BAL fluid from patients contain high numbers of activated neutrophils [74, 75]. In contrast, the increase in neutrophil count is relatively little in the airways or lung parenchyma [76], indicating a rapid transport through the airways.
The disease progression is associated with neutrophilia; the concentration of neutrophils in the BAL fluid and sputum increases with reduced FEV_1 (forced expiratory volume in 1 second) and a longer history of smoking [77, 78]. These neutrophilic numbers are further

increased in the peripheral blood [79, 80] and sputum [79, 81] during exacerbation, with levels correlating with the severity of exacerbation [79].

It has been postulated that these neutrophils migrate into the lungs as a reaction to the CXCL8 released by smoke-activated macrophages and epithelial cells [8]. After transmigration, the neutrophil releases and produces different mediators, including chemokines, serine proteases, myeloperoxidase (MPO), ROS and MMPs [36], which leads to tissue breakdown, mucus hypersecretion, neutrophil recruitment and degranulation and furthering inflammation. In COPD, the levels of the chemokine CXCL8 are increased in sputum and BAL fluid and correlate with the increased number of neutrophils found in the lungs [74, 75, 77]. The recruited neutrophils to the airways of COPD patients are activated, since levels of MPO, a neutrophil activation marker, are increased in the supernatant [82]. These MPO levels increase even further during exacerbation [83]. The neutrophils also show an increase in the respiratory burst response (the rapid release of ROS), which correlates with reduced FEV_1 [84]. In COPD, neutrophils release serine proteases, including elastase, as well as MMP8 and MMP9, which contribute to alveolar destruction [10]. The levels of both CXCL8 and neutrophil elastase (NE) are elevated in BAL fluid [85] from smokers who acquire COPD. Neutrophil elastase is stored in primary granules in neutrophils and this serine proteinase is able to cause structural changes in the lung, including impairment of mucociliary clearance (the self-clearing mechanism of the bronchi) and host defense and induction of mucus secretion [86, 87]. It is inhibited by α_1-AT, and NE/α_1-AT complexes are elevated in BAL fluid from COPD patients [88], and this is correlated with the rate of decline in FEV_1 [89]. Upon activation, neutrophils also release MMPs, including MMP8 and MMP9. In COPD, the sputum levels of these proteolytic enzymes are elevated as compared to non-smokers and healthy smokers [90]. Moreover, during exacerbation, the NE activity and the MMP8 level are significantly increased in sputum [91].

Neutrophilic inflammation in CD

In CD, patients have activated and impaired innate and adaptive immune responses and a loss of tolerance to commensal bacteria [26]. Crohn's disease activity index is associated with increased CD64 expression, a high-affinity Fc receptor on the neutrophil [92]. Neutrophils migrate into the intestinal mucosa as a reaction to the CXCL8 and TNF-α released by macrophages, endothelial cells, intestinal epithelial cells [14, 93]. Neutrophils are predominantly present in the exudates of the crypt abscesses [94].

During active inflammation, in a manner regulated by IL-10, neutrophils release cytokines IL-1β and TNF-α [95]. Moreover, these neutrophils produce CXCL8 [94]. This pro-inflammatory cytokine and chemokine production leads to a further influx of neutrophils and other immune cells. The expression of the CXCL8 gene in CD is related to the grade of active inflammation;

cells expressing CXCL8 were mainly located at the base of ulcers, in inflammatory exudates on mucosal surfaces, in crypt abscesses, and at the border of fistulae. Analysis points to macrophages, neutrophils, and epithelial cells as possible sources of this chemokine in active inflammatory bowel disease [94].

In active CD, neutrophils cause tissue damage by the release of nonspecific inflammatory mediators [25], including proteases [96], ROS [97] and lipid mediators [98]. NE is significantly increased during relapsing periods in CD. The NE plasma levels can be used as a useful independent marker of disease activity, especially for identifying patients in remission [96]. In addition, during acute inflammation in CD, neutrophils express MMP9 and these levels are increased in intestinal fistulae during acute inflammation [99].

The gastrointestinal tract has a powerful enzymatic machinery, which is necessary to form large amounts of ROS [100]. In active CD, neutrophils have enhanced ROS production [101], leading to oxidative stress and a decreased antioxidant status [102]. As the patients improved and became clinically stable, the oxidative parameters decreased, approaching normal values [102].

It has been proposed that the development of CD is a result of the incapacity of macrophages to generate adequate CXCL8 levels. In this hypothesis bacteria and bowel contents go through the permeable mucosal layer into the tissue. In people resistant to CD, the resident macrophages have an inherently high inflammatory response, resulting in high CXCL8 levels and the recruitment of neutrophils, which engulf, digest and expel the exogenous material from the body. However in people susceptible for CD, macrophages respond inherently weak to the exogenous agents; low CXCL8 levels lead to poor neutrophil accumulation and subsequently to macrophage sequestration and the formation of granulomas. Moreover, compensatory pathways, such as signaling through PPRs NOD or TLR, are also weakened. The formation of granulomata elicits a local and systemic inflammation, which is characteristic of Crohn's disease [35, 103-106].

Outline of this thesis

The scope of this thesis is to investigate the neutrophilic inflammation in chronic disease and the self-enforcement of the inflammatory state in the pathogenesis of chronic disease. In this thesis, the neutrophilic migration from the blood stream into the interstitial space is studied.

Chapter 1, the general introduction, describes the scientific rationale and the aims of the thesis. This is further substantiated in **chapter 2**, which provides a review on chemokine receptors in inflammatory diseases of the airway and the intestinal tract.

In **chapter 3** and **4** the human neutrophilic migration is assessed using adhesion and transmigration assays. Neutrophils migrate from the blood stream into the tissue via the

activation of integrins and integrin-dependent firm adhesion, ultimately leading to transendothelial migration. Since cigarette smoking is the major risk factor for COPD and tissue destruction is a hallmark of COPD, the effects of cigarette smoke and N-ac-PGP on neutrophil migration were investigated. **Chapter 3** describes the effect of cigarette smoke on β_2-integrin activation and function in neutrophilic transmigration through endothelium. In **chapter 4**, the influence of N-ac-PGP on β_2-integrin activation and function in neutrophilic firm adhesion to endothelium is presented.

After transmigration, neutrophils are activated to release mediators in the tissue. This thesis continues with describing direct effects of compounds, such as cigarette smoke or N-ac-PGP, on neutrophil activation and mediator release. In **Chapter 5** and **6** the mechanism of N-ac-PGP-induced chemotaxis is investigated in more detail. In **chapter 5** the effect of the collagen fragment on CXCL8 release by human neutrophils is described, thereby investigating the role of N-ac-PGP in the activation of neutrophils which may lead to a self-maintaining situation. In **chapter 6**, the role of CXCR2 in N-ac-PGP-induced neutrophilic migration and activation is assessed in a neutrophil inflammatory mouse model.

Since the processes of neutrophil migration and collagen breakdown contribute to the progression of COPD, this thesis continues with studying the self-propagating cycle of neutrophil infiltration. In **chapter 7**, the effect of cigarette smoke and N-ac-PGP on protease release (MMP8, MMP9 and PE) by human neutrophils is investigated and the generation N-ac-PGP from whole collagen is studied. Furthermore, the effect of N-ac-PGP on the release of the proteases of its proteolytic cascade by human neutrophils was investigated and a PE activity assay was performed to investigate whether this activity is different in neutrophils from COPD patients in comparison to neutrophils from healthy people.

Chapter 8 focuses on another neutrophil-mediated disease and describes the neutrophilic migration and CXCL8 levels in patients with CD. Moreover, the levels of the proteolytic enzymes responsible for PGP generation were assessed in neutrophils from CD patients and healthy donors.

Chapter 9 provides a summarizing discussion of the most relevant findings of this thesis.

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Chapter 2

Chemokine receptors in inflammatory diseases

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Abstract

The traffic of the different types of immune cells is an important aspect of the immune response. Chemokines are soluble peptides that are able to attract cells by interaction with chemokine receptors on their target cells. Several different chemokines and receptors exist enabling the specific trafficking of different immune cells. In chronic inflammatory disorders there is an abundance of immune cells present at the inflammatory site. This chapter focuses on the role of chemokine receptors in chronic inflammatory disorders of the lungs and intestine and the potential of targeting these receptors as therapeutic intervention in these disorders.

Introduction

An immune response is a complex reaction to tissue injury and infection, characterized by the classic response of rubor (redness), calor (heat), tumor (swelling), dolor (pain) and functio laesa (loss of function). The immune system consists of cells and soluble factors that mediate the reaction in order to eliminate the immune stimulus and initiate the process of immunological memory. Immunological diseases occur due to inappropriate inflammation or when the normal immune response progresses to chronic inflammation, either because of a long-term inappropriate response to stimuli (for example allergies) or because the offending agent is not removed (for example autoimmunity).

The major events in chronic inflammatory responses are continuous activating tissue resident immune cells and ongoing infiltration of circulating immune cells after which mechanisms of innate and adaptive immunity serve to neutralize and remove the inflammatory stimulus. Chemokines are a subset of chemoattractant cytokines that promote immune cell trafficking to sites of inflammation [1]. In this review the role of chemokines and their receptors in inflammatory diseases of the airways (COPD) and the intestinal tract (inflammatory bowel disease) is discussed. Investigations of receptor-mediated and intracellular signal pathways in chemokine-receptor interactions might help to develop more effective therapeutic approaches for chronic inflammatory diseases.

Chemokines and their receptors

Chemokines are small (8-15 kD) homologous proteins that are subdivided into families based on the relative position of Cysteine (Cys) residues in the NH_2 terminus of the protein. The two major structural families are distinguished by the arrangement of these Cys residues, either separated by a single amino acid (CXC) or in adjacent positions (CC). Other families are the C and CX3C chemokines [1]. The systemic nomenclature of the chemokines (including mouse homologues) is shown in table 1. Chemokines control cellular trafficking under physiological and pathological conditions, by interaction with their cognate receptors on their target cells. These chemokine receptors comprise a large family of seven-transmembrane domain G protein-coupled receptors, defined by their ability to signal upon binding to one or more chemokines [2]. This leads to an increase in intracellular calcium and subsequent chemotactic migration of the target cells. Chemokine receptors are subdivided into two major classes defined by their ability to bind either CXC or CC chemokines. Several chemokines are able to bind to more than one receptor, and few receptors bind only a single ligand (see table 2, 3 and 4), enabling a high level of redundancy in chemokine receptor function, especially in inflammatory responses.

Table 1. The CXC, CC, C and CX3C chemokines, their functional names and mouse homologues.

Systemic name	Functional ligand	Mouse ligand
CXCL1	GRO-α, MGSA-α	GRO/MIP-2/KC
CXCL2	GRO-β, MGSA-β	GRO/MIP-2/KC
CXCL3	GRO-γ, MGSA-γ	GRO/MIP-2/KC
CXCL4	PF-4	PF4
CXCL5	ENA-78	GCP-2/LIX
CXCL6	GCP-2	GCP-2/LIX
CXCL7	NAP-2	?
CXCL8	IL-8	?
CXCL9	Mig	Mig
CXCL10	IP-10	IP-10/CRG-2
CXCL11	I-TAC	
CXCL12	SDF-1α/β	SDF-1/PBSF
CXCL13	BCA-1	BLC
CXCL14	BRAK/bolekine	BRAK
CXCL15	?	Lungkine/WECHE
CXCL16		
CCL1	I-309	TCA-3
CCL2	MCP-1 / MCAF/TDCF	JE
CCL3	MIP-1α / LD78α	MIP-1α
CCL4	MIP-1β	MIP-1β
CCL5	RANTES	RANTES
CCL6	?	C10/MRP-2
CCL7	MCP-3	MARC
CCL8	MCP-2	MCP-2
CCL9	?	MRP-2/CCF18/MIP-1γ
CCL10	?	MRP-2/CCF18/MIP-1γ
CCL11	Eotaxin	Eotaxin
CCL12	?	MCP-5
CCL13	MCP-4	?
CCL14	HCC-1	?
CCL15	HHC-2	?
CCL16	HCC-4 / LEC	?
CCL17	TARC	TARC/ABCD-2
CCL18	DC-CK1 / PARC / AMAC-1	?
CCL19	MIP-3β / ELC / Exodus-3	MIP-3β / ELC / Exodus-3
CCL20	MIP-3α / LARC / Exodus-1	MIP-3α / LARC / Exodus-1
CCL21	6Ckine / SLC / Exodus-2	6Ckine / SLC / Exodus-2
CCL22	MDC / STCP-1	AMCD-1
CCL23	MPIF-1 / CKβ8	?
CCL24	MPIF-2 / Eotaxin-2	MPIF-2
CCL25	TECK	TECK
CCL26	Eotaxin-3	?
CCL27	CTACK / ILC	ESKine
CCL28	MEC	
XCL1	Lymphotactin/SCM-1α/ATAC	Lymphotactin
XCL2	SCM-1β	?
CX3CL1	Fractalkine	Neurotactine/ABCD-3

Adapted from [3].

Chemokine receptors on immune cells

There are many different cell types in the immune system and these cells interact in a complex reaction of signaling and communication to create the overall response. The cells of the immune system derive from two types of cells in the bone marrow; myeloid stem cells and lymphoid stem cells. Myeloid cells give rise to precursor cells of the innate immune system, whereas lymphoid cells generate precursors of cells of the adaptive immune system. Cells of the innate immune system are the first responders of the immune system. Innate immune cells perform three important tasks:

1) neutralization of infectious agents by secretion of cytotoxic proteins or phagocytosis
2) antigen presentation for activation of the adaptive immune system
3) secretion of numerous cytokines that further amplify the immune response.

The major cell types of the innate immune system include monocytes (blood precursor cells of antigen-presenting cells), antigen-presenting cells (macrophages and dendritic cells (DCs)), granulocytes (neutrophils, eosinophils and basophils), mast cells and natural killer (NK) cells.

While innate immune mechanisms contribute to the first line of defense, at the same time, pathogens are taken up and presented by antigen-presenting cells to adaptive immune cells to allow the induction of an antigen-specific immune response directed against distinctive molecular targets. This process will lead either to a humoral immune response, where antigen-specific immunoglobulins produced by B lymphocytes play a central role, or to a cellular immune response, where antigen-specific CD4+ T lymphocytes (T helper type-1 (T_H1), T helper type-2 (T_H2) and T helper type-17 (T_H17) cells) or CD8+ T cells (cytotoxic T (T_C) cells) are the central players.

Almost all immune cells express chemokine receptors and their migration is dependent on chemokines of which they themselves are an important source. In general, CXC chemokines are attractants for neutrophils and B and T lymphocytes (table 2), while CC chemokines induce chemotaxis of multiple subsets of white blood cells, such as monocytes, basophils, DCs, macrophages, NK cells and T cells (table 3). Chemokines are important for travelling of T cells to the thymus and the CX3C chemokine fractalkine, also acting as an adhesion molecule, seems to be important for the infiltration of T cells, NK cells and monocytes (table 4).

Table 2. CXC chemokine receptor family and inflammatory cells.

Receptor	Ligands	Expression
CXCR1	CXCL6, CXCL7, CXCL8	neutrophil, basophil, monocyt/macrophage, immature DC, NK cell, mast cell
CXCR2	CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8	neutrophil, eosinophil, monocyt/macrophage, immature DC, microvascular endothelial cell, T cell, NK cell, mast cell
CXCR3A	CXCL9, CXCL10, CXCL11	neutrophil, eosinophil, DC, T_H1 cell, B cell, NK cell, mast cell
CXCR3B	CXCL4, CXCL9, CXCL10, CXCL11	microvascular endothelial cell, neoplastic cell
CXCR4	CXCL12	neutrophil, eosinophil, basophil, monocyt/ macrophage, DC, T_0 cell, memory T cell, T_H1 cell, T_H2 cell, T_H17 cell, T_{reg} cell, B cell, NK cell, platelets
CXCR5	CXCL13	B cell, T cell
CXCR6	CXCL16	memory T cell, T_H1 cell, NK cell
CXCR7	CXCL11, CXCL12	tumor cell

[2, 4-13]

Table 3. CC chemokine receptor family and inflammatory cells.

Receptor	Ligands	Expression
CCR1	CCL2, CCL3, CCL3LI, CCL4, CCL5, CCL7, CCL8, CCL13, CCL14, CCL15, CCL16, CCL23	neutrophil, eosinophil, basophil, monocyt/ macrophage, immature DC, memory T cell, B cell, NK cell, mast cell
CCR2	CCL2, CCL7, CCL8, CCL13, CCL16	neutrophil, eosinophil, basophil, monocyt/ macrophage, immature DC, DC, B cell, memory T cell, T_{reg} cell, NK cell
CCR3	CCL5, CCL7, CCL8, CCL11, CCL13, CCL15, CCL16, CCL24, CCL26, CCL28	neutrophil, eosinophil, basophil, T cell, T_H2 cell, mast cell, platelets, endothelial cell
CCR4	CCL17, CCL22	eosinophil, basophil,, monocyt/macrophage, DC, T_H2 cell, T_{reg} cell, NK cell, thymocyte, platelets
CCR5	CCL3, CCL3LI, CCL4, CCL5, CCL7, CCL8, CCL11, CCL13, CCL14, CCL16	monocyt/macrophage, DC, T_H1 cell, T_{reg} cell, B cell, NK cell, thymocyte
CCR6	CCL20	immature DC, memory T cell, T_H17 cell, T_{reg} cell, B cell
CCR7	CCL19, CCL21	DC, T_0 cell, T_H1 cell, T_H2 cell, T_{reg} cell, B cell, NK cell
CCR8	CCL1	neutrophil, monocyt/macrophage, DC, T_H2 cell, T_{reg} cell, B cell, thymocyte
CCR9	CCL25	memory T cell, thymocyte, epithelial cell, IgA+ plasma cell
CCR10	CCL27, CCL28	memory T cell, B cell, fibroblast, epithelial cell

[2, 4, 6, 7, 9-13]

Table 4. C and CX_3C chemokine receptor and inflammatory cells.

Receptor	Ligands	Expression
XCR1	XCL1, XCL2	T cell, NK cell, mast cell
CX_3CR1	CX3CL1	neutrophil, monocyte/macrophage, DC, T_H1 cell, NK cell, endothelial cell

[2, 4, 6, 9-14]

Monocytes and macrophages

Monocytes originate in the bone marrow from a common myeloid progenitor, and are released into the peripheral blood, where they circulate for several days before entering the tissue. In response to inflammation signals, monocytes are quickly recruited from the blood into the tissue were they differentiate into macrophages and dendritic cells, thereby inducing the immune response [15].

Monocytes are divided in two subsets based on the expression of CD14, a component of the lipopolysaccharide receptor complex, and CD16, also known as the FcγRIII immunoglobulin receptor [16]. The first subset is characterized by a high expression of CD14 ($CD14^{hi}CD16^{-}$; $CD14^{+}$ monocyte), whereas the second subset co-expresses CD16 and low levels of CD14 antigens ($CD14^{+}CD16^{+}$; $CD16^{+}$ monocyte) [17]. These subsets express different adhesion, immunoglobulin and scavenger receptors [15]. In addition, the two subsets express distinct chemokine receptors on the cell surface, thereby reacting differently in migration.

The $CD14^{+}$ monocytes, which are also called the classic monocytes, express high levels of the chemokine receptors CCR1, CCR2, CCR4, CCR7, CXCR1 and CXCR2. In addition, these monocytes also express CXCR4 and CX_3CR1, although in lower levels than the pro-inflammatory $CD16^{+}$ monocytes, which also express CCR5. The $CD14^{+}$ monocytes therefore migrate into the tissue under the influence of CCL2, whereas the $CD16^{+}$ monocytes respond to CX3CL1 and CXCL12 [15-17].

The monocytes and macrophages are a huge source for chemokine ligands themselves. Upon stimulation, these cells can release the following chemokine ligands: CCL1-10, CCL12, CCL15-18, CCL20, CCL22-24, CXCL1-3, CXCL6, CXCL8-11, CXCL13, CXCL14 and CXCL16 [9, 11].

Dendritic cells

Dendritic cells (DCs) are antigen-presenting cells which function as sentinels of the immune system [11]. After antigen phagocytosis, the DCs present the antigen to helper T cells, killer T cells and B cells, thereby activating the adaptive immune system [11, 18].

There are two major subsets identified in humans; myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). The two subsets differ in function and can be recruited to promote variable

types of immunologic reactions. mDCs resemble monocytes and secrete IL-12 upon activation [19], whereas pDCs are morphologically similar to plasma cells and differ functionally from mDCs by their ability to produce massive amounts of type I interferons in response to viral and microbial stimuli [19, 20].

mDC precursors in the peripheral blood express CCR2 and CXCR4. After migration into peripheral tissues, they differentiate to become immature mDCs. The chemokine receptor repertoire changes drastically from two into a whole range of chemokine receptors including CCR1, CCR2, CCR5, CCR6, CXCR1, CXCR2 and CXCR4. After activation and changing in the mature phenotype, the cells lose the ability to express almost all the chemokine receptors except CCR7 and CXCR4 [11, 18].

Blood pDCs express a different chemokine receptor pattern, including CCR2, CCR5, CCR7, CXCR3, and CXCR4. However, only CXCR3 and CXCR4 have been demonstrated chemotactic for the pDCs [20].

DCs can be a substantial source of chemokine ligands. Upon stimulation, immature mDCs can release CCL3-5, CXCL8 and CXCL10, whereas mature mDCs release CCL17-19, CCL22, and CX3CL1 to attract NK, T and B cells [11, 18, 21]. Following stimulation, pDCs mostly release CCL3, CCL4, CCL22 and CXCL8 [22].

Neutrophils

Neutrophils are polymorph nuclear cells that are critical for defense against bacterial and fungal infections. Neutrophils are traditionally known to express only a very limited number of chemokine receptors and the recruitment of these phagocytes to the side of infection is mainly directed by the CXC chemokine subfamily, in particular CXCR1 and CXCR2 [12, 14, 23]. However, the expression pattern of the chemokine receptors on the neutrophil is subjected to change. Hartl *et al.* described that neutrophils, while infiltrating at the pulmonary and synovial site of inflammation, acquire a distinct chemokine receptor expression repertoire enabling them to adapt to chronic inflammatory conditions. In addition to the expression of CXCR1 and CXCR2, the infiltrated neutrophils from patients with chronic inflammatory lung diseases and rheumatoid arthritis express CCR1, CCR2, CCR3, CCR5, CXCR3, and CXCR4 [14].

Neutrophils can not only bind chemokine ligands via the chemokine receptors. Upon stimulation, neutrophils can also be a source for various chemokine ligands by releasing CCL2-4, CCL19, CCL20, CXCL1-3 and CXCL8-11 [9, 11].

Eosinophils

Eosinophils are polymorph nuclear cells that are associated with allergy and are thought to be responsible for the defense against parasites. These granulocytic leukocytes derive from

the bone marrow under the influence of IL-3, IL-5 and granulocyte-macrophage colony-stimulating factor [9]. Under homeostatic conditions, eosinophils leave the bone marrow and migrate to the gastrointestinal tract. However during inflammation, both maturated eosinophils and eosinophil progenitors leave the bone marrow and enter the bloodstream under the influence of chemotactic signals released by activated endothelial cells [24]. The cells leave the bloodstream near sites of allergic inflammation and migrate into the tissue toward the site of inflammation. After activation by cytokines released by T_H2 cells, these cells differentiate to mature effector cells, after which they release toxic granule proteins and free radicals, thereby killing microorganisms and parasites [9, 24].

Eosinophils can express several chemokine receptors, such as CCR1, CCR2, CCR4, CXCR2, CXCR3 and CXCR4 [2, 6, 7, 10-12]. However, CCR3 is the chemokine receptor which is most highly expressed by eosinophils [2, 6, 7, 9-12].

The presence of eosinophils in the lung tissue and alveolar space is one of the most profound characteristics of allergic asthma. The migration of these cells out of the blood and into the lung tissue is mediated by a gradient of CCL3, CCL7 and CCL22, expressed by lung macrophages. This gradient changes near the airway epithelium, where CCL5 and CCL11 are highly expressed [11, 25]. However, CCL5 and CCL11 are not solely responsible for the eosinophil migration to the lungs. The other CCR3 ligands CCL7, CCL8 and CCL13 might also play a role in the migration [9, 11, 25]. CCL11 uniquely binds to CCR3 on the eosinophils. In addition, two other CCR3 ligands, CCL24 and CCL26, are also known to serve as a specific eosinophil chemoattractant acting via CCR3. However, the expression of CCL24 and CCL26 has not been reported in asthmatics [25].

Activated eosinophils augment the immune response by releasing chemical mediators, such as prostaglandins, leukotrienes, cytokines and chemokines. The chemokines released by eosinophils are CCL3, CCL4, CCL11 and CXCR5 [9, 11].

Basophils

Basophils are derived from the bone marrow and are the least abundant granulocytes. Their function is poorly understood. Normally, these cells are rarely found in tissue. However, during allergic reactions in for instance the lungs or the skin, their numbers increase considerably [26].

Basophils can express several chemokine receptors, such as CCR1, CCR2, CCR3, CCR4, CXCR1 and CXCR4 [2, 6, 7, 10-12, 27]. Uguccioni *et al.* described that CCR3 expressed on the basophil is mainly responsible for the chemotaxis from the blood to the site of inflammation. These basophils migrate in response to CCL5 and CCL13. Interestingly, this study also showed that CCL11, known to be a specific eosinophil chemoattractant, is

chemotactic for basophils [27]. Basophils can be a source for the chemokine ligands CCL4 and CXCL8 [9, 11].

Mast cells

Mast cells arise from bone marrow-derived progenitor cells that circulate as undifferentiated $CD34^+$ mononuclear cells in the peripheral circulation. Subsequently, these undifferentiated $CD34^+$ cells migrate into tissue and mature under the influence of locally derived growth factors and cytokines.

Mast cells contain large granules that store various mediators, such as histamine. Upon stimulation, mast cells release the contents of the granules, leading to a hypersensitivity reaction. Mast cells have a pivotal role in allergic diseases such as asthma [28].

Human mast cells, cultured from different sources including the human lung, cord blood progenitors and bone marrow progenitors, have distinct chemokine patterns. For instance, human lung mast cells express CCR1, CCR3, CCR4, CCR7, CXCR1, CXCR3, CXCR4 and CXCR6 [9, 28], whereas the cord blood mast cells express also CCR5, CXCR1 and CXCR2, but no CCR7, CXCR3 and CXCR6 [28, 29] and the bone marrow mast cells express CCR1, CCR4, CCR7, CXCR2, CXCR4 and CXCR6 [28]. Moreover, in addition to the previously mentioned chemokine receptors, human mast cells can also express CX_3CR1 and XCR1 [9, 11].

Human mast cells can release chemokines after cross-linking of the IgE receptor. These chemokines are CCL1-5, CCL7, CCL11, CCL19, CXCL1, CXCL2, CXCL5, CXCL8 and XCL1 [9, 11, 25].

Natural killer cells

Natural killer (NK) cells are cytotoxic lymphocytes, which kill tumors and virus-infected cells. Two major NK cell subsets have been identified in humans: $CD56^{dim}$ $CD16^+$ ($CD56^{dim}$; ~ 90% of all NK cells) and $CD56^{bright}$ $CD16^-$ ($CD56^{bright}$; ~ 10% of all NK cells) [30, 31]. The two subsets have distinct functional activity, cell-surface antigen expression and chemokine receptor expression [30]. Freshly isolated, unstimulated $CD56^{dim}$ NK cells mediate natural killing and antibody-dependent cellular cytotoxicity. In a resting state, they express CXCR1, CXCR2, CXCR3, CXCR4, and CX_3CR1 and their migration is mainly mediated by CXCL12 and CXC_3L1. There are no detectable levels of CC chemokine receptors on the cell surface [30].

Freshly isolated $CD56^{bright}$ cells have weak cytolytic activity and are thought to predominantly regulate other cells through cytokine production. In contrast to the $CD56^{dim}$ NK cells, resting $CD56^{bright}$ NK cells express little CXCR1, CXCR2, and CXC_3R1 but high levels of CCR5 and

CCR7. These NK cells migrate in response to CCL19, CCL21, CXCL10, CXCL11, and CXCL12 [30].

In addition, other studies reveal that NK cells can also express other chemokine receptors. Pokkali *et al.* demonstrated that NK cells during a *Mycobacterium tuberculosis* infection change their chemokine pattern; $CD56^{dim}$ NK cells express higher levels of CCR5, whereas $CD56^{bright}$ cells increase the expression of CCR1, CCR2 and CCR7 [31]. Moreover, NK cells are also known to express CCR4, CCR6 and XCR1 [2, 9-12]. NK cells can produce several chemokines upon stimulation such as CCL1, CCL3-5, CCL15, CCL22, CXCL8 and XCL1-2 [9, 11, 30].

B lymphocytes

B cells are one of the two major types of lymphocyte. These bone marrow derived cells express a cell-surface immunoglobulin, also referred to as a B cell receptor. Upon activation by an antigen, B cells differentiate into plasma cells and produce antibodies of the same type as their receptor [32].

After being released from the bone marrow, mature B cells migrate to the secondary lymphoid organs in a process called homing, which is a CXCR5/CXCL13 dependent process. All mature B cells, including re-circulating follicular B cells, marginal zone (MZ) B cells, and peritoneal B1 B cells express CXCR5. The B cells migrate to the lymph node under the influence of CXCL13, a chemokine ligand released by B cell follicles [33].

After antigen activation, B cells upregulate CCR7 and subsequently relocate from the follicle to the B/T zone. After interaction with antigen-specific T cells, B cells differentiate into activated antibody-secreting plasma cells. Due to the downregulation of CCR7 and CXCR5 and an upregulation of CXCR4, the plasma cells relocate to the red pulp of the spleen and the medullary cords of lymph nodes. CXCR4 expression is also essential for plasma cell homing to the bone marrow [12, 32, 33].

However, B cells are also capable of expressing other chemokine receptors than previously mentioned. Studies have demonstrated that B cells can also express CCR1, CCR2, CCR5, CCR6, CCR8-10 and CXCR3 [2, 6, 7, 10-12, 34]. B cells can produce several chemokines upon stimulation such as CCL15, CCL22, CXCL14 and CXCL16 [9, 11].

T lymphocytes

T cells are bone marrow derived lymphocytes. There are six different types of T cells identified in humans; helper T cells (T_H), cytotoxic T cells (T_c), memory T cells, regulatory T cells (T_{reg}), natural killer T cells (NKT) and $\gamma\sigma$ T cells. All types have different roles in the adaptive immune systems and consequentially distinct chemokine receptor expression patterns.

Naïve $CD4^+$ T cells leave the thymus expressing abundant amounts CCR7, migrating in response to CCL19 and CCL21, which are produced by the high endothelial venules of lymph nodes. In addition, naïve $CD4^+$ T cells also express CXCR4. However, this receptor is not essential for entering the lymph nodes [9, 25, 32, 33]. Shortly after activation, the naïve $CD4^+$ T cells differentiate into T_H0 cells, expressing CCR7, CCR5 and CXCR3. Subsequently, the T_H0 cells develop in T_H1 or T_H 2 cells, depending on the environment. These helper T cells have distinct chemokine patterns; T_H1 cells express CCR5 and CXCR3, whereas T_H2 cells express CCR3, CCR4, CCR8 and CXCR4 [9, 25]. However, when the helper T cells or cytotoxic T cells differentiate into effector T cells, the chemokine receptor profile changes drastically, enabling them to migrate into the tertiary lymphoid tissues. This migration is organ specific and chemokines provide a "homing signal" for the effector cells expressing the correct chemokine receptor [9, 35].

$CD4^+$ T_H0 cells can also develop in T_H3 (or T_r1; adaptive $CD4^+$ regulatory T cell), $CD4^+CD25^+FoxP3^+$ regulatory T cells ($CD4^+$ T_{reg} cells), T_H17 or T_HF (Follicular helper T cell) cells, who all have a distinct chemokine receptor pattern. T_H3 cells can express CCR6 [36], whereas $CD4^+$ Treg cells can express CCR2, CCR4, CCR5, CCR7 and CXCR4 [37]. T_H17 cells are capable of co-expressing CCR6 and CCR4 [36, 38] and T_HF cells can express CXCR5 [39].

Memory $CD4^+$ and $CD8^+$ T cells can be divided in "effector" and "central" memory T cells. Effector memory cells are thought to home to non-lymphoid tissues, whereas the central memory T cells localize more to secondary lymphoid tissue [35]. Although originally distinguished by the expression of CCR7 by the central memory T cells, it is clear that both subsets express CCR7 [35, 40]. However, it is thought that these memory cells can express every appropriate chemokine receptor [35].

Most peripheral blood NKT cells express CCR1, CCR2, CCR5, CCR6, CXCR3, CXCR4 and CXCR6, which mediate homing to extra-lymphoid tissue or sites of inflammation. However, a few NKT cells express lymphoid tissue homing chemokine receptors CCR7 and CXCR5 [9].

γσ T cells represent a small subset of T cells that express a distinct T cell receptor (TCR) repertoire on their surface; these cells have a TCR composed of a *γ* and a *σ* chain instead of an *α* and *β* chain. *γσ* T cells can express CCR1-3, CCR5, CCR9, CXCR1-3 and CXCR5 [39, 41, 42]. All T cells are capable of releasing chemokine ligands. After the appropriate stimulation, T cells can produce CCL1, CCL5, CCL15, CCL20, CCL22, CCL24, CXCL8, CX3CL1, XCL1 and XCL2 [9, 11].

Chemokine receptors in chronic inflammatory diseases

Chemokine receptors in COPD

COPD, a term referring to two lung diseases: chronic bronchitis and emphysema, is characterized by airflow limitation that is not fully reversible, is usually progressive, and is associated with an abnormal inflammatory response of the lungs to noxious particles or gases [43]. COPD is primarily associated with cigarette smoke where recurrent lung inflammation leads to a progressive decline in lung function. The inflammatory cascade starts with exposure to cigarette smoke or other irritants, which activate the epithelial cells and the macrophages in the respiratory tract. When these cells are activated they acquire the capacity to release several chemokines which induce inflammatory cell migration to the airways (see fig. 1). Most of the pathologic changes caused by inflammation are found in the small airways and in lung parenchyma [44].

COPD is a complex inflammatory disease that involves different inflammatory cell types, like macrophages, neutrophils, and $CD8^+$ T cells [45, 46]. CCL2 and the receptor CCR2 are involved in the recruitment of monocytes into the airway epithelium in COPD. During migration into tissues, these monocytes differentiate into macrophages*;* an increased number of macrophage is observed in the lungs of COPD patients [47-49] and CCL2 levels are increased in sputum, bronchoalveolar lavage (BAL) fluid and lungs of patients suffering from COPD [49].

One of the most important chemokines in the recruitment of inflammatory cells in COPD is CXCL8 [46]. CXCL8 is secreted by several cell types, like macrophages, neutrophils and airway epithelial cells and is a powerful chemotactic mediator for neutrophils (fig. 1). CXCL8 binds to both CXCR1 and CXCR2 chemokine receptors, which are expressed on a broad range of leukocytes, predominantly neutrophils. COPD patients have increased expression of CXCR1 on circulating neutrophils compared to healthy controls [50], and the expression of CXCR2 in bronchial biopsies of COPD patients is increased [51]. The CXCR2 receptor is also selectively activated by CXCL1, which is secreted by alveolar macrophages and airway epithelial cells (Fig. 1). The concentration of neutrophils is increased in the sputum and BAL fluid of COPD patients and this is related to the increased production of CXCL8 and CXCL1 [46, 48, 52-54]. The CXCR2 receptor responds not only to CXCL8 and CXCL1, but also to other chemokines including, CXCL2, CXCL3, CXCL5, CXCL6 and CXCL7 (table 2). CXCL5 is predominantly derived from epithelial cells and BAL fluid cells from smokers release more CXCL5 than cells from non-smokers [55]. CXCL7 is chemotactic for neutrophils as well as for monocytes and shows an enhanced chemotactic activity for monocytes from COPD patients which is similar to the chemotactic activity of CXCL1 [56].

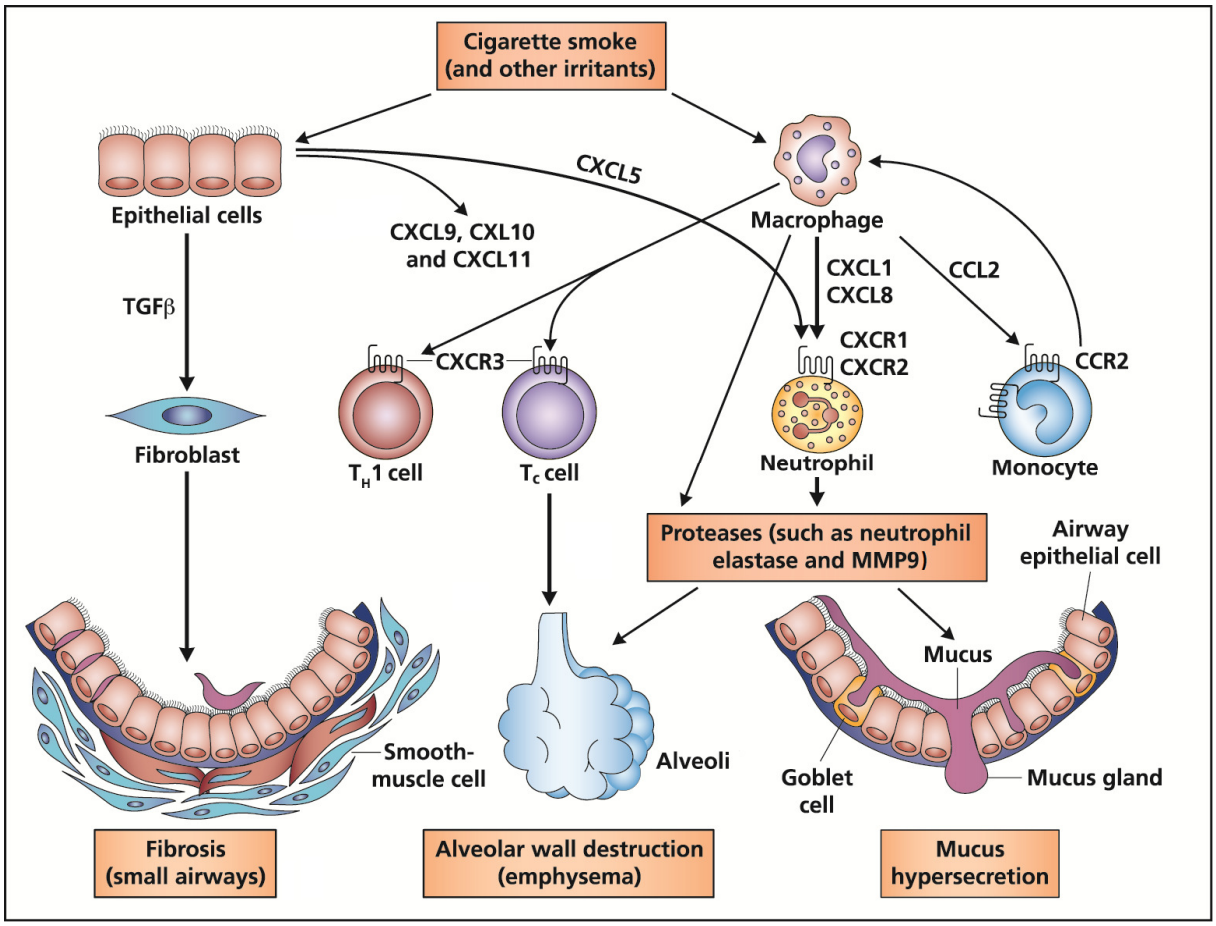

Figure 1. Involvement of chemokines and chemokine receptors and different cell types in the inflammation of COPD.

Chemokines released from epithelial cells and macrophages in the lung recruit inflammatory cells from the circulation leading to the development of COPD. Adapted from [46].

The tripeptide Proline-Glycine-Proline (PGP), a collagen breakdown product which is extensively formed during airway remodeling in COPD, is also a powerful neutrophil attractant via CXCR2 [57-60]. Blocking CXCR2 by small-molecule inhibitors/antagonists reduces neutrophilic infiltration in the lungs of mice either induced by intratracheal PGP installation [61] or cigarette smoke exposure [62, 63]. Moreover, CXCR2 blockage reduces neutrophilic infiltration induced by ozone in the lungs of healthy subjects [64]. Recently, two clinical trial phase I studies with the CXCR2 antagonist, GSK 1325756, were completed. The first study aimed at evaluating safety, pharmacokinetics and pharmacodynamics of oral single and repeated doses of GSK 1325756 to healthy male volunteers (Clinicaltrials.gov: NCT01209052). The second study investigated the effects of age, gender and food on the pharmacokinetics of oral administered GSK 1325756 (Clinicaltrials.gov: NCT01209104). Unfortunately, the results of these studies are at present not available.

In addition to macrophages and neutrophils, T cells are also important in the initial inflammatory process leading to COPD, reflected by increased number of T cells in the airways and in lung parenchyma of COPD patients, especially in CD8+ T cells [65, 66]. $CD4^+$ T_H1 and $CD8^+$ T cells express CXCR3 (Fig. 1). In the airways of COPD patients an increase in the number of $CXCR3^+$ T cells and an increased expression of CXCR3 was observed [67]. T cells may be attracted to the lungs by interferon(IFN)-γ-induced CXCR3 receptor ligands such as CXCL9, CXCL10 and CXCL11, that are present at high levels in COPD airways [67, 68]. Kelsen *et al.* demonstrated that human airway epithelial cells also express CXCR3 and that activation of CXCR3 by CXCL9, CXCL10 and CXCL11 may contribute to airway inflammation/remodeling in the development of COPD [69]. Furthermore, $CXCR3^{-/-}$ mice showed less lung inflammation induced by cigarette smoke exposure compared to wildtype mice [70]. The CCR5 receptor is also expressed on T cells and might have a cooperative role with CXCR3 in the recruitment of these cells into the lungs [71]. The CCR5 ligand CCL5 is elevated in sputum from COPD patients, and this increase is also observed in the airways and sputum of COPD patients during exacerbations [68, 72].

Due to the activity of these inflammatory cells as well as the epithelial cells the inflammatory response in COPD is further augmented leading to the induction and release of different proteases, including, matrix metalloproteinases (MMPs, e.g. MMP9) and neutrophil elastase. This proteolytic cascade leads to the remodeling of the lung tissue by collagen and elastin degradation (emphysema) and mucus hypersecretion (chronic bronchitis). The protease-antiprotease imbalance hypothesis is thought to play a key role in the development of COPD [52, 73, 74]. Finally, epithelial cells and macrophages in the small airways also regulate the proliferation of fibroblasts by releasing transforming growth factor(TGF)-β, resulting in fibrosis, a clinical feature of COPD [75].

Chemokine receptors in inflammatory bowel diseases

The term inflammatory bowel disease (IBD) is used to describe chronic inflammatory conditions of the gastro-intestinal tract. Crohn's disease (CD) and ulcerative colitis (UC) are the two major forms of IBD, and although the clinical pathological phenotypes are similar, they can be separated by different localization of the inflammation in the gastro-intestinal tract and immunological and histological pattern. CD is characterized by a transmural inflammation in any part of the gastrointestinal tract while UC is a mucosal inflammation restricted to the colon. CD is postulated to be a T_H1 and T_H17-mediated disease [76, 77], whereas UC is mainly a T_H2-mediated disorder [78]. The exact etiology of IBD remains unknown but is thought to be a complex interaction of genetic, environmental (i.e. enteric microflora) and immunological factors [79, 80]. See figure 2 for the involvement of chemokines, chemokine receptors and different cell types in the development of IBD.

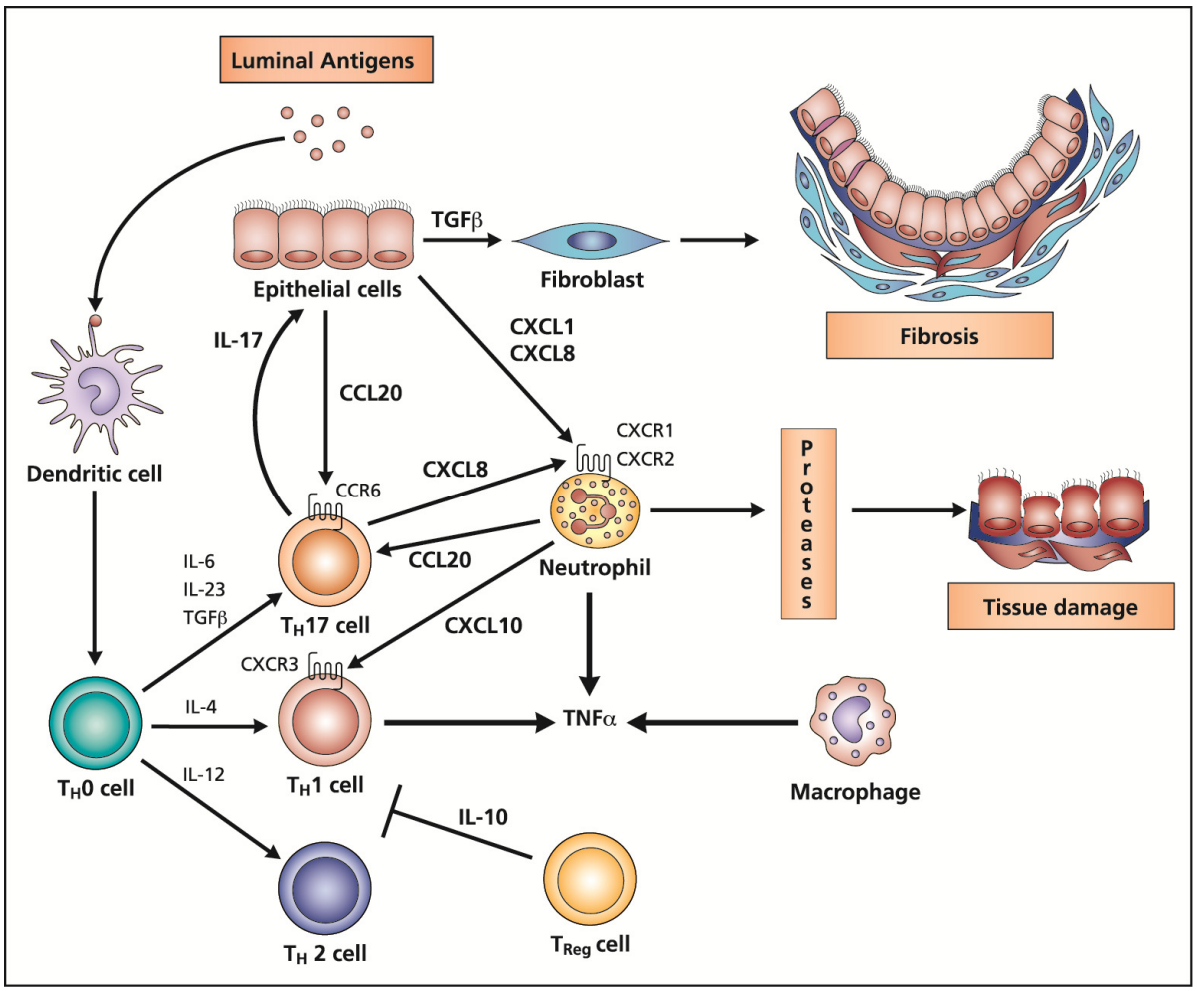

Figure 2. Involvement of chemokines, chemokine receptors and different cell types in the development of IBD.

Tumor Necrosis Factor (TNF)-α, produced by macrophages, DCs, neutrophils and T_H1 cells, is responsible for pro-inflammatory signaling pathways, like cytokine production and apoptosis. Anti-TNF-α treatment is one of the most promising therapies in IBD nowadays [81, 82]. The contribution of chemokines to the pathogenesis of IBD stems from a series of clinical and animal model studies [83, 84]. Several chemokines have been described in both UC and CD and their expression is consistently increased during the active phase of disease. Especially CXCL8, expressed by intestinal epithelial cells, macrophages, fibroblasts and neutrophils, is significantly increased in the mucosa of IBD patients [85-89], and also the corresponding receptors, CXCR1 and CXCR2, are increased [90]. Circulating neutrophils from IBD patients show increased mRNA levels of CXCR1 and CXCR2, and show an enhanced migratory capacity compared with neutrophils from healthy subjects [91]. Also other CXCR2 chemokines, CXCL1 and CXCL5 are up regulated in IBD [92, 93]. In experimental mouse models for IBD the expression of CXCL1 (the mouse homologue for CXCL8) and CXCL2 is increased and associated with neutrophil recruitment [94]. Antibodies

against CXCR2 or CXCR2 antagonists are effective in inhibiting acute and chronic intestinal inflammation in mouse IBD models [95-97] and $CXCR2^{-/-}$ mice are less susceptible to the induction of colitis [94].

CXCL9, CXCL10 and CXCL11 are increased in IBD and attract $CXCR3^{+}$ T_H1 and NK cells [83, 98-100]. The expression of CXCR3 is also increased in mucosal specimens of IBD patients, especially in T cells, and the expression of CXCR3 in intestinal epithelial cells is increased upon stimulation with pro-inflammatory cytokines. Targeting T_H1 cells via CXCR3 by neutralization of CXCL10, with anti-CXCL10 antibodies, lead to a decrease in intestinal inflammation and subsequent damage [100], although others could not confirm this [101]. Phase II clinical trials with a fully human anti-CXCL10 antibody (MDX 1100) in patients with IBD are currently conducted [102]. In addition, the CCR1-3 and 5 ligands CCL2 and CCL3, CCL4, CCL5 and CCL7 and CCL8 are reported to be increased in IBD [83, 84, 98, 100]. $CCR2^{-/-}$ and $CCR5^{-/-}$ mice exhibited a reduction in colonic inflammation [103], and blockade of CCR2, CCR5 and CXCR3 by a non-peptide antagonist (TAK 799) showed clinical improvement in experimental colitis [104]. Notably, CCL5 has been shown to be crucial in the transition from acute to chronic disease [105].

T_H17 cells are getting more and more attention in relation to IBD, especially in CD [77]. CCR6 has been identified on T_H17 cells [106] in inflammatory lesions of CD patients [107], and colonic epithelial cells from IBD patients show an increased expression of CCL20, the ligand for CCR6 [64, 108]. Activated neutrophils can also attract both T_H17 and T_H1 cells by the production of CCL20 and CXCL10. T_H17 cells are in turn able to attract more neutrophils by the production of CXCL8, providing an accumulation loop and co-localizing neutrophils and T_H17 cells in the intestines of CD patients [109]. Neutralizing CCL20 significantly ameliorated murine colitis, which was associated with a decreased influx of $CCR6^{+}$ T_H17 cells in the lamina propria [110]. CCR9 is also abundantly expressed on intraepithelial and lamina propria T cells, and infiltration of these $CCR9^{+}$ T cells into the intestinal mucosa has been shown to play a role in IBD. These mucosal $CCR9^{+}$ T cells might be T_H17 cells, since it has been demonstrated that $CCR9^{+}$ T cells can produce IL-17 [111]. Recently, the results of a phase II clinical trial using an orally bioavailable CCR9-specific antagonist (Traficet-EN) in CD patients were presented [112]. Oral treatment with the CCR9 receptor antagonist resulted in reduced disease severity. However, it was also shown that $CCR9^{-/-}$ mice are more susceptible to the induction of experimental colitis [113], and that CCL25-CCR9 signaling induces the migration of T_{reg} cells to the intestine [114], raising concerns about the disruption of this pathway as treatment of IBD.

T_{reg} cells are equipped with immunosuppressive activities and it has been hypothesized that reduced T_{reg} function is related to exacerbations in IBD. Transferring T cells without the T_{reg} population into immunodeficient mice results in colitis, which is used as an experimental IBD

model: the transfer model [115, 116]. This can be cured by co-transferring the T_{reg} cells [117]. The suppressive capacity of T_{reg} cells in IBD depends on their migration into the intestine and this is a promising target for the therapy of IBD. Three reports describe the critical role of CCR4, CCR6 and CCR7 in the homing of T_{reg} cells to the intestine, as $CCR4^{-/-}$, $CCR6^{-/-}$ and $CCR7^{-/-}$ T_{reg} cells are not able to cure or prevent colitis in the transfer model [118-120]. IL-10 is an important cytokine in the function of T_{reg} cells. IL-10 -/- mice develop spontaneous colitis highlighting the role of this cytokine in intestinal tissue homeostasis and IBD [121].

Both CXCL12 and CXCR4 are up regulated in IBD. CXCL12 is expressed by intestinal epithelial cells and has a chemotactic effect on CXCR4 expressing T cell subsets which could be blocked by anti-CXCR4 antibodies [122]. In mouse models for IBD in which CXCL12 and CXCR4 are up regulated, a CXCR4 antagonist could decrease infiltration of T cells and reduce damage [123, 124].

CX3CL1, probably originating from epithelial cells, and CX_3CR1^+ T cells are enhanced in active CD [125, 126]. CX_3CR1 is also expressed by macrophages and DCs in the intestine. Treatment of murine colitis models by neutralizing CX3CL1, with an anti-CX3CL1 antibody, reduced clinical signs [127] and $CXC3R^{-/-}$ mice are less susceptible to Dextran Sodium Sulfate (DSS)-induced colitis [128].

Similar to COPD the activity of the attracted immune cells leads to tissue destruction by proteases, mainly produced by neutrophils. A variety of cells, including epithelial cells, release TGF-β thereby regulating the proliferation and trans-differentiation of fibroblasts and the development of T_H17 cells [129, 130].

Chemokine receptor expression is not restricted to immune or epithelial cells but is also found on neurons. Neurons and glial cells can produce chemokines such as CXCL8 and CCL4 during intestinal inflammation [131]. *In vitro* studies have shown that intestinal epithelial cells can up regulate neuronal CXCL8 and CCL3 mRNA expression, mediated by the IL-1β receptor, and that pretreatment of neuronal cells with IL-1β results in the CXCL8 dependent chemotaxis of peripheral blood mononuclear cells [131]. Production of neuronal chemokines could therefore be responsible for the presence of leukocytes, monocytes and lymphocytes in the enteric nervous system, which could ultimately lead to the development of enteric neuropathies and central nervous system-mediated symptoms as observed in IBD.

Conclusion

Taken together, chemokines and their respective receptors play an important role in chronic inflammatory diseases, like COPD and IBD. Chemokine receptors can therefore be considered as promising drug targets in these diseases. However, most of those compounds targeting chemokine receptors shown to be successful in *in vitro* and animal disease models have failed in clinical trials as single therapy in these diseases. Further research should focus on a more multi-targeted approach via antagonizing more than one chemokine receptor or targeting the chemokines themselves.

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Chapter 3

Cigarette smoke induces β_2-integrin-dependent neutrophil migration across human endothelium

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Abstract

Cigarette smoking induces peripheral inflammatory responses in all smokers and is the major risk factor for neutrophilic lung disease such as chronic obstructive pulmonary disease (COPD). The aim of this study was to investigate the effect of cigarette smoke on neutrophil migration and on β_2-integrin activation and function in neutrophilic transmigration through endothelium.

In this report, we demonstrated that cigarette smoke extract (CSE) dose dependently induced migration of freshly isolated human neutrophils *in vitro*. Moreover, CSE promoted neutrophil adherence to fibrinogen. Using functional blocking antibodies against CD11b and CD18, it was demonstrated that Mac-1 (CD11b/CD18) is responsible for the cigarette smoke-induced firm adhesion of neutrophils to fibrinogen. Furthermore, neutrophils transmigrated through endothelium by cigarette smoke due to the activation of β_2-integrins, since pre-incubation of neutrophils with functional blocking antibodies against CD11b and CD18 attenuated this transmigration.

This is the first study to describe that cigarette smoke extract induces a direct migratory effect on neutrophils and that CSE is an activator of β_2-integrins on the cell surface. Blocking this activation of β_2-integrins might be an important target in cigarette smoke induced neutrophilic diseases.

Introduction

Neutrophils play a pivotal role in pulmonary inflammatory diseases such as chronic obstructive pulmonary disease (COPD). COPD is a progressive disease, which is characterized by two major pathological processes, namely bronchitis and emphysema. The neutrophils accumulate in the affected tissues and contribute to the chronic inflammatory reaction, eventually leading to lung destruction [1, 2]. It is generally accepted that cigarette smoking is the most important risk factor for the development of COPD. The WHO estimated that 73% of COPD mortality is related to smoking [3]. However, not only COPD patients have increased neutrophil counts in bronchoalveolar lavage (BAL) fluid and sputum [4-7]; increased neutrophil numbers are also observed in sputum of smokers without respiratory problems [5, 8]. To migrate from the blood stream to the lung, neutrophils use a specific set of adhesion and chemokine receptors [9-11]. This multistep process of adhesive and migratory events includes selectin-mediated rolling, chemokine-induced activation of integrins and integrin-dependent firm adhesion leading to transendothelial migration. During rolling, neutrophils interact with the endothelial cell surface via selectins binding weakly to mucin-like structures bearing specific carbohydrate moieties [9-11]. These rolling interactions allow neutrophils to sense CXC-chemokines, such as CXCL8, which are bound to the endothelial cells via heparin-like structures. These chemokines activate the neutrophils via G protein-coupled receptors, ultimately leading to firm adhesion. Neutrophil firm adhesion to endothelial cells is mediated via interaction between integrins, such as β_2-(CD18-)integrins Lymphocyte Function-associated Antigen 1 (LFA-1; CD11a/CD18; $\alpha_L\beta_2$) and Macrophage 1 Antigen (Mac-1; CD11b/CD18; $\alpha_M\beta_2$) on neutrophils and members of the immunoglobulin superfamily, such as ICAM-1 and ICAM-2 present on endothelial cells [9-12]. The β_2-integrins are heterodimeric receptors, consisting of an α- and a β-chain that together form a ligand-binding head region with two legs that contain the transmembrane and cytoplasmic domains of each chain [13, 14]. During inflammation, activation of the β_2-integrins is essential, since this leads to a conformational change in structure, going from an inactive, low affinity state to an active, high-affinity state [13]. These conformational changes can be initiated via stimuli received by receptors for chemokines, cytokines or foreign antigens inducing intracellular signals (inside-out signaling) [14] and further strengthened by integrin clustering, transferring signals from the extracellular domain to the cytoplasm (outside-in signaling) [9]. The aim of this study was to investigate the effect of cigarette smoke on neutrophil movement and on β_2-integrin activation and function in neutrophilic transmigration through endothelium. Our findings indicate that cigarette smoke has a direct effect on the migration of neutrophils and that cigarette smoke is an activator of β_2-integrins on the cell surface, leading to firm adhesion and transmigration of neutrophils through endothelium.

Material and methods

Chemicals and reagents

2R4F reference cigarettes were from Kentucky Tobacco Research Institute (Lexington, KY, USA). Recombinant human CXCL8 and rabbit IgG (AB-105-C) were supplied by R&D Systems Europe Ltd. (Abingdon, United Kingdom). $MgSO_4$, glucose, diaminobenzidene and formyl Met-Leu-Phe (fMLP) was purchased from Sigma Aldrich Chemie BV (Zwijndrecht, the Netherlands). Human fibrinogen was supplied by Kordia Life Sciences (South Bend, IN, USA). Human Serum Albumin (HSA) was purchased from Sanquin Blood Bank (Amsterdam, the Netherlands). Trypsin-EDTA (0.05%), fibronectin from human plasma and calcein-AM (1 mM solution in DMSO) were obtained from Invitrogen (Breda, the Netherlands). TNF-α was from Endogen (Woburn, MA, USA). HEPES was obtained from Agros Organics (Geel, Belgium). NaCl, KCl, $K_2HPO_4 \cdot 3H_2O$, $CaCl_2$, NH_4Cl, $KHCO_3$, EDTA (Triplex III), H_2O_2, Mayers' haematoxylin and trisodium citrate dihydrate were purchased from Merck KGaA (Darmstadt, Germany). Ficoll-Paque™ PLUS was purchased from GE Healthcare (Eindhoven, the Netherlands). PBS, Roswell Park Memorial Institute (RPMI) 1640 medium (without L-glutamine and phenol red) and Endothelial cell Growth Medium-2 (EGM-2) BulletKit were obtained from Lonza Verviers SPRL (Verviers, Belgium). FBS was from Perbio Science Nederland BV (Etten-Leur, the Netherlands). Pentobarbital (Nembutal™) was from Ceva Santé Animale (Naaldwijk, the Netherlands). The 5% goat serum and the goat anti-rabbit antibody were obtained from Dakocytomation (Glostrup, Denmark). The rabbit polyclonal CD11b antibody (ab75476) was purchased from Abcam. The streptavidin–biotin complex/ horseradish peroxidase (Vectastain Elite ABC) was from Vector Laboratories. Permount was purchased from Fisher Scientific.

Antibodies

Functional blocking monoclonal antibodies IB4 (mouse IgG_{2a}; α-CD18) and 44A (mouse IgG_1; α-CD11b) were isolated from the supernatant of mouse hybridomas purchased from the American Type Culture Collection (ATCC). Mouse IgG_1 and IgG_{2a} isotype controls were obtained from R&D Systems Europe Ltd. (Abingdon, United Kingdom). Treatment of neutrophils with isotype control antibodies showed no difference in adhesion or transmigration as compared to buffer control, CSE control or fMLP control. Antibody concentrations and pre-incubation periods were adapted from [15-17].

Cells

Isolation of human polymorphonuclear leukocytes

Human polymorphonuclear leukocytes (PMNs) were isolated as previously described [18] from fresh whole blood, for which healthy donors signed written consent forms. The study population consisted of 21 participants, 38.1% men and 61.9% women. The median age was 48 (range: 27-60). Resulting PMN preparations consisted of approximately 95-97% PMNs, based on PMNs physical parameters analyzed by flow cytometry and CD16 expression. The preparations were negative for CD14, meaning that the preparations did not contain monocytes.

HUVEC culture

Human umbilical vein endothelial cells (HUVECs) were isolated as previously described [19] and were provided by J.H. van Kats-Renaud (UMC Utrecht, the Netherlands). The cells were cultured in EGM-2 and were grown to confluence in 5–7 days. Cell passages two to three were used for all experiments.

Cigarette smoke extract (CSE)

CSE was prepared by using a smoking machine (Teague Enterprises, Davis, Ca, USA) as previously described [20]. Direct and side stream smoke from one 2R4F cigarette was directed via a tube through 5 ml PBS using a peristaltic pump. Subsequently, the optical density (OD) of this extract was determined using a spectrometer (UV-mini 1240, Shimadzu) measuring at wavelength 320 nm. Freshly prepared CSE was used in all experiments. Non-toxic solutions ranging from 0.03 to 0.24 OD were used in the present study as determined by Annexin-V staining and FACS analysis. These concentrations correspond to serum values < 10 to 65 ng/ml cotinine, as measured with ELISA. These values of the breakdown product of nicotine are associated with light cigarette smoking or moderate passive exposure, according to the Foundation of Blood Research [21].

Migration assay

The migration assay was performed as previously described [22, 23]. Briefly, indicated reagents were placed in the bottom wells of a 3-µm 96-well polycarbonate filter plate (Millipore BV, Amsterdam, the Netherlands) in RPMI 1640 medium (without L-glutamine and phenol red) supplemented 1% heat-inactivated FBS. 2 x 10^5 PMNs, isolated from fresh whole blood, were added to the top portion. The plate was incubated for 1 h at 37 °C in 5% CO_2. After removing the upper portion, the cells in each bottom well were counted for 30 sec using a BD FACSCalibur Flow Cytometer with CellQuest Pro Software (version 5.2.1.).

Adhesion assay

The adhesion assay was performed according to the procedure as described before [24, 25] with some minor modifications. Briefly, wells of a 96-wells special optics black plate with a clear bottom (Sigma Aldrich Chemie BV, Zwijndrecht, the Netherlands) were coated with 100 µl fibrinogen (10 µg/ml) overnight at 4 °C. Non-specific binding sites were blocked with blocking buffer (0.5% HSA in PBS) for 1 h at 37 °C. Freshly isolated PMNs were loaded with the non-fluorescent dye calcein-AM (1µM) by incubating the cells for 30 min at 37 °C. After labeling, the cells were washed with a HEPES incubation buffer (20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM K_2HPO_4 supplemented with 5 mM glucose, 1 mM $CaCl_2$ and 0.5% (w/v) HSA) and resuspended in this buffer to a concentration of 10^6 cells/ml. Subsequently, 5 x 10^4 cells were added to each well and incubated with indicated reagents for 30 min at 37 °C. After incubation, fluorescence was measured at 485 nm excitation and 535 nm emission using a Mithras LB 940 fluorescence meter (Berthold Technologies, Belgium) before and after three washes with HEPES incubation buffer. The percentage adherence was determined by calculating the fluorescence after three washes as a percentage of the total fluorescence before washing.

Transendothelial migration assay

The transendothelial migration of neutrophils was evaluated using 3 µm 24-transwell system filter plate (Sigma Aldrich Chemie BV, Zwijndrecht, the Netherlands). HUVECs were subcultured to confluent monolayers on the inserts precoated with fibronectin (0.5 µg/ml; 100 µl per insert) and pre-incubated with TNF-α (2 ng/ml; 100 µl per insert) for 5 h. Freshly isolated PMNs were labeled with the non-fluorescent dye calcein-AM (3 µM) by incubating the cells for 20 min at room temperature under gentle agitation. Subsequently, 5 x 10^5 PMNs were added to the upper chamber whereas the indicated reagents were placed in the lower chambers. The plate was incubated for 1 h at 37 °C in 5% CO_2. After removing the inserts, fluorescence was measured of 100 µl suspension of each lower chamber at 485 nm excitation and 535 nm emission using a Mithras LB 940 fluorometer (Berthold Technologies, Belgium). The percentage of transmigration was defined as the fluorescence value of the migrated PMNs divided by the value of total fluorescence, multiplied by 100.

Animals

Male Balb/c mice, 5-6 weeks old were obtained from Charles River Laboratories and housed under controlled conditions in standard laboratory cages in the animal facility. They were provided free access to water and food. All *in vivo* experimental protocols were approved by the local Ethics Committee and were performed under strict governmental and international guidelines on animal experimentation.

Cigarette smoke exposure

The one-week cigarette smoke exposure protocol was performed as previously described [26]. The mice were exposed in whole-body chambers to air (sham) or to air-diluted mainstream cigarette smoke from the reference cigarettes 2R4F using a peristaltic pump. Just before the experiments, filters were cut from the cigarettes. Each cigarette was smoked in 5 min at a rate of 5l/h in a ratio with 60l/h air. The mice were exposed to cigarette smoke using 5 cigarettes twice daily for five consecutive days, except for the first day when they were exposed to 3 cigarettes. The mice were sacrificed 16 h after the last air or smoke exposure.

Immunohistochemistry

Immunohistochemistry was performed according to the procedure as described before [26] with some minor modifications. Mice used for morphometric analysis, were sacrificed by an i.p. injection with an overdose of pentobarbital. The left lung was dissected and fixed with 10% formalin for at least 24 h, after which it was embedded in paraffin. Paraffin sections were deparaffinized, endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol for 30 min at room temperature and rehydrated in a graded ethanol series to PBS. For antigen retrieval, the slides were boiled in 10 mM citrate buffer (pH 6.0) for 10 min in a microwave. The slides were cooled down to room temperature, rinsed with PBS (3x) and blocked with 5% goat serum in 1% BSA in PBS for 30 min at room temperature. Sections were incubated with the primary antibody (rabbit polyclonal CD11b antibody, 1.1 µg/ml) in 1% BSA/PBS overnight at 4 °C. The slides were rinsed with PBS (3x) and incubated with the biotinylated secondary antibody (goat anti-rabbit, 1:200) in 1% BSA/PBS for 45 min at room temperature. The slides were rinsed with PBS (3x) and the biotinylated proteins were visualized by incubation with streptavidin–biotin complex/horseradish peroxidase for 45 min at room temperature, followed by 0.015% H_2O_2/0.05% diaminobenzidene/0.05 M Tris–HCl (pH 7.6) for 10 min at room temperature. Sections were counterstained with Mayers' haematoxylin, dehydrated and mounted in Permount. Negative controls without the primary antibody and normal rabbit IgG were included as controls. Photomicrographs were taken with an Olympus BX50 microscope equipped with a Leica DFC 320 digital camera.

Statistical analyses

For all statistical analyses, GraphPad Prism version 4.0 was used. Two-tailed paired Student *t*-tests were used for comparing two groups. For comparing three or more groups, the data were analyzed using a one-way repeated measures ANOVA followed by Tukey post hoc analysis. Data were considered significant at $P < 0.05$. All results are expressed as mean ± SEM.

Results

CD11b expression in the airways of mice after 5 days smoke exposure

Cigarette smoke exposure to mice leads to a neutrophil influx in BAL fluid and lung tissue [26]. To investigate the expression of CD11b *in vivo*, male Balb/C mice were exposed to air or smoke for 5 days and immunohistological staining for CD11b was performed on lung sections. Smoke exposure resulted in an increase of CD11b expressing cells (fig. 1B), whereas air exposure did not result in CD11b positive cell accumulation in the lung (fig. 1A). Focusing on the morphology of these CD11b expressing cells, it became clear that these cells were neutrophils since these cells showed segmented nuclei characteristic for neutrophils (fig. 1C). These findings indicate that CD11b expressing neutrophils are present in the airways of mice exposed to cigarette smoke.

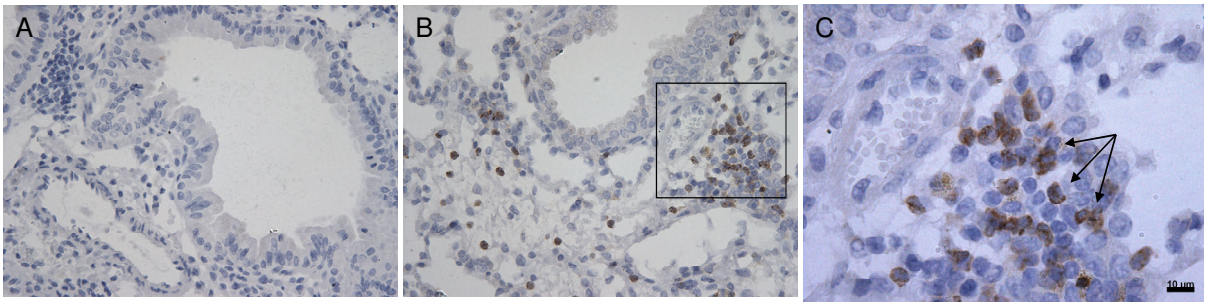

Figure 1. CD11b expression in the lung after 1 week smoke exposure.

*Representative photomicrographs (n = 3) of an immunohistological staining for CD11b (brown color, DAB staining) in lung tissue of air-exposed mice (**A**) and smoke-exposed mice (**B** and **C** (magnified from fig. B)). CD11b expressing neutrophils (indicated with arrows in C) are present in mice exposed to cigarette smoke, whereas air exposed mice did not show an influx of these cells.*

Cigarette smoke extract induces migration of PMNs *in vitro*

The *in vivo* findings described above, led us to the hypothesis that cigarette smoke may induce the migration of neutrophils. A transwell migration system was used to evaluate this effect of CSE on PMNs from fresh whole blood. After 1 h incubation, the migration of PMNs to the lower chamber was quantified. For all experiments, non-toxic concentrations of CSE were used. CSE (OD 0.03-0.24) was dose-dependently chemotactic for PMNs (fig. 2). CXCL8 (10 ng/ml) was tested as a positive control.

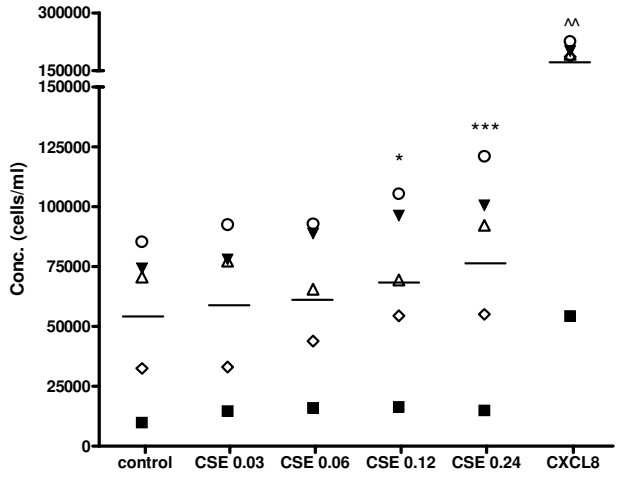

Figure 2. Cigarette smoke extract induces migration of PMNs in vitro.

2 x 10^5 freshly isolated PMNs were placed in the top well and the cigarette smoke extract (CSE) was added to the bottom well of a 96-wells Millipore Filtration Plate System. After 1 h incubation at 37 °C in 5% CO_2, the cells from each bottom well were counted. CSE (OD 0.03-0.24) induces migration of PMNs (, P < 0.05; ***, P < 0.001 CSE vs. control). Legend: each symbol represents a different donor (N = 5, n = 3-9). Horizontal bars represent mean values.*

Cigarette smoke extract induced adhesion of PMNs to fibrinogen is mediated by the activation of Mac-1

Neutrophils migrate from the blood into the extracellular matrix by activating β_2-integrins, such as Mac-1 [9, 10]. This led to the hypothesis that besides its migratory effect, cigarette smoke may activate the integrin Mac-1 on neutrophils, leading *in vivo* to a situation where more neutrophils transmigrate from the blood stream into the lung tissue.

To examine this hypothesis, the effect of CSE on the Mac-1-fibrinogen interaction was investigated. Incubating PMNs with CSE (OD = 0.06-0.24) caused a significant increase in adhesion to fibrinogen compared to medium incubation (fig. 3A). Functionally blocking CD11b or CD18 on the PMN resulted in a complete inhibition of the CSE-induced activation and showed no significant difference with the control (fig. 3B). FMLP (10^{-8}M) was tested as a positive control.

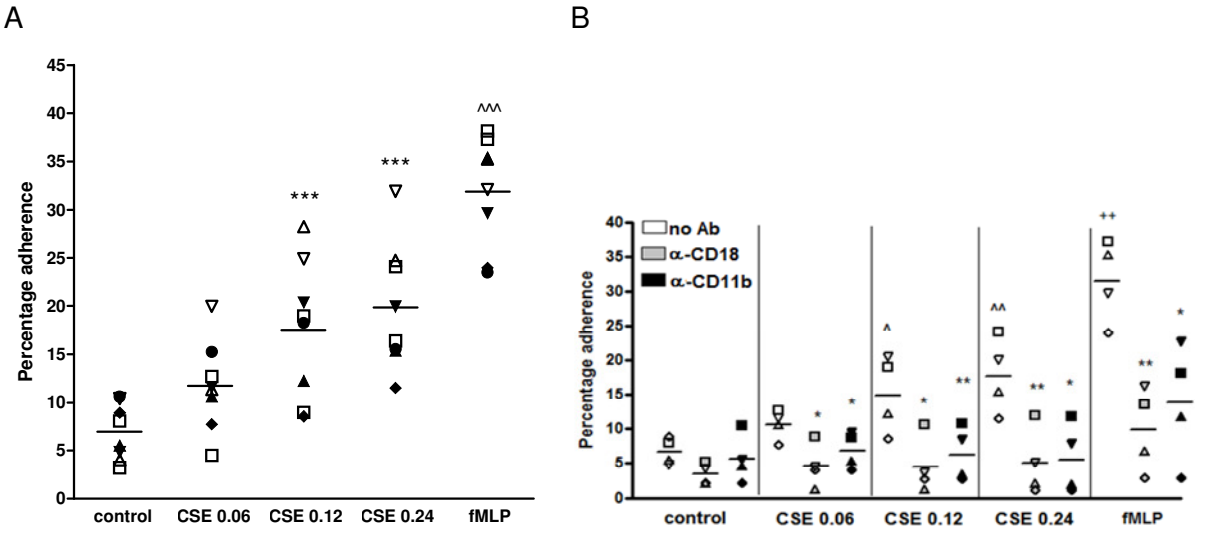

Figure 3. CSE-induced adhesion of PMNs to fibrinogen is inhibited after pre-incubation with α-CD11b and α-CD18.

*Calcein-AM labeled PMNs were added to fibrinogen-coated wells and incubated with indicated reagents for 30 min at 37 °C. After incubation, fluorescence was measured before and after three washes with HEPES incubation buffer. (**A**) Cigarette smoke extract (CSE; OD 0.06-0.24) induces adhesion of PMNs to fibrinogen (***, P < 0.001 CSE vs. control). Legend: each symbol represents a different donor (N = 8, n = 3-8). (**B**) After labeling, PMNs were pre-incubated for 20 min with medium (white symbols), α-CD18 (10 µg/ml; grey symbols) or α-CD11b (10 µg/ml; black symbols) at 37 °C prior to incubation with medium or cigarette smoke extract (CSE) for 30 min at 37 °C. Cigarette smoke extract (CSE; OD 0.06-0.24) induces adhesion of PMNs to fibrinogen (^, P < 0.05; ^^, P < 0.01 CSE vs. control), which is blocked by α-CD18 and α-CD11b (*, P < 0.05; **, P < 0.01 CSE α-CD11b/CD18 vs. CSE no Ab). FMLP (10^{-8} M) induces adhesion of PMNs to fibrinogen ($^{++}$, P < 0.01 fMLP vs. control), which is blocked by α-CD18 and α-CD11b (*, P < 0.05; **, P < 0.01 fMLP α-CD11b/CD18 vs. fMLP no Ab). Legend: each symbol represents a different donor (N = 4, n = 3). Horizontal bars in both pictures represent mean values.*

Functionally blocking CD11b and CD18 attenuates cigarette smoke extract-induced transmigration of neutrophils through HUVECs

Figures 2 and 3 show that cigarette smoke induces neutrophil movement and that it can activate β_2-integrins on the PMN. A transmigration assay was performed to investigate the effect of cigarette smoke on the next step in neutrophil recruitment. Administration of the CSE did not affect the permeability of the HUVEC layer in this assay since the electrical resistance of the confluent HUVEC layer did not change after CSE exposure (Table 1). The transendothelial migration of PMNs was significantly higher in response to CSE (OD = 0.06-0.24) than to medium *in vitro* (fig. 4A and 4B). Performing this assay in absence of the HUVEC layer resulted in a spontaneous transmigration of 38.97% ± 6.58%, whereas neutrophilic transmigration towards fMLP (10^{-8} M) was 73.83% ± 4.63% (N = 4-6). This leads

to the conclusion that neutrophils actively migrate through an endothelial layer in response to CSE.

To assess the role of CD11b and CD18 in the transmigration of PMNs through HUVECs, PMNs were pre-incubated with antibodies against CD11b or CD18 after which the transmigration assay was performed. Functionally blocking CD11b or CD18 resulted in a decrease in CSE-induced transmigration of PMNs of approximately 35% and 50% resp. (fig. 5A and 5B). FMLP (10^{-8} M) was tested as a positive control.

Table 1. Transendothelial electric resistance of the HUVEC layer.

Antibody	**TER ($\Omega \times cm^2$) after medium exposure**	**TER ($\Omega \times cm^2$) after CSE exposure**
No Ab	158	148
α-CD18	155	149
IgG_{2a} isotype	150	153
α-CD11b	153	157

No differences in transendothelial electrical resistance (TER) of the HUVEC layer were observed after antibody incubation or CSE (OD = 0.12) exposure as compared to medium exposure. The TER of filters alone was 82 $\Omega \times cm^2$. Data in table 1 are representative of three separate experiments.

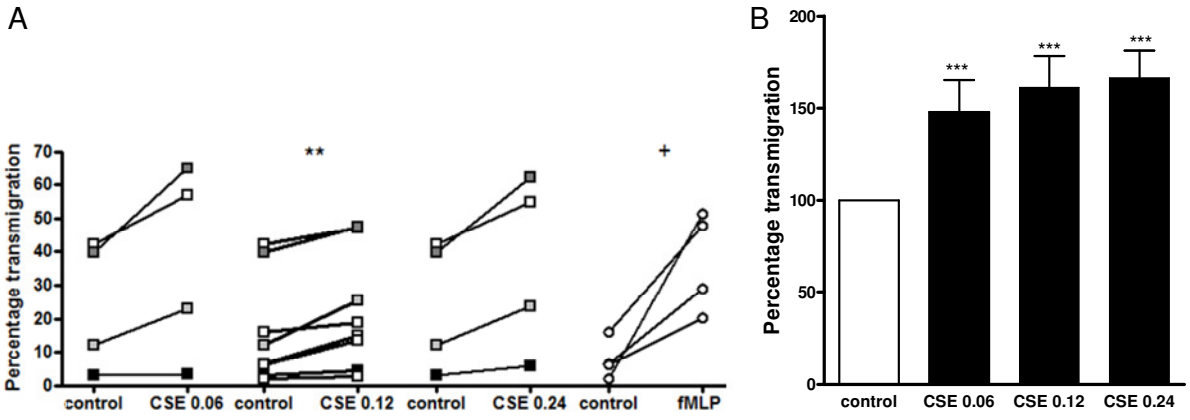

Figure 4. CSE induces transmigration of neutrophils through HUVECs.

*5 x 10^5 calcein-AM labeled PMNs were placed in the top well on HUVEC layers and the indicated reagent (CSE or fMLP) was added to the bottom well of the transmigration system. After 1 h incubation at 37 °C in 5% CO_2, fluorescence in 100 µl from each bottom well was measured. Data was standardized to the fluorescence of 100 µl PMNs (5 x 10^5 cells). (**A**) CSE (OD 0.06-0.24) induces transmigration of PMNs (**, P < 0.01 CSE vs. control). FMLP was tested as a positive control ($^+$, P < 0.01 fMLP vs. control). Legend: each color represents a different donor (N = 4-8, n = 2). (**B**) Standardizing the data of figure 4A to an index where the transmigration under control situation is 100%, CSE incubation leads to approximately a 1.5-fold higher migratory capacity (***, P < 0.001 CSE vs. control). Horizontal bars represent mean values and SEM.*

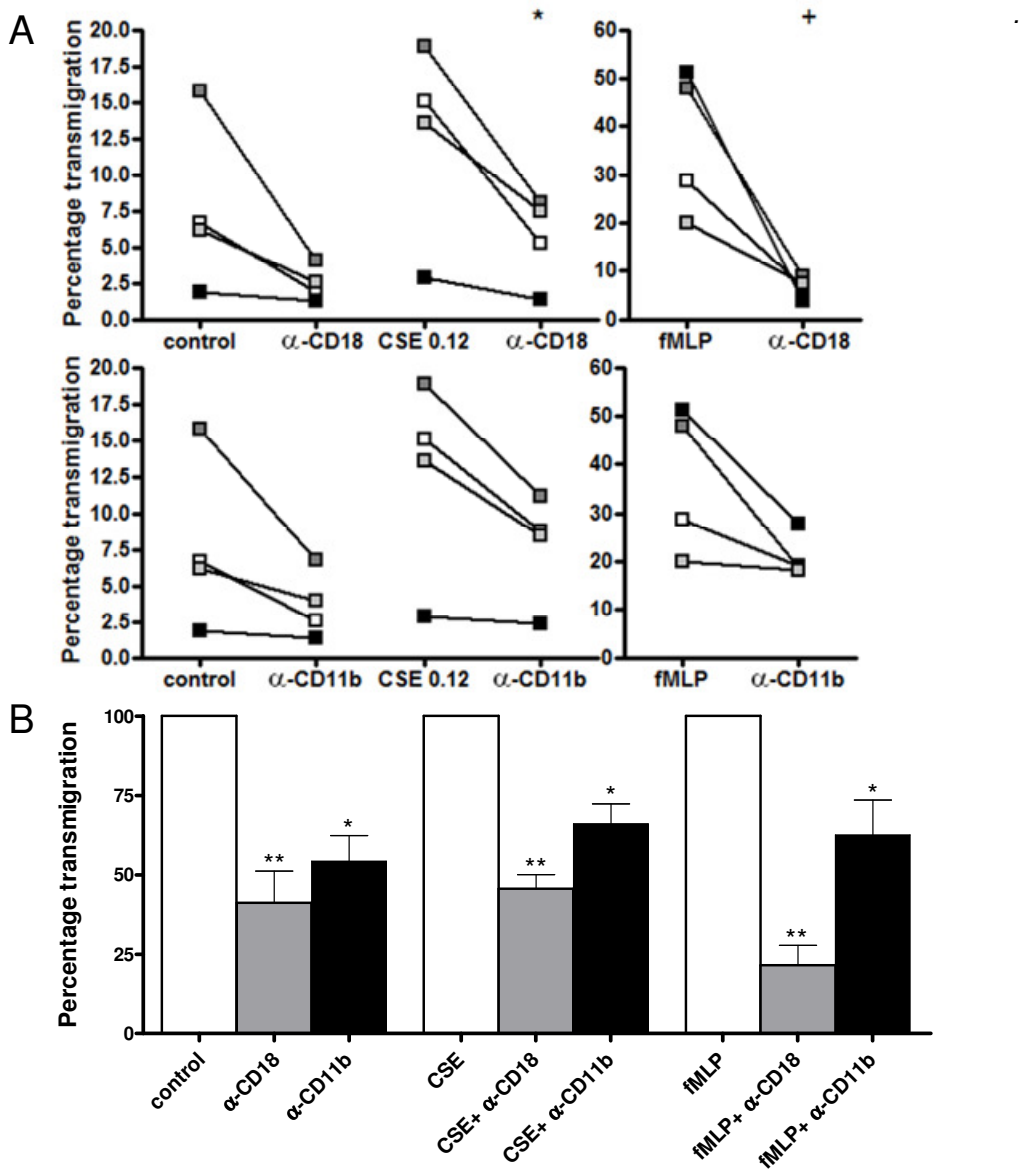

Figure 5. CSE-induced transmigration of neutrophils through HUVECs is decreased after pre-incubation with α-CD11b or α-CD18.

*(**A**) Prior to the transmigration assay, PMNs were pre-incubated for 20 min with medium, α-CD18 (10 µg/ml; upper panel) or α-CD11b (10 µg/ml; lower panel) at 37 °C. CSE-induced transmigration of PMNs is decreased after pre-incubation with α-CD11b or α-CD18 (*, P < 0.05 CSE α-CD18 vs. CSE no Ab). FMLP (10^{-8} M) was tested as a positive control (†, P < 0.01 fMLP vs. control). Legend: each color represents a different donor (N = 4, n = 2). (**B**) Standardizing the data of fig. 5A to an index where the transmigration after CSE incubation is 100%, pre-incubation with α-CD11b or α-CD18 leads to a decrease in transmigration of approximately 35% and 50% resp. (*, P < 0.05; **, P < 0.01 CSE α-CD18/α-CD11b vs. CSE no Ab). Spontaneous transmigration of PMNs was also inhibited by blocking CD11b and CD18 (*, P < 0.05; **, P < 0.01 control α-CD18/α-CD11b vs. control no Ab). FMLP (10^{-8} M) was tested as a positive control (*, P < 0.05; **, P < 0.01 fMLP α-CD18/α-CD11b vs. fMLP no Ab). Horizontal bars represent mean values and SEM.*

Discussion

In this report, the direct effect of cigarette smoke on neutrophil migration and on β_2-integrin activation and function in the endothelial transmigration of neutrophils was investigated. Exposing mice to cigarette smoke for 5 days resulted in the presence of CD11b expressing neutrophils in the lungs, showing that cigarette smoke attracts neutrophils. For a more in depth study, we exposed freshly isolated human neutrophils to CSE to study possible migratory effects and involvement of Mac-1. To our knowledge, this is the first study to show that CSE can induce the migration of neutrophils *in vitro*. Moreover, CSE activated the β_2-integrin Mac-1 on the neutrophil leading to firm adhesion to fibrinogen. Furthermore, neutrophils transmigrated through endothelium in response to CSE via the activation of β_2-integrins, since functionally blocking CD11b and CD18 decreased this transmigration. Taken together, our data provide evidence for a critical role of β_2-integrins in the firm adhesion and transmigration of neutrophils in response to cigarette smoke *in vitro* and *in vivo*.

Cigarette smoke consists of more than 4000 compounds, known to be mutagenic, carcinogenic, antigenic and cytotoxic [27, 28] and it induces a peripheral inflammatory response in all smokers [29]. This is reflected in increased macrophage and neutrophil numbers, changes in expression surface markers, elevated levels of chemokines (amongst which are CXCL1 and CXCL8) and cytokines (such as TNF-α and IL-1β) and increased production of proteolytic enzymes by different immune cells, such as macrophages and neutrophils [18, 28, 30].

Exposing mice for five days to cigarette smoke results in an influx of CD11b expressing cells. We concluded that these cells were neutrophils due to the characteristic segmented nuclei. Other studies also observed increased numbers of neutrophils in BAL fluid and lung tissue after one week [26, 31] and five months cigarette smoke exposure in mice [32]. These *in vivo* findings led to the hypothesis that cigarette smoke may have a direct migratory effect. Interestingly, it has recently been suggested by Barnes that smoke may induce neutrophil movement [33]. In the present study, we confirm this hypothesis and demonstrate that CSE induces the migration of neutrophils. Although it has been reported that nicotine, a major constituent of cigarette smoke, can have a chemotactic effect on neutrophils [34], other groups found no effect or even an inhibitory effect [35-37] or describe this effect as very weak and state that nicotine enhances neutrophil responsiveness to other chemoattractants [38]. Another major constituent of smoke, acrolein, inhibits chemotaxis [36, 39]. Since CSE consist of more than 4000 components, it is likely that other chemical compounds or combinations of soluble compounds are responsible for the migratory effect on neutrophils.

To our knowledge, we are the first to show a direct migratory effect of CSE. Other groups have investigated the priming effect of cigarette smoke. Bridges & Hsieh demonstrated that

water-soluble fractions (WSF) and cigarette smoke condensates (CSC) were able to inhibit endotoxin-activated serum-induced chemotaxis of neutrophils [40]. Selby *et al.* described that exposure of human neutrophils *in vitro* to cigarette smoke resulted in impaired cell spreading and chemokinesis, but did not influence zymosan-activated serum-induced chemotaxis [41]. However, both groups did not investigate the direct migratory effect of cigarette smoke on neutrophils. The inhibitory effects of cigarette smoke on activated serum-induced chemotaxis of neutrophils, as described in the studies of Bridges & Hsieh and Selby *et al.* [40, 41], may be explained by the fact that adding the cigarette smoke to the neutrophils leads to immobilization of the cells; the cells are attracted by the cigarette smoke in the upper chamber and do not actively need to go to the lower chamber.

Neutrophils use β_2-integrins, such as Mac-1, for nearly every step in transmigration [13]. During inflammation, β_2-integrins on the neutrophil are activated by chemokines such as CXCL8, leading to neutrophilic firm adhesion to endothelial cells and subsequently to transmigration. This activation is essential, since it leads to a conformational change in structure, going from an inactive, low affinity state to an active, high-affinity state [13]. We show that CSE activates β_2-integrins on the neutrophil leading to firm adhesion to fibrinogen and transmigration through an endothelial cell layer, since functionally blocking CD11b and CD18 decreased these effects of cigarette smoke. Consistent with previous studies describing β_2-integrin expression patterns of smokers [29, 42, 43], we found that CSE-induced adhesion and transmigration of neutrophils are the result of β_2-integrin activation, and more specifically Mac-1 activation. It may be possible that cigarette smoke exposure leads to an up-regulation of β_2-integrin expression on the neutrophil. Koethe *et al.* described that pre-incubating PMNs with CSC resulted in a 2.5 fold increase in CD11b/CD18 expression as compared to the control [44]. From these results it can be suggested that besides the activation of β_2-integrins, CSE incubation may result in an up-regulation of the CD11b/CD18 expression. However, Selby *et al.* reported that acute *in vivo* cigarette smoking of up to 4 cigarettes did not change the expression patterns of CD18 on the neutrophils [41].

Cigarette smoke enhances the expression of E- and P-selectin [45] and ICAM-1 and VCAM-1 [46] on HUVECs, thereby increasing rolling interactions and subsequent firm adhesion of neutrophils to HUVECs via β_2-integrins Mac-1 and LFA-1 and β_1-integrin VLA-4 [9]. In our experiments, neutrophils migrated through an endothelial cell layer in response to CSE. Administration of cigarette smoke did not affect the permeability of the HUVEC layer in the transmigration assay since the electrical resistance of the confluent HUVEC layer did not change before and after CSE exposure (table 1). This leads to the conclusion that neutrophils actively migrate through the layer in response to cigarette smoke.

Although CXCL8 has a stronger migratory effect and fMLP is a stronger activator of β_2-integrins, cigarette smoke exposure leads to a 1.5 fold increase in migration and to a 1.5 to 3

fold increase in adhesion as compared to the control. Mortaz and colleagues have recently shown that CSE exposure to human neutrophils results in the release of CXCL8 [18]. Furthermore, earlier studies have demonstrated that exposure of neutrophils to CXCL8 leads to the phosphorylation of focal adhesion kinase (FAK) and CXCL8 induces FAK cellular redistribution [47]. FAK activation is involved in cellular adhesion and spreading processes of neutrophils [48]. Thus in the *in vivo* situation we cannot rule out the additional chemotactic effects of cigarette smoke-induced released inflammatory mediators from for example epithelial cells, pulmonary neutrophils and resident macrophages.

Taken together, this is the first study that describes CSE as a potent inducer of neutrophil movement. Besides this migratory effect, CSE plays a critical role in β_2-integrin activation, which leads to an increase in firm adhesion and transmigration through HUVECs. These observations may contribute to a better understanding of neutrophilic transmigration in smokers and COPD patients and potentially offer a new target in disease management.

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Chapter 4

Chemoattractant N-acetyl proline-glycine-proline induces CD11b/CD18-dependent neutrophil adhesion

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Abstract

Neutrophils play a pivotal role in pulmonary inflammatory diseases, such as chronic obstructive pulmonary disease (COPD). In this progressive disease neutrophils migrate from the blood and accumulate in the affected tissues where they release mediators and proteases. The chronic inflammation leading to lung tissue destruction is hypothesized to be due to the formation of chemoattractant N-acetyl proline-glycine-proline (N-ac-PGP), a collagen breakdown product. In the current study we investigate whether N-ac-PGP influences β_2-integrin activation and function in neutrophilic firm adhesion to endothelium.

Human neutrophils transmigrated through an endothelial cell layer in response to basolateral N-ac-PGP. N-ac-PGP induced also a neutrophil adherence to fibrinogen. Using functional blocking antibodies against CD11b and CD18, it was demonstrated that Mac-1 was responsible for the N-ac-PGP-induced firm adhesion of neutrophils to fibrinogen. Pertussis toxin decreased the Mac-1 activation indicating the involvement of G-proteins. N-ac-PGP most likely activated Mac-1 by initiating a conformational change, since the expression pattern of Mac-1 on the cell surface did not change significantly.

In conclusion, this is the first study to describe that the chemoattractant N-ac-PGP also activates Mac-1 on the surface of neutrophils, which can additionally contribute to neutrophilic transmigration into the lung tissue during lung inflammation.

Introduction

Neutrophils play a pivotal role in pulmonary inflammatory diseases, such as chronic obstructive pulmonary disease (COPD) [1]. This disease, which includes bronchitis and emphysema, is an important cause of morbidity worldwide [2, 3] and is characterized by irreversible progressive development of airflow limitation [1]. Neutrophils migrate from the blood and accumulate in the affected tissues where they release mediators and proteases, contributing to the chronic inflammatory reaction that ultimately leads to tissue destruction [1, 4]. The most prominent chemokine in COPD is CXCL8. In patients, the levels of this chemokine are increased in sputum and correlate with the increased number of neutrophils found in the lungs [4]. Recently, a role has been demonstrated for another chemoattractant, namely N-acetyl proline-glycine-proline (N-ac-PGP) in this complicated and progressive disease. This tripeptide is generated from the breakdown of extracellular matrix collagen and is specifically chemotactic for neutrophils *in vivo* and *in vitro* [5-9] and chronic airway exposure to N-ac-PGP causes lung emphysema in mice [9, 10]. This collagen fragment has therefore been suggested to be a new biomarker and therapeutic target for COPD [11].

Neutrophils migrate from the blood stream to the lung interstitium via a multistep process of adhesive and migratory events, which includes selectin-mediated rolling, chemokine-induced activation of integrins and integrin-dependent firm adhesion leading to transendothelial migration [12-14]. Neutrophil firm adhesion to endothelial cells is mediated via interaction between integrins, such as β_2-integrins Lymphocyte Function-associated Antigen 1 (LFA-1; CD11a/CD18; $\alpha_L\beta_2$) and Macrophage 1 antigen (Mac-1; CD11b/CD18; $\alpha_M\beta_2$) on neutrophils and members of the immunoglobulin superfamily, such as ICAM-1 and ICAM-2 present on endothelial cells [12-15]. The β_2-(CD18-) integrins are heterodimeric receptors, consisting of an α- and a β-chain that together form a ligand-binding head region with two legs that contain the transmembrane and cytoplasmic domains of each chain [16, 17]. During inflammation, activation of these β_2-integrins is essential, since it leads to a conformational change in structure, going from an inactive, low affinity state to an active, high-affinity state [16]. These conformational changes can be initiated via stimuli received by activated receptors for chemokines, cytokines or foreign antigens inducing intracellular signals (inside-out signaling) [17] and further strengthened by integrin clustering, transferring signals from the extracellular domain to the cytoplasm (outside-in signaling) [12].

The aim of this study was to investigate the effect of N-ac-PGP on β_2-integrin activation and function in neutrophils. In this report, we demonstrate that neutrophils adhere to fibrinogen and transmigrate through endothelial layers upon activation with N-ac-PGP.

Material and methods

Chemicals and antibodies

N-ac-PGP was purchased from AnaSpec (San Jose, CA, USA) and controlled for purity by high-performance liquid chromatography and mass spectrometry. Functional blocking monoclonal antibodies (mAb) IB4 (mouse IgG_{2a}; α-CD18) and 44A (mouse IgG_1; α-CD11b) were isolated from the supernatant of mouse hybridomas purchased from the American Type Culture Collection (ATCC). Mouse IgG_1 & IgG_{2a} isotype controls and mAb ICAM-1 were obtained from R&D Systems Europe Ltd. (Abingdon, United Kingdom). Treatment of PMNs with isotype control antibodies showed no difference in adhesion and transmigration as compared to control. Antibody concentrations and pre-incubation periods were adapted from previously published articles [18-20].

Isolation of human polymorphonuclear leukocytes.

Human polymorphonuclear leukocytes (PMNs) were isolated from fresh whole blood as previously described [7]. Resulting PMN preparations consisted of approximately 95-97% PMNs, based on PMNs physical parameters analyzed by flow cytometry and CD16 expression. The preparations were negative for CD14, meaning that the preparations did not contain monocytes.

HUVEC culture

Human umbilical vein endothelial cells (HUVECs) were isolated as previously described [21] and were kindly provided by J.H. van Kats-Renaud (UMC Utrecht). The cells were cultured in endothelial cell growth medium-2 (Lonza Verviers SPRL, Belgium) and were grown to confluence in 5–7 days. Cell passages 2-3 were used for all experiments.

Transendothelial migration assay

The transendothelial migration assay was performed according to the procedure as described in [22]. The percentage of transmigration was defined as the fluorescence value of the migrated PMNs divided by the value of total fluorescence, multiplied by 100. Data were standardized to an index where the adhesion under control situation is 100%.

Adhesion assay

The adhesion assay was performed according to the procedure as described in [22]. The percentage adherence was determined by calculating the fluorescence after three washes as a percentage of the total fluorescence before washing.

Flow cytometry

Freshly isolated PMNs or HUVECs were pre-incubated with indicated reagents for 5 and 60 min at 37 °C. Subsequently, PMNs were incubated with mAb against CD16 (R&D Systems, Abingdon, UK; diluted 1:100) and CD11b (Beckman Coulter, Woerden, the Netherlands; diluted 1:100) for 45 min at 4 °C in the dark. HUVECs were incubated with mAb ICAM-1 (1:100). After washing, the fluorescent intensity of 10^4 PMNs or HUVECs was analyzed using a FACSCalibur flow cytometer (Becton Dickinson) with CellQuest Pro Software (version 5.2.1.). PMN gating was based on forward and side scatter parameters and CD16 expression.

Statistical analyses

For all statistical analyses, GraphPad Prism version 4.0 was used. Two-tailed paired Student *t*-tests were used for comparing two groups. For comparing three or more groups, the data were analyzed using a one-way repeated measures ANOVA followed by Tukey post hoc analysis. Data were considered significant at $P < 0.05$. All results are expressed as means ± SEM.

Results and Discussion

Chemoattractant N-ac-PGP induces transmigration of PMNs through HUVEC cells

It has been previously shown by different groups that N-ac-PGP is chemotactic for neutrophils *in vitro* by using trans-well systems, allowing neutrophils to pass through a filter [6, 7, 9]. Here we are the first to investigate the chemotactic effect of N-ac-PGP in a physiological environment, namely the transmigration of PMNs through a HUVEC monolayer. The transendothelial migration of PMNs was significantly higher in response to N-ac-PGP (10^{-3} M) than to medium *in vitro* (fig. 1). In the transmigration assay, HUVEC cells were pre-incubated with TNF-α to activate adhesion molecule ICAM-1 and to increase permeability *in vitro*, thereby modeling vascular permeability during inflammation *in vivo*. Our results indicate that N-ac-PGP can diffuse from the basolateral to the apical side of the endothelial layer. This is in agreement with a previously published experiment where serum levels of the tripeptide were measured in COPD patients [11], meaning that the breakdown product PGP released from the extracellular matrix can diffuse through the endothelial layer into the blood stream.

Since neutrophils use β_2-integrins for nearly every step in transmigration [16], the effect of N-ac-PGP on β_2-integrin Mac-1 activation on the neutrophil was further investigated.

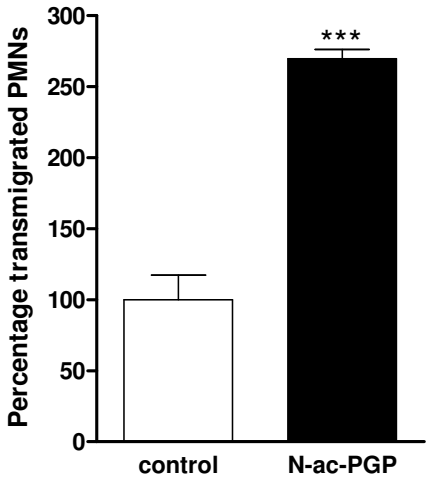

Figure 1. N-ac-PGP induces transmigration of PMNs through the HUVEC layer.

*5 x 10^5 calcein-AM labeled PMNs were placed in the top well on TNF-α-primed HUVEC layers and N-ac-PGP (10^{-3}M) was added to the bottom well of the transmigration system. After 1 h incubation at 37 °C in 5% CO_2, fluorescence in 100 µl from each bottom well was measured. Control was standardized to 100%. N-ac-PGP induces transmigration of PMNs (***, P < 0.001; N-ac-PGP vs. control; n=3).*

N-ac-PGP-induced adhesion of PMNs to fibrinogen is mediated via the activation of Mac-1 and G protein-coupled receptors

During inflammation, β_2-integrins on the neutrophil are activated by chemokines such as CXCL8, subsequently leading to neutrophilic firm adhesion to endothelial cells and followed by transmigration [12, 13]. This activation is essential, since it leads to a conformational change in structure, going from an inactive, low affinity state to an active, high-affinity state [16]. This led to the hypothesis that the N-ac-PGP-induced migration is due to the activation of the β_2-integrin Mac-1 on neutrophils.

To examine this hypothesis, the effect of N-ac-PGP on the Mac-1-fibrinogen interaction was investigated. Incubating PMNs with N-ac-PGP (10^{-5}-10^{-3} M) caused a significant increase in adhesion to fibrinogen compared to medium incubation (fig. 2A). FMLP (10^{-8} M) was tested as a positive control, which also increased the adhesion to fibrinogen. Functionally blocking CD11b or CD18 on the PMN resulted in a profound inhibition of the N-ac-PGP-induced adhesion (fig. 2B).

To assess the role of G protein-coupled receptors (GPCRs) in N-ac-PGP-induced adhesion, the effect of pertussis toxin (PTX) was investigated. PTX incubation decreased the N-ac-PGP-induced adhesion to fibrinogen (fig. 2C), suggesting that N-ac-PGP induces firm adhesion by promoting activation of Mac-1 via inside-out signaling via a GPCR or via direct G-protein activation.

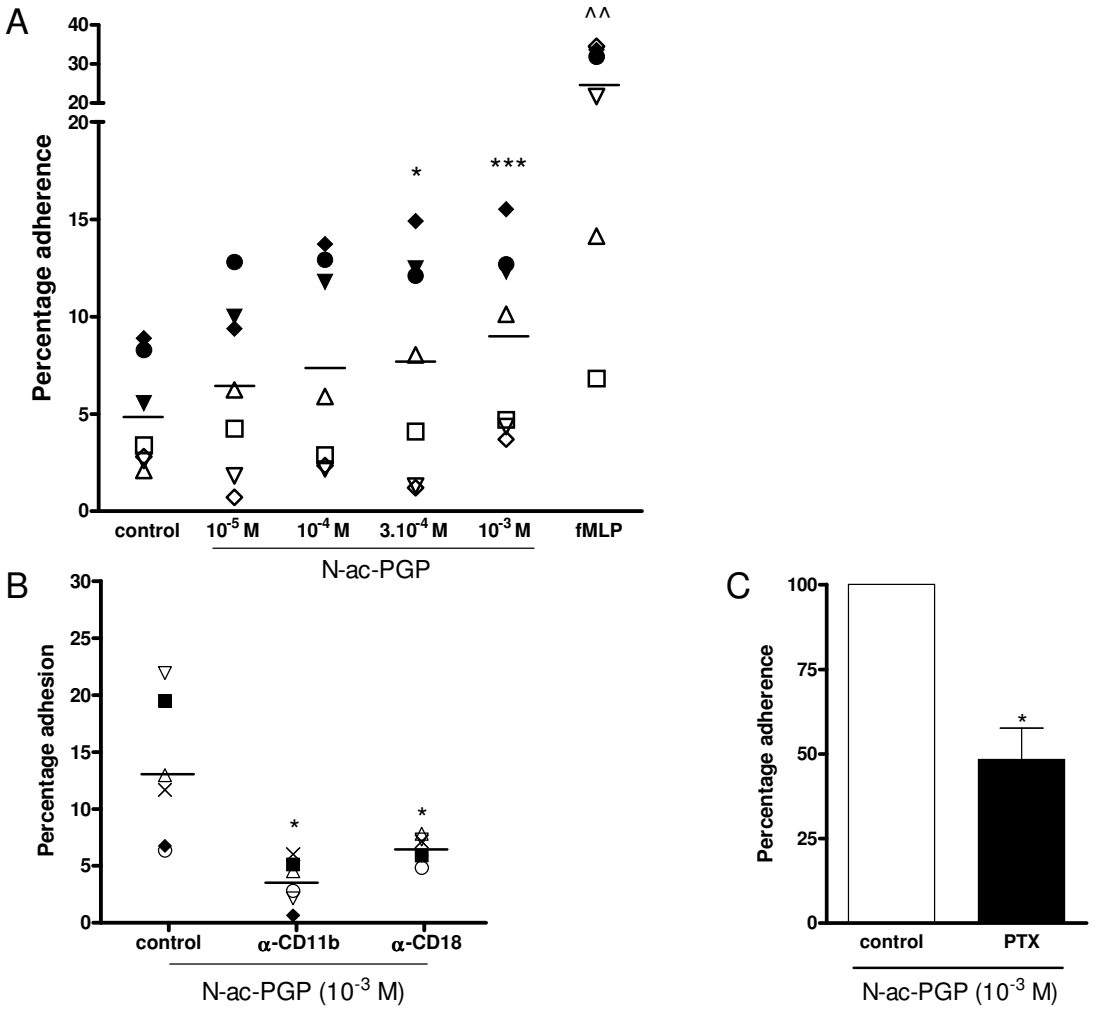

Figure 2. N-ac-PGP-induced adhesion of PMNs to fibrinogen is inhibited after pre-incubation with α-CD11b and α-CD18 or pertussis toxin (PTX).

*Calcein-AM labeled PMNs were added to fibrinogen-coated wells and incubated with indicated reagents for 30 min at 37 °C. After incubation, fluorescence was measured before and after three washes with HEPES incubation buffer. (**A**) Control was standardized to 100%. N-ac-PGP (10^{-5} – 10^{-3} M) induces adhesion of PMNs to fibrinogen (*, P < 0.05; ***, P < 0.001 N-ac-PGP vs. control). FMLP (10^{-8} M) was used as a positive control (^^, P < 0.01 fMLP vs. control). N=7 healthy donors; each symbol represents an individual donor. Horizontal bars represent mean values. (**B**) After labeling, PMNs were pre-incubated for 20 min with medium, α-CD18 (10 µg/ml) or α-CD11b (10 µg/ml) at 37 °C prior to incubation with medium or N-ac-PGP for 30 min at 37 °C. N-ac-PGP (10^{-3} M) induces adhesion of PMNs to fibrinogen, which is blocked by α-CD18 and α-CD11b (*, P < 0.05 N-ac-PGP α-CD11b/CD18 vs. N-ac-PGP no Ab). N=6 healthy donors; each symbol represents an individual donor. Horizontal bars represent mean values. (**C**) Freshly isolated PMNs were incubated with medium or pertussis toxin (PTX; 500 ng/ml) for 90 min at 37 °C in 5% CO_2. Subsequently, the adhesion assay was performed. N-ac-PGP adhesion was standardized to 100%. N-ac-PGP (10^{-3} M) induced adhesion (white bar) is inhibited in PMNs pre-incubated with PTX (black bar) (*, P < 0.05 N-ac-PGP vs. PTX N-ac-PGP). N=3 healthy donors. Horizontal bars represent mean values and SEM.*

N-ac-PGP incubation does not affect the CD11b expression on the PMN

Figure 2A and 2B showed that N-ac-PGP incubation leads to firm adhesion to fibrinogen via β_2-integrins. To investigate whether this adhesion is regulated via a change in CD11b expression on the cell membrane during transmigration *in vitro*, PMNs were incubated with N-ac-PGP. CD11b expression was measured using flow cytometry. N-ac-PGP incubation did not significantly affect the CD11b expression, whereas TNF-α (positive control) increased the expression of CD11b on the cell membrane (fig. 3A and 3B).

Since Mac-1 on the neutrophil is a ligand for ICAM-1 on endothelial cells, the effect of N-ac-PGP on the expression of ICAM-1 was studied. ICAM-1 is constitutively present on endothelial cells, but its expression is increased by prolonged exposure to pro-inflammatory cytokines such as TNF-α [23]. However, incubating HUVEC cells with N-ac-PGP or TNF-α for 60 min did not affect the ICAM-1 expression (fig. 3C).

To summarize, our results indicate that the tripeptide N-ac-PGP activates Mac-1 by initiating a conformational change, rather than by inducing a change in expression pattern.

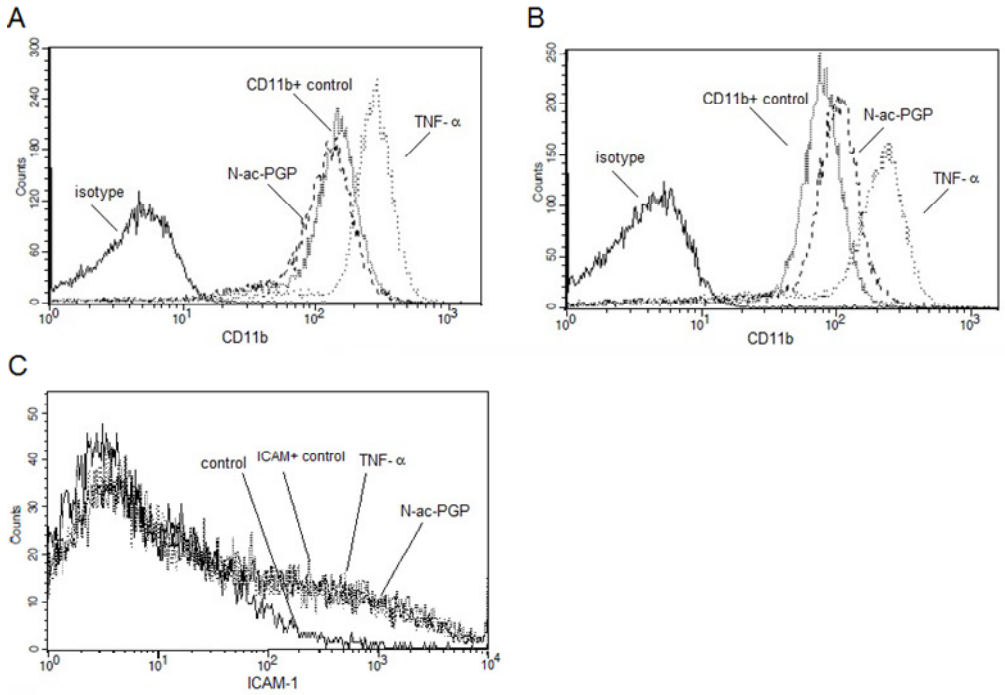

Figure 3. N-ac-PGP incubation does not affect cell surface expression of CD11b on PMNs and ICAM-1 on HUVECs.

*(**A**) Incubating PMNs for 5 min with N-ac-PGP does not increase the expression of CD11b, whereas this expression is increased after TNF-α incubation (2 ng/ml; positive control). (**B**) N-ac-PGP incubation leads to a small increase in CD11b expression after 60 min, whereas TNF-α incubation increases this expression to a large extent. (**C**) Incubating HUVEC cells for 60 min with N-ac-PGP or TNF-α does not increase the expression of ICAM-1. These graphs are representative of three separate experiments. Legend: isotype control cells (solid line); CD11b/ICAM-1 positive control cells (⋯); CD11b/ICAM-1 positive N-ac-PGP incubated cells (---); CD11b/ICAM-1 positive TNF-α incubated cells (……).*

Concluding remarks

In the last decade, the collagen breakdown product N-ac-PGP has been implicated as a biomarker and therapeutic target for COPD [11]. In this neutrophilic inflammatory lung disease neutrophils migrate from the blood stream into the lung tissue via the activation of integrins and integrin-dependent firm adhesion, ultimately leading to transendothelial migration. In this report, we investigated the effect of N-ac-PGP on β_2-integrin activation and function in neutrophilic firm adhesion to fibrinogen.

Here we show that freshly isolated neutrophils transmigrated through endothelium in response to N-ac-PGP. For a more in depth study, freshly isolated human neutrophils were exposed to N-ac-PGP to study possible adhesive effects and involvement of Mac-1. Neutrophils adhered to fibrinogen in response to N-ac-PGP via the activation of β_2-integrin Mac-1, since functionally blocking CD11b and CD18 inhibited this adhesion. Moreover, PTX incubation profoundly inhibited the N-ac-PGP-induced adhesion to fibrinogen, indicating that this adhesion is mediated via pertussis toxin sensitive $G_{i/o}$ proteins. In addition, N-ac-PGP incubation does not change the cell surface expression of Mac-1 on the PMN, which points to a conformational change of the integrin on the PMN. Furthermore, N-ac-PGP incubation does not affect the ICAM-1 expression on HUVECs.

In COPD, there appears to be a relationship between N-ac-PGP formation and cigarette smoking. Cigarette smoking is the major risk factor for neutrophilic lung disease such as COPD [24] and it induces peripheral inflammatory responses in all smokers [25, 26]. Previous findings and the findings described here point to a self-sustaining pathway of neutrophilic inflammation and lung destruction, initiated by cigarette smoke exposure (fig. 4); cigarette smoke attracts neutrophils and activates β_2-integrins, such as Mac-1 on the cell surface, which leads to neutrophil transmigration [22]. Subsequently, cigarette smoke-induced neutrophil activation leads to the release of CXCL8 [27], thereby attracting more neutrophils to the lung. Moreover, this neutrophil activation causes the release of matrix metalloproteinase (MMP) 8 and 9. Together with prolyl endopeptidase (PE), these MMPs breakdown whole lung which ultimately leads to the formation of collagen fragment N-ac-PGP [27]. During inflammation vascular permeability is increased, allowing N-ac-PGP to diffuse from the basolateral to the apical side of the endothelial layer to establish a concentration gradient. Subsequently, N-ac-PGP attracts neutrophils to the lung and induces firm adhesion by promoting activation of Mac-1 via inside-out signaling, which leads to neutrophil transmigration. In the lung tissue, N-ac-PGP exposure activates the neutrophil to release CXCL8 and MMP8 and MMP9 [27], which results in a self-sustaining cycle of neutrophil infiltration and collagen destruction.

Taken together, our data provide evidence for a role of β_2-integrins in the firm adhesion of neutrophils in response to N-ac-PGP *in vitro*, leading to neutrophilic transmigration into the lung tissue.

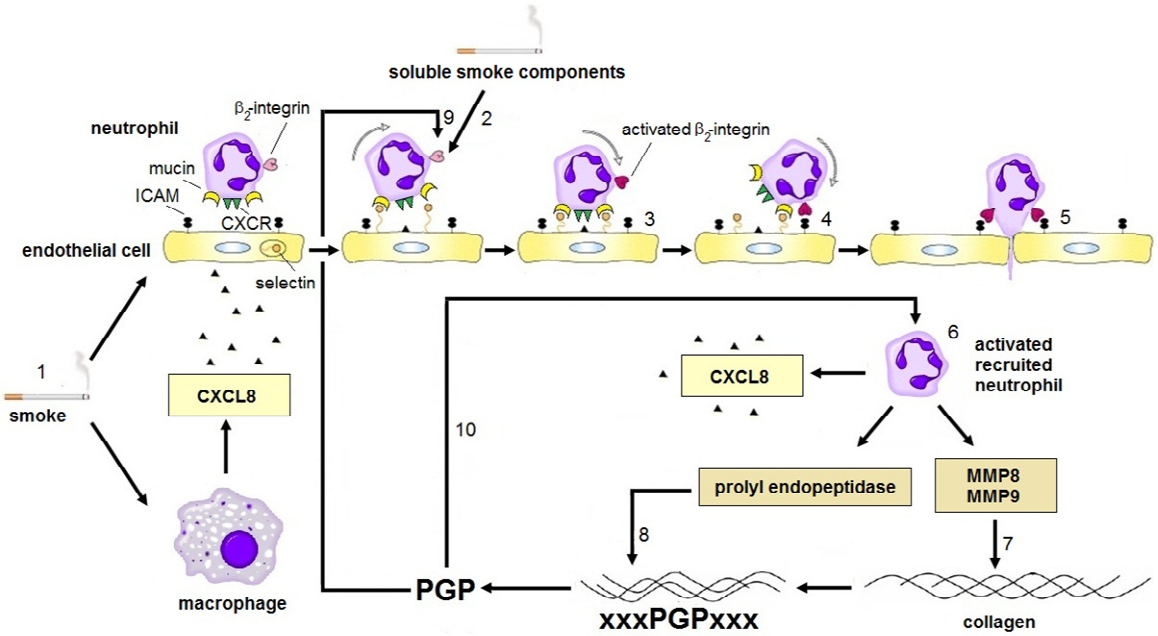

Figure 4. In COPD, cigarette smoking initiates a vicious circle of events leading to collagen breakdown, PGP generation and PGP-induced neutrophilic transmigration.

*Cigarette smoking activates resident macrophages and epithelial cells to secrete chemokines such as CXCL8 (**1**). These chemokines are retained on matrix and cell-surface heparan sulfate proteoglycans and on the surface of the endothelium, establishing a chemokine concentration gradient. Neutrophils rolling on the endothelium in a selectin-mediated process are brought into contact with CXCL8, leading to the activation of β_2-intergrins, such as Mac-1. Also soluble smoke components can activate these β_2-intergrins (**2-3**), leading to firm adherence (**4**) and extravasation (**5**). In the lung tissue, the recruited neutrophil is activated by cigarette smoke which diffused through the damaged epithelial cell layer (**6**), leading to the release of CXCL8. Moreover, this activation leads to the release of MMP8 and MMP9. These proteases denature and proteolytically cleave collagen to fragments 30–100 amino acids in length (**7**). These collagen fragments are then further cleaved into PGP by prolyl endopeptidase (**8**). Subsequently, this tripeptide attracts neutrophils to the lung and induces firm adhesion by promoting activation of Mac-1, which leads to neutrophil transmigration (**9**). In the lung tissue, N-ac-PGP exposure activates the neutrophil to release CXCL8 and MMP8 and MMP9 (**10**), which results in a self-sustaining cycle of neutrophil infiltration and collagen destruction.*

Acknowledgements

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Chapter 5

N-acetyl Proline-Glycine-Proline induced G protein-dependent chemotaxis of neutrophils is independent of CXCL8 release

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Eur J Pharmacol, 2011, in press

Abstract

Chronic inflammation in lung diseases contributes to lung tissue destruction leading to the formation of chemotactic collagen fragments such as N-acetyl Proline-Glycine-Proline (N-ac-PGP). In this study, we investigated in more detail the mechanism of action of N-ac-PGP in neutrophilic inflammation. N-ac-PGP was chemotactic for human neutrophils via pertussis toxin sensitive GPCRs *in vitro* and directly activated this cell type, which led to cytosolic calcium mobilization and release of CXCL8. Furthermore, using a selective CXCR2 antagonist confirmed that N-ac-PGP-induced neutrophil chemotaxis is mediated through CXCR2 activation. To determine whether N-ac-PGP was solely responsible for the migration and activation of human neutrophils *in vitro* and not the released CXCL8 upon stimulation with N-ac-PGP, an antibody directed against CXCL8 was used. Performing chemotaxis and calcium influx assays in the presence of this antibody did not alter the effects of N-ac-PGP whereas effects of CXCL8 were attenuated. These experiments indicate that N-ac-PGP, in addition to the direct induction of chemotaxis, also directly activates neutrophils to release CXCL8. *In vivo*, this may lead in the long term to a self-maintaining situation enhanced by both N-ac-PGP and CXCL8, leading to a further increase in neutrophil infiltration and chronic inflammation.

Introduction

Neutrophils play a pivotal role in pulmonary inflammatory diseases, such as chronic obstructive pulmonary disease (COPD) [1-3]. These defensive cells are the first to be recruited to the site of inflammation. During inflammation, the major attractants for neutrophils are ELR^+ CXC-chemokines, such as CXCL8 [4]. However, it has long been known that fragments of the extracellular matrix, such as collagen fragments, have chemotactic properties [5, 6]. One of these fragments is N-acetyl Proline-Glycine-Proline (N-ac-PGP), which was first identified by Pfister *et al*. They described a rabbit model in which it was demonstrated that alkali degradation of whole corneal protein generated a tripeptide, N-ac-PGP [7]. Injecting N-ac-PGP in normal rabbit corneas resulted in a rapid and severe neutrophil invasion leading to corneal ulceration and perforation, resembling the neutrophil infiltration in the alkali-injured eye [7]. Interestingly, N-ac-PGP has been found in the sputum of COPD [8, 9] and cystic fibrosis (CF) patients [10]. In collaboration with Weathington *et al*., we investigated the possible molecular mechanisms of N-ac-PGP's activity in the lung. Intratracheal administration of the collagen fragment in mice resulted in a direct neutrophil influx into the airways. Furthermore, chronic intra-airway exposure of N-ac-PGP resulted in COPD-like characteristics, such as enlargement of alveoli and right ventricular hypertrophy [8]. It was proposed that the N-ac-PGP activity is mediated via CXCR1/2, since the tripeptide shares sequence homology with the highly conserved GP motif in ELR^+ CXC-chemokines, which is an essential motif for neutrophil cell binding and cell activation [11], and blocking these chemokine receptors resulted in inhibition of the chemotactic N-ac-PGP response [8]. However, in collaboration with De Kruijf *et al*., we recently reported that N-ac-PGP does not directly activate or interact with these receptors, since the peptide was unable to activate G protein (in)dependent signaling and was unsuccessful in displacing the radioligands [^{125}I]-CXCL8 and [^{3}H]-SB265610 from CXCR1- and CXCR2-expressing HEK293T cells or neutrophils. These observations led to the hypothesis that N-ac-PGP interacts indirectly with CXCR1/2 via the release of chemokines known to bind these receptors, or through activation of other receptors on the neutrophil to induce chemotaxis [12]. Therefore, the aim of our study was to investigate the mechanism of N-ac-PGP-induced chemotaxis in more detail. Here, we demonstrated that N-ac-PGP dose dependently induces pertussis toxin-sensitive chemotaxis of neutrophils, which is associated with a calcium (Ca^{2+}) influx and that N-ac-PGP activation induces release of CXCL8. However, using an antibody against CXCL8, we demonstrated that the released CXCL8 is not responsible for N-ac-PGP's directly induced chemotaxis and Ca^{2+} influx. Yet, the CXCR2 antagonist Compound 1 was able to attenuate the N-ac-PGP induced neutrophilic chemotactic response. These findings indicate that N-ac-PGP is a chemoattractant for neutrophils in which CXCR2 but not CXCL8 plays a role.

Material and methods

Chemicals and reagents

N-ac-PGP was purchased from AnaSpec (San Jose, CA, USA) and checked for purity by high-performance liquid chromatography and mass spectrometry. Proline-Glycine-Glycine (PGG) was purchased from Bachem AG (Budendorf, Germany). CXCR2 antagonist Compound 1 ((*S*)-2-(2-(1*H*-imidazol-1-yl)-6-(octylthio)pyrimidin-4-ylamino)-*N*-(3-ethoxypropyl) -4-methylpentanamide) was synthesized at the Schering-Plough Research Institute (Oss, the Netherlands; for molecular structure, see [13] and [12]. Recombinant human CXCL8 and human α-CXCL8 (antibody directed against CXCL8) were supplied by R&D Systems Europe Ltd. (Abingdon, United Kingdom). LPS, fMLP, pertussis toxin (PTX) and propidium iodide (PI) were purchased from Sigma Aldrich Chemie BV (Zwijndrecht, the Netherlands). The human CXCL8 ELISA kit was from BD Biosciences (Alphen a/d Rijn, the Netherlands). NH_4Cl, $KHCO_3$, EDTA (Triplex III) and trisodium citrate dihydrate were purchased from Merck KGaA (Darmstadt, Germany). Fluo-3 AM (1 mM solution in DMSO) and probenecid (water soluble) were obtained from Molecular Probes, Invitrogen (Breda, the Netherlands). Ficoll-Paque^{TM} PLUS was purchased from GE Healthcare (Eindhoven, the Netherlands). Heparin was purchased from LEO-Pharma (5,000 IU/ml; Weesp, the Netherlands). Phosphate Buffered Saline (PBS) and Roswell Park Memorial Institute (RPMI) 1640 medium (without L-glutamine and phenol red) were obtained from Lonza Verviers SPRL (Verviers, Belgium). Fetal Bovine Serum (FBS) was from Perbio Science Nederland BV (Etten-Leur, the Netherlands).

Isolation of human polymorphonuclear leukocytes

Human polymorphonuclear leukocytes (PMNs) were isolated from fresh whole blood, for which donors signed written consent forms, or from buffy coats, which were purchased from Sanquin Blood Bank (Amsterdam, the Netherlands). The PMNs were obtained by centrifugation on Ficoll-Paque^{TM} PLUS (density: 1.077 g/ml), followed by hypotonic lysis of erythrocytes with sterile lysis buffer (0.15 M NH_4Cl, 0.01 M $KHCO_3$ and 0.1 mM EDTA, pH at 4 °C is 7.4). After lysis, the PMNs were washed with PBS and finally resuspended in RPMI 1640 medium (without L-glutamine and phenol red) supplemented with 1% heat-inactivated FBS. Resulting PMN preparations consisted of approximately 95-97% PMNs, based on PMNs physical parameters analyzed by flow cytometry and CD16 expression. The preparations were negative for CD14, meaning that the preparations did not contain monocytes.

Cell viability

Cell viability was determined by monitoring the fluorescence enhancement of PI in PMNs. This fluorescent probe can only cross the plasma membrane of non-viable cells, after which the fluorescence of this probe is enhanced 20- to 30-fold due to nucleic acid binding. PMNs were incubated for several time points with indicated reagents. After incubation, cells were washed with PBS and resuspended in 300 µl RPMI 1640 medium (without L-glutamine and phenol red) supplemented 1% heat-inactivated FBS and loaded with 3 µl PI (0.5 mg/ml). PI fluorescence was monitored in the FL2 channel using a BD FACSCalibur Flow Cytometer with CellQuest Pro Software (version 5.2.1.).

CXCL8 ELISA

Buffy coat isolated PMNs (10^5 cells/well) were incubated for several time points with indicated reagents. After incubation, the supernatants were collected and CXCL8 levels were measured using a human CXCL8 ELISA kit according to manufacturer's instructions.

Chemotaxis assay

The chemotaxis assay was performed as previously described by Weathington *et al.* [8]. Briefly, indicated reagents were placed in the bottom wells of a 3-µm 96-well polycarbonate filter plate (Millipore BV, Amsterdam, the Netherlands) in RPMI 1640 medium (without L-glutamine and phenol red) supplemented with 1% heat-inactivated FBS. 2 x 10^5 PMNs, isolated from fresh whole blood, were added to the top portion. The plate was incubated for 1 h at 37 ℃ in 5% CO_2. After removing the upper portion, the cells in each bottom well were counted for 30 sec using a BD FACSCalibur Flow Cytometer with CellQuest Pro Software (version 5.2.1.). Data were standardized to a chemotactic index (= cells per well migrating to chemoattractant/cells per well migrating to medium).

Calcium mobilization assay

PMNs, isolated from fresh whole blood, were resuspended in RPMI 1640 medium (without L-glutamine and phenol red) supplemented with 1% heat-inactivated FBS. The cells were loaded with the fluorescent dye FLUO-3 AM (1 µM) by incubating the cells for 20 min at room temperature in the presence of 2.5 mM probenecid under gentle agitation. After washing with PBS the cells were resuspended to a concentration of 10^6 cells/ml. For each measurement a baseline was obtained after which the cells were stimulated with the indicated reagents. The Ca^{2+} mobilization was measured using a BD FACSCalibur Flow Cytometer with CellQuest Pro Software (version 5.2.1.) and analyzed using the program FCS Press (version 1.4 b).

Statistical analyses

For all statistical analyses, GraphPad Prism version 4.0 was used. One-tailed Student *t*-tests were used for comparing two groups. For comparing three or more groups, the data were analyzed using a one-way ANOVA followed by Tukey post hoc analysis. Data were considered significant at $P < 0.05$. All results are expressed as means ± SEM.

Results

N-ac-PGP dose dependently induces chemotaxis of PMNs via a PTX sensitive G protein-coupled receptor and a Ca^{2+} influx in vitro

A transwell chemotaxis system was used to evaluate the chemotactic effect of N-ac-PGP on PMNs from fresh human whole blood. After 1 h incubation, the migration of PMNs to the lower chamber was quantified and standardized to a chemotactic index. Collagen fragment N-ac-PGP (10^{-7} - $3{\cdot}10^{-3}$ M) was dose dependently chemotactic for PMNs (fig.1A), whereas PGG ($3{\cdot}10^{-3}$ M) was not.

The chemoattractant CXCL8 initiates its biological activity by binding to G protein-coupled receptors (GPCRs) CXCR1 and CXCR2, leading to the mobilization of cytosolic Ca^{2+} and ultimately to the migration of the cell to the site of inflammation [14, 15]. Therefore, we hypothesized that N-ac-PGP, resembling CXCL8, was able to induce neutrophilic migration via GPCRs and instantly increase cytosolic Ca^{2+} in PMNs, which is a measure of cell activation.

To assess the role of GPCRs in N-ac-PGP induced chemotaxis, the effect of pertussis toxin (PTX) was investigated. Figure 1B shows that pre-incubation of PMNs for 1.5 h with 500 ng/ml PTX completely abolished neutrophil migration by N-ac-PGP and CXCL8. Figure 1C shows that N-ac-PGP (10^{-4} M - $3{\cdot}10^{-3}$ M) dose dependently induced an instant Ca^{2+} influx. PMN stimulation with $3{\cdot}10^{-3}$ M PGG did not lead to Ca^{2+} mobilization, whereas 50 ng/ml CXCL8 stimulation resulted in an increase in cytosolic Ca^{2+}.

To evaluate the role of G protein-coupled CXCR2 in N-ac-PGP induced neutrophilic chemotaxis, PMNs were pre-incubated with the CXCR2 antagonist Compound 1 for 45 min after which the chemotaxis assay was performed. As shown in figure 1D, N-ac-PGP ($3{\cdot}10^{-3}$ M) and CXCL8 (10 ng/ml) induced a chemotactic response, which could be attenuated for both chemoattractants due to incubation with Compound 1.

These data demonstrate that N-ac-PGP instantly activates the PMNs via PTX sensitive GPCRs and that blockade of CXCR2 receptors resulted in a complete inhibition of the chemotactic response to N-ac-PGP.

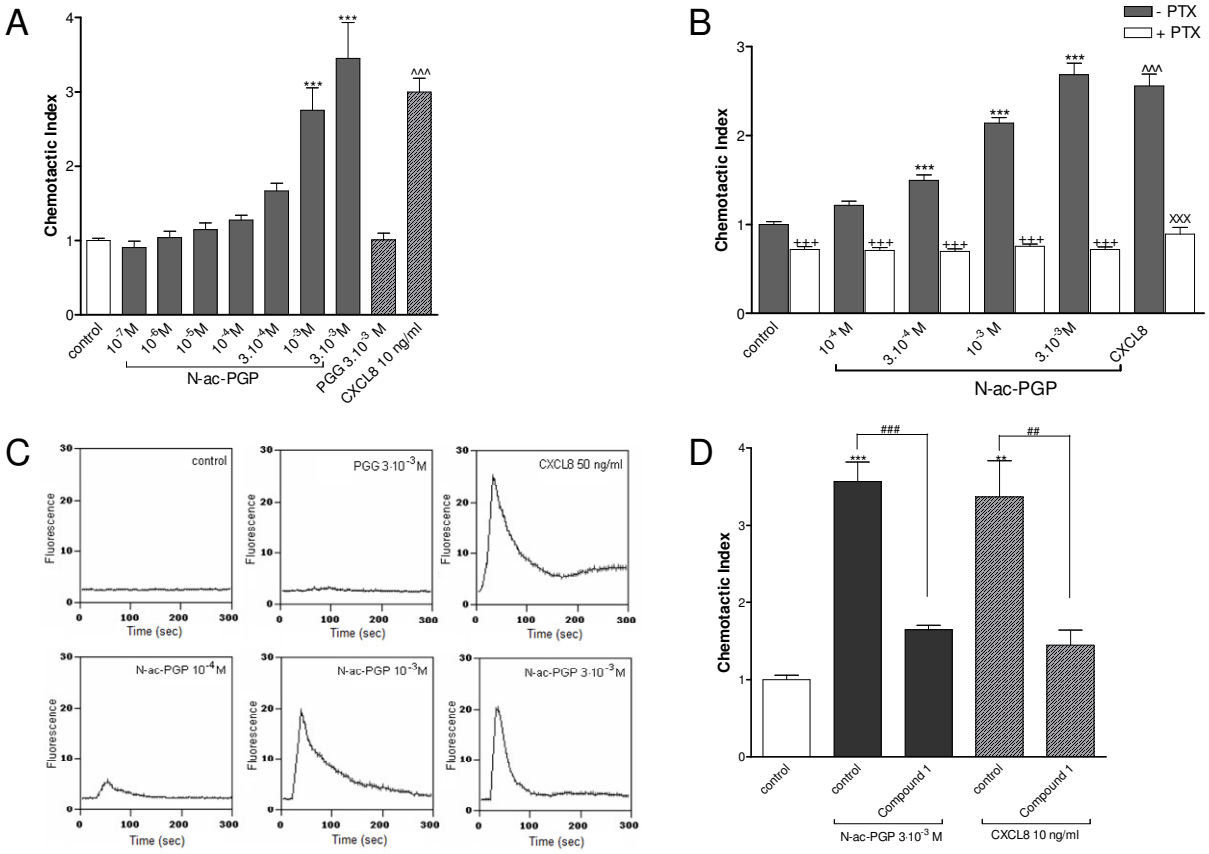

Figure 1. N-ac-PGP induces chemotaxis of PMNs via PTX sensitive GPCR CXCR2 and a Ca^{2+} influx in vitro.

*(**A**) N-ac-PGP (10^{-7} – $3{\cdot}10^{-3}$ M) induces chemotaxis in PMNs (***, P < 0.001 N-ac-PGP vs. control), whereas PGG ($3{\cdot}10^{-3}$ M) is not chemotactic. CXCL8 (10 ng/ml) was tested as a positive control (^^^, P < 0.001 CXCL8 vs. control). (**B**) Freshly isolated PMNs were incubated with medium or PTX (500 ng/ml) for 90 min at 37 °C in 5% CO_2. Subsequently, the chemotaxis assay was performed. N-ac-PGP (10^{-4} – $3{\cdot}10^{-3}$ M) induces chemotaxis in PMNs pre-incubated with medium (black bars; ***, P < 0.001 N-ac-PGP vs. control). This migration is completely inhibited in PMNs pre-incubated with PTX (white bars; $^{+++}$, P < 0.001 N-ac-PGP vs. PTX N-ac-PGP), which is not significantly different from PTX control level. Chemotaxis induced by CXCL8 (10 ng/ml; ^^^, P < 0.001 CXCL8 vs. control) can be decreased by pre-incubating PMNs with PTX (^{xxx}, P < 0.001 CXCL8 vs. PTX CXCL8). (**C**) 2.25 x 10^5 freshly isolated PMNs were loaded with FLUO-3 AM. For each measurement, a baseline for 15 sec was obtained, after which the cells were stimulated with medium, N-ac-PGP (10^{-4} – $3{\cdot}10^{-3}$ M), PGG ($3{\cdot}10^{-3}$ M) or CXCL8 (50 ng/ml). N-ac-PGP dose dependently induces an instant Ca^{2+} influx (**lower panel if 1C**). Medium control and PGG were used as negative controls and CXCL8 was used as a positive control (**upper panel fig. 1C**). (**D**) Prior to chemotaxis, cells were pre-incubated with medium or CXCR2-antagonist Compound 1 (10^{-5} M) for 45 min at 37 °C. N-ac-PGP and CXCL8 induce chemotaxis in PMNs pre-incubated with medium (**, P < 0.01; ***, P < 0.001 control vs. N-ac-PGP control or CXCL8 control). For both chemoattractants, this migration is decreased in PMNs pre-incubated with CXCR2-antagonist Compound 1 ($^{\#\#}$, P < 0.001 N-ac-PGP control vs. Compound 1 N-ac-PGP; $^{\#\#}$, P < 0.01; CXCL8 control vs. Compound 1 CXCL8).*

PMNs release CXCL8 upon activation with collagen fragment N-ac-PGP

Early in inflammation, neutrophils migrate from the capillary into the interstitial space, following a chemotactic gradient of CXCL8 [15]. At the site of inflammation, neutrophils are activated by stimulants released by macrophages, such as CXCL8 and leukotriene B_4 [1, 16]. Upon activation, neutrophils release CXCL8, leading to a self-perpetuating inflammatory state where neutrophils attract more neutrophils via CXCR1 and CXCR2 [4, 17, 18]. This led to the hypothesis that N-ac-PGP, formed after collagen breakdown and working via the same mechanism as CXCL8, activates the neutrophil to synthesize CXCL8, acting in an autocrine/paracrine fashion.

To examine this hypothesis, PMNs were incubated with N-ac-PGP for different time points from 1 to 8 h. Figure 2 A and B show that stimulation of PMNs with increasing concentrations of N-ac-PGP results in CXCL8 release by these cells at all time points. Significant amounts were produced after stimulation of the cells with 10^{-3} M - $3 \cdot 10^{-3}$ M N-ac-PGP. Incubation with $3 \cdot 10^{-3}$ M PGG did not lead to a CXCL8 release, whereas 1 µg/ml LPS exposure did.

Cell viability was assessed by flow cytometry by staining the cells with propidium iodide after different incubation time periods with N-ac-PGP, PGG or LPS (table 1). At all time points, there were no significant differences between cells incubated with medium or cells incubated with $3 \cdot 10^{-3}$ M PGG or 10^{-7} M - $3 \cdot 10^{-3}$ M N-ac-PGP. These results indicate that N-ac-PGP treatment does not negatively influence cell viability.

N-ac-PGP activated the neutrophil to release CXCL8 and CXCL8 itself works via CXCR1 and CXCR2 on the neutrophil. This led to the suggestion that not N-ac-PGP but CXCL8 released after N-ac-PGP exposure was responsible for effects seen on neutrophil chemotaxis and Ca^{2+} mobilization. To determine whether N-ac-PGP exerts these effects directly or indirectly on the neutrophil, Ca^{2+} influx assays and chemotaxis studies in the presence of an antibody against CXCL8 (α-CXCL8) were conducted.

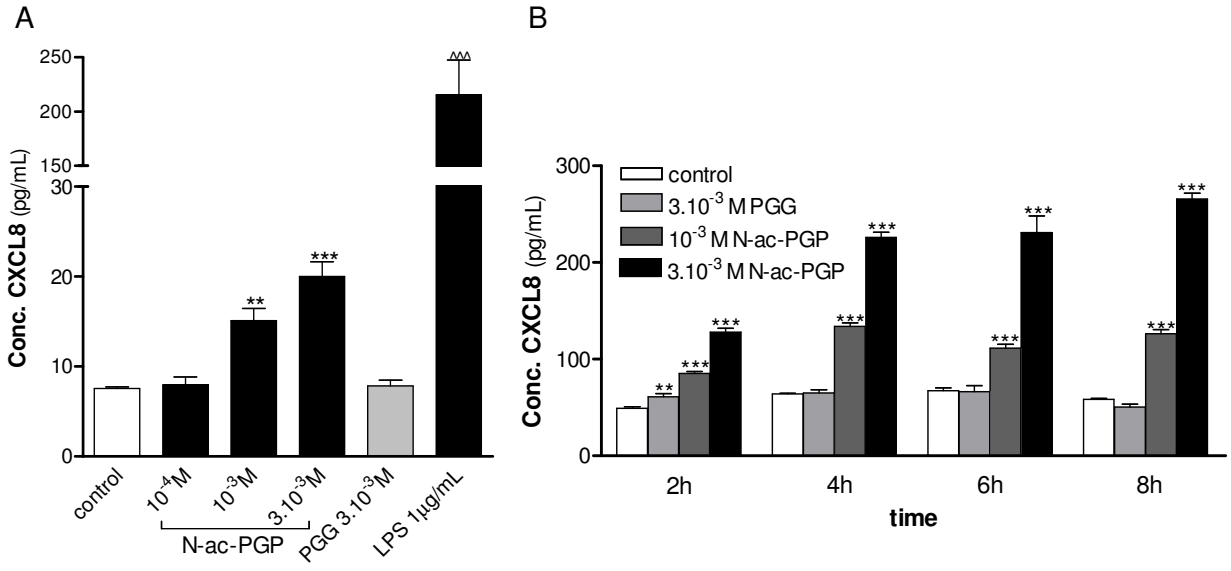

Figure 2. N-ac-PGP induces the release of CXCL8.

*(**A**) 10^5 freshly isolated PMNs were stimulated for 1 h with N-ac-PGP (10^{-4} – $3{\cdot}10^{-3}$ M), PGG ($3{\cdot}10^{-3}$ M) or LPS (1 µg/ml). A CXCL8 ELISA was performed on the supernatants. N-ac-PGP induced the release of CXCL8 from fresh cells within 1 h (**P < 0.01; ***/^^^, P < 0.001 vs. control). (**B**) 10^5 buffy coat isolated neutrophils were stimulated for 2, 4, 6 and 8 h with N-ac-PGP (10^{-7} – $3{\cdot}10^{-3}$ M), PGG ($3{\cdot}10^{-3}$ M) or LPS (1 µg/ml). N-ac-PGP (10^{-3} M and $3{\cdot}10^{-3}$ M) induced the release of CXCL8 from fresh cells for all measured time points (**, P < 0.01; ***, P < 0.001 N-ac-PGP vs. control). PGG was negative for all time points, whereas LPS was positive for all time points. Only the concentrations $3{\cdot}10^{-4}$ – $3{\cdot}10^{-3}$ M are shown here; the cells stimulated with lower concentrations N-ac-PGP did not release more CXCL8 than the control cells.*

Antibodies directed against CXCL8 do not inhibit N-ac-PGP-induced Ca^{2+} mobilization in PMNs

To investigate if N-ac-PGP directly activates PMNs with or without intervention of released CXCL8, a Ca^{2+} mobilization assay was performed. Prior to stimulation with the indicated reagents, α-CXCL8 was added to the PMNs to capture spontaneously released CXCL8. The cells were stimulated with 20 ng/ml CXCL8 or $3{\cdot}10^{-3}$ M N-ac-PGP (fig. 3). At lower concentrations, the α-CXCL8 antibody blocked the CXCL8-induced Ca^{2+} influx partially, whereas at higher concentrations of the antibody, it blocked the mobilization completely. However, the antibody did not inhibit the Ca^{2+} response generated upon N-ac-PGP stimulation, indicating that CXCL8 released by PMNs after N-ac-PGP exposure was not responsible for the observed instant Ca^{2+} mobilization in PMNs.

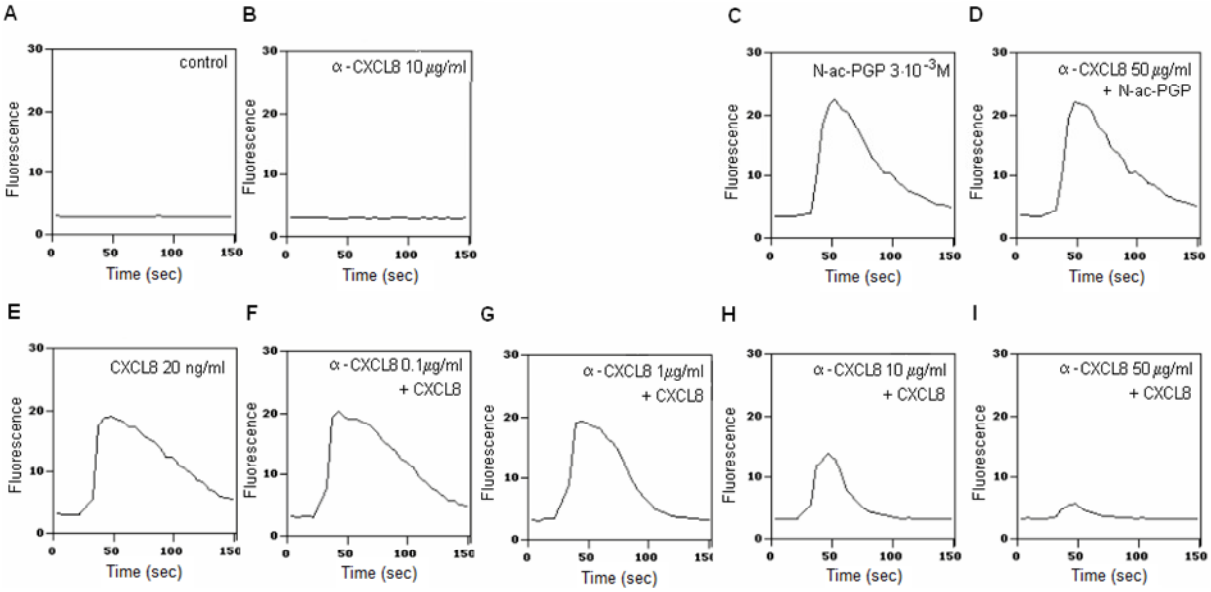

Figure 3. Antibody directed against CXCL8 does not block N-ac-PGP-induced Ca^{2+} mobilization in PMNs.

*2.25 x 10^5 freshly isolated PMNs were loaded with FLUO-3 AM. Before measuring, medium (**A-C** and **E**) or α-CXCL8 (**D** and **F-I**) was added to the cells. After 20 sec baseline measurement, the cells were stimulated with medium (**A**), N-ac-PGP (3·10^{-3} M; **C** and **D**) CXCL8 (20 ng/ml; **E-I**) or α-CXCL8 (10 µg/ml; **B**). The antibody α-CXCL8 does not stimulate the PMN to release Ca^{2+} into the cytosol (**B**). N-ac-PGP (**C**) and CXCL8 (**E**) induce Ca^{2+}mobilization. The N-ac-PGP induced Ca^{2+} mobilization was not influenced by α-CXCL8 incubation (**D**), whereas the influx induced by CXCL8 could be attenuated dose dependently by α-CXCL8 incubation (0.1-50 µg/ml; **F-I**).*

CXCL8 is not responsible for N-ac-PGP-induced chemotaxis *in vitro*

To determine if N-ac-PGP was solely responsible for the migration of neutrophils or whether CXCL8 released by these cells upon stimulation was contributing to migratory signals, cells were incubated with α-CXCL8 antibody for 1 h at 37 ℃ in 5% CO_2. Subsequently, the chemotaxis assay was performed. N-ac-PGP (3·10^{-3} M) and CXCL8 (10 ng/ml) induced a chemotactic response, as shown in figure 4. The CXCL8 induced chemotaxis was attenuated after α-CXCL8 antibody incubation, whereas the chemotactic effect of N-ac-PGP was not affected.

As shown in figure 2A, LPS activation led to a significant CXCL8 release from PMNs. To determine whether LPS was able to induce chemotaxis via CXCL8 release, the chemotaxis assay was performed. Figure 5 shows no chemotactic effect for LPS on PMNs. At higher concentrations of LPS, there was even a significant reduction in basal chemotactic activity, possibly due to overstimulation of the cell, leading to cell death (table 1). These data support the findings that N-ac-PGP directly activates the PMN, without intervention of released CXCL8.

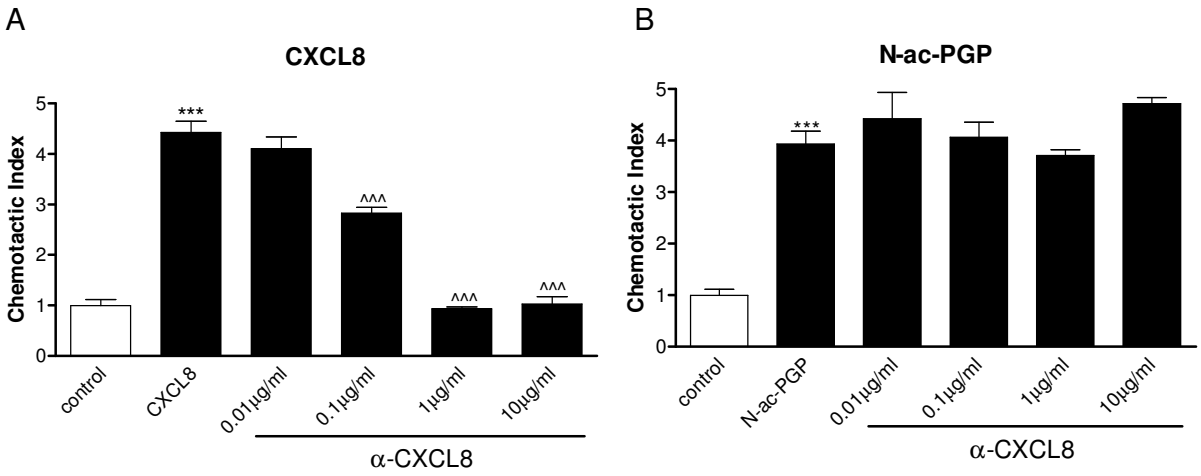

Figure 4. CXCL8 is not responsible for N-ac-PGP induced chemotaxis in vitro.

*$2x10^5$ freshly isolated neutrophils were incubated for 1 h at 37 °C with various concentrations of α-CXCL8 (0.01-10 µg/ml). The cells were placed in the top well and the chemoattractant (N-ac-PGP; $3 \cdot 10^{-3}$ M or CXCL8; 10 ng/ml) was present in the bottom well of a 96-wells Millipore Filtration Plate System. After 1 h incubation at 37 °C, the cells were counted with FACS. CXCL8 (**A**) and N-ac-PGP (**B**) induce chemotaxis in PMNs (***, P < 0.001 vs. control). The chemotaxis induced by N-ac-PGP is not inhibited by α-CXCL8 (**B**), whereas incubation of the PMNs with the antibody resulted in a complete inhibition of the CXCL8 induced chemotaxis (**A**) (^^^, P < 0.001 α-CXCL vs. CXCL8).*

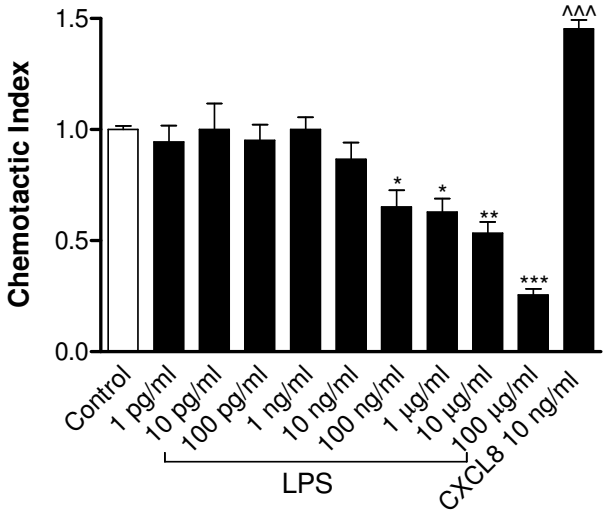

Figure 5. LPS is not chemotactic for PMNs in vitro.

$2x10^5$ freshly isolated neutrophils were placed in the top well and the chemoattractant was present in the bottom well of a 96-wells Millipore Filtration Plate System. After 1 h incubation at 37°C in 5% CO_2, the cells from each bottom well were counted. Data were standardized to a chemotactic index. LPS did not induce chemotaxis in PMNs. Concentrations from 100 ng/ml to 100 µg/ml led to a significantly lower chemotactic index, possibly due to cell death by overstimulation (, P < 0.05; **, P < 0.01; ***/^^^, P < 0.001 LPS/CXCL8 vs. control).*

Table 1. Cell viability of PMNs after N-ac-PGP, PGG and LPS incubation.

	1h	2h	4h	6h	8h	19h	24h
Control							
%PMNs	95.7	95.6	95.5	94.3	93.0	86.2	76.4
% PI^+ PMNs	2.7	1.9	2.3	2.8	3.9	21.5	41.6
N-ac-PGP $3 \cdot 10^{-4}$ M							
%PMNs	96.7	96.6	95.6	95.0	94.7	87.3	78.7
% PI^+ PMNs	2.2	2.0	2.7	2.5	3.5	24.9	49.4
N-ac-PGP 10^{-3} M							
%PMNs	97.0	96.6	95.9	94.8	94.9	89.8	84.1
% PI^+ PMNs	2.0	1.9	2.4	2.4	3.9	19.5	45.8
N-ac-PGP $3 \cdot 10^{-3}$ M							
%PMNs	96.9	96.5	95.7	95.1	95.1	91.5	87.4
% PI^+ PMNs	2.1	2.0	2.1	2.6	3.3	13.2	34.2
PGG $3 \cdot 10^{-3}$ M							
%PMNs	97.1	96.5	96.3	94.5	93.7	86.7	76.6
% PI^+ PMNs	2.0	2.0	2.1	2.6	3.0	22.9	45.4
LPS 1 µg/ml							
%PMNs	86.0	94.2	90.7	88.3	86.9	60.5	47.4
% PI^+ PMNs	9.1	4.2	5.5	6.0	9.2	49.8	74.4

7×10^5 buffy coat isolated PMNs resuspended in 300 µl RPMI medium with 1% FCS were loaded with 3 µl PI (5 µg/ml). A gate based on PMNs physical parameters (forward and side scatter) was set to exclude debris. Within this gate, the PI positive population was monitored in the FL2 channel.

Discussion

In this report, we show that N-ac-PGP is chemotactic for neutrophils via PTX sensitive $G_{i/o}$ proteins and that this tripeptide can directly activate this cell type, leading to cytosolic Ca^{2+} mobilization and the release of CXCL8. However, this CXCL8 release is not involved in the observed chemotactic effects of N-ac-PGP *in vitro*, because studies using an antibody directed against CXCL8 demonstrated that the released CXCL8 is not responsible for the N-ac-PGP induced chemotaxis and Ca^{2+} influx. CXCL8 is one of the most prominent chemokines found in neutrophilic inflammatory diseases, such as COPD. The levels of this chemokine are increased in sputum of COPD patients and correlate with the increased number of neutrophils found in the lungs [1]. These levels further increase during exacerbation of this disease, leading to increased numbers of neutrophils in the lungs [1]. However, antagonizing CXCL8 with an α-CXCL8 antibody, and blocking leukotrienes, such as leukotriene B_4, with an antagonist incompletely prevents neutrophil chemotaxis in COPD patients [19]. This suggests that other unidentified chemoattractants are involved in neutrophil migration in COPD.

During inflammation, mediator release from inflammatory cells such as neutrophils and macrophages leads to extracellular matrix breakdown and subsequently, to the formation of collagen fragments [15, 20]. Some of these fragments can have chemotactic properties, as described in several reports [6, 7, 14]. One of the most potent collagen fragments with chemotactic properties is N-ac-PGP, which was first identified in a rabbit model investigating alkali injury to the cornea [7]. Moreover, this collagen fragment can attract neutrophils to the lung, leading to lung damage [8], and has been found in patients suffering from COPD [8, 9] and CF [10]. In these diseases, neutrophils secrete proteases, including neutrophil elastase and matrix metalloproteinases (MMPs) 8 and 9 [1, 3]. Proteolytic cleavage of alveolar collagen by MMPs and prolyl endopeptidase and subsequent acetylation, generates N-ac-PGP [9, 10], thereby presumably prolonging the neutrophilic influx [15].

In the present study, we demonstrate that N-ac-PGP has pro-inflammatory properties. Consistent with previous studies [7, 8], we found that N-ac-PGP was chemotactic for the neutrophil *in vitro*. Concentrations in the millimolar range of N-ac-PGP were used, which were in a similar range as the concentrations used for chemotaxis and polarization assays by other groups [7]. Moreover, these concentrations did not induce cell death (table 1).

The concentrations of N-ac-PGP and CXCL8 have been measured separately in several COPD and CF patient studies and, to our knowledge, there are no reports in which both CXCL8 and N-ac-PGP were measured using human disease material. However, the levels of both N-ac-PGP and CXCL1 (KC; keratinocyte cell-derived chemokine, a CXCL8 homologue in mice) have been measured in a murine model where A/J mice were exposed to cigarette

smoke for five months [21]. After this exposure, emphysema-like characteristics such as alveolar enlargement and heart hypertrophy were detected. This lung tissue degradation was also reflected by the levels of N-ac-PGP in the bronchoalveolar lavage (BAL) fluid, which increased after 5 months smoke from 0.06 ng/ml to 12.4 ng/ml [21], whereas the concentration of CXCL1 was approximately 0.02 ng/ml (Braber, personal communication). Moreover, it was shown in a murine study using BALB/c mice that the N-ac-PGP level was more than 30 times higher than the level of CXCL1 12 h after LPS aerosolization [8]. From these murine studies it can be concluded that the concentrations of N-ac-PGP and the CXCL8 homologue are physiologically relevant and that both chemoattractants play an important role in the pathology of neutrophilic diseases, such as COPD. In addition, the N-ac-PGP concentrations measured in BAL fluid may not be representative for the possible higher concentrations in the tissues, which was not assessed in these reports.

N-ac-PGP induced chemotaxis via PTX sensitive G proteins and mobilization of cytosolic Ca^{2+} by a similar mechanism as other chemoattractants such as fMLP [14, 22, 23] and CXCL8 [14, 23, 24]. Pre-incubation of the PMNs with 500 ng/ml PTX, a specific inhibitor of $G_{i/o}$ type G proteins, completely abolished the N-ac-PGP induced migration, without affecting the cell viability. As expected, chemotaxis induced by CXCL8 was also decreased by pre-incubating PMNs with PTX. Moreover, pre-incubation of neutrophils with CXCR2 antagonist Compound 1 attenuated the chemotactic effect of N-ac-PGP and CXCL8, demonstrating a role for CXCR2 in the chemotaxis induced by these chemoattractants. N-ac-PGP may resemble CXCL8 in initiating the classical pathway in which its binding to GPCRs or other membrane-bound proteins ultimately leads to the release of Ca^{2+} from the endoplasmatic reticulum into the cytosol. This Ca^{2+} mobilization is required for processes such as chemotaxis [24-26].

Previously, we have proposed in collaboration with De Kruijf and colleagues that N-ac-PGP may interact indirectly with CXCR1 and CXCR2 via the release of chemokines, known to bind these receptors, or through activation of other receptors on the neutrophil to induce chemotaxis, since the peptide was unable to activate G protein-dependent and G protein-independent signaling and unsuccessful in displacing the radioligands [^{125}I]CXCL8 and [^{3}H]-SB265610 from CXCR1 and CXCR2 expressing HEK293T cells or neutrophils [12].

In this report we demonstrated that N-ac-PGP driven chemotaxis for neutrophils is PTX sensitive. In addition, we showed that N-ac-PGP directly induces Ca^{2+} mobilization. Moreover, we performed a chemotaxis assay using L1.2 B cells transiently expressing CXCR2. These L1.2 B cells did not show any chemotactic response upon exposure to N-ac-PGP, while CXCL8 did (suppl. fig. 1). These data indicate that N-ac-PGP is able to activate signaling in neutrophils, which is not apparent in HEK293T or L1.2 B cells. The effect of N-

ac-PGP appears therefore cell type dependent and the results of CXCR signaling studies performed on HEK293T cells, L1.2 B cells or neutrophils may therefore differ in outcome.
Moreover, it is possible that N-ac-PGP-induced chemotaxis is mediated through a phosphatidylinositol 3-kinase (PI3K)-γ-independent pathway [25]. CXCL8-induced chemotaxis is mediated through PI3K-γ-independent and –dependent pathways. In the PI3K-γ-independent pathway, phospholipase C is activated, which in turn produces inositol trisphosphate, leading ultimately to Ca^{2+} mobilization from non-mitochondrial stores [25]. However, Ca^{2+} mobilization can also be induced via inositol trisphosphate independent signal transduction pathways, such as via the activation of store-operated channels. These channels are activated by depletion of Ca^{2+} stores through the Ca^{2+} influx factor- Ca^{2+}-independent phospholipase A_2 mechanism [27]. It may be possible that N-ac-PGP and CXCL8 induce chemotaxis through different signal transduction pathways.
Here we report that neutrophils release CXCL8 upon activation with N-ac-PGP. In order to establish whether the released CXCL8 were responsible for the observed N-ac-PGP effects *in vitro*, PMNs were incubated with α-CXCL8 antibody prior to Ca^{2+} mobilization measurement and chemotaxis. This incubation with the antibody did not result in a change in N-ac-PGP induced Ca^{2+} mobilization or chemotaxis, even when high concentrations of α-CXCL8 antibody were used. In contrast, α-CXCL8 antibody attenuated the CXCL8-induced Ca^{2+} influx and chemotactic effect. Therefore, it can be concluded that the released CXCL8 by neutrophils was not responsible for the observed N-ac-PGP activating and chemotactic effects. Although N-ac-PGP induces CXCL8-independent chemotaxis of neutrophils, it can be hypothesized that N-ac-PGP may interact with other membrane bound proteins as is described for MIF and CD74-CXCR2 and CD74-CXCR4 [28] or that N-ac-PGP induces the release of other chemokines known to bind to CXCRs [29]. In addition, N-ac-PGP may act indirectly on the CXCR by activating the growth factor receptor tyrosine kinase, thereby inducing the transactivation of GPCRs [30].
Neutrophilic CXCL8 release induced by N-ac-PGP may lead in the long term to a self-perpetuating situation where neutrophils can attract and activate more neutrophils via CXCR1 and CXCR2. In our studies, differences in CXCL8 release and chemotactic properties between N-ac-PGP-stimulated and unstimulated PMNs were measured within 1 h. Although CXCL8 is not stored in neutrophils [18], we presume that isolation from blood already activates the neutrophil [31] to synthesize CXCL8. After stimulation with an activator, such as N-ac-PGP or LPS, CXCL8 release can then be induced from PMNs.
Finally, our data indicate that besides chemotaxis, N-ac-PGP stimulates the neutrophils to release CXCL8, which *in vivo* may lead to a self-perpetuating situation where N-ac-PGP and CXCL8 work in concert, leading to enhanced neutrophil inflammation and lung inflammation.

Acknowledgements

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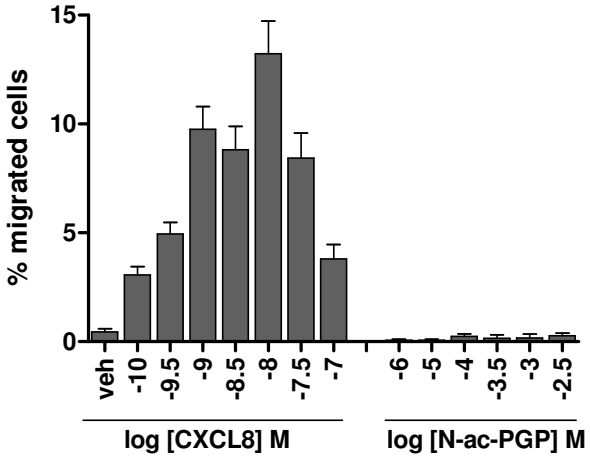
Supplementary data



Suppl. figure 1. Chemotactic response to CXCL8 or N-a-PGP of L1.2 cells expressing hCXCR2.

Cell culturing and transfection

The murine L1.2 pre-B cells (kindly provided by Dr. Pease, Imperial College London, UK) were grown in RPMI 1640 medium with GlutaMax-I and 25 mM HEPES, supplemented with 10% heat-inactivated certified FBS, penicillin, streptomycin, glutamine, nonessential amino acids, 2-mercaptoethanol, and sodium pyruvate. L1.2 cells were transfected with 10 µg receptor/1x10^7 cells using a Bio-Rad Gene Pulser Xcell (330 V and 975 µF), and subsequently grown overnight in culture medium supplemented with 10 mM sodium butyrate.

Chemotaxis L1.2 B cells

Twenty-four hours after transfection, migration of L1.2 B cells towards CXCL8 or N-ac-PGP was determined using 5-µm pore ChemoTx 96-well plates (Neuro Probe). First the lower wells of the ChemoTx plates were blocked for 30 min using RPMI 1640 medium with GlutaMAX-I and 25 mM HEPES supplemented with 1% (w/v) BSA. CXCL8 or N-ac-PGP was diluted in the same medium supplemented with 0.1% (w/v) BSA, and dispensed in the bottom wells of the chemotaxis plate after removing the blocking buffer. The membrane was placed on top of these wells and 2.5 x10^5 cells in the same buffer were applied to the upper surface (total 31 µl) and incubated for 4 h in a humidified chamber at 37 ℃ in the presence of 5% CO_2. The number of cells that traversed the 5-µm pore membrane and migrated into the bottom wells was quantified on the Victor2 1420 multilabel plate reader upon the incorporation of the calcein AM dye (Invitrogen).

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Chapter 6

CXCR2 antagonists block the N-acetyl Proline-Glycine-Proline-induced neutrophil influx in the airways of mice, but not the production of the chemokine CXCL1

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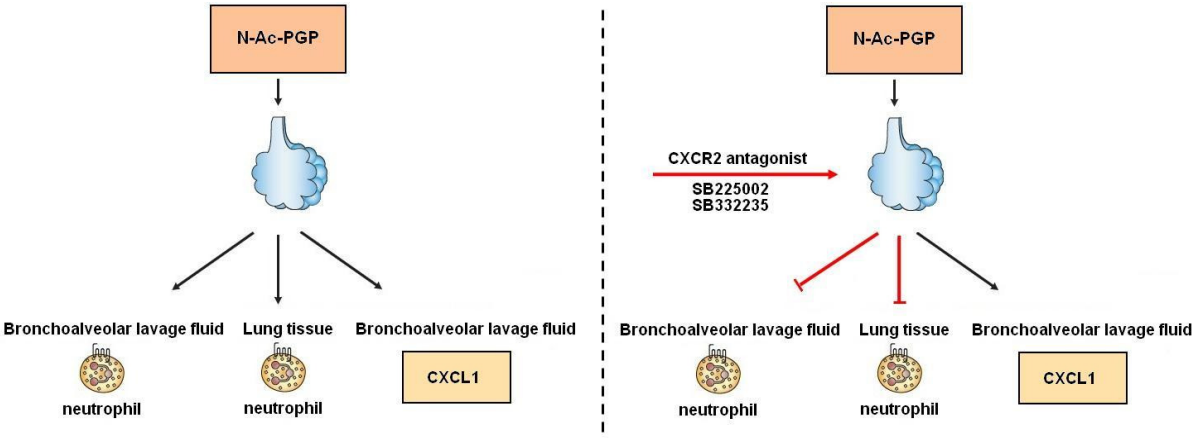
Abstract

Neutrophils are innate immune cells in chronic inflammatory diseases including chronic obstructive pulmonary disease (COPD) and can be attracted to the site of inflammation via the collagen breakdown product N-acetyl proline-glycine-proline (N-ac-PGP). To elucidate whether CXCR2 is involved in N-ac-PGP-induced neutrophil migration and activation, studies using specific antagonists were performed *in vivo*. N-ac-PGP and keratinocyte cell-derived chemokine (KC, CXCL1) were administered in C57Bl/6 mice via oropharyngeal aspiration. Intraperitoneal applications of CXCR2 antagonist SB225002 or SB332235 were administered 1 h prior and 1 h after oropharyngeal aspiration. Six hours after oropharyngeal aspiration mice were sacrificed. Neutrophil counts and CXCL1 levels were determined in bronchoalveolar lavage (BAL) fluid, myeloperoxidase (MPO) levels were measured in lung tissue homogenates and an immunohistological staining for neutrophils was performed on lung tissue. N-ac-PGP and CXCL1 induced a neutrophil influx in the BAL fluid and lung tissue, which was also reflected by increased MPO levels in lung tissue. The N-ac-PGP- and CXCL1-induced neutrophil influx and the increased pulmonary tissue MPO levels were inhibited by the CXCR2 antagonists SB225002 and SB332235. Moreover, N-ac-PGP administration enhanced the CXCL1 levels in BAL fluid, which could not be attenuated by both CXCR2 antagonists.

In conclusion, neutrophil migration induced by N-ac-PGP is mediated via direct CXCR2 interaction. The N-ac-PGP-induced release of CXCL1 is independent of CXCR2. Related to the maximal effect of CXCL1, N-ac-PGP is more potent at inducing neutrophil migration in the pulmonary tissue than into the BAL fluid, or N-ac-PGP may be more potent at inducing MPO levels in the lung tissue.



N-Ac-PGP-induced neutrophil migration into the lungs of mice is inhibited by CXCR2 antagonists, while the N-Ac-PGP-induced release of CXCL1 is independent of CXCR2.

Introduction

Neutrophils are innate inflammatory cells in chronic inflammatory diseases including chronic obstructive pulmonary disease (COPD) and are attracted to the site of inflammation via chemoattractants, such as interleukin-8 (CXCL8) in humans and keratinocyte cell-derived chemokine (KC; CXCL1) or macrophage inflammatory protein-2 (MIP-2, CXCL2) in mice [1]. CXCL8, CXCL1 and CXCL2 bind to the CXCR2 that is predominantly expressed by neutrophils. Activated neutrophils release proteases, which contribute to collagen breakdown leading to lung emphysema. In our hypothesis, the increased protease activity leads not only to alveolar wall destruction but also to the formation of N-acetyl proline-glycine-proline (N-ac-PGP) from collagen [2]. N-ac-PGP is chemotactic for neutrophils *in vitro* as well as *in vivo* [3] and activates human neutrophils to release CXCL8 [4]. The importance of N-ac-PGP in inflammatory diseases, such as COPD, is reinforced by several studies. Clinical data demonstrated that N-ac-PGP can be detected in the bronchoalveolar lavage (BAL) fluid, sputum and serum of COPD patients [3, 5]. Chronic airway exposure to N-ac-PGP causes neutrophil infiltration and lung emphysema in mice [2, 3].

It has been suggested that the basis for N-ac-PGP effects lies in its structural homology with the GP motif present in all ELR^+ CXC chemokines, such as CXCL8. This GP motif is essential for cell activation and ligand binding to CXCR1/2 receptors on neutrophils [3, 6]. Overbeek *et al.* [4] and Weathington *et al.* [3] reported that N-ac-PGP activity is mediated via the G protein-coupled receptor CXCR2, since CXCR2 antibodies suppressed the N-ac-PGP-induced neutrophil chemotaxis *in vitro*. Furthermore, the accumulation of neutrophils seen in mice upon intratracheal administration of N-ac-PGP, was not detected in $CXCR2^{-/-}$ mice [3].

The aim of this study was to investigate whether the N-ac-PGP-induced neutrophil influx was mediated via CXCR2 *in vivo.* We investigated the effects of two different selective nonpeptide CXCR2 antagonists, SB225002 and SB332235, in a murine model where a neutrophil influx was induced via oropharyngeal administration of N-ac-PGP or CXCL1. In this report, we demonstrate that N-ac-PGP and CXCL1 can induce a neutrophil influx in lung tissue and BAL fluid of mice, which can be inhibited by CXCR2 antagonists. The neutrophil migration induced by N-ac-PGP is mediated via direct interaction with CXCR2 and by a small, if any, release of CXCL1. The N-ac-PGP-induced release of CXCL1 is independent of the CXCR2.

Material and methods

Animals

Male C57Bl/6 mice, 6-7 weeks old were obtained from Charles River Laboratories and housed under controlled conditions in standard laboratory cages in the animal facility. They were provided free access to water and food. All *in vivo* experimental protocols were approved by the local Ethics Committee and were performed under strict governmental and international guidelines on animal experimentation.

Oropharyngeal aspiration

Oropharyngeal aspiration of CXCL1 (0.5 µg in 70 µl PBS) (R&D systems, Minneapolis, USA), N-ac-PGP (500 µg in 70 µl PBS) (Anaspec, San Jose, USA) or vehicle (PBS) was performed after induction of light isoflurane anesthesia. The dosages used for CXCL1 and N-ac-PGP are previously described by Frevert *et al.* [7] and Van Houwelingen *et al.* [2]. The anesthetized mice were held vertically and the tongue was gently pulled out of the mouth using forceps in order to visualize the base of the tongue and the pharynx. The CXCL1, N-ac-PGP or PBS solution was pipetted onto the back of the tongue and to force breathing through the mouth, the nose was pinched. Respiration was monitored to ensure the solution was fully aspirated before the tongue was released [8, 9].

CXCR2 antagonist administration

The selective nonpeptide CXCR2 antagonist SB225002 (*N*-(2-hydroxy-4-nitrophenyl)-*N'*-(2-bromophenyl)urea) was purchased from Alexis Biochemicals (Nottingham, UK). The CXCR2 antagonist SB332235 (*N*-(2-hydroxy-3-sulfamyl-4-chlorophenyl)-*N'*-(2,3-dichlorophenyl)urea) was synthesized at Merck Research Laboratories as described in the International Application published under the Patent Cooperation Treaty (2000, WO 00/35442).

SB225002 was dissolved according to manufacturer's instructions at a stock concentration of 10 mg/ml ($28x10^{-3}$ M). For *in vivo* application, aliquots of SB225002 were diluted in vehicle (0.9% NaCl solution containing 0.33% Tween-80) just before use [10].

SB332235 was dissolved according to manufacturer's instructions at a stock concentration of 150 mg/ml ($380 \cdot 10^{-3}$ M). For *in vivo* application, aliquots of SB332235 were diluted in vehicle (0.9% NaCl solution containing 5% castor oil) just before use [11].

The mice received intraperitoneal applications of vehicle, SB225002 (50 µg ($1.4 \cdot 10^{-7}$ moles) in 200 µl per animal) as previously described by Herbold *et al.* (2010) or SB332235 (20 µg ($0.5 \cdot 10^{-7}$ moles), 100 µg ($2.5 \cdot 10^{-7}$ moles) and 300 µg ($7.6 \cdot 10^{-7}$ moles) in 200 µl per animal) as previously described by Mihara *et al.* (2005) 1 h prior and 1 h after oropharyngeal aspiration of CXCL1, N-ac-PGP or PBS.

Bronchoalveolar lavage (BAL)

Six hours after the oropharyngeal aspiration of CXCL1, N-ac-PGP or PBS, the mice were i.p. injected with an overdose of pentobarbital. The lungs of these mice were lavaged 4 times through a tracheal cannula with 1 ml saline (NaCl 0.9%), pre-warmed at 37 °C. The first lavage was performed with 1 ml saline containing a mixture of protease inhibitors (Complete Mini, Roche Applied Science, Penzberg, Germany). After centrifugation of the BAL fluid at 4 °C (400 g, 5 min), the supernatant of the first ml was used for CXCL1 analysis and the cell pellets of the 4 lavages were used for neutrophil cell counts. The 4 cell pellets, kept on ice, were pooled per animal and resuspended in 150 µl cold saline. After staining with Türk solution, total cell counts per lung were made under light microscopy using a Burker-Türk chamber. Differential cell counts were performed on cytospin preparations stained by DiffQuick™ (Dade A.G., Düdingen, Switzerland). Cells were identified as neutrophils according to standard morphology. At least 200 cells were counted and the absolute number of neutrophils was calculated [12].

Measurement of CXCL1

A standard mouse cytokine/chemokine kit was used to determine CXCL1 concentrations in the BAL fluid according to the manufacturer's instructions (Millipore, Billerica, USA). The concentrations of CXCL1 were expressed as pg/ml BAL fluid.

Preparation of lung homogenates

Right lung samples were homogenized in a potter glass tube with a Teflon pestle in 1 ml ice cold PBS. Homogenates were centrifuged at 14,000 g for 5 min and supernatants were collected. The protein concentration of each sample was assayed using the Pierce BCA protein assay kit standardized to BSA, according to the manufacturer's protocol (Thermo Fisher Scientific**,** Rockford, IL, USA). The homogenates were diluted to a final concentration of 2 µg protein/µl [13].

Myleoperoxidase (MPO) ELISA

MPO levels were measured in lung homogenate supernatants by ELISA using the Mouse MPO ELISA kit (Hycult Biotechnology, Uden, the Netherlands) according to manufacturer's instructions.

Immunohistochemistry

Left lung samples were fixed with 10% formalin for at least 24h, after which the left lung was embedded in paraffin. Paraffin sections were deparaffinized, endogenous peroxidase activity was blocked with 0.3% H_2O_2 (Merck, Darmstadt, Germany) in methanol for 30 min at room temperature and rehydrated in a graded ethanol series to PBS. For antigen retrieval, the slides were boiled in 10 mM citrate buffer (pH 6.0) for 10 min in a microwave. The slides were cooled down to room temperature, rinsed with PBS (3x) and blocked with 5% rabbit serum (Dakocytomation, Glostrup, Denmark) in PBS containing 1% bovine serum albumin for 30 min at room temperature. Sections were incubated with the primary antibody (rat-anti-mouse neutrophils (MCA771GA), 1:2000, AbD serotec, UK) in PBS containing 1% bovine serum albumin overnight at 4 °C. The slides were rinsed with PBS (3x) and incubated with the biotinylated secondary antibody (rabbit-anti-rat, 1:200, Dakocytomation) in 1% bovine serum albumin/PBS for 45 min at room temperature. The slides were rinsed with PBS (3x) and the biotinylated proteins were visualized by incubation with streptavidin–biotin complex horseradish peroxidase (Vectastain Elite ABC, Vector Laboratories) for 45 min at room temperature, followed by 0.015% H_2O_2/0.05% diaminobenzidene (Sigma, Schneldorf, Germany)/0.05 M Tris–HCl (pH 7.6) for 10 min at room temperature. Sections were counterstained with Mayers' hematoxylin (Merck), dehydrated and mounted in Permount (Fisher Scientific). Negative controls without the primary antibody were included as controls. Photomicrographs were taken with an Olympus BX50 microscope equipped with a Leica DFC 320 digital camera [13].

Statistical analysis

Experimental results were expressed as mean ± SEM. Differences between groups were statistically determined by an unpaired two-tailed Student's *t*-test using GraphPad Prism (Version 4.0). Results were considered statistically significant when $P < 0.05$.

Results

N-ac-PGP-induced neutrophil infiltration in lung tissue is more pronounced than the influx into the BAL fluid

Oropharyngeal aspiration of N-ac-PGP and CXCL1 resulted in a neutrophilic airway inflammation, since an increased number of neutrophils was observed in the BAL fluid of N-ac-PGP- and CXCL1-treated mice compared to control mice (fig. 1 and 2). However, N-ac-PGP at a dose of 500 µg/mouse only induced 5% of the amount of neutrophils in BAL fluid compared to that observed after treatment with 0.5 µg CXCL1 per mouse. Next, we examined whether the appearance of neutrophils in BAL fluid represents the infiltration of neutrophils in the pulmonary tissue. Neutrophil accumulation in lung tissue was assessed by immunohistological analysis and quantitated by measuring MPO levels in lung tissue homogenate supernatants. Besides an increased neutrophil influx in the BAL fluid, oropharyngeal aspiration of N-ac-PGP induced a neutrophil accumulation in the lung tissue (fig. 3B) and increased MPO levels were observed in the lung homogenate supernatants of N-ac-PGP-treated mice compared to control mice (fig. 4). In addition, CXCL1 also induced a neutrophil accumulation in lung tissue (fig. 3E) and elevated MPO levels were observed in supernatants of lung tissue homogenates of CXCL1-treated mice when compared to control mice (fig. 5). When compared to CXCL1-induced infiltration of neutrophils in lung tissue, N-ac-PGP was able to induce 31% compared to the amount of pulmonary tissue MPO levels observed after CXCL1 treatment.

N-ac-PGP-induced neutrophil influx in the BAL fluid is significantly decreased after CXCR2 antagonist administration

Since CXCL1 is one of the main CXCR2 ligands in mice [14], CXCL1 was used as positive control for testing two CXCR2 antagonists: SB225002 and SB332235. SB225002 and SB332235 on their own had no effect on the number of neutrophils in BAL fluid of control mice (data not shown). Intraperitoneal administration of mice with either SB225002 or SB332235, given 1 h before and 1 h after oropharyngeal aspiration of CXCL1, inhibited the CXCL1-induced neutrophil influx in the BAL fluid with 42% and 72%, respectively (fig. 2). To investigate whether N-ac-PGP-induced neutrophil infiltration in BAL fluid is mediated via CXCR2, the two different CXCR2 antagonists (SB225002 and SB332235) were administered to N-ac-PGP-treated mice. Intraperitoneal treatment of mice with SB225002 (50 µg/animal) inhibited the N-ac-PGP-induced neutrophil influx in the BAL fluid with 77% (fig. 1). Moreover, the N-ac-PGP-induced neutrophil influx in the BAL fluid of mice was also significantly decreased after different doses of SB332235 (20 µg, 100 µg and 300 µg/animal) (fig. 1). The SB332235-induced inhibition was for all doses tested more than 90%.

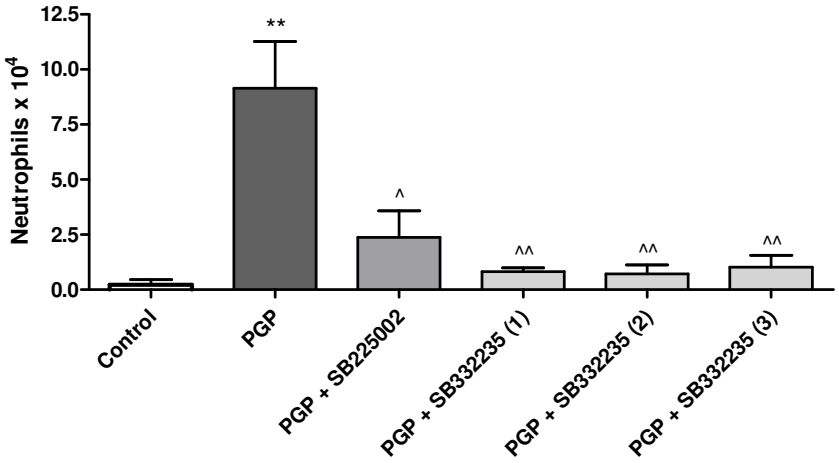


Figure 1. N-ac-PGP-induced neutrophil influx in the BAL fluid is significantly decreased after CXCR2 antagonist administration.

*Neutrophil numbers in the BAL fluid of C57Bl/6 mice after oropharyngeal aspiration of PBS or N-ac-PGP (500 µg/70 µl PBS). The PBS and N-ac-PGP-treated mice received i.p. injections with vehicle, SB225002 (50 µg/animal) or SB332235 (20 µg (1), 100 µg (2) and 300 µg (3)/animal) 1 h before and 1 h after treatment. n = 6 animals per group. Values are expressed as mean ± SEM. (**, P < 0.01; significantly different from the control group. ^, P < 0.05; ^^, P < 0.01 significantly different from the N-c-PGP-treated group)*

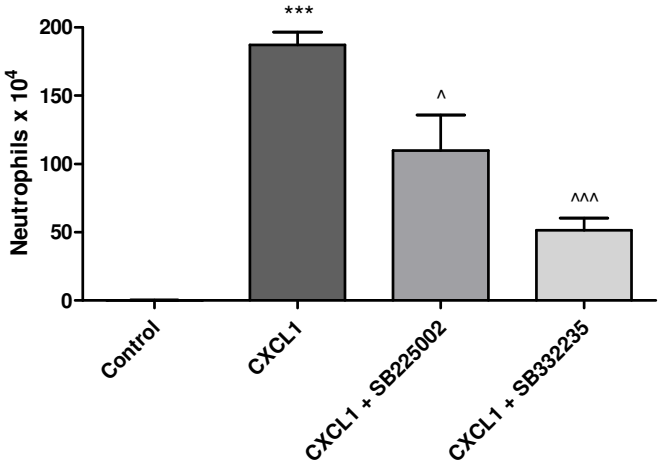


Figure 2. CXCL1-induced neutrophil influx in the BAL fluid is significantly decreased after CXCR2 antagonist administration.

*Neutrophil numbers in the BAL fluid of C57Bl/6 mice after oropharyngeal aspiration of PBS or CXCL1 (0.5 µg/70 µl PBS). The PBS and CXCL1-treated mice received i.p. injections with vehicle, SB225002 (50 µg/animal) or SB332235 (300 µg/animal) 1 h before and 1 h after treatment. n = 6 animals per group. Values are expressed as mean ± SEM. (***, P < 0.001; significantly different from the control group. ^, P < 0.05; ^^^, P < 0.001 significantly different from the CXCL1-treated group)*

N-ac-PGP-induced neutrophil infiltration in pulmonary tissue is decreased after CXCR2 antagonist administration

Immunohistochemical analysis was performed to observe neutrophils in the lung tissue of PBS-, CXCL1- and N-ac-PGP-treated animals with or without CXCR2 antagonist administration. As stated before, this immunological staining confirmed the results obtained from the MPO measurement in the lung homogenates, since an enormous neutrophil influx was observed in the lung tissue after oropharyngeal aspiration of CXCL1 (fig. 3E) compared to the control mice (fig. 3A). Furthermore, N-ac-PGP also induced a neutrophil accumulation in the lung tissue (fig. 3B).

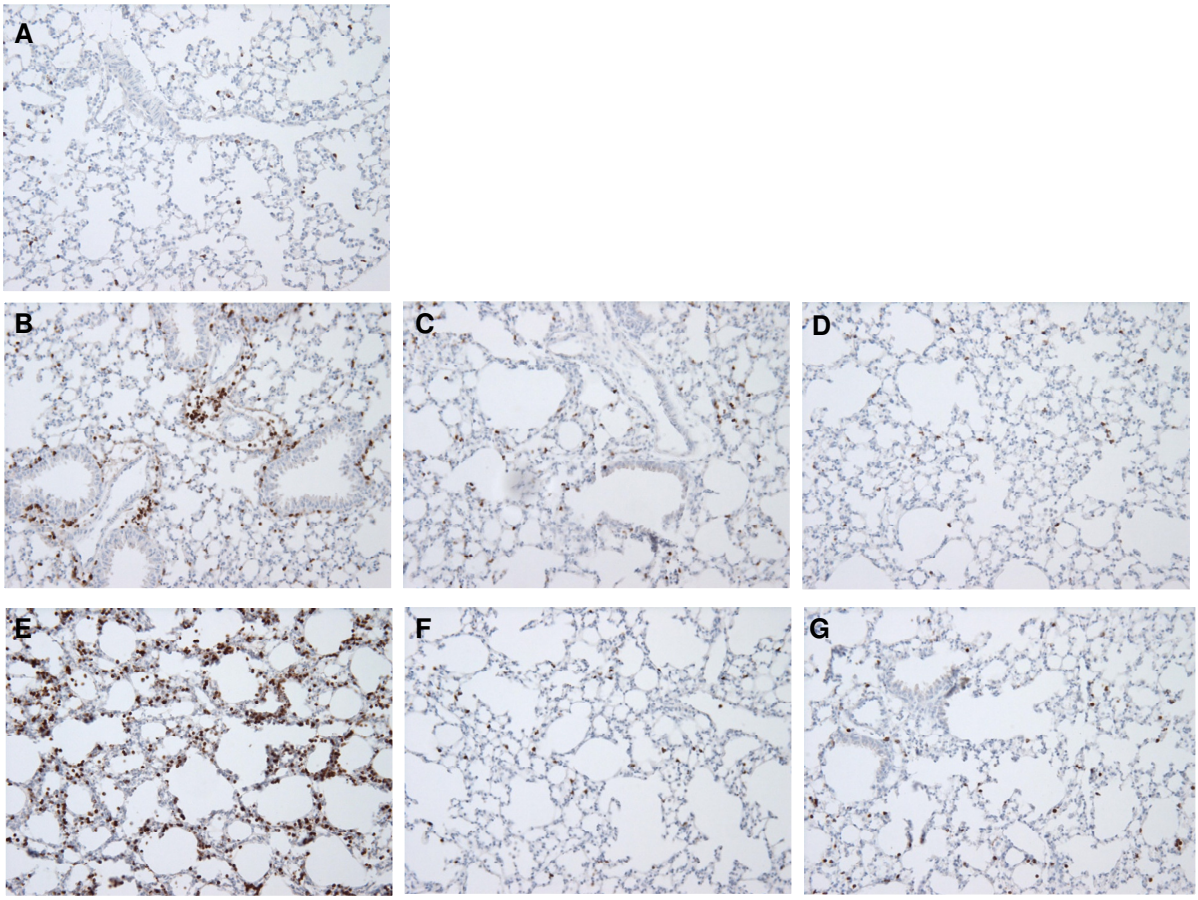


Figure 3. N-ac-PGP- and CXCL1-induced neutrophil influx in the lung is decreased after CXCR2 antagonist administration.

*Representative photomicrographs of an immunohistological staining for neutrophils (brown color, DAB staining) in lung tissue of PBS-treated mice (**A**), N-ac-PGP-treated mice (**B**) and CXCL1-treated mice (**E**). The neutrophils in lung tissue of N-ac-PGP-treated mice are decreased after i.p. administration with SB225002 (50 µg/animal) (**C**) or SB332235 (300 µg/animal) (**D**). The neutrophil accumulation in lung tissue of CXCL1-treated mice are inhibited by i.p. administration with SB225002 (50 µg/animal) (**F**) or SB332235 (100 µg/animal) (**G**). Magnification, 200x.*

The N-ac-PGP-induced neutrophil infiltration was primarily located in peribronchial and perivascular areas in the airways, whereas CXCL1 induced a more scattered pattern throughout the pulmonary tissue (fig. 3B and E). Intraperitoneal treatment of mice with SB225002 (50 µg/animal) reduced the amount of neutrophils in the lung tissue induced by CXCL1 (fig. 3F). The increased neutrophil numbers induced by CXCL1 were also decreased after application of SB332235 (300 µg/animal) (fig. 3G). Moreover, the N-ac-PGP-induced neutrophil influx in the lung tissue was inhibited after treatment with as well SB225002 (50 µg/animal) (fig. 3C) as SB332235 (100 µg/animal) (fig. 3D). SB225002 and SB332235 on their own had no effect on the neutrophil influx in pulmonary tissue of control mice (data not shown).

N-ac-PGP-induced increased MPO levels in the lung tissue homogenates are significantly decreased after CXCR2 antagonist administration

In addition to the examination of the neutrophil influx in BAL fluid, the effect of the two CXCR2 antagonists was investigated on the N-ac-PGP- and CXCL1-induced neutrophil infiltration into the lung tissue by determining MPO levels in lung tissue homogenate supernatants. SB225002 and SB332235 had no effect on basal MPO levels in lung tissue of PBS-treated mice (data not shown).

Again, CXCL1 was used as positive control for testing two CXCR2 antagonists. Treatment of mice with SB225002 (50 µg/animal) inhibited the increased MPO levels in lung homogenates with 62% induced by CXCL1 (fig. 5). Moreover, after application of SB332235 (300 µg/animal), the increased MPO levels induced by CXCL1 were also significantly decreased by 54% (fig. 5).

Treatment of mice with SB225002 (50 µg/animal) completely abolished the increased MPO levels in lung tissue homogenate supernatants induced by N-ac-PGP (fig. 4). Furthermore, the increased MPO levels induced by N-ac-PGP were also decreased after different doses of SB332235 (20 µg, 100 µg and 300 µg/animal) by 66%, 88% and 42%, respectively. The most pronounced effect in reducing the MPO levels by the CXCR2 antagonist SB332235 appeared after administration of the 100 µg dose (fig. 4).

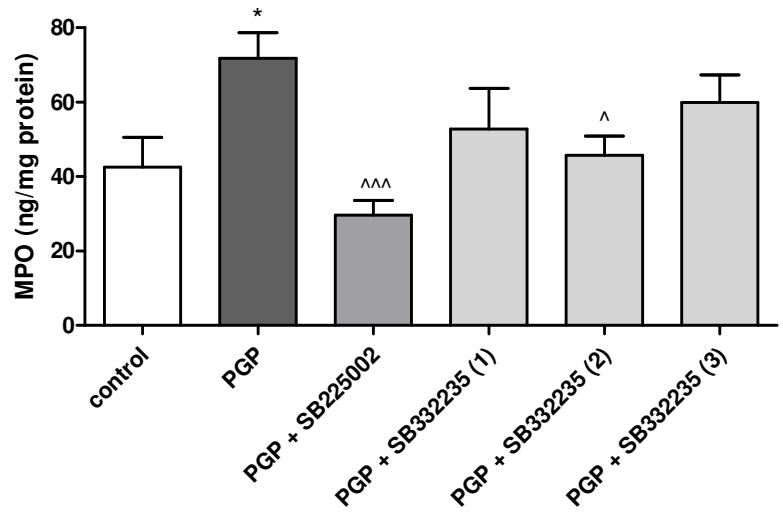


Figure 4. N-ac-PGP-induced MPO levels in the lung homogenates are decreased after CXCR2 antagonist administration.

MPO levels in lung homogenates of C57Bl/6 mice after oropharyngeal aspiration of PBS or N-ac-PGP (500 µg/70 µl PBS). The PBS and N-ac-PGP-treated mice received i.p. injections with vehicle, SB225002 (50 µg/animal) or SB332235 (20 µg (1), 100 µg (2) and 300 µg (3)/animal) 1 h before and 1 h after treatment. n = 6 animals per group. Values are expressed as mean ± SEM .(, P < 0.05; significantly different from the control group. ^, P < 0.05; ^^^, P < 0.001 significantly different from the N-ac-PGP-treated group)*

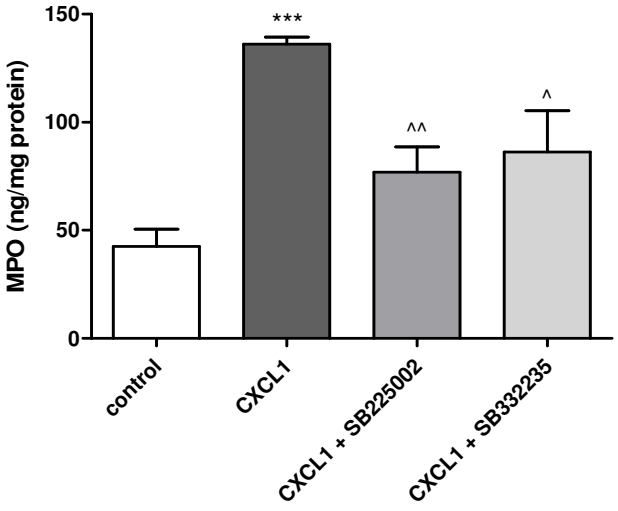


Figure 5. CXCL1-induced MPO levels in the lung homogenates are significantly decreased after CXCR2 antagonist administration.

*MPO levels in lung homogenates of C57Bl/6 mice after oropharyngeal aspiration of PBS or CXCL1 (0.5 µg/70 µl PBS). The PBS and CXCL1-treated mice received i.p. injections with vehicle, SB225002 (50 µg/animal) or SB332235 (300 µg/animal) 1 h before and 1 h after treatment. n = 6 animals per group. Values are expressed as mean ± SEM. (***, P < 0.001; significantly different from the control group. ^, P < 0.05; ^^, P < 0.01 significantly different from the CXCL1-treated group)*

CXCR2 antagonists did not significantly attenuate the N-ac-PGP-induced CXCL1 release in the BAL fluid

The levels of the chemokine CXCL1 were measured in the BAL fluid of PBS and N-ac-PGP-treated mice with or without administration of the CXCR2 antagonists SB225002 or SB332235. The concentrations of CXCL1 were significantly elevated in the BAL fluid of N-ac-PGP-treated mice compared to the PBS-treated mice (fig. 6). The N-ac-PGP-induced CXCL1 levels in the BAL fluid were not significantly decreased after treatment with the CXCR2 antagonist SB225002 (50 µg/animal). Moreover, the N-ac-PGP-induced CXCL1 levels were not reduced after applications of different doses of SB332235 (20 µg, 100 µg and 300 µg/animal) (fig. 6). Although at a dose of 100 µg, SB332235 demonstrated a small but not significant reduction of N-ac-PGP-induced CXCL1 release in BAL fluid.

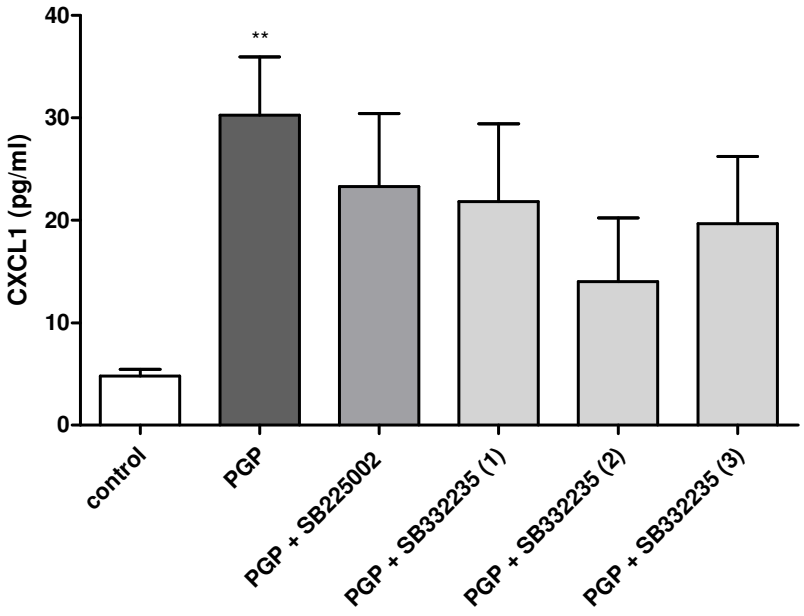


Figure 6. CXCR2 antagonists do not significantly affect N-ac-PGP-induced CXCL1 release in the BAL fluid.

*Levels of the chemokine CXCL1 in the BAL fluid of C57Bl/6 mice after oropharyngeal aspiration of PBS or N-ac-PGP (500 µg/70 µl PBS). The PBS and N-ac-PGP-treated mice received i.p. injections with vehicle, SB225002 (50 µg/animal) or SB332235 (20 µg (1), 100 µg (2) and 300 µg (3)/animal) 1 h before and 1 h after treatment. n = 6 animals per group. Values are expressed as mean ± SEM. (**, P < 0.01; significantly different from the control group)*

Discussion

The aim of this study was to investigate the role of CXCR2 in neutrophilic airway inflammation induced by N-ac-PGP. Neutrophils are the first cells to be recruited to the site of inflammation in response to chemoattractants, such as CXCL8 in humans and CXCL1 and CXCL2 in mice [1]. In chronically inflamed tissues, neutrophilic mediator release leads to extracellular matrix breakdown and subsequently to the formation of collagen fragments with chemotactic properties, such as N-ac-PGP [15-17]. It has been demonstrated that N-ac-PGP is chemotactic for neutrophils *in vitro* as well as *in vivo* [3, 4, 18, 19]. The importance of N-ac-PGP in chronic lung diseases is pointed out in different murine and human studies [2, 3, 5]. It has been proposed that N-ac-PGP acts as a neutrophilic chemoattractant via CXCR1 and CXCR2 *in vitro* and *in vivo* [3, 4].

The most important murine ligands for CXCR2 in the early state of inflammation are CXCL1 and CXCL2 and these ligands bind with high affinity to mouse CXCR2, whereas the affinity for CXCR1 is low [3, 20-22]. Upon stimulation with cigarette smoke, the major risk factor in COPD, neutrophils are major producers of CXCL1 and CXCL2 [23]. Since CXCL1 levels are more prominent than CXCL2 levels in airway inflammation [3], CXCL1 was chosen in this study as a positive control. The effect of oropharyngeal aspiration of CXCL1 in mice lungs was tested and a significant increased neutrophilic airway accumulation was observed in the BAL fluid and lung tissue and the MPO levels in the supernatant of lung tissue homogenates were increased. This finding is in agreement with the results of Frevert *et al.* [7], who demonstrated a dose dependent increase of neutrophils in the BAL fluid of rats after intratracheal administration of recombinant CXCL1.

N-ac-PGP mimicked CXCL1 in attracting neutrophils into the BAL fluid 6 h after oropharyngeal aspiration. This observation is in accordance with the study of Van Houwelingen *et al.* [2], who described that intratracheal N-ac-PGP administration provoked neutrophilic airway inflammation that was dependent on the N-ac-PGP concentration and the time after application. Besides an increase in neutrophil numbers in BAL fluid caused by N-ac-PGP administration, we also observed a neutrophil accumulation in the lung tissue of N-ac-PGP-treated mice as measured indirectly by MPO in lung tissue homogenates and directly via an immunohistological staining for neutrophils. The histological lung sections of the N-ac-PGP-treated animals demonstrated an increase in neutrophils in mainly peribronchial and perivascular areas in the airways while histological lung sections of CXCL1-treated mice showed a more diffused neutrophilic distribution throughout the airways.

In general, the N-ac-PGP-induced neutrophil levels in BAL fluid and lung tissue were less pronounced than the neutrophil levels measured after oropharyngeal aspiration of CXCL1. An explanation for this can be that N-ac-PGP contacts a single site on the CXCR2 [3],

whereas CXCL1 contacts multiple binding sites on the receptor [24]. Strikingly, N-ac-PGP was more potent at inducing neutrophil infiltration in lung tissue than in BAL fluid, when related to the maximal effect of CXCL1. An alternative explanation is that N-ac-PGP may be more potent at inducing MPO levels in the lung tissue. When compared to the CXCL1-induced influx of neutrophils in pulmonary tissue and BAL fluid, N-ac-PGP treatment resulted in a 31% and only 5% of the CXCL1 response, respectively.

This study confirmed that N-ac-PGP has chemotactic activities as measured by the neutrophils in the BAL fluid and lung tissue. Furthermore, we show that CXCL1 levels in the BAL fluid of N-ac-PGP-treated mice were significantly enhanced compared to control mice, suggesting that N-ac-PGP may indirectly initiate a continuous inflammatory response in the lung caused by the release of CXCL1, probably by infiltrated macrophages and neutrophils in BAL fluid. However, the CXCL1 levels in BAL fluid induced by N-ac-PGP were more than 10,000 fold lower than the amount CXCL1 used for inducing neutrophil migration into the lung. Therefore, it is unlikely that N-ac-PGP is acting via CXCL1.

The N-ac-PGP- as well as the CXCL1-induced neutrophil infiltration in the pulmonary tissue and the influx in the BAL fluid was decreased by the CXCR2 antagonists SB225002 and SB332235. Both CXCR2 antagonists were equally effective in reducing the CXCL1-induced neutrophil accumulation in pulmonary tissue and seemed to be more potent at inhibiting the N-ac-PGP response in the lung tissue as well as in the BAL fluid. SB225002 was more effective in inhibiting N-ac-PGP-induced pulmonary tissue neutrophilia than SB332235, since lower as well as higher doses of SB332235 did not lead to the reduction in MPO levels as observed after SB225002 administration. Contrary, with regard to the N-ac-PGP-induced neutrophil influx in BAL fluid, SB332235 appears to be more effective than SB225002, since lower and higher doses of SB332235 caused a more pronounced effect.

The effect of the selective antagonists SB225002 and SB332235 has been described in other inflammatory diseases where neutrophils play a major role. Application of SB225002 resulted in decreased alveolar neutrophil recruitment in mice suffering from a pneumococcal lung infection [10] and this CXCR2 antagonist was able to attenuate the inflammatory cell influx and tissue damage in a murine colitis model [25]. In addition, SB225002 selectively blocked CXCL8-induced neutrophil chemotaxis in ears of rabbits [26]. *In vitro*, SB225002 potently inhibited human and rabbit CXCL8-induced neutrophil chemotaxis [26, 27]. SB332235 effectively inhibited cigarette smoke-induced neutrophilia in a dose-dependent manner in rats [28]. Moreover, it was reported that SB332235 can inhibit acute and chronic models of arthritis in rabbits [29] and an inhibition of CXCL8 neutrophil migration into the ear of hCXCR2 knock-in mice was observed after SB332235 administration [11]. To our knowledge, we are the first to show that the CXCR2 antagonists affect the neutrophilic airway inflammation induced by CXCL1 and N-ac-PGP as indicated by the decreased

neutrophil influx in the lung tissue (observed by the reduced MPO levels in the lung tissue homogenates and the immunohistological staining for neutrophils) as well as in the BAL fluid. This shows that the observed N-ac-PGP-induced neutrophil influx is mediated via CXCR2. A role for CXCR2 in N-ac-PGP-induced neutrophilic airway inflammation has been confirmed by other studies, since antibodies directed against CXCR2 suppressed the N-ac-PGP-induced neutrophilic chemotaxis *in vitro* [3, 4]. Furthermore, the accumulation of neutrophils seen in mice upon intratracheal administration of N-ac-PGP was not detected in $CXCR2^{-/-}$ mice [3].

We also show that oropharyngeal aspiration of N-ac-PGP induces a CXCL1 release *in vivo*. This is in agreement with our *in vitro* experiments, where we demonstrated that human neutrophils release CXCL8 after N-ac-PGP incubation [4]. In that study, it was demonstrated that the N-ac-PGP-induced release of CXCL8 was not responsible for the N-ac-PGP-induced chemotaxis. Although, the concentration of CXCL1 (30 pg/ml) observed in the BAL fluid after N-ac-PGP administration is much lower than the concentration realized after oropharyngeal aspiration of CXCL1 (0.5 µg/70 µl), we cannot exclude from our studies that a small part of the N-ac-PGP induced neutrophil infiltration is via the release of CXCL1. Finally, administration of CXCR2 antagonists SB225002 and SB332235 did not result in significantly decreased N-ac-PGP-induced CXCL1 levels in the BAL fluid.

In summary, it can be concluded that the N-ac-PGP-induced neutrophil influx in lung tissue and BAL fluid can be inhibited by CXCR2 antagonists, showing that the neutrophil migration induced by N-ac-PGP is mediated via direct interaction with CXCR2 and by a small, if any, release of CXCL1. The N-ac-PGP-induced release of CXCL1 is independent of the CXCR2. Related to the maximal effect of CXCL1, N-ac-PGP is more potent at inducing the neutrophil migration in the pulmonary tissue than into the BAL fluid. In addition, N-ac-PGP may be more potent at inducing MPO levels in the lung tissue.

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Chapter 7

Cigarette smoke-induced collagen destruction; key to chronic neutrophilic airway inflammation?

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Abstract

Cigarette smoking induces inflammatory responses in all smokers and is the major risk factor for lung diseases such as chronic obstructive pulmonary disease (COPD). In this progressive disease, chronic inflammation in the lung contributes to lung tissue destruction leading to the formation of chemotactic collagen fragments such as N-acetyl Proline-Glycine-Proline (N-ac-PGP). The generation of this tripeptide is mediated by a multistep pathway involving matrix metalloproteinases (MMPs) 8 and 9 and prolyl endopeptidase (PE). Here we investigated whether cigarette smoke extract (CSE) stimulates neutrophils to breakdown whole matrix collagen leading to the generation of the chemotactic collagen fragment N-ac-PGP.

Incubating human polymorph nuclear cells (PMNs) with CSE led to the release of the chemoattractant CXCL8 and the proteases MMP8 and MMP9. PMNs constitutively exhibited PE activity as well as PE protein expression. Incubating CSE-primed PMNs with collagen resulted in collagen breakdown and in the generation of N-ac-PGP. Incubation of PMNs with the tripeptide N-ac-PGP resulted in the release of CXCL8, MMP8 and MMP9. Moreover, we tested whether PMNs from COPD patients are different from PMNs from healthy donors. Here we show that the basal intracellular PE activity of PMNs from COPD patients is a 25-fold higher when compared to PMNs from healthy donors. Immunohistological staining of human lung tissue for PE showed that besides neutrophils, macrophages and epithelial cells also express PE protein. In addition, exposure of human bronchial epithelial cells to cigarette smoke extract *in vitro* led to an elevated PE activity in the supernatant.

In conclusion, this study indicates that neutrophils activated by cigarette smoke extract can breakdown collagen into N-ac-PGP and that this collagen fragment itself can activate neutrophils, which may lead *in vivo* to a self-propagating cycle of neutrophil infiltration, chronic inflammation and lung emphysema.

Introduction

Chronic inflammation is observed in lung diseases such as chronic obstructive pulmonary disease (COPD) [1]. This disease, referring to bronchitis and emphysema, is an important cause of morbidity worldwide [2, 3] and is characterized by irreversible progressive development of airflow limitation [4]. Neutrophils are a notable component of the inflammation in COPD; they release mediators and proteases, thereby contributing to the chronic inflammatory reaction that ultimately may lead to lung destruction [1, 4]. It is generally accepted that cigarette smoking is the main risk factor for the development of COPD. The World Health Organization estimated that 73% of COPD mortality is related to smoking [5]. Although smoking cessation will beneficially affect disease progression, there is currently no specific therapy for COPD. Since this prevalent disease is an enormous health burden, major efforts have been directed towards understanding the pathophysiology of this complicated disease [2]. One of the most prominent chemokines in COPD is CXCL8. The levels of this chemokine are increased in sputum from COPD patients and correlate with the increased number of neutrophils found in the lungs [1]. Antagonizing CXCL8 with an α-CXCL8 antibody and blocking leukotrienes such as LTB_4 with an antagonist, incompletely prevents neutrophil chemotaxis in COPD patients [6], suggesting that other chemoattractants are involved in neutrophil migration in COPD. An example of such a chemoattractant is N-acetyl Proline-Glycine-Proline (N-ac-PGP). This tripeptide has been implicated as a new biomarker and therapeutic target for COPD [7]. N-ac-PGP is generated from the breakdown of extracellular matrix collagen and is specifically chemotactic for neutrophils *in vivo* and *in vitro*, as has been shown by different groups [8-12]. Moreover, chronic airway exposure to N-ac-PGP causes emphysema in mice [12]. In COPD patients, N-ac-PGP was detected in induced sputum samples, whereas this tripeptide was undetectable in healthy individuals and asthmatics [7]. Gaggar *et al.* described the proteolytic cascade that generates the tripeptide PGP from collagen in cystic fibrosis (CF), a disease where chronic neutrophilic inflammation is present in the lungs. Using sputum from CF patients, it was shown that matrix metalloproteinases (MMPs) 8 and 9 and prolyl endopeptidase (PE) are involved in this multistep pathway [9].

The aim of this study is to investigate the effect of cigarette smoke on the generation of N-ac-PGP from whole collagen by human neutrophils. In addition, here we investigated the PE activity in COPD. In this report, we show that neutrophils activated by cigarette smoke extract (CSE) can breakdown collagen into N-ac-PGP and that this collagen fragment itself can activate neutrophils, which may lead to a further increase in neutrophil infiltration, chronic inflammation and lung destruction. Moreover, we propose that PE can play an important role in lung collagen breakdown leading to the development of COPD.

Material and methods

Chemicals and reagents

2R4F reference cigarettes were from Kentucky Tobacco Research Institute (Lexington, KY, USA). Recombinant human CXCL8 and PE, human MMP8 and MMP9 ELISA kits and rabbit IgG antibodies were supplied by R&D Systems Europe Ltd. (Abingdon, United Kingdom). Z-Gly-Pro-7-amido-4-methylcoumarin (Z-G-P-AMC) was purchased from Bachem. LPS, BSA, Triton X100, diaminobenzidene, selenite, Hoechst stain solution and collagen type I and II were purchased from Sigma Aldrich Chemie BV (Zwijndrecht, the Netherlands). The human CXCL8 ELISA kit was from BD Biosciences (Alphen a/d Rijn, the Netherlands). HEPES was obtained from Agros Organics (Geel, Belgium). Mayers' haematoxylin, H_2O_2, NaCl, KCl, $K_2HPO_4 \cdot 3H_2O$, $CaCl_2$, NH_4Cl, $KHCO_3$, EDTA (Triplex III) and trisodium citrate dihydrate were purchased from Merck KGaA (Darmstadt, Germany). Ficoll-Paque^{TM} PLUS was purchased from GE Healthcare (Eindhoven, the Netherlands). FITC-labeled goat anti-rabbit secondary antibody was purchased from Southern Biotechnology (Birmingham, AL, USA), whereas the rabbit anti-human PE antibody was from ProteinTech Group (Manchester, UK), the 5% goat serum and the biotinylated secondary antibody goat anti-rabbit from Dakocytomation (Glostrup, Denmark) and the goat anti-rabbit-HRP antibody from DAKO (Enschede, the Netherlands). Vectastain Elite ABC was obtained from Vector Laboratories (Burlingame, USA). Permount was from Fisher Scientific. PBS and Roswell Park Memorial Institute (RPMI) 1640 medium (without L-glutamine and phenol red) were obtained from Lonza Verviers SPRL (Verviers, Belgium). FBS was from Perbio Science Nederland BV (Etten-Leur, the Netherlands).

Lung tissue specimens

The characteristics of the human subjects included in the study are presented in Table 1.

Tissue specimens from the current smokers and ex-smokers were obtained from noninvolved lung tissue from patients undergoing resective surgery for pulmonary carcinoma. These patients had no airway obstruction and no chronic airway symptoms, such as cough and sputum production. Material was always taken from as far away as possible from the tumor, or from a noninvolved lobe. No histopathological lesions were present in these tissue specimens.

Tissue specimens of GOLD stage II COPD patients were obtained from noninvolved lung tissue from patients undergoing resective surgery for pulmonary carcinoma. Tissue was always taken as far away as possible from the tumor or from a noninvolved lobe.

Table 1. Characteristics of COPD patients and controls

	CURRENT SMOKER	**EX-SMOKER**	**COPD PATIENT GOLD STAGE II**	**COPD PATIENT GOLD STAGE IV**
Gender (m/f, n)	0/6	0/6	5/3	0/6
Age (yrs)	58 (49-67)	59 (51-67)	67 (58-76)	54 (49-60)
Current smoker / not current smoker (n/n)	6/0	0/6	4/4	0/6
Pack years (yrs)	30 (25-45)	31 (18-44)	38 (21-55)	34 (21-47)
FEV_1, % predicted	94.2 (84.7-103.7)	88.2 (83.2-93.2)	65.6 (55.6-75.6)	19.5 (13.7-25.3)
FEV_1/FVC, %	78.4 (71.0-85.8)	71.9 (62.9-80.9)	58.7 (50.2-67.2)	35.6 (20.9-50.3)

Data are presented as median (range). FEV1: Forced Expiratory Volume in one sec. FVC: Forced Vital Capacity. The FEV1 of the current smoker and ex-smoker is based on prebronchodilator values and the FEV1 of the COPD patients is based on post-bronchodilator values.

Histopathologically emphysematous lesions were present in these tissue specimens, however, of limited but varying severity. The moderate forms can be histopathologically demonstrated by the finding of isolated or free lying segments of viable alveolar septal tissue or isolated cross sections of pulmonary vessels.

Tissue specimens of GOLD stage IV COPD patients were obtained from patients with COPD undergoing surgery for lung transplantation or lung volume reduction. All these patients had to quit smoking for at least 1 yr before surgery. The resected tissue showed both macroscopically and microscopically severe emphysematous lesions, often accompanied by bullae. Subpleural, fibrous areas were avoided. The study protocol was consistent with national ethical and professional guidelines ("Code of Conduct; Dutch Federation of Biomedical Scientific Societies"; http://www.federa.org/?s=1&m= 68). Immunohistochemistry was performed on 3 µm formalin-fixed, paraffin-embedded lung tissue.

Human polymorphonuclear leukocytes

Human polymorphonuclear leukocytes (PMNs) were isolated as previously described from fresh whole blood [10], for which healthy donors signed written informed consent forms or from buffy coats, which were purchased from Sanquin Blood Bank (Amsterdam, the Netherlands). Resulting PMN preparations consisted of 95-97% PMNs, based on PMNs physical parameters analyzed by flow cytometry and CD16 expression. The preparations were negative for CD14, indicating that the preparations did not contain monocytes.

PMNs from COPD patients were collected and were tested for CXCL8 release and PE activity. For this PMN study, the PMNs from fresh whole blood of healthy donors and COPD patients were used. The characteristics of the human subjects included in the PMN study are presented in Table 2.

Table 2. Characteristics of COPD patients and healthy controls

	HEALTHY DONOR	**COPD PATIENT GOLD STAGE I-III**
Gender (m/f, n)	3/5	7/4
Age (yrs)	45 (26-63)	60 (35-72)
Current smoker / not current smoker (n/n)	0/8	5/6
Pack years (yrs)	-	31 (22-67)
FEV_1, % predicted	-	55.7 (27.9-99.9)
FEV_1/FVC, %	-	47 (27-70)

Data are presented as median (range).

Human bronchial epithelial cell culture.

Human bronchial epithelial (HBE) cells (type 16 HBE-14o) were cultured as previously described [13]. Briefly, HBE cells were grown in a MEM culture medium containing 0.292g/l L-glutamine, 1 g/l glucose, 2.2 g/l $NaHCO_3$ and 10% FBS and antibiotics (complete culture medium) in 75 cm^2 cell culture flasks coated with Vitrogen 100 (3mg/ml collagen I, bovine) and human fibronectin (1 mg/ml). At confluence, cells were washed with PBS and cultured overnight in serum-free MEM-ITS. Before each experiment, cells were trypsinized and washed with PBS and taken up in fresh serum-free MEM-ITS.

Cigarette smoke extract (CSE)

CSE was prepared by using a smoking machine (Teague Enterprises, Davis, Ca, USA) as previously described [14]. Direct and side stream smoke from one 2R4F cigarette was directed via a tube through 5 ml PBS using a peristaltic pump. The optical density (OD) of this extract was determined using a spectrometer (UV-mini 1240, Shimadzu) measuring at wavelength 320 nm. Freshly prepared CSE was used in all experiments. Non-toxic solutions ranging from 0.03 to 0.24 OD were used in the present study as determined by Annexin-V staining and FACS analysis.

CXCL8, MMP8 and MMP9 ELISA's

PMNs (10^5 cells/well) were incubated for several time points with indicated reagents. After incubation, the supernatants were collected and CXCL8, MMP8 and MMP9 levels were measured using a human CXCL8, MMP8 or MMP9 ELISA kit according to manufacturer's instructions.

Immunofluorescence microscopy

Cytospin preparations of PMNs on glass slides were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100. After blocking with 3% BSA in PBS, PMNs were incubated with anti-PE antibody (45 µg/ml in PBS/1% BSA), preimmune rabbit antibody or anti-PE antibody which had been preadsorbed with rhPE (200 µg/ml) for 2 h at room temperature. After a sec blocking step with 3% BSA, PMNs were incubated with FITC-labeled goat anti-rabbit secondary antibody (1:12,000 in PBS/1% BSA) for 1 h. Nuclei were stained with Hoechst (1:2000) and PMNs examined by immunofluorescence microscopy.

PE activity assay.

Freshly isolated PMNs or HBE cells (10^6 cells) were stimulated with indicated reagents. Supernatant and cell lysates (lysated with 50 mM HEPES (pH 7.4), 150 mM NaCl, 15 mM $MgCl_2$, 1 mM EDTA, 10% glycerol and 1% Triton-X 100 in Milli Q water) were harvested and

frozen until use. The protein concentration of each lysate was assayed using the Pierce BCA protein assay kit standardized to BSA according to the manufacturer's protocol (Thermo Fisher Scientific, Rockford, IL). PE activity was measured in these supernatants and lysates using the fluorogenic substrate Z-Gly-Pro-7-amido-4-methylcoumarin (2-G-P-AMC) (Bachem). Twenty microliters of cell lysate or supernatant was added to each well in a black 96-well flat-bottom plate, followed by addition of 80 µl of assay buffer (25 mM Tris, 0.25 M NaCl, pH 7.5, 2 mM DTT) containing 100 µM substrate Z-Gly-Pro-AMC. The fluorescence from liberated AMC was monitored every 1 min over 60 min at 37 °C using a Fluostar reader at excitation wavelength of 355 nm and an emission wavelength of 460 nm. Fluorometric intensities observed were converted to pmol AMC released per min using appropriate AMC standard curves.

Western blotting

Freshly isolated PMNs (10^6 cells) were stimulated for 8 h with indicated reagents. Supernatant and cell lysates (lysated with 50 mM HEPES (pH 7.4), 150 mM NaCl, 15 mM $MgCl_2$, 1 mM EDTA, 10% glycerol and 1% Triton-X in Milli Q water) were harvested and frozen until use. Equal amounts of proteins of boiled nonreduced samples were separated electrophoretically (SDS-PAGE 10%) and transferred onto nitrocellulose membranes. The membranes were blocked with PBS-0.05% Tween-20 (PBST) containing 5% milk proteins for 1 h at room temperature. After blocking, primary antibody rabbit anti-human PE (1:500) in PBST containing 5% milk proteins was applied overnight at 4 °C. Subsequently, the membranes were incubated with goat anti-rabbit-HRP antibodies (1:2000) in PBST containing 5% milk proteins for 1 h. The antibodies were visualized using commercial ECL reagents and exposed to photographic film.

Immunohistochemistry

Paraffin sections of human lung specimens were deparaffinized, endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol for 30 min at room temperature and rehydrated in a graded ethanol series to PBS. For antigen retrieval, the slides were boiled in 10 mM citrate buffer (pH 6.0) for 10 min in a microwave. The slides were cooled down to room temperature, rinsed with PBS (3x) and blocked with 5% goat serum in 1% bovine serum albumin in PBS for 30 min at room temperature. Sections were incubated with the primary antibody (rabbit anti-PE, 0.6 µg/ml) in 1% bovine serum albumin/PBS overnight at 4 °C. The slides were rinsed with PBS (3x) and incubated with the biotinylated secondary antibody (1:200) in 1% bovine serum albumin/PBS for 45 min at room temperature. The slides were rinsed with PBS (3x) and the biotinylated proteins were visualized by incubation with streptavidin–biotin complex/horseradish peroxidase for 45 min at room temperature,

followed by 0.015% H_2O_2/0.05% diaminobenzidene/0.05 M Tris–HCl (pH 7.6) for 10 min at room temperature. Sections were counterstained with Mayers' haematoxylin, dehydrated and mounted in Permount. Negative controls without the primary antibody and normal rabbit IgG were included as controls. Photomicrographs were taken with an Olympus BX50 microscope equipped with a Leica DFC 320 digital camera.

PGP generation assay

Freshly isolated PMNs (10^6 cells) were incubated with 15 µl of a 1 mg/ml solution of type I or type II collagen in PBS containing bestatin (5 mg/ml) and indicated reagents for 16 h at 37 ℃. The collagen was extensively dialyzed beforehand to remove PGP. After incubation, samples were 10 kDa filtered, washed with 40 µl of 1 N HCl, and analyzed by ESI-LC-MS/MS for levels of N-ac-PGP.

Electrospray ionization liquid chromatography–MS/MS (ESI-LC/MS/MS) for PGP and N-ac-PGP detection.

PGP and N-ac-PGP were measured as previous described [15] using a MDS Sciex (Applied Biosystems, Foster City, CA) API-4000 spectrometer equipped with a Shimadzu HPLC (Columbia, MD). HPLC was done using a 2.0 x 150-mm Jupiter 4u Proteo column (Phenomenex, Torrance, CA) with *buffer A* (0.1% HCOOH) and *buffer B* (MeCN + 0.1% HCOOH): 0 min-0.5 min 5% *buffer B*/95% *buffer A*, then increased over 0.5–2.5 min to 100% *buffer B*/0% *buffer A*. Background was removed by flushing with 100% isopropanol/0.1% formic acid. Positive electrospray mass transitions were at 270–70, 270–116, and 270–173 for PGP and 312–140 and 312–112 of N-ac-PGP.

Statistical analyses

For all statistical analyses, GraphPad Prism version 4.0 was used. When data passed the normality test; two-tailed Student *t*-tests were used for comparing control and CSE paired groups and one-tailed Student *t*-tests were used for comparing control and N-ac-PGP paired groups. For comparing three or more paired groups, parametric data were analyzed using a repeated measures ANOVA followed by Tukey post hoc analysis. When data did not pass the normality test; non-parametric *t*-tests followed by Mann-Whitney post hoc analysis were used for comparing two unpaired groups. Data were considered significant at $P < 0.05$. All results are expressed as means ± SEM.

Results

Incubation of PMNs with CSE induces the release of CXCL8, MMP8 and MMP9

To examine the role of cigarette smoke in neutrophil activation, PMNs were incubated with CSE for 9 h. Figure 1A shows that significant amounts of CXCL8 were produced after stimulation of the cells with CSE OD 0.06 and 0.12. Although the concentration of OD 0.24 is not toxic for PMNs as tested with Annexin-V/PI, the CXCL8 level produced after 9 h incubation with this concentration of CSE is lower. Figures 1B and 1C show that incubation of PMNs with increasing concentrations CSE resulted in MMP8 and MMP9 release. Significant amounts of MMP8 and MMP9 were produced after stimulation for 9 h of the cells with CSE OD 0.12. LPS was used as a positive control and induces a significant production of all proteins at 9 h.

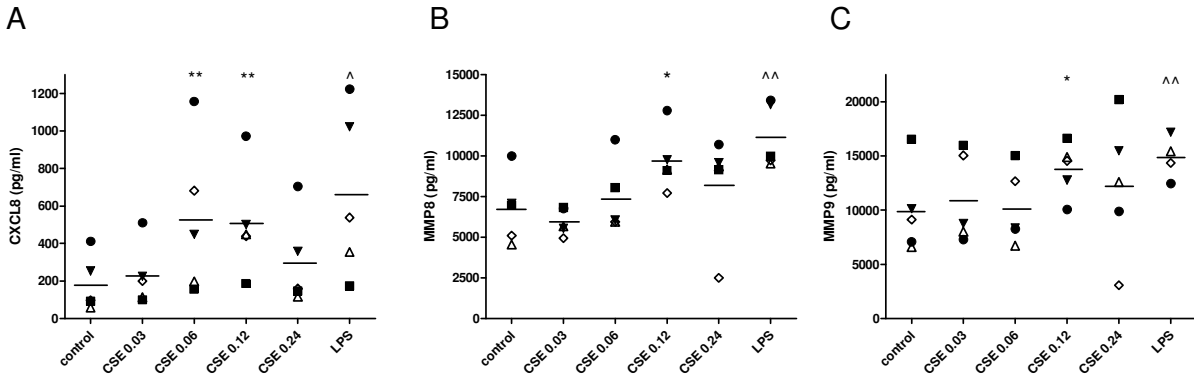

Figure 1. CSE induces the release of CXCL8, MMP8 and MMP9 from human PMNs.

*(**A**) 10^5 freshly isolated buffy coat PMNs were stimulated for 9 h with cigarette smoke extract (CSE; OD 0.03-0.24) or LPS (100 ng/ml; positive control). CXCL8, MMP8 and MMP9 ELISA's were performed on the supernatants. CSE induced the release of CXCL8 from fresh cells (**, P < 0.01 repeated measures ANOVA + Tukey CSE vs. control; ^, P < 0.01 paired t-test LPS vs. control). (**B**) CSE induced the release of MMP8 from fresh cells (*, P < 0.05 repeated measures ANOVA + Tukey CSE vs. control; ^^, P < 0.01 t-test LPS vs. control). (**C**) CSE induced the release of MMP9 from fresh cells (*, P < 0.05 paired t-test CSE OD 0.12 vs. control; ^^, P < 0.01 paired t-test LPS vs. control). Legend: each symbol represents a different donor (n = 5). Individual data are shown, horizontal bars represent mean values. The data presented here all passed the normality test.*

The activity and intracellular levels of PE are unaffected by CSE incubation of PMNs

It has recently been published that neutrophils contain PE [16], an enzyme capable of cleaving the carboxyl side of proline residues in oligopeptides. Immunofluorescence microscopy on PE in PMNs of healthy donors was performed to confirm these results. Figure 2A and 2B show that PE was located in the cytoplasm of PMNs in a granular pattern.

To investigate whether CSE influences the levels of intracellular PE protein and PE protein in the supernatant, PMNs were incubated with CSE and subsequently PE was determined in lysates and supernatants by Western blotting. PE protein levels within the cells did not change after incubation with CSE for 8 h (fig. 3A and 3B) and the levels were also unaffected after 1 µg/ml LPS exposure (data not shown). Furthermore, figure 3C shows that intracellular PE activity did not change after CSE incubation for 16 h (control = 12.49 pmol AMC/min; OD 0.06 = 12.40 pmol AMC/min; OD 0.12 = 12.79 pmol AMC/min).

Moreover, the PE activity was measured in PMN supernatants of healthy donors. The PE activity measured in the supernatant was very low after CSE incubation for 16 h (control = 0.73 pmol AMC/min; OD 0.06 = 0.27 pmol AMC/min; OD 0.12 = 0.42 pmol AMC/min). Furthermore, PE protein could not be detected in the supernatant of PMNs incubated with CSE or LPS (1 µg/ml) for 8 h, leading to the conclusion that although PMN supernatants demonstrate little PE activity, the PE protein levels are probably too low to be measured by Western blotting techniques (data not shown).

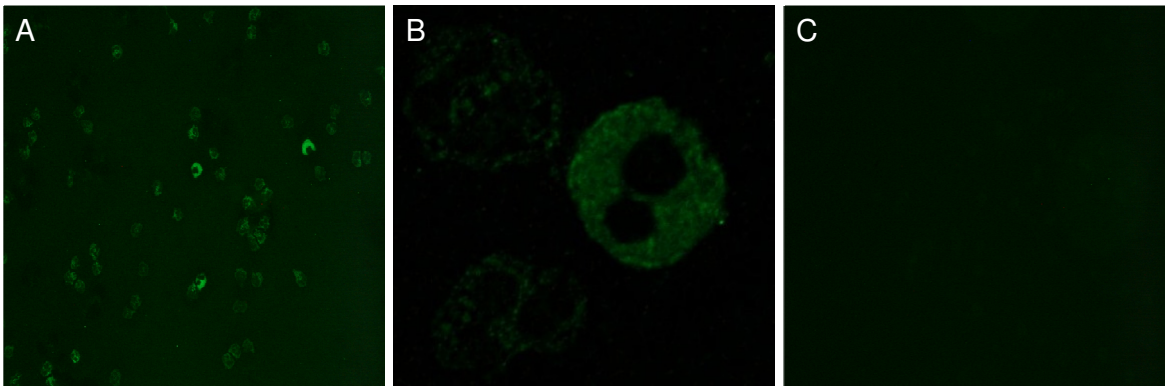

Figure 2. Human PMNs contain prolyl endopeptidase (PE).

Representative photomicrographs (n = 3) of an immunofluorescent staining for PE (green color) in PMNs of healthy volunteers (A and B (magnified from fig. A)). Panel C displays unstained cells (negative control). PE is located in the cytoplasm in a granular pattern. Magnification 200x.

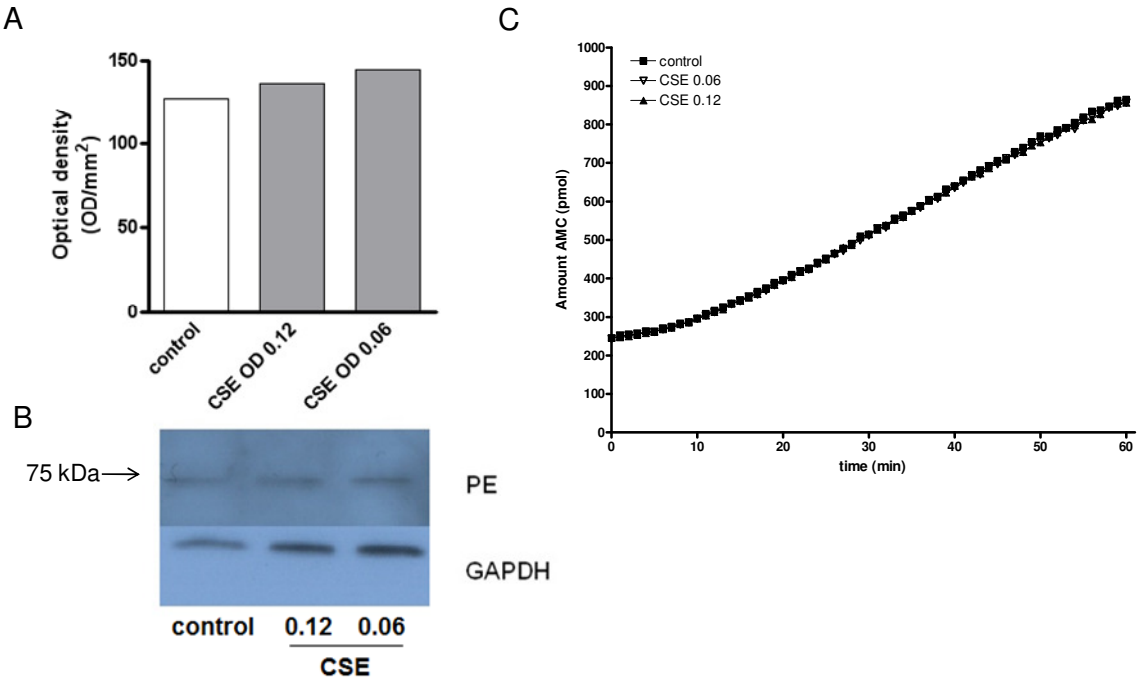

Figure 3. Human PMN incubation with CSE does not affect the level and activity of PE in cell lysates.

*(**A, B**) 10^6 isolated PMNs were stimulated for 8 h with CSE (OD 0.06 or 0.12). PE and GAPDH Western blots were performed on the cell lysates. PE in human neutrophils was a monomer and migrated at 75 kDa, which was similar to rhPE (not depicted). Incubation of PMNs with CSE did not change the optical density of the bands when compared to the control. (**C**) Freshly isolated PMNs (10^6 cells) were stimulated for 16 h with indicated reagents. PE activity was measured in lysates using Z-Gly-Pro-AMC as a substrate. Intracellular PE activity does not change after CSE exposure when compared to the control.*

Human PMNs can generate N-ac-PGP from whole collagen upon activation with CSE

The findings described before and shown in the figures 1 to 3 led to the hypothesis that cigarette smoke may stimulate neutrophils to breakdown collagen into smaller fragments, and more specifically, to PGP and N-ac-PGP. For these experiments, collagen type I was used since this is the prominent type of collagen seen in the airways [17]. PMNs were incubated with dialyzed collagen type I and CSE (OD 0.06 or 0.12). To prevent any new formed PGP from degrading, bestatin was added every 3 h. Bestatin inhibits leukotriene A_4 hydrolase, an enzyme known to degrade PGP [18]. At time point 16 h, the N-ac-PGP levels were determined in supernatants from PMNs stimulated with PBS or CSE (0.06 and 0.12). CSE OD 0.06 and 0.12 induced a 3-4 fold production of N-ac-PGP from whole collagen type I; the N-ac-PGP levels were 0.194 ng/ml and 0.217 ng/ml respectively (fig. 4).

To investigate whether the collagen breakdown process is general to other collagen types, collagen type II was also used. Interestingly, PMNs stimulated with CSE OD 0.06 or 0.12 generated N-ac-PGP levels of 0.217 ng/ml and 0.909 ng/ml, respectively, whereas PBS incubated PMNs did not generate N-ac-PGP levels above detection limit. In addition, the supernatants were examined for non-acetylated PGP levels and were tested negative (data not shown), meaning that all generated PGP is readily acetylated (see discussion).

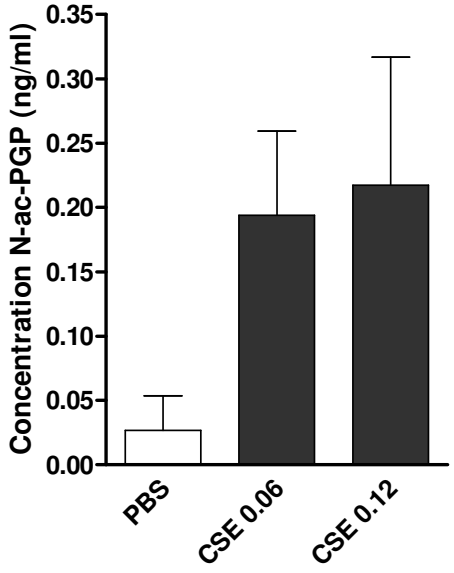

Figure 4. Human PMNs can generate N-ac-PGP de novo from collagen type I.

10^6 PMNs were incubated for 16 h at 37 °C with collagen type I dialyzed solution (1 mg/ml) and CSE (OD 0.06 or 0.12). Bestatin (50m µg/ml) was added every 3 h. At time point 16 h, the N-ac-PGP levels were determined in supernatants from the incubated PMNs. All samples were filtered through a 10-kDa filter, washed with 20 µl of 1 N HCl and analyzed using ESI-LC-MS/MS for levels of N-ac-PGP. Detection limit N-ac-PGP: < 0.025 ng/ml. (n=2)

N-ac-PGP activates PMNs to release CXCL8 and proteolytic enzymes MMP8 and MMP9

The ability of PMNs to generate N-ac-PGP from whole collagen upon stimulation with CSE led to the question whether the peptide itself may activate the PMN to release CXCL8 and MMP8 and 9. *In vivo,* this may lead to a self-perpetuating situation where newly produced N-ac-PGP can attract PMNs to the site of inflammation and activate these cells to produce more N-ac-PGP, thereby enhancing inflammation.

Figure 5A shows that PMNs incubated for 9 h with N-ac-PGP released CXCL8. Significant amounts were produced after stimulation of the cells with $3 \cdot 10^{-3}$ M N-ac-PGP. Figures 5B and 5C demonstrate that N-ac-PGP incubation led to MMP8 and MMP9 release. Significant amounts of MMP8 and MMP9 were produced after stimulation for 9 h with $3 \cdot 10^{-3}$ M N-ac-PGP.

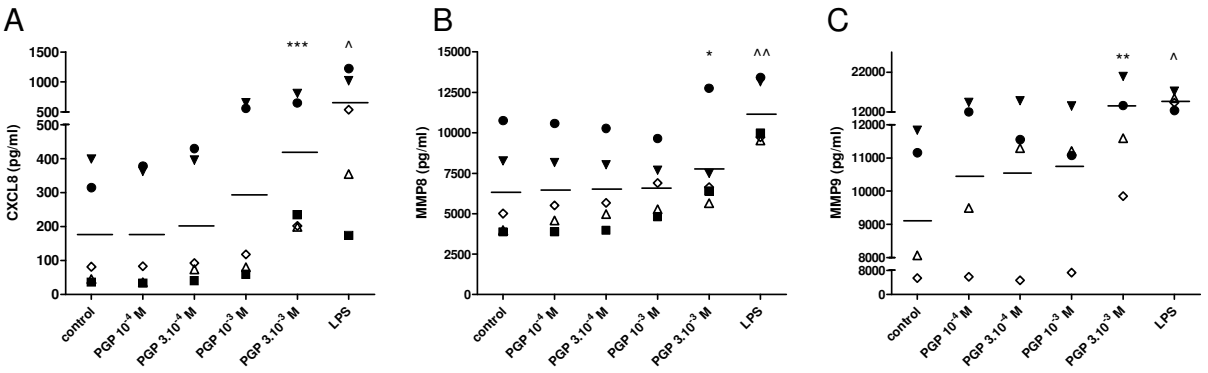

Figure 5. N-ac-PGP induces the release of CXCL8, MMP8 and MMP9 from human PMNs.

*10^5 freshly isolated buffy coat PMNs were stimulated for 9 h with N-ac-PGP (10^{-4} - $3{\cdot}10^{-3}$ M) or LPS (100 ng/ml). CXCL8, MMP8 and MMP9 ELISA's were performed on the supernatants. (**A**) N-ac-PGP induced the release of CXCL8 from fresh cells (***, P < 0.001 repeated measures ANOVA + Tukey N-ac-PGP vs. control; ^, P < 0.05 paired t-test LPS vs. control). (**B**) N-ac-PGP induced the release of MMP8 from fresh cells (*, P < 0.05 paired t-test CSE OD 0.12 vs. control; ^^, P < 0.01 paired t-test LPS vs. control). (**C**) N-ac-PGP induced the release of MMP9 from fresh cells (**, P < 0.01 repeated measures ANOVA + Tukey N-ac-PGP vs. control; ^ P, < 0.01 paired t-test LPS vs. control). Legend: each symbol represents a different donor (n = 4-5). Individual data are shown, horizontal bars represent mean values. The data presented here all passed the normality test.*

PE activity of PMNs is unaffected by N-ac-PGP incubation

To investigate whether N-ac-PGP influences PE activity, PMNs were incubated with N-ac-PGP for 16 h and subsequently PE activity was measured. Figure 6 shows that intracellular PE activity did not change after N-ac-PGP incubation (N-ac-PGP $3{\cdot}10^{-4}$ M = 13.41 pmol AMC/min; N-ac-PGP 10^{-3} M = 13.04 pmol AMC/min; N-ac-PGP $3{\cdot}10^{-3}$ M = 12.40 pmol AMC/min) when compared to the control (12.49 pmol AMC/min).

Additionally, the PE activity was measured in PMN supernatants of healthy donors. The PE activity measured in the supernatant was very low after N-ac-PGP incubation for 16 h (control = 0.73 pmol AMC/min; N-ac-PGP $3{\cdot}10^{-4}$ M = 0.80 pmol AMC/min; N-ac-PGP 10^{-3} M = 0.58 pmol AMC/min; N-ac-PGP $3{\cdot}10^{-3}$ M = 0.44 pmol AMC/min).

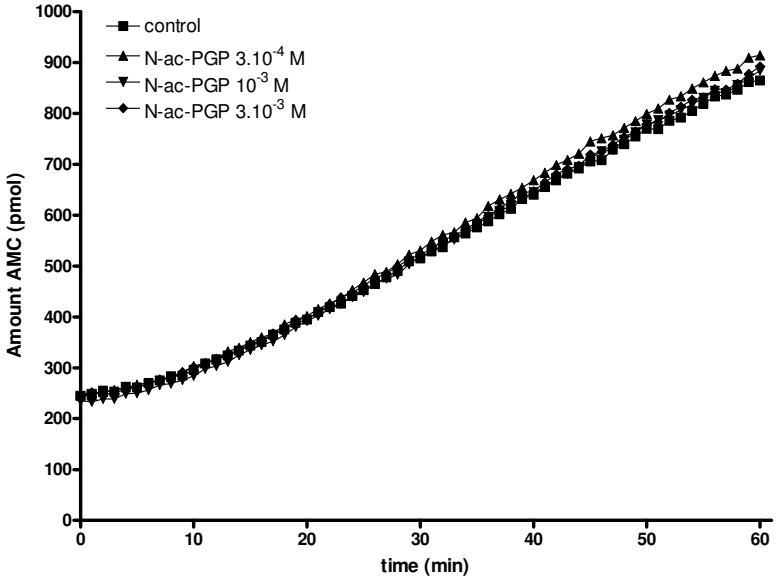

Figure 6. Human PMN incubation with N-ac-PGP does not affect the activity of released or intracellular PE.

*Freshly isolated PMNs (10^6 cells) were stimulated for 16 h with indicated reagents. PE activity was measured in supernatants and lysates using Z-Gly-Pro-AMC as a substrate. (**A**) Incubation of PMNs with N-ac-PGP ($3{\cdot}10^{-4}$-$3{\cdot}10^{-3}$ M) leads to the release active PE, which is not different from the control. (**B**) Intracellular PE activity does not change after N-ac-PGP exposure when compared to the control.*

CSE-stimulated PMNs from COPD patients tend to release more CXCL8 than healthy PMNs

To investigate whether PMNs isolated from fresh blood from COPD patients are intrinsically different from healthy donors, PMNs were exposed for 6 h to increasing concentrations CSE. Figure 7A shows that PMNs obtained from COPD patients tended to produce more CXCL8 upon stimulation with CSE than PMNs obtained from healthy controls, however, significance was not reached due to the large variation within the COPD-PMN measurements.

The basal intracellular PE activity of PMNs from COPD patients is a 25-fold higher when compared to healthy donors

The PE activity assay was performed to investigate whether the intracellular PE activity of PMNs from COPD patients is different from healthy donors. Figure 7B shows that the basal PE activation in PMNs obtained from COPD patients was significantly higher than the PE activity in PMNs from healthy donors.

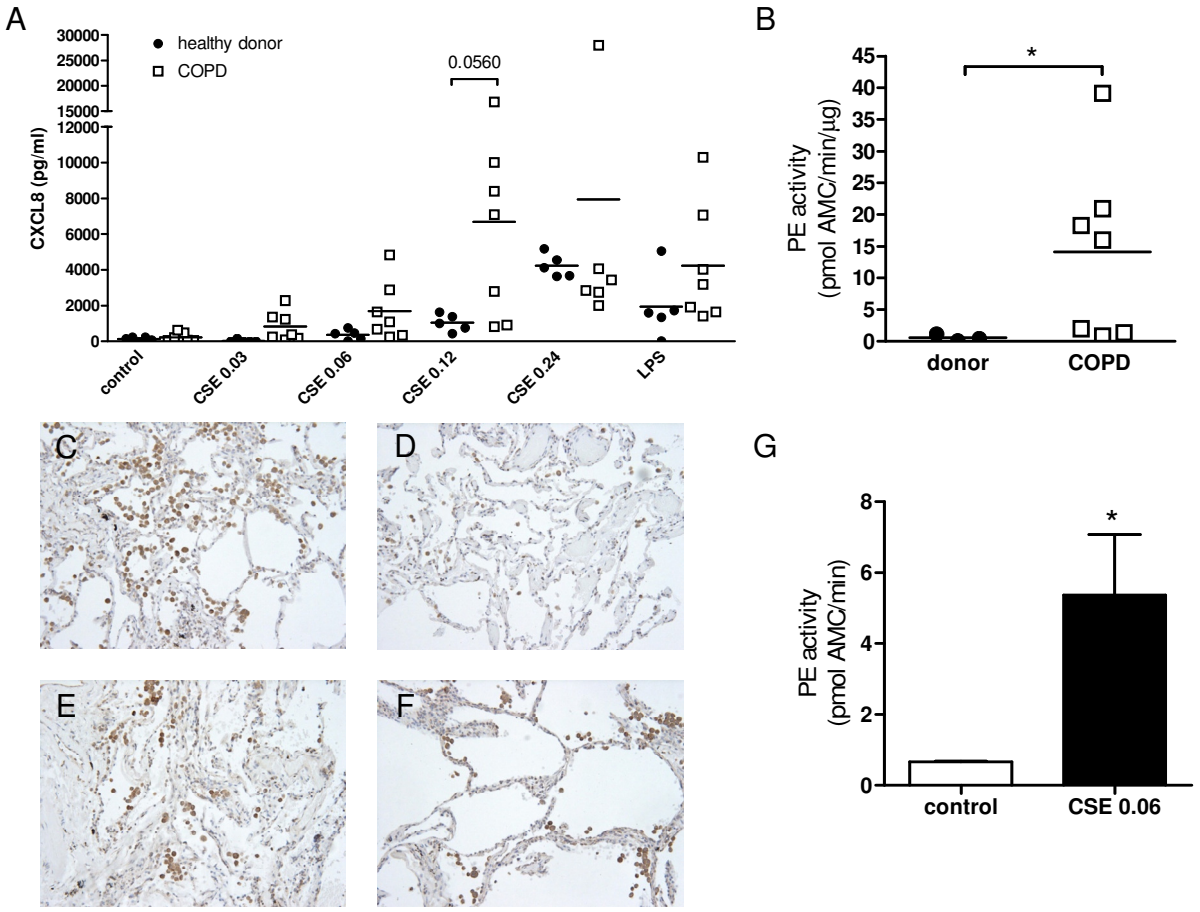

Figure 7. The basal PE activity of PMNs from COPD patients is a 25-fold higher when compared to healthy donors.

*(**A**) 10^5 freshly isolated PMNs from healthy donors (block dots, n=5) and COPD patients (white squares, n=7) were stimulated for 6 h with cigarette smoke extract (CSE; OD 0.03-0.24). A CXCL8 ELISA was performed on the supernatants. PMNs from COPD patients tend to produce higher amounts of CXCL8 after CSE incubation (P = 0.0560, t-test CSE OD 0.12 donor vs. COPD). Individual data are shown, horizontal bars represent mean values. (**B**) The PE activity was measured in lysates of unstimulated PMNs (10^6 cells) using Z-Gly-Pro-AMC as a substrate. The basal PE activation of PMNs from COPD patients (white squares, n=7) is significantly higher than the PE activity of PMNs from healthy donors (block dots, n=3) (*, P < 0.05 Mann-Whitney). (**C-F**) Localization of PE in the human lung. Representative photomicrographs of an immunohistological staining for PE (brown color, DAB staining) in lung tissue of (**C**) a current smoker, (**D**) ex-smoker, (**E**) COPD patient with GOLD stage II and (**F**) a COPD patient with GOLD stage IV. Magnification, x 200. (**G**)10^6 HBE cells were incubated with indicated reagents, after which the PE activity was measured in supernatants. Exposing HBE cells to cigarette smoke extract for 16 h in vitro leads to an elevated PE activity in the supernatant.*

Inflammatory cells in lung tissue of smokers and COPD patients express PE protein

Immunohistochemical analysis was performed to compare the PE protein expression in lung tissue specimens of current smokers, ex-smokers, COPD patients with GOLD stage II and COPD patients with GOLD stage IV. In the lung tissue specimens of current smokers the massive amount of inflammatory cells, such as neutrophils and macrophages, highly expressed PE protein (fig. 7C). The number of inflammatory cells and consequently the PE expression was decreased in the lung tissue specimens of ex-smokers (fig. 7D). Furthermore, the inflammatory cells observed in the lung tissue specimens from COPD patients with GOLD stage II and IV also expressed high levels of PE protein (fig. 7E and 7F). Additionally, the bronchial epithelial cells in the human lung tissues expressed PE protein. Furthermore, exposing human bronchial epithelial (HBE) cells to cigarette smoke extract for 16 h *in vitro* led to an elevated PE activity in the supernatant (fig. 7G).

Discussion

COPD is a lung disease characterized by progressive airflow limitation due to the destruction of alveolar walls. Neutrophils are a notable component of the inflammation in COPD. Since one of the main risks to develop COPD is cigarette smoke exposure, we investigated the effect of CSE on the breakdown of whole pulmonary matrix collagen into the chemotactic collagen fragment N-ac-PGP by human neutrophils.

We are the first to show that cigarette smoke can activate neutrophils to generate chemotactic collagen fragment N-ac-PGP from whole collagen *in vitro*. Incubating PMNs with CSE led to the activation of these cells, which resulted in the release of chemoattractant CXCL8 and proteases MMP8 and MMP9. Simultaneous incubation of PMNs with CSE and collagen resulted in N-ac-PGP generation. In addition, PMNs constitutively exhibited PE activity as well protein. Simultaneous incubation of PMNs with the tripeptide N-ac-PGP resulted in the release of CXCL8, MMP8 and MMP9.

Moreover, we tested whether PMNs from COPD patients are different from PMNs from healthy donors. Although the incubation with different CSE concentrations led to more release of CXCL8 from COPD PMNs, this did not reached the level of significance when compared to the healthy donors. Interestingly, here we show that the basal intracellular PE activity of PMNs from COPD patients is a 25-fold higher when compared to healthy donors. Immunohistological staining of human lung tissue specimens for PE protein showed that besides neutrophils, macrophages and epithelial cells also express significant levels of PE.

Early in inflammation, neutrophils migrate from the capillary into the interstitial space, following a chemotactic gradient of CXCL8 [19]. At the site of inflammation neutrophils are activated, leading to the release of more CXCL8 [1, 20]. This release leads to a self-perpetuating inflammatory state where neutrophils attract more neutrophils via chemokine receptors CXCR1 and CXCR2 [21-23]. Recently, we showed that cigarette smoke extract (CSE) can act as a chemoattractant for PMNs [24]. This led to the question of whether CSE may activate the neutrophil to synthesize CXCL8, acting in an autocrine/paracrine fashion. Figure 1 shows that the activation of PMNs by CSE exposure leads to the release CXCL8. We hypothesize that once infiltrated in the lung tissue, cigarette smoke activates the infiltrated neutrophil. This activation results in a CXCL8 release by the neutrophil, which in turn will attract more neutrophils into the airways.

The increased expression of MMPs is considered to be a key factor in the development of COPD. In this study, the MMP8 and MMP9 release by PMNs was elevated after cigarette smoke and N-ac-PGP exposure to human neutrophils. These results are in accordance with clinical data from different groups. It was shown that although MMP8 and MMP9 levels are

lower in smokers when compared to COPD patients [25, 26], the MMP levels from both groups are elevated when compared to non-smokers [25-28].

Besides MMP8 and MMP9, PE is needed to generate PGP from whole collagen; the MMPs cleave whole collagen into fragments of 30 to 100 amino acids in length, after which PE specifically cleaves PGP from these smaller fragments [9]. Recently, it was published that neutrophils contain PE [16], which is confirmed in this study. PE activity was measured in lysates of PMNs. Incubation of PMNs with CSE or N-ac-PGP did not affect intracellular PE activity, which suggests that PE is constitutively active. Although PE activity could be measured in the supernatant of CSE or N-ac-PGP incubated PMNs, these levels were very low. We hypothesize that cigarette smoking causes a locally restricted lung inflammation where necrotic neutrophils release PE to the exterior, which contributes to PGP generation. It is possible that other cells besides neutrophils play a role in collagen destruction by supplying PE. Figure 7 shows that pulmonary alveolar macrophages express PE protein as well. Neutrophils and macrophages present in lung tissue of current smokers and COPD patients with GOLD stage II and IV highly expressed PE protein, while the number of inflammatory cells and consequently the PE expression was decreased in the lung tissue of ex-smokers. Moreover, here we show that epithelial cells of human lung tissue contain PE, indicating that epithelial cells may be an important source for PE in the lung. Exposure of HBE cells to cigarette smoke extract for 16 h *in vitro* leads to an elevated PE activity in the supernatant, suggesting that cigarette smoke exposure leads to a release of PE. It cannot be excluded however that PE could also be released as a result of airway epithelial necrosis or necrosis of other inflammatory cell types containing PE after cigarette smoke exposure.

The next step was to investigate the effect of CSE on the breakdown of whole collagen into collagen fragment N-ac-PGP by human neutrophils. The multistep pathway of collagen breakdown has been studied in a murine model of cigarette smoke-induced lung emphysema in our group by Braber *et al.* [29]. It was demonstrated that all relevant components (neutrophils, MMP8, MMP9 and PE) involved in this pathway to generate (N-ac-)PGP from collagen were upregulated in the airways exposed to cigarette smoke, suggesting that activation of cells by cigarette smoke leads to the release of proteases and extracellular matrix breakdown. Although this murine model showed that (N-ac-)PGP is formed after cigarette smoke exposure in the airways, here we demonstrate using *in vitro* techniques that upon stimulation with CSE the neutrophil is able to breakdown collagen into N-ac-PGP fragments. Our results are reinforced with previous *in vitro* findings describing that human neutrophils were capable to generate PGP from whole collagen after LPS exposure [16].

Neutrophils contain all necessary components for PGP generation and in this report we demonstrated that simultaneous incubation of these cells with CSE and collagen leads to PGP generation. Although N-ac-PGP levels were measurable after 16 h incubation, non-

acetylated PGP could not be detected in these supernatants. An explanation can be found in the results described by Snelgrove *et al.* They demonstrated that cigarette smoke itself is responsible for N-terminally acetylating PGP, enhancing its chemotactic capacity [18].
It was shown that smoking cessation improved lung function although elevated neutrophil counts and the protease burden in the airways continued for months [27, 30] . An explanation for the elevated neutrophil influx and protease levels after smoke cessation is that the continued neutrophil chemotaxis and activation is mediated via N-ac-PGP. Here we have demonstrated that this tripeptide can activate neutrophils to release CXCL8 that will lead to increased neutrophilic migration. In addition, N-ac-PGP also induced the release of MMP8 and MMP9 from neutrophils, which will result in more collagen breakdown and formation of N-ac-PGP. It was recently published that N-ac-PGP can induce the release of MMP9, which is confirmed in this study. It was indicated that extracellular matrix-derived N-ac-PGP could result in a feed-forward cycle by releasing MMP9 from activated PMNs through the ligation of CXCR1 and CXCR2 and subsequent activation of the ERK1/2 MAPK [31].
Tissue destruction is a hallmark of COPD. Since PE is essential in the collagen breakdown process, we measured the basal intracellular PE activity in PMNs from COPD patients. Interestingly, the basal PE activity of PMNs from COPD patients was remarkably higher than in PMNs from healthy donors, which suggests that PE can play an important role in lung collagen breakdown leading to the development of COPD. Moreover, here we suggest that the PMNs from COPD patients are activated to a greater extend, since the CXCL8 levels released by these PMNs appeared to be higher than from PMNs from healthy controls.
Finally, to our knowledge, this is the first *in vitro* study that indicates that neutrophils activated by cigarette smoke can destruct collagen into N-ac-PGP and that this collagen fragment can activate neutrophils, which may lead *in vivo* to a self-propagating cycle of neutrophil infiltration, chronic inflammation and lung emphysema. In addition, here we propose that PE can play an important role in lung collagen breakdown leading to the development of COPD since the basal intracellular PE activity of neutrophils was a 25-fold higher in COPD, making PE a promising therapeutic target.

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Chapter 8

Neutrophils from Crohn's disease patients are significantly different from healthy donors in basal migration and collagen breakdown.

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Abstract

Crohn's disease (CD) is a disease of the intestinal tract which relapses and remits throughout its course. Since luminal disease activity is accompanied with extensive neutrophil migration, we investigated this neutrophil migration and the components that play a role in this process in more detail.

Although neutrophils from CD patients had a higher absolute migration capacity under basal conditions in comparison to neutrophils from healthy donors, there was no difference between these groups in relative migration towards CXCL8. Surprisingly, CXCL8 protein secretion and CXCL8 mRNA expression levels were significantly decreased in PMNs from CD patients, while CXCR1 and CXCR2 mRNA expression levels tended to be increased. In addition, decreased levels of CXCL8 protein were found in homogenates of non-inflamed intestinal tissue from CD patients, compared with non-inflamed colorectal cancer controls, whereas in inflamed CD tissue the CXCL8 level was strongly elevated. In contrast to the lower CXCL8 production, neutrophils from CD patients produced higher amounts of MMP8 and MMP9. Moreover, the ability of neutrophils to produce the chemoattractant tripeptide PGP from collagen was increased, suggesting a role for PGP in neutrophil accumulation and subsequent neutrophil-mediated tissue damage in this bowel disease.

In conclusion, neutrophils from CD patients are significantly different from healthy donors; these neutrophils show increased absolute migration and collagen breakdown capacities *in vitro*. Since CXCL8 secretion and mRNA expression levels were decreased in PMNs and in non-inflamed tissue from CD patients, we postulate that Crohn's pathogenesis may result of an impaired neutrophilic innate immunity.

Introduction

Crohn's disease (CD) is one of the major inflammatory bowel diseases (IBD) and is characterized by transmural inflammation involving any part of the gastrointestinal tract [1, 2]. Although the precise mechanism underlying the development of CD remains unknown, it has been postulated that it results from a complex interaction of genetic, environmental and immunologic factors [3]. Importantly, alterations in intestinal barrier function may play an essential role in the pathogenesis [1, 3, 4]. In CD, patients' symptoms, epithelial injury and disease activity are correlated with extensive neutrophilic infiltration in affected parts of the intestine. During the inflammatory response, neutrophils transmigrate from the blood into the intestinal (sub)mucosal and serosal tissue were they release nonspecific inflammatory mediators, which ultimately can lead to tissue damage [3-6].

At the inflammatory site, the attractants for neutrophils are ELR^+ CXC-chemokines, of which CXCL8 is the most potent chemoattractant [7]. The role of this CXCR1 and CXCR2 ligand is extensively studied; several groups have described that the CXCL8 production is significantly increased in inflamed mucosal tissue samples of CD patients when compared to healthy controls [8-10]. In addition, since the seventies it is known that breakdown products of the extracellular matrix, such as collagen fragments, can also have chemotactic properties [11, 12]. One of the collagen fragments is Proline-Glycine-Proline (PGP), which was first identified by Pfister and colleagues [13]. This tripeptide has specific chemotactic properties for neutrophils *in vivo* and *in vitro* [14-18]. Recently, PGP and its acetylated form, N-ac-PGP, have been proposed as new biomarkers and therapeutic targets for the neutrophil-mediated lung diseases: chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) [15, 19]. Moreover, the proteolytic cascade that generates PGP from collagen has been described in CF [15] and in a murine model of cigarette-smoke-induced lung emphysema [20]. Both studies showed that matrix metalloproteinases (MMPs) 8 and 9 and prolyl endopeptidase (PE) are involved in this multistep pathway of collagen breakdown [15, 20]. Neutrophils, activated by an inflammatory stimulus, release MMP8 and MMP9, which cleave whole collagen into smaller fragments of 30 to 100 amino acids in length, allowing PE to cleave these fragments specifically into PGP fragments [15, 20].

The aim of this study was to investigate the migration of neutrophils obtained from peripheral blood of patients with CD and healthy controls. Moreover, the neutrophilic mRNA expression and release of CXCL8 and the expression of its receptors CXCR1 and CXCR2 on these PMNs were studied in these groups. Furthermore, CXCL8 levels were determined in intestinal tissue specimens from CD patients and controls. In addition, the ability of neutrophils from CD patients to produce the chemoattractant PGP from whole collagen was investigated.

In this report, we show that the neutrophils from CD patients are significantly different from healthy donors. Furthermore, our study indicates that Crohn's pathogenesis may result from an impaired innate immunity. In contrast, neutrophils from CD patients produced higher amounts of PGP from whole collagen, suggesting a role for PGP in neutrophil accumulation and subsequent neutrophil-mediated tissue damage in this bowel disease.

Material and methods

In this study, human polymorphonuclear leukocytes (PMNs) and bowel tissue samples from CD patients and controls subjects were analyzed.

PMN study

CD patients and healthy donor characteristics

The characteristics of the human subjects included in the PMN study are presented in Table 1. All patients were recruited through the outpatient clinic at the department of Gastroenterology in the Academic Medical Centre (AMC) Amsterdam, the Netherlands as part of the Elephant Study. All patients signed informed consent and the Elephant Study was approved by the ethics review committee of the AMC. Blood of healthy controls was taken with informed consent using protocols approved by University Medical Center Utrecht.

Table 1. Characteristics of CD patients and healthy donors

Characteristics	**CD**	**healthy donors**
Total	36	12
Gender		
- Male (%)	15 (42)	5 (42)
- Female (%)	21 (58)	7 (58)
Median age	38.5	47
- (range)	(21-70)	(24-60)

Isolation of human PMNs

Human PMNs were isolated as previously described from fresh whole blood [16], Resulting PMN preparations consisted of approximately 95-97% PMNs, based on PMNs physical parameters analyzed by flow cytometry and CD16 expression. The preparations were negative for CD14, meaning that the preparations did not contain monocytes.

Chemotaxis assay

The chemotaxis assay was performed as previously described [16] using a 3-µm 96-well polycarbonate filter plate (Millipore BV, Amsterdam, the Netherlands). After incubating the plate for 1 h at 37 °C in 5% CO_2, the cells in the bottom well were counted for 30 sec using a BD FACSCalibur Flow Cytometer with CellQuest Pro Software (version 5.2.1.). Data were standardized to a chemotactic index (= cells per well migrating to chemoattractant/cells per well migrating to medium).

CXCL8 ELISA

Freshly isolated PMNs (10^5 cells/well) were incubated with indicated reagents for 6 h. Supernatants were harvested and frozen until use. CXCL8 levels were measured using a human CXCL8 ELISA kit (BD Biosciences, Alphen a/d Rijn, the Netherlands) according to manufacturer's instructions.

RNA isolation

TRIzol was used to extract total RNA from PMNs. After defrosting, the samples were incubated for 3 min at room temperate (RT). Next, 200 µl chloroform was added to 1 ml TRIzol reagent. After vigorously shaking for 15 sec, the samples were again incubated for 3 min at RT and then centrifuged at 12,000 g for 15 min at 4°C. The aqueous phase was transferred to a new tube and ice-cold 500 µl isopropyl alcohol was added to this tube. After vigorously shaking for 15 sec, the samples were incubated for 30 min at -20 °C. Next, the samples were centrifuged at 18,000 g for 10 min at 4°C. Subsequently, the supernatant was removed and washed with 1 ml 70% ice-cold ethanol and centrifuged at 18,000 g for 5 min at 4°C. After removing the supernatant, the RNA pellet was dried for 5-10 min. Finally, the pellet was resuspended in 30 µl nuclease-free water.

cDNA synthesis

A NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA) was used to both determine the concentration and the purity of RNA. Furthermore, the quality of the RNA was checked by running a 2% agarose gel (detection of 28S, 18S and 5S bands). 200-500 ng total RNA was treated for 30 min at 37 °C with DNAse. To inactivate the DNAse, 1 µl EDTA was added and incubated for 10 min at 65 °C. Subsequently, the RNA was directly subjected to cDNA synthesis (Iscript, Bio-Rad) for 60 min at 42 °C, followed by 5 min at 85 °C. The synthesized cDNA was diluted 10 times in nuclease-free water and stored at -20 °C until use.

Quantitative RT-PCR

Quantitative RT-PCR was performed in duplicate using a MyiQ detection system (Biorad, Hercules, USA), and consisted of 3 min initial denaturation at 95 °C, followed by 40 thermal cycles of 10 sec at 95 °C and 30 sec at 60 °C. To confirm the specificity of the PCR amplification, a dissociation curve (65 °C 95 °C, each 10 sec increase temperature with 0.5 °C) was taken as well. The RT-PCR reactions were carried out in 96-well Clear-Hard-Shells (HSS-9601) from Biorad, using iQ™ SYBR® Green and primers (10 pmol, SA Biosciences). Determination of β-actin expression was included to normalize the results of the different

samples. The relative expression per gene of interest was analyzed by setting β-actin arbitrary on 10,000 and by use of the formula: $10{,}000*2^{(\text{Ct-value B-actin} - \text{Ct value gene of interest})}$.

MMP analysis

Freshly isolated PMNs (10^5 cells/well) were incubated in medium for 6 h. Supernatants were harvested and frozen until use. MMP8 and MMP9 were quantitated using an R&D Systems Fluorokine MAP Human Base Kit (R&D Systems, Minneapolis, USA; cat. no. LMP 000) according to the manufacturer's instructions and were measured on a Luminex® 200 TM (Luminex corporation, Austin, USA).

PE activity assay

Freshly isolated PMNs (10^5 cells/well) were incubated in medium for 6 h. Supernatants were harvested and frozen until use. PE activity was measured in these supernatants using the fluorogenic substrate Z-Gly-Pro-7-amido-4-methylcoumarin (Z-G-P-AMC) (Bachem AG, Budendorf, Germany). Twenty microliters of supernatant was added to each well in a black 96-well flat-bottom plate, followed by addition of 80 µl of assay buffer (25 mM Tris, 0.25 M NaCl, pH 7.5, 2 mM DTT) containing 100 µM substrate Z-Gly-Pro-AMC. The fluorescence from liberated AMC was monitored every 1 min over 60 min at 37 °C using a Fluostar reader at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Fluorometric intensities observed were converted to pmol AMC released per min using appropriate AMC standard curves.

PGP generation assay and total PGP detection

80 µl supernatants of isolated PMNs (10^5 cells/100 µl medium for 6 h) were incubated with 20 µl of a 1 mg/ml solution of type I collagen in PBS for 24 h at 37 °C. The collagen was extensively dialyzed beforehand to remove small collagen fragments (molecular weight cut off: 12,000-14,000 Da). After incubation, samples were 10 kDa filtered, washed with 40 µl of 1 N HCl, and analyzed by ESI-LC-MS/MS for levels of total PGP (PGP and N-α-PGP) as previous described [21] using a MDS Sciex (Applied Biosystems, Foster City, CA) API-4000 spectrometer equipped with a Shimadzu HPLC (Columbia, MD). HPLC was done using a 2.0 × 150-mm Jupiter 4u Proteo column (Phenomenex, Torrance, CA) with *buffer A*: 0.1% HCOOH and *buffer B*: MeCN + 0.1% HCOOH: 0 min-0.5 min 5% *buffer B*/95% *buffer A*, then increased over 0.5–2.5 min to 100% *buffer B*/0% *buffer A*. Background was removed by flushing with 100% isopropanol/0.1% formic acid. Positive electrospray mass transitions were at 270–70, 270–116, and 270–173 for PGP and 312–140 and 312–112 of N-ac-PGP.

To test if the dialyzed collagen was destructible by proteases, dialyzed collagen was incubated with rhMMP8 (Western blotting standard R&D; 45 µl) + PE (R&D; 0.4 µg) or

rhMMP9 (Western blotting standard R&D; 45 µl) + PE. The total PGP concentrations were 2.16 and 1.56 ng/ml respectively.

CD tissue study

CD patient and control characteristics

The characteristics of the human subjects included in the tissue specimen study are presented in Table 2. The study was performed according to the guidelines from the Medical Ethics Committee of the Leiden University Medical Center. The samples in this study were obtained from surgical resection specimens and include pairs of macroscopically inflamed and normal-appearing (non-inflamed) mucosa from patients with CD, clinically and histologically confirmed, with normal tissue from patients with a colorectal carcinoma, at least 10 cm from the tumor, as controls. Details on patients characteristics, all tissue specimens included, and the preparation of the tissue homogenates are described in previous studies [22, 23].

Table 2. Characteristics of CD patients and controls

Characteristics	**CD**	**controls**
Total	41	20
Gender - Male (%) - Female (%)	 14 (34) 27 (66)	 9 (45) 11 (55)
Median age at surgery (range)	33 (12-79)	59 (35-85)
Inflamed tissue (colon, ileum)	62 (30, 32)	-
Total patients with inflamed tissue	40	-
Non-inflamed tissue (colon, ileum)	46 (19, 27)	22 (17, 5)
Total patients with non-inflamed tissue	34	20

CXCL8 ELISA

CXCL8 levels were measured in tissue homogenates (50 µg total protein) using a human CXCL8 ELISA kit according to manufacturer's instructions.

Immunohistochemistry

Cryostate sections (5 µm) of the intestinal tissues of five CD patients were cut, fixated in ice-cold acetone for 10 min, blocked with 5% rabbit serum in 1% BSA/PBS and afterwards incubated with goat anti-CXCL8 (1:50, R&D AF-208-NA) and mouse anti-human neutrophil elastase, (1:00 Dakocytomation Clone NP57) in 1% BSA/PBS overnight at 4 ℃. After subsequent washings with PBS, the antibodies were visualized by incubation with rabbit anti-mouse-Alexa488 and rabbit anti-goat-Alexa568 (both 1:500, Invitrogen, Breda, the Netherlands) in 1% BSA/PBS for 1 h at RT. Counterstaining was done with Hoechst 33342 (Sigma Aldrich Chemie BV, Zwijndrecht, the Netherlands) for 5 min at RT and slides were sealed with Prolong gold anti-fade reagent (Invitrogen). Photomicrographs were taken with a Zeiss microscoop equiped with a camera.

Statistical analyses

For all statistical analyses, GraphPad Prism version 4.0 was used. When data passed the normality test; two-tailed Student *t*-tests were used for comparing CD and control groups. For intestinal tissue CXCL8 levels data did not pass the normality test and Mann-Whitney and Wilcoxon signed rank tests were performed. Data were considered significant at $P < 0.05$. All results are expressed as means + SEM.

Results

Peripheral blood PMNs from CD patients have an increased basal migratory capacity than PMNs from healthy subjects

A transwell chemotaxis system was used to evaluate the migratory effect of PMNs from healthy subjects or patients with CD. The migration of PMNs to the lower chamber was quantified. Figure 1A shows the migration of the PMNs of either group under basal conditions; freshly isolated PMNs were added to the top well and medium was added to the bottom well. The PMNs from patients with CD showed a 5-times higher spontaneous migration from the top to the bottom well when compared to PMNs from healthy controls ($P < 0.001$). Although adding the chemoattractant CXCL8 to the assay led to a concentration dependent migration, this did not result in a significant difference between the CD and control group in the extent of the reaction towards this chemoattractant (fig. 1B). In these experiments, the basal migration of each individual was set to 1 and all other data of that individual were standardized to this to calculate the chemotactic index (relative migration).

In summary, although the PMNs from CD patients had a higher absolute migration capacity under basal conditions when compared to healthy PMNs, in the presence of various concentrations of CXCL8 there was no significant difference in relative migration of both groups towards various concentrations of CXCL8 *in vitro*.

CXCL8 protein secretion and CXCL8 mRNA expression level under basal conditions are decreased in PMNs from CD patients

Since CXCL8 is the strongest chemoattractant for neutrophils [7, 8], we have investigated whether there was any difference in CXCL8 release between CD and healthy PMNs. PMNs from both groups were incubated with medium, mimicking basal conditions, or LPS, mimicking a bacterial stimulus. Figure 2A shows that PMNs from healthy controls spontaneously released significantly higher amounts of CXCL8 than PMNs from CD patients. Although incubating the PMNs of either group with LPS did result in an extreme elevation in CXCL8 release when compared to control, there was no difference in CXCL8 release between both groups.

Moreover, the CXCL8 mRNA expression level was measured in PMNs from both groups. Figure 2B shows that PMNs from healthy subjects had a significantly higher CXCL8 mRNA expression level than PMNs from CD patients.

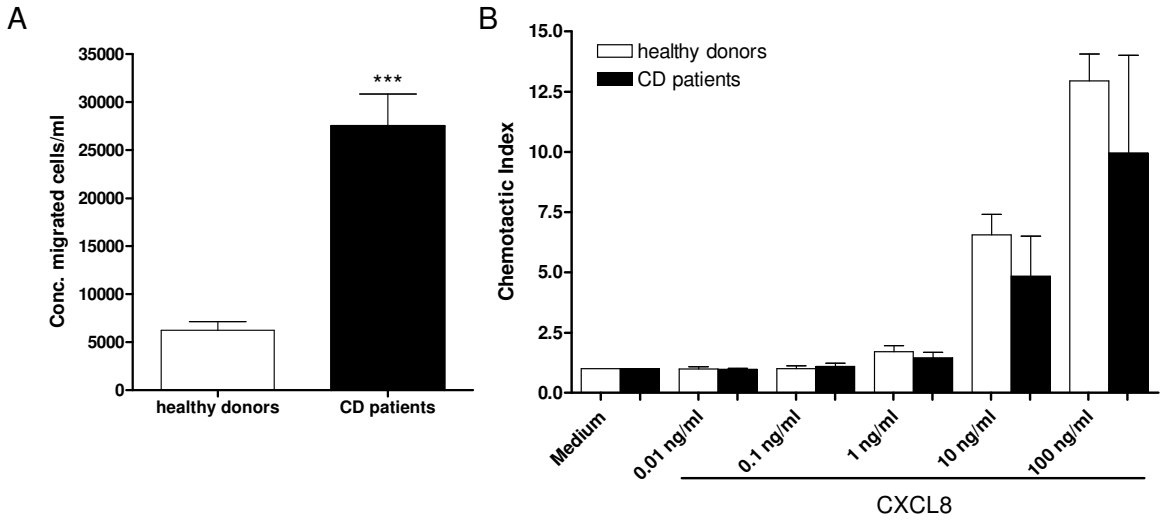


Figure 1. The basal migratory capacity of PMNs from CD patients is a 5-fold higher when compared to healthy donors, but both react to the same extent to CXCL8.

*(**A**) Freshly isolated PMNs (10^5) were added to the top well and medium was added to the bottom well. Subsequently, the chemotaxis assay was performed. The neutrophilic basal migratory capacity is a 5-fold higher in CD as compared to healthy donors (***, unpaired t-test, P < 0.001). (mean + SEM, healthy donors: n=6; CD patients: n=3) (**B**) There is no significant difference in the chemotactic response to various concentrations of CXCL8 between the two groups, when data are standardized to a chemotactic index (= cells per well migrating to chemoattractant/cells per well migrating to medium). (mean + SEM, healthy donors: n=6; CD patients: n=14)*

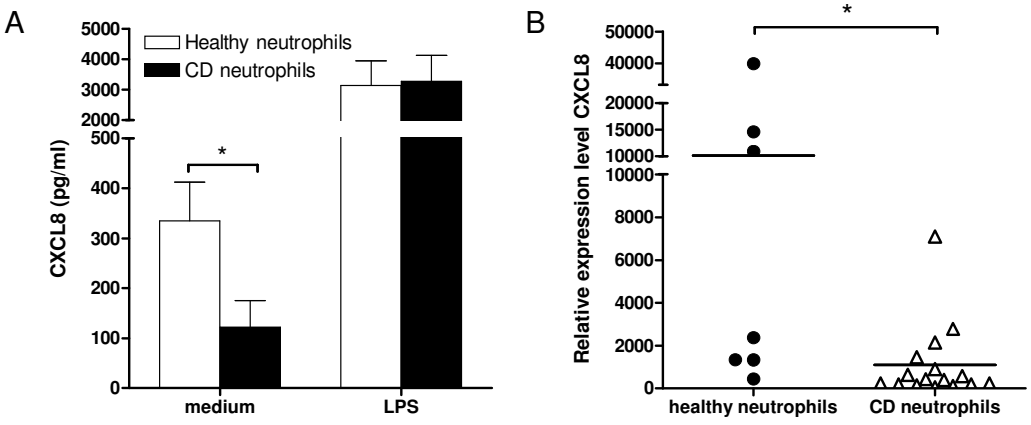


Figure 2. Neutrophilic CXCL8 secretion and CXCL8 expression levels are decreased in CD patients.

*(**A**) 10^5 freshly isolated PMNs were incubated for 6 h with medium or LPS (10 ng/ml). PMNs from CD patients release lower concentrations of CXCL8 as compared to healthy donors (*, unpaired t-test, P < 0.05). Although LPS induces an increase in CXCL8 release in both groups, there is no difference in response to LPS between the two groups. (mean + SEM, healthy donors: n=12; CD patients: n=13) (**B**) PMNs from CD patients have a lower basal expression of CXCL8 when compared to PMNs from healthy donors (*, unpaired t-test, P < 0.05). Individual data are shown, horizontal bars represent mean (+ SEM) values. (healthy donors: n=7; CD patients: n=16)*

CXCR1 and CXCR2 mRNA expression levels are elevated, though not significantly, in PMNs from CD patients

To investigate if the mRNA expression level of the two CXCL8 receptors CXCR1 and CXCR2 is different in the two groups, total RNA was extracted from PMNs from healthy and CD patients and quantitative real-time PCR was performed. Figure 3A and 3B show that PMNs obtained from peripheral blood of CD patients tended to express more CXCR1 and CXCR2 mRNA than PMNs from healthy subjects.

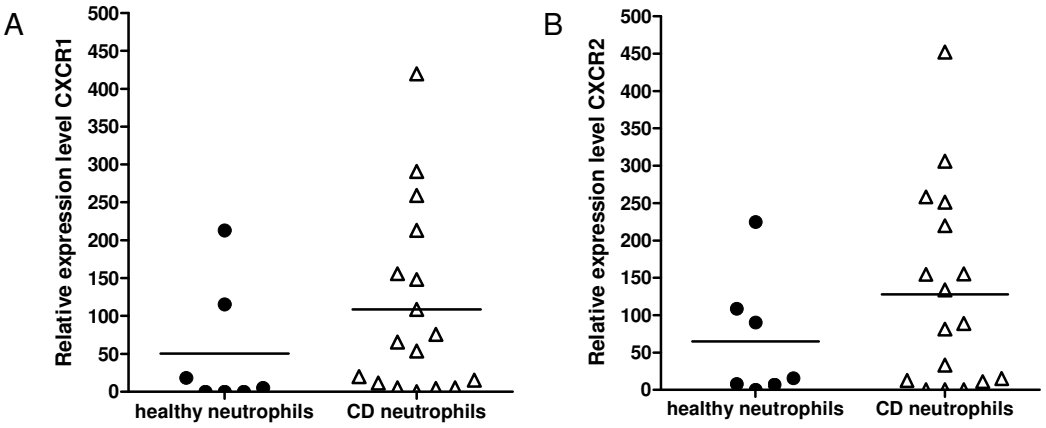


Figure 3. Neutrophilic CXCR1 and CXCR2 expression levels are not significantly increased in Crohn's disease patients.

*(**A**) CXCR1 and (**B**) CXCR2 mRNA levels were measured in PMNs from healthy subjects and patients with CD. Individual data are shown, horizontal bars represent mean values. PMNs from CD patients have no significant increased expression of CXCR1 and CXCR2 when compared to healthy subjects (P = 0.26, unpaired t-test). (healthy donors: n=7; CD patients: n=17)*

CXCL8 levels in non-inflamed CD tissue specimens are lower in comparison with control tissue, whereas in inflamed tissue the CXCL8 levels are strongly elevated

In Figures 1 and 2 we described that PMNs from CD patients had an increased movement capacity under basal conditions and tended to express more CXCR1/2 receptors. In addition, the PMNs from CD patients basically expressed and secreted lower levels of CXCL8 than PMNs from healthy controls. Therefore, our next step was to measure the CXCL8 levels in inflamed and non-inflamed tissue specimens obtained from CD patients to compare with CXCL8 levels measured in non-inflamed control tissue material obtained from patients undergoing colorectal carcinoma surgery. Figure 4A shows that CXCL8 levels were significantly lower in non-inflamed CD tissue when compared to the control tissue. In contrast, the CXCL8 levels were strongly increased in inflamed tissue specimens when compared with non-inflamed tissue samples within patients.

To investigate which cells express CXCL8 in CD tissue specimens, immunohistochemical staining for CXCL8 was performed. Figure 4B shows that CXCL8 was primarily located in epithelial cells, although other cells, including neutrophils, sporadically expressed CXCL8.

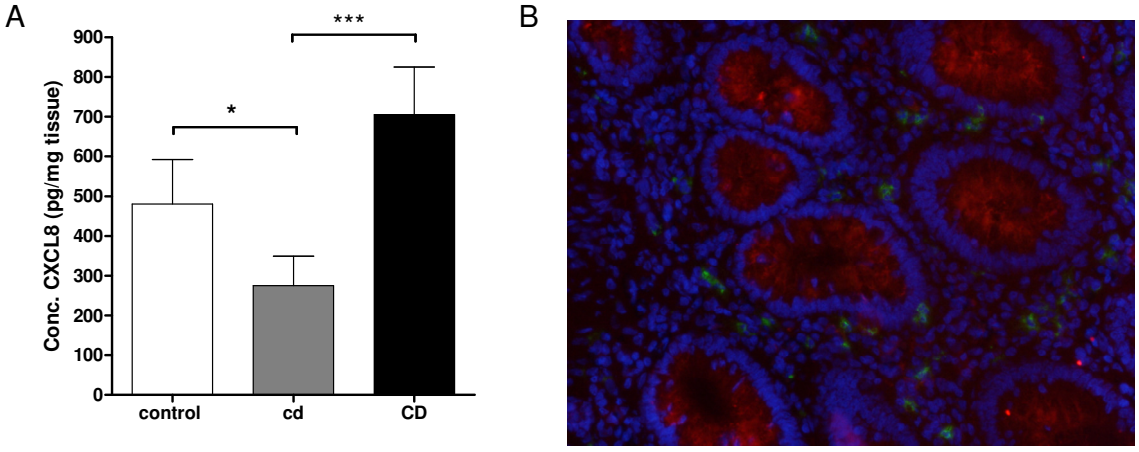


Figure 4. The concentration of CXCL8 is decreased in non-inflamed tissue from CD patients when compared to control tissue, whereas in inflamed tissue the CXCL8 concentration is strongly elevated.

*(**A**) CXCL8 levels were measured in resected tissue homogenates from patients with CD and normal looking control tissue from patients with a colorectal carcinoma. In non-inflamed CD (grey bar, "cd") the amount of CXCL8 levels are significantly lower when compared to the control tissue (white bar, "control") (*, Mann Whitney test, P < 0.05). The amount of CXCL8 in inflamed tissue (black bar, "CD") was near 3-fold higher when compared to the paired non-inflamed tissue (***, Wilcoxon signed rank test, P < 0.001). Individual data are shown, horizontal bars represent mean (+ SEM) values. (control: n=20; cd: n=30; CD: n=41) (**B**) Immunohistochemical staining of tissue from a CD patient. In these patients, epithelial cells are a great source of CXCL8 (in red). Moreover, staining of cell nuclei (in blue, Hoechst staining) and neutrophils (in green, human neutrophil elastase) are shown. This is a typical example of 5 patients.*

MMP8, MMP9 and PE secretion is higher from PMNs obtained from CD patients when compared to PMNs from healthy subjects

To investigate whether specific proteases involved in collagen breakdown and PGP generation are expressed by PMNs from CD patients and healthy subjects, PMNs were incubated for 6 h with medium. Subsequently, MMP8, MMP9 and PE levels were measured in the supernatants. Figure 5A and 5B show that PMNs from CD patients released significantly higher amounts of MMP8 and MMP9 under basal conditions when compared to the healthy control PMNs (both $P < 0.01$). Figure 5C shows that the PE activity tended to be elevated in the supernatant of PMNs from CD patients ($P = 0.076$).

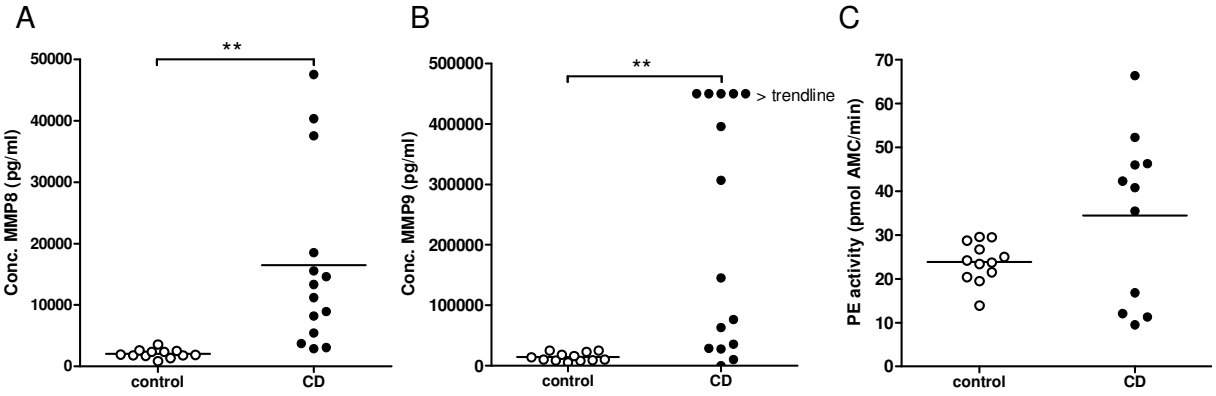


Figure 5. PMNs from CD patients release higher concentrations of MMP8 and MMP9 and have elevated intracellular prolyl endopeptidase activity when compared to the PMNs from healthy subjects.

*MMP8, MMP9 and PE activity levels were measured in supernatants of 10^5 PMNs, which were incubated for 6 h with medium. Individual data are shown, horizontal bars represent mean values. PMNs from CD patients release elevated amounts of MMP8 (**A**) and MMP9 (**B**) under basal conditions when compared to the healthy controls (**, unpaired t-test, P < 0.01). The 5 patients with MMP9 levels above the trend line were not included in the statistics for MMP9. (MMP8/MMP9; healthy donors: n=12/12; CD patients: n=14/15) (**C**) The PE activity was measured using Z-Gly-Pro-AMC as a substrate. The basal PE activation of PMNs from CD patients is not significantly higher than the PE activity of PMNs from healthy donors (P = 0.076; unpaired t-test). (healthy donors: n=12; CD patients: n=11)*

PMNs from CD patients breakdown collagen more efficiently and produce higher amounts of PGP than PMNs from healthy subjects

Since the PMN can secrete all the essential proteases to generate PGP from collagen (fig. 5), the next step was to test whether supernatants of PMNs from CD patients were capable of producing PGP when incubated with collagen. For these experiments, collagen type I was used since this is the prominent type of collagen seen in the gastro-intestinal tract [24]. Supernatants of PMNs incubated for 6 h with medium were incubated with dialyzed collagen type I for 24 h, and the total PGP protein levels were determined. Figure 6 shows that PMNs from CD patients produce higher amounts of total PGP protein under basal conditions than PMNs from healthy controls.

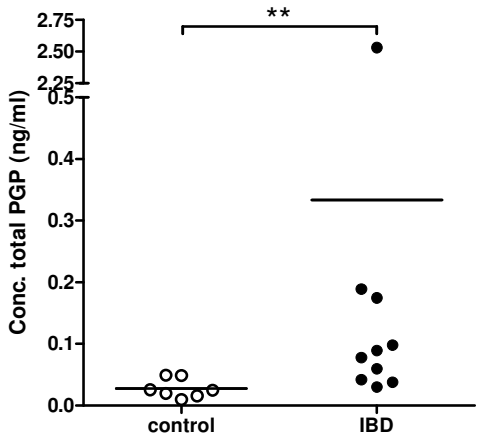


Figure 6. Supernatant components of PMNs from CD patients generate higher amounts of total PGP protein from collagen type I under basal conditions than PMNs from healthy controls.

*Supernatants of 10^6 PMNs, which were stimulated for 6 h with medium, were incubated for 24 h with collagen type I dialyzed solution (1 mg/ml). After incubation, the PGP levels were determined. All samples were filtered through a 10-kDa filter, washed with 20 µl of 1 N HCl and analyzed using ESI-LC-MS/MS for levels of total PGP protein (detection limit < 0.025 ng/ml). Individual data are shown, horizontal bars represent mean values. PMNs from CD patients are superior in generating PGP fragments from whole collagen than healthy controls (**, Mann-Whitney, P < 0.01). (healthy donors: n=7; CD patients: n=11)*

Discussion

CD is a transmural intestinal inflammatory disease which relapses and remits throughout its course. It affects any part of the gastrointestinal tract from mouth to anus and has a patchy nature. Since luminal disease activity is accompanied by extensive neutrophil infiltration and activation, we investigated if there were any differences between CD patients and controls in neutrophilic migration, CXCL8 release and CXCR1/2 expression. Furthermore, CXCL8 levels were determined in tissue specimens from CD patients and controls. Moreover, the ability of neutrophils to produce PGP from collagen was investigated to study whether there is a role for PGP in neutrophil accumulation and subsequent neutrophil-mediated tissue damage in this bowel disease.

In this report, we show that PMNs from CD patients have an increased basal migratory capacity in comparison with PMNs from healthy donors. PMNs from CD patients have a decreased basal CXCL8 mRNA expression and CXCL8 release in comparison to PMNs from healthy donors. Moreover, CXCR1 and CXCR2 mRNA expression levels are elevated, however not significantly, in PMNs obtained from CD patients. In addition, decreased basal CXCL8 levels were measured in non-inflamed tissue from CD patients when compared to control tissue, whereas in inflamed CD tissue the CXCL8 concentration was strongly elevated. Since the collagen breakdown product PGP has been shown to be a ligand for CXCR1/2 besides CXCL8, we have investigated levels of proteases known to play an essential role in the generation of PGP in CD and healthy PMNs. PMNs from CD patients secrete under basal conditions higher amounts of MMP8 and MMP9 in comparison with PMNs from healthy subjects, whereas the PE activity is slightly enhanced. In addition, supernatant of CD PMNs generates higher amounts of PGP from collagen type I under basal conditions than PMNs from healthy controls.

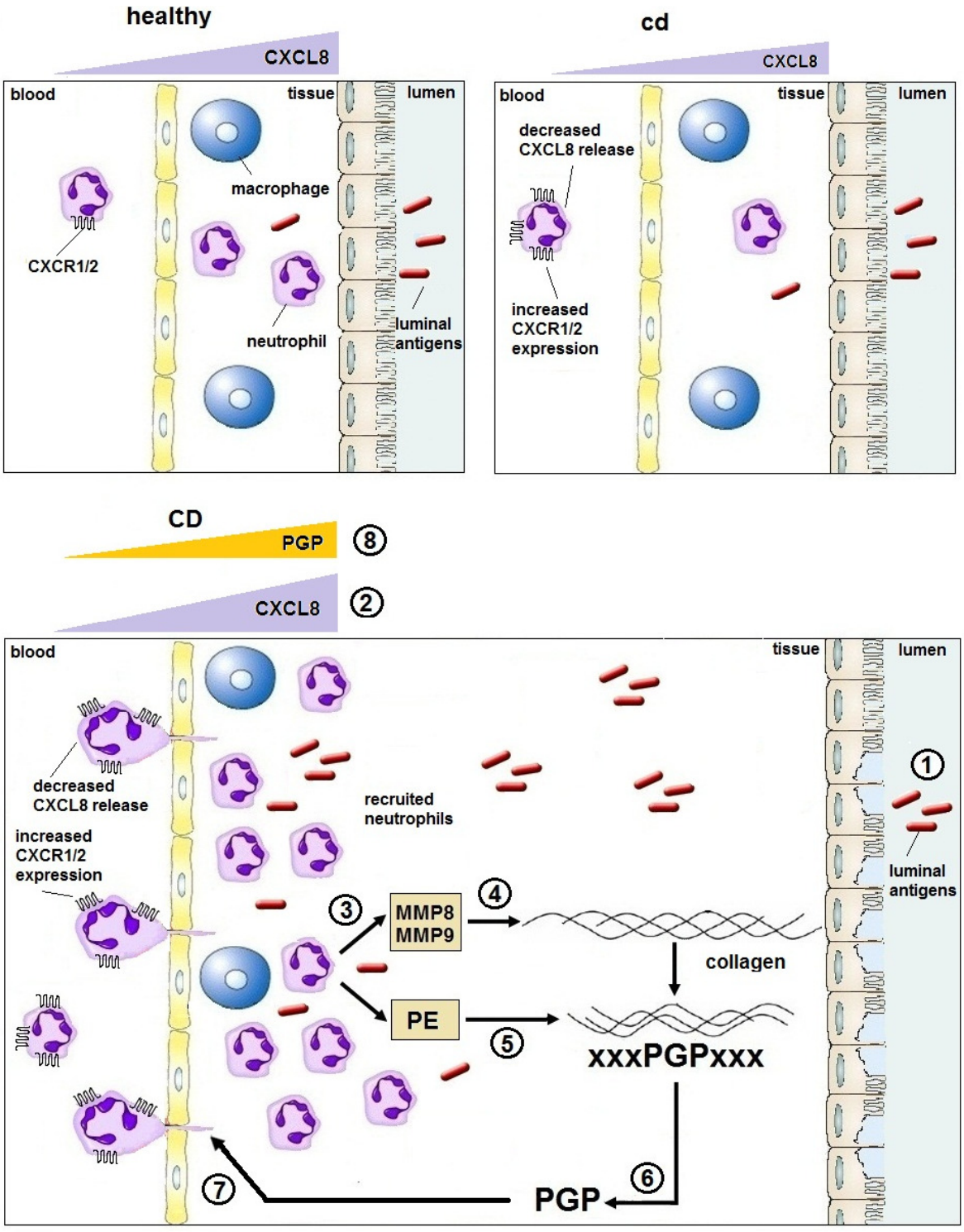
CXCL8 is one of the major chemotactic mediators in the inflammatory response. During the active disease state, this ligand for the receptors CXCR1 and CXCR2 is produced by several cell types, such as neutrophils, macrophages, fibroblasts and intestinal epithelial cells [8, 25]. CXCL8 induces the transmigration of neutrophils from the blood into the intestinal wall (sub)mucosa and serosa [26] (fig. 7A). After extravasation, these activated neutrophils secrete oxygen radicals and proteolytic enzymes, which ultimately can lead to tissue damage [3]. Since disease activity is positively linked to extensive neutrophil migration [3-6], we hypothesized that PMNs isolated from the blood of CD patients have increased chemotactic properties and express elevated levels of the proteases involved in collagen breakdown. Supporting this hypothesis, figure 1A shows that under basal conditions freshly isolated CD PMNs have a 5-fold increased absolute migratory capacity in comparison to healthy control PMNs. Moreover, elevated CXCL8 levels were measured in inflamed CD tissue samples.

Interestingly, PMNs obtained from blood from CD patients secrete less CXCL8 and CXCL8 mRNA expression levels are lower than in healthy PMNs. These observations may lead to a strong chemotactic CXCL8 gradient towards inflamed intestinal areas (fig. 4 and fig. 7C). Other groups have previously described increased CXCL8 levels in inflamed mucosal tissue specimens of CD patients when compared to controls [8-10].

Here we demonstrate that CXCL8 levels are significantly lower in non-inflamed tissue specimens from CD patients in comparison to inflamed tissue specimens and tissue specimens from the control group (fig. 4). These findings are in accordance to previous findings in studies performed by Marks *et al.* in CD patients that were in complete remission [27]. In these CD patients a failure of neutrophil accumulation and CXCL8 production was found at sites of acute trauma induced by biopsy in the non-involved bowel and in the skin. These results were repeated by Smith *et al*. In this study killed Escherichia coli was injected subcutaneously into the forearm of human subjects. They found that the accumulation of the neutrophils at these sites and the clearance of the bacteria from these sites were markedly impaired in CD [28]. The diminished recruitment of neutrophils into the traumatized skin was not due to abnormalities in their motility, since the neutrophils responded normally to CXCL8. In contrast, at the site of trauma there were reduced levels of CXCL8 due to an inappropriate CXCL8 release by macrophages [27]. This is in accordance with our findings in non-involved (non-inflamed) groups of the intestinal tract of CD patients. We describe that neutrophils from CD patients have an increased basal migratory capacity and that these CD PMNs tend to have a higher expression levels of CXCR1 and CXCR2 mRNA in comparison to healthy PMNs (fig. 7C). Chemokine receptors are able to gain the ability to signal in a constitutively active manner. This phenomenon of ligand-independent activation has been described for CXCR2 [29, 30]. The observed increased chemokine receptor expression might therefore be an explanation for the higher basal migratory capacity of CD PMNs.

Furthermore, we mimicked the active disease state by adding various concentrations of CXCL8 to the migration assay, which did not lead to a significant difference in relative migration of PMNs from CD patients and healthy subjects towards this chemokine (Fig. 1B). Taking these observations together, it can be concluded that the PMN itself has different characteristics in CD when compared to the healthy situation.

In chronic inflammatory diseases where neutrophils are a notable component of the inflammation, such as in COPD or CF, chemotactic collagen breakdown products play an additional role in disease progress. Proline-Glycine-Proline (PGP) and its acetylated form N-ac-PGP are collagen fragments liberated from whole collagen by proteases MMP8, MMP9 and PE [15] and have chemotactic properties specifically for neutrophils via the activation of CXCR1 and CXCR2 receptors [14-18]. Here we propose a similar role for PGP in CD. PMNs from CD patients secreted higher MMP8 and MMP9 levels in comparison to healthy control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increased CXCR1/2 expression
CD
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collagen
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PMNs. MMP9 levels were enhanced in inflamed intestinal tissue of CD patients in comparison to non-inflamed tissue [22, 31] or controls [22, 32]. Neutrophils were confirmed as the likely origin of this protease [22, 32] and the enhanced MMP9 levels in CD tissue seem to be actively involved in the inflammatory and remodeling processes [22, 31]. Neutrophils are also a major source of secreted MMP8, which is known to be one of the predominant MMPs that are capable of cleaving native fibrillar collagens of types I [33]. To our knowledge the pathogenetic role of MMP8 secreted by neutrophils in CD has not been extensively studied yet. However, it was shown in a dextran sulfate sodium (DSS)-induced mouse model that during active IBD, MMP8 is one of the MMPs whose expression is markedly upregulated, whereas this expression is undetectable in the normal colon [34], indicating a role for MMP8 in inflammation in the bowel.

Figure 7 (previous page). In CD, intestinal damage initiates a vicious circle of events leading to collagen breakdown, PGP generation and PGP-induced neutrophilic transmigration. *(**A**) In healthy individuals, triggering of the mucosal layer by bacteria, their products or harmful food components leads to an inherently strong release of CXCL8 by macrophages and epithelia and an appropriate CXCL8 gradient (purple triangle). This CXCL8 release leads to neutrophil recruitment and a subsequent clearance of bacteria. (**B**) Peripheral blood neutrophils of CD patients show under basal conditions more motility and tend to express more CXCR1 and CXCR2. In addition, these blood neutrophils release lower amounts of CXCL8 than neutrophils from healthy individuals. In non-involved areas of the intestinal tract of CD patients, no epithelial damage is found. The extreme low levels of CXCL8 will not result in a normal CXCL8 gradient (purple triangle), upon luminal antigen infiltration into the gut wall. Subsequently, less neutrophils will infiltrate from the blood into these areas.*

*(**C**) In inflamed regions of the intestinal tract of CD patients, there is a strongly increased CXCL8 gradient from the circulation towards the affected gut wall tissue. Due to epithelial damage, high loads of luminal antigens activate resident macrophages to secrete high amounts of chemokines, such as CXCL8 (1). The peripheral blood neutrophils, that are already constitutively more activated and release less CXCL8, migrate following this CXCL8 gradient (2) from the blood stream into the tissue. In the intestinal tissue, the recruited neutrophils are activated, which leads to the release of more CXCL8. Moreover, this activation leads to the release of MMP8, MMP9 and PE (3). The matrix metalloproteinases denature and proteolytically cleave collagen into fragments 30–100 amino acids in length (4). These collagen fragments are then further cleaved to PGP by PE (5/6). In addition, the neutrophil activation by PGP also leads to CXCL8 release (7). Neutrophils migrate following the PGP and CXCL8 gradient (yellow triangle) (8). This leads in the long term to a self-maintaining situation enhanced by both PGP and CXCL8, leading to a further increase in neutrophil infiltration and chronic inflammation.*

Besides MMP8 and MMP9, PE is needed to generate PGP from whole collagen. In the present manuscript, supernatants of PMNs obtained from blood of CD patients had an increased PE activity. The next step was to measure collagen breakdown by PMNs from CD patients *in vitro*. For this experiment collagen type 1 was used, since this is the major collagen type in the intestine [24]. Here we show for the first time that supernatants of PMNs from CD patients contain all components of the PGP proteolytic cascade and that these supernatant components generated higher amounts of total PGP protein from collagen type I under basal conditions than the supernatants from PMNs from healthy controls. Since neutrophil influx and neutrophil-mediated tissue damage are major characteristics of activated disease state, PGP may play a significant role in CD (fig. 7C).

In conclusion, our interpretations regarding the neutrophilic migration and activation in healthy people, non-inflamed CD and inflamed CD are summarized in figure 7A-C. In healthy individuals appropriate PMN recruitment occurs in the intestinal mucosa to defend against pathogenic bacteria, their products or harmful food components that enter the intestinal tissue regularly. In quiescent (non-inflamed) CD impaired PMN recruitment leads to an weak defense against these antigens. During exacerbation, high expression of CXCL8 leads to a strong chemotactic gradient. Neutrophils in the blood stream, that under basal conditions show more motility and tend to express more CXCR1 and CXCR2, will infiltrate massively into the affected intestinal wall. The infiltrated neutrophils will locally release MMP8, MMP9 and PE that in turn will breakdown collagen resulting in the generation of a novel CXCR1/2 ligand, PGP. In addition, in the tissue infiltrated neutrophils will also release more CXCL8. Together with PGP, a more pronounced infiltration of neutrophils and extensive tissue damage will be the result as observed in the CD associated transmural inflammation.

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Chapter 9

Summarizing discussion

Introduction

The experiments described in this thesis were performed to gain more knowledge about the neutrophilic inflammation in chronic obstructive pulmonary disease (COPD) and Crohn's disease (CD) and the self-enforcement of the inflammatory state in the pathogenesis of these two diseases. In these chronic diseases, neutrophils are a notable component of the inflammation and disease progression and severity are associated with increased neutrophil numbers [1, 2]. Neutrophils accumulate in the affected tissues and contribute to the chronic inflammatory reaction by releasing many inflammatory mediators [1, 3], which are involved in disease progression. In this summarizing discussion the most important findings are discussed in the same order as the numbered events depicted in figures 1 and 2, which explain our new hypothesis on the pathogenesis of COPD and CD respectively.

The involvement of cigarette smoke in neutrophilic transmigration

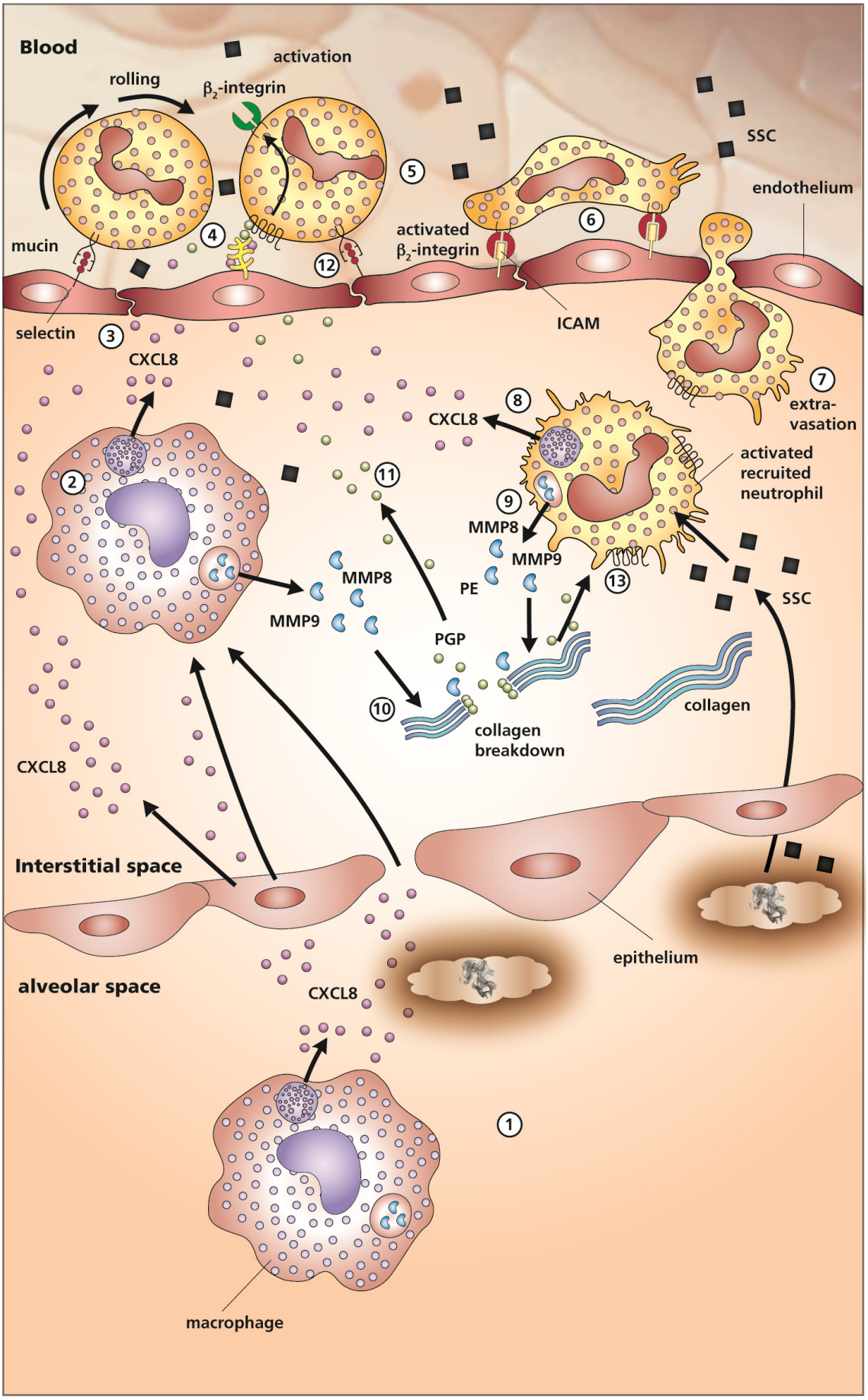
It is generally accepted that cigarette smoking is the most important risk factor for the development of COPD. The World Health Organization (WHO) estimated that 73% of COPD mortality is related to smoking [4]. However, increased neutrophil counts in bronchoalveolar lavage (BAL) fluid and sputum is not restricted to COPD patients [5-8]; increased neutrophil numbers are also observed in sputum of smokers without respiratory problems [6, 9], indicating that cigarette smoke induces lung inflammation. Since cigarette smoke is the main risk factor for COPD and since this disease is associated with a substantial increase in neutrophil numbers in BAL fluid and sputum [10, 11], the effect of cigarette smoke on the neutrophil transmigration was investigated (**chapter 3**). The increased neutrophil counts in BAL fluid and sputum in COPD patients and smokers described in literature were confirmed with murine model data: exposing mice to cigarette smoke for 5 days resulted in the presence of neutrophils in the lungs, showing that cigarette smoke may have an effect on the migration of these cells. This is in accordance with other studies that observed increased numbers of neutrophils in BAL fluid and lung tissue after one week [12, 13] and five months cigarette smoke exposure in mice [14]. Moreover, in **chapter 3** it was found that cigarette smoke extract (CSE) can directly induce the migration of neutrophils *in vitro* using a transwell migration system; an effect, which has recently been suggested by Barnes [15].

Neutrophils migrate from the blood stream to the lung in a multistep process, using a specific set of adhesion and chemokine receptors [16-18]. In this transendothelial migration process, the β_2-integrin family, which includes Mac-1 (CD11b/CD18), plays an important role since the activation of these β_2-integrins leads to neutrophilic firm adhesion and subsequent transendothelial migration [16, 17]. In **chapter 3** the direct effect of cigarette smoke on neutrophil β_2-integrin activation and function in the endothelial transmigration of neutrophils

was described. CSE activated the β_2-integrin Mac-1 on the neutrophil leading to firm adhesion to fibrinogen. Furthermore, neutrophils transmigrated through endothelium in response to CSE via the activation of β_2-integrins, since functionally blocking CD11b and CD18 decreased this transmigration. Taken together, **chapter 3** provides evidence for a critical role of β_2-integrins in the firm adhesion and transmigration of neutrophils in response to cigarette smoke *in vitro* (fig. 1, events 5-7).

Cigarette smoke can activate the neutrophil to degranulate and synthesize inflammatory mediators

Early in inflammation, neutrophils migrate from the capillary into the interstitial space, following a chemotactic gradient of CXCL8 [19]. At the site of inflammation neutrophils are activated, leading to the release of more CXCL8 [20, 21]. This release leads to a self-perpetuating inflammatory state where neutrophils attract more neutrophils via chemokine receptors CXCR1 and CXCR2 on these cells [22-24]. Since it was shown that CSE can induce the migration of neutrophils (**chapter 3**), the next question was whether CSE may activate the neutrophil to synthesize and release CXCL8, acting in an autocrine/paracrine fashion. This question was answered in **chapter 7**; it was demonstrated that the activation of neutrophils by CSE exposure leads to the release CXCL8. We hypothesized that once infiltrated in the lung tissue cigarette smoke activates the infiltrated neutrophil. This activation results in a CXCL8 release by the neutrophil, which in turn will attract more neutrophils into the airways. Moreover, **chapter 7** describes that CSE induces the release of matrix metalloproteinase (MMP)8 and MMP9. In COPD, these proteolytic enzymes have been proposed to contribute to alveolar destruction and remodeling, because of their capacity to cleave structural proteins, such as collagen and elastin [20, 25]. This induction of the MMP8 and MMP9 release by cigarette smoke described in **chapter 7** was substantiated with clinical data. It was shown that although MMP8 and MMP9 induced sputum levels are lower in smokers when compared to COPD patients [26, 27], the MMP levels from both groups are elevated when compared to non-smokers [26-29]. The main findings of **chapter 7** are depicted in figure 1, events 8 and 9.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PE
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Interstitial space
epithelium
alveolar space
CXCL8
1
macrophage

Neutrophils activated by cigarette smoke play a significant role in collagen breakdown and the generation of neutrophilic chemoattractant N-acetyl Proline-Glycine-Proline

A longer history of cigarette smoking is associated with an increase in neutrophil levels in BAL fluid and sputum, an increase in MMP8 and MMP9 levels in sputum and a reduction of FEV_1 (forced expiratory volume in 1 second) [27, 30, 31], which means that cigarette smoking can lead to tissue destruction and airway remodeling and ultimately to the development of COPD. During the destruction of the extracellular matrix collagen fragments can be formed and it has long been known that these fragments have chemotactic properties [32, 33]. One of these fragments is N-acetyl Proline-Glycine-Proline (N-ac-PGP), which was first identified by Pfister and colleagues [34]. This tripeptide is specifically chemotactic for neutrophils *in vitro* [35, 36] and *in vivo* [37], as shown in **chapter 5** and **6** respectively. N-ac-PGP may play a significant role in the development and progression of COPD, since N-ac-PGP was detected in induced sputum samples, whereas this tripeptide was undetectable in healthy individuals and asthmatics [38]. Furthermore, chronic airway exposure to N-ac-PGP causes emphysema in mice [35]. The generation of this tripeptide is mediated by a multistep pathway involving MMP8, MMP9 and prolyl endopeptidase (PE) and was first described by Gagger *et al.* in cystic fibrosis [36], a disease where chronic neutrophilic

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Figure 1 (previous page). A new hypothesis of COPD; cigarette smoking initiates a vicious circle of events leading to collagen breakdown, PGP generation and PGP-induced neutrophilic transmigration.

*Cigarette smoking activates resident macrophages and epithelial cells to the secrete chemokines, such as CXCL8 (**1, 2**). These chemokines are retained on matrix and cell-surface heparan sulfate proteoglycans and on the surface of the endothelium, establishing a chemokine concentration gradient (**3**). Neutrophils rolling on the endothelium in a selectin-mediated process are brought into contact with CXCL8 (**4**), leading to the activation of β_2-intergrins, such as Mac-1 (**5**). Soluble smoke components (SSC) also can activate these β_2-intergrins. This activation leads to firm adherence (**6**) and extravasation (**7**). In the lung tissue, the recruited neutrophil is activated by cigarette smoke which diffuses through the damaged epithelial cell layer, leading to the release of CXCL8 (**8**). Moreover, this activation leads to the release of matrix metalloproteinase (MMP) 8 and 9 (**9**), which denature and proteolytically cleave collagen to fragments 30–100 amino acids in length. These collagen fragments are then further cleaved into PGP by prolyl endopeptidase (PE) (**10**). Subsequently, this tripeptide attracts neutrophils to the lung (**11**) and induces firm adhesion by promoting activation of Mac-1 (**12**), which leads to neutrophil transmigration. In the lung tissue, N-ac-PGP exposure activates the neutrophil to release CXCL8 and MMP8 and MMP9 (**13**), which results in a self-sustaining cycle of neutrophil infiltration and collagen destruction.*

inflammation is present in the lungs. The multistep pathway of collagen breakdown has been studied in a murine model of cigarette smoke-induced lung emphysema in our group by Braber *et al.* [39]. All relevant components (neutrophils, MMP8, MMP9 and PE) involved in this pathway to generate (N-ac-)PGP from collagen were upregulated in the airways exposed to cigarette smoke, suggesting that activation of cells by cigarette smoke leads to the release of proteases and extracellular matrix breakdown. In **chapter 7** we show that the neutrophil may be a key player in alveolar destruction in COPD patients, as activated neutrophils release proteolytic enzymes. Since lungs mainly consist of collagen and collagen type I is the prominent type of collagen seen in the airways [40], it was investigated whether cigarette smoke may stimulate neutrophils to breakdown collagen type 1 in smaller fragments, and more specifically, to (N-ac-)PGP. Neutrophils activated by CSE can indeed breakdown collagen into N-ac-PGP.

One of the crucial proteases of the PGP generation cascade is serine protease PE. Besides MMP8 and MMP9, PE is needed to generate PGP from whole collagen; the MMPs cleave whole collagen into fragments of 30 to 100 amino acids in length, after which PE specifically cleaves PGP from these smaller fragments [36]. Although this proteolytic enzyme is relatively new in studies on neutrophilic lung diseases [36, 39], a role for PE has been established in learning, memory and mood and it is implicated in psychiatric conditions such as depression [41]. Neutrophils contain PE [42], which was confirmed in **chapter 7**. PE activity was present in lysates of neutrophils but incubation of neutrophils with CSE did not affect intracellular PE activity, which suggests that PE is constitutively active. PE activity could not be measured in the supernatant of CSE incubated neutrophils, probably because PE levels were below detection limit of the assay used. In addition, we hypothesize that cigarette smoking causes a locally restricted lung inflammation where damaged and necrotic neutrophils release PE to the exterior, which contributes to PGP generation.

Since increased MMP8 and MMP9 levels in sputum and BAL fluid are present in COPD patients and tissue destruction is characteristic for COPD, the basal intracellular PE activity in neutrophils from COPD patients was measured (**chapter 7**). Interestingly, the basal PE activity in these cells was remarkably higher than in neutrophils from healthy donors, indicating that PE might play an important role in lung collagen breakdown leading to the development of COPD. Moreover, the neutrophils from COPD patients were activated to a greater extend, since the CXCL8 levels released by these neutrophils appeared to be higher than from neutrophils from healthy controls.

Other cells besides neutrophils might contribute to collagen destruction by supplying PE. It was described in **chapter 7** that also pulmonary alveolar macrophages express PE. Moreover, it was shown that epithelial cells of human lung tissue contain PE, indicating that epithelial cells may be an important source for PE. Exposure of bronchial epithelial cells to

CSE for 16 h *in vitro* led to an elevated PE activity in the supernatant, indicating that epithelial cells can be activated by cigarette smoke exposure to a release of PE in vivo.
The main findings of **chapter 7** are depicted in figure 1, events 9 and 10.

Collagen fragment N-ac-PGP induces a self-sustaining pathway of inflammation

From the results described in **chapter 3** and **7** it can be concluded that cigarette smoking activates the human neutrophil to release proteolytic enzymes, which leads to collagen breakdown and N-ac-PGP generation. Non-acetylated PGP levels could not be detected in the supernatants of neutrophils which were simultaneous incubated with CSE and collagen. An explanation can be found in the results described by Snelgrove *et al.*, were it was demonstrated that cigarette smoke itself is responsible for N-terminally acetylating PGP, thereby enhancing its chemotactic capacity [43].
In **chapter 5** it was demonstrated that N-ac-PGP was chemotactic for human neutrophils via pertussis toxin sensitive G protein-coupled receptors *in vitro*. Furthermore, using a selective CXCR2 antagonist it was confirmed that N-ac-PGP-induced neutrophil chemotaxis is mediated through CXCR2 activation. This was also demonstrated in a neutrophil inflammatory mouse model in **chapter 6**; the neutrophil migration in lung tissue and BAL fluid induced by N-ac-PGP is mediated via direct CXCR2 interaction. Moreover, the chemotactic effect of N-ac-PGP was shown in an environment closer resembling the human pathophysiological situation where neutrophils transmigrated through a TNF-α preincubated human endothelial cell layer in response to basolateral N-ac-PGP (**chapter 4**).
In addition to its chemotactic effect on neutrophils, the effect of N-ac-PGP on β_2-integrin activation and function in neutrophils was studied in **chapter 4**. The activation of these β_2-integrins is essential during inflammation, since it leads to a conformational change in structure, going from an inactive, low affinity state to an active, high-affinity state [44]. Neutrophils adhered to fibrinogen in response to N-ac-PGP via the activation of β_2-integrin Mac-1, since functionally blocking CD11b and CD18 inhibited this adhesion. Moreover, pertussis toxin incubation profoundly inhibited the N-ac-PGP-induced adhesion to fibrinogen, indicating that this adhesion is mediated via pertussis toxin sensitive $G_{i/o}$ proteins. In addition, N-ac-PGP incubation did not change the cell surface expression of Mac-1 on the neutrophil, which points to a conformational change of the integrin on the neutrophil.
After extravasation, neutrophils migrate along a concentration gradient of chemokines, such as CXCL8 or N-ac-PGP. At the site of infection/inflammation, neutrophils get further activated by the inflammatory mediators in this environment, which results in the production of various cytokines, chemokines, and growth factors [22, 45-47]. In addition to the chemotactic effect and the activation of Mac-1 on the neutrophil, N-ac-PGP induced a direct calcium influx (**chapter 5**) and the release of CXCL8 (**chapter 5** and **7**) by neutrophils. However, using an

antibody against CXCL8 (α-CXCL8) we demonstrated that the released CXCL8 was not responsible for N-ac-PGP's directly induced chemotaxis and calcium influx (**chapter 5**). The *in vitro* release of CXCL8 by human neutrophils was in accordance with the results from the neutrophil inflammatory mouse model in **chapter 6**; oropharyngeal aspiration of N-ac-PGP induced a CXCL1 (a human CXCL8 homologue) release *in vivo*. Since antibodies against CXCL1 were not studied in mice exposed to N-ac-PGP, it cannot be excluded that a small part of the N-ac-PGP induced neutrophil infiltration is mediated via the release of CXCL1. However, the concentration of CXCL1 (30 pg/ml) observed in the BAL fluid after N-ac-PGP administration was much lower than the concentration realized after oropharyngeal aspiration of CXCL1 (0.5 µg/70 µl), making it likely that N-ac-PGP is solely responsible for its chemotactic effect *in vivo* via CXCR2.

During inflammation, N-ac-PGP can activate neutrophils to release CXCL8, which will lead to an increase in neutrophilic migration. In addition, N-ac-PGP also induced the release of MMP8 and MMP9 from neutrophils (**chapter 7**). These results indicate that this N-ac-PGP-induced MMP release may result in more collagen breakdown and formation of N-ac-PGP, which may lead *in vivo* to a self-propagating cycle of neutrophil infiltration, chronic inflammation and lung emphysema.

The main findings of **chapter 5-7** are depicted in figure 1, events 9-13.

Neutrophils and N-ac-PGP in Crohn's disease

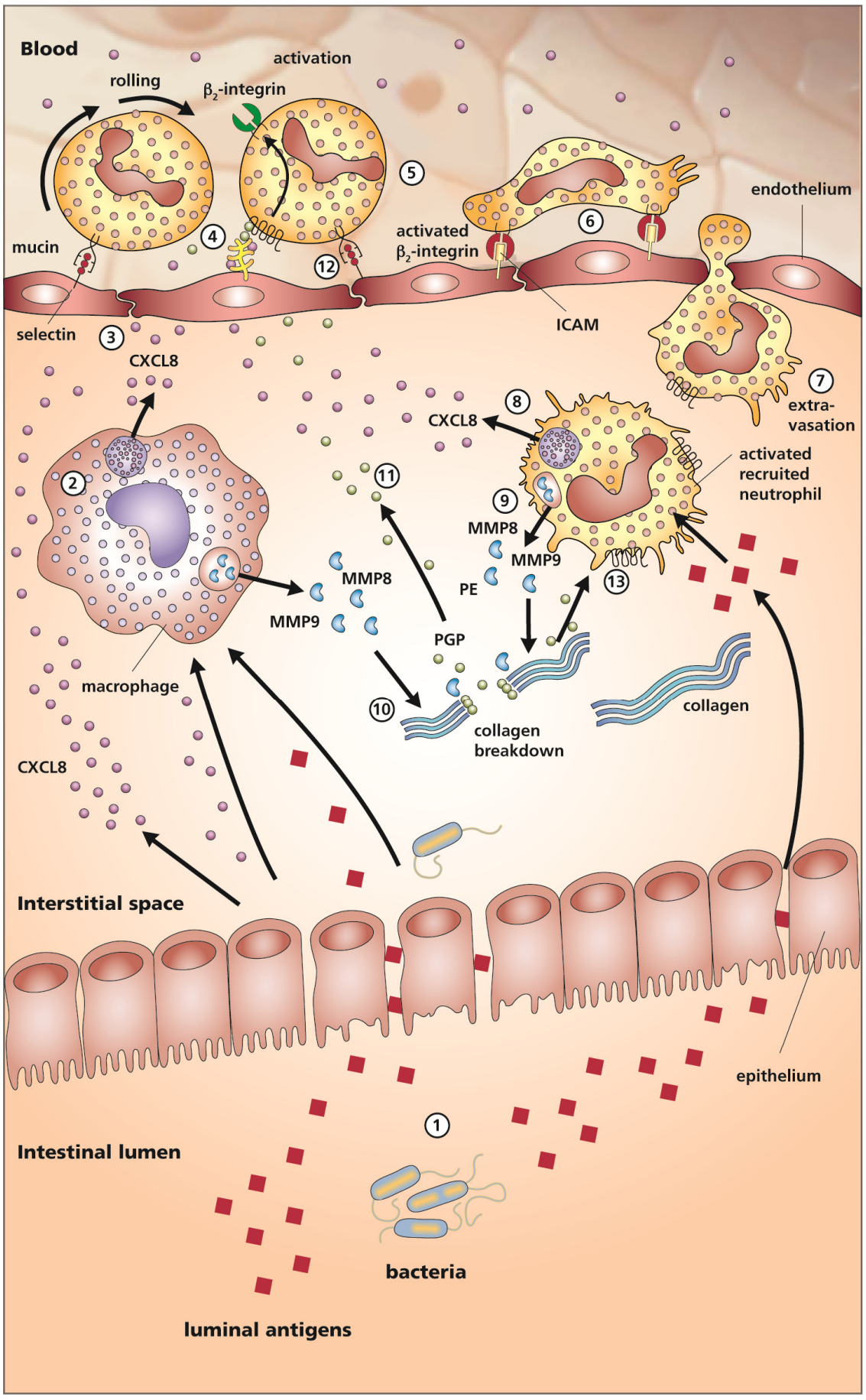
CD is a transmural inflammatory disease which relapses and remits throughout its course. Since disease activity is correlated with extensive neutrophil migration [3], the neutrophil migration and the components, such as CXCL8 and N-ac-PGP, that play a role in this process were investigated in more detail in **chapter 8**. Neutrophils from CD patients may be intrinsically different from healthy donors since these neutrophils showed increased absolute migration and collagen breakdown capacities *in vitro*. Moreover, neutrophils isolated from blood of CD patients tended to express more CXCR1 and CXCR2 mRNA than healthy subjects. CXCL8 protein secretion and CXCL8 mRNA expression levels were decreased in neutrophils from CD patients. In addition, decreased levels of CXCL8 protein were found in homogenates of non-inflamed intestinal tissue from CD patients when compared with non-inflamed colorectal cancer controls, whereas in inflamed CD tissue the CXCL8 level was strongly elevated. These findings indicate that Crohn's pathogenesis may result of an impaired innate immunity, as suggested by Marks & Segal [48]. They hypothesize that rather than Crohn's disease being caused by excessive inflammation, the primary mechanism is actually that of an immunodeficiency. The most profound evidence for this theory is the observation of granuloma formation in these patients [49]. The development of CD may be a result of the incapacity of macrophages to generate adequate CXCL8 levels. Bacteria and

bowel contents go through the permeable mucosal layer into the intestinal tissue. In people resistant to CD, the resident macrophages have an inherently high inflammatory response, resulting in high CXCL8 levels and the recruitment of neutrophils, which engulf, digest and expel the exogenous material from the body. However in people susceptible for CD, macrophages respond inherently weak to the exogenous agents; low CXCL8 levels lead to poor neutrophil accumulation and subsequently to macrophage sequestration and the formation of granulomata leading to chronic inflammation [48, 50]. Moreover, compensatory pathways, such as signaling through pattern recognition receptors NOD or TLR, are also weakened [48, 49, 51-53].

N-ac-PGP can also play a role in CD, since the proteolytic enzymes necessary for collagen breakdown are also present in inflammatory bowel disease (IBD) [54]. In **chapter 8** the generation of N-ac-PGP was investigated. Blood neutrophils from CD patients secreted under basal conditions higher amounts of MMP8 and MMP9 in comparison with neutrophils from healthy subjects, whereas the PE activity was slightly enhanced in the supernatant. In addition, supernatants from CD neutrophils generate higher amounts of PGP from collagen type I under basal conditions than supernatants from neutrophils of healthy controls. These interesting results indicate that the neutrophil plays a key role in the generation of N-ac-PGP and the self-sustaining inflammation in CD, as already indicated for COPD.

Finally, in **chapter 8** it was hypothesized that triggering of the intestinal mucosal layer by bacteria or food components leads to an inherently strong release of CXCL8 by macrophages and epithelia in healthy individuals. This CXCL8 release leads to neutrophil recruitment and a subsequent clearance of bacteria and the induction of tolerance to harmless food components.

In CD patients, peripheral blood neutrophils show under basal conditions more motility and tend to express more CXCR1 and CXCR2. In addition, these blood neutrophils release lower amounts of CXCL8 than neutrophils from healthy individuals. At the inflamed intestinal regions of CD patients, luminal antigens or bacterial components will infiltrate into the gut wall and this will lead to an increase in CXCL8 release by activated resident macrophages and ‘active’ CD-neutrophils migrate towards the strong CXCL8 gradient into affected intestinal tissue segments. Contrary to the hypothesis of Marks & Segal, we have found an increased expression of CXCL8 in inflamed intestinal specimens obtained from CD patients. We do not know whether this CXCL8 is derived from macrophages or infiltrated neutrophils. Of interest, we observed that in non-inflamed intestinal tissue specimens obtained from CD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patients, low levels of CXCL8 were found, even when compared to healthy intestinal tissue. In the inflamed intestinal tissue segments of CD patients, the recruited neutrophils will be activated, which leads to the release of more CXCL8. Moreover, this activation leads also to the release of MMP8, MMP9 and PE and the subsequent generation of chemotactic PGP (as described in the paragraph above). In addition, the neutrophil activation by PGP leads to more CXCL8 release. This leads in the long term to a self-maintaining situation enhanced by both PGP and CXCL8, leading to a further increase in neutrophil infiltration and chronic inflammation.

The main findings of **chapter 8** are described in figure 2.

Is N-ac-PGP a ligand for CXCR1 and CXCR2?

There has been some discussion in literature regarding the receptor of N-ac-PGP. It was proposed that the N-ac-PGP activity is mediated via CXCR1/2, since the tripeptide shares sequence homology with the highly conserved GP motif in ELR^+ CXC-chemokines, which is an essential motif for chemokine receptor binding and neutrophil activation [55]. Moreover, blocking these chemokine receptors with blocking antibodies or preincubating neutrophils with pertussis toxin resulted in inhibition of the neutrophilic chemotactic N-ac-PGP response *in vitro*, as shown in **chapter 5** and by Weathington *et al.* [35]. Furthermore, performing a receptor competition assay with [^{125}I]- radiolabeled CXCL8 on stably transfected CXCR1 or

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Figure 2 (previous page). In CD, intestinal damage initiates a vicious circle of events leading to collagen breakdown, PGP generation and PGP-induced neutrophilic transmigration.

*Bacteria and luminal antigens activate resident macrophages and epithelial cells to the secrete chemokines, such as CXCL8 (**1, 2**). These chemokines are retained on matrix and cell-surface heparan sulfate proteoglycans and on the surface of the endothelium, establishing a chemokine concentration gradient (**3**). Neutrophils rolling on the endothelium in a selectin-mediated process are brought into contact with CXCL8 (**4**), leading to the activation of β_2-intergrins, such as Mac-1 (**5**). This activation leads to firm adherence (**6**) and extravasation (**7**). In the lung tissue, the recruited neutrophil is activated by bacteria and luminal antigens which diffused through the damaged epithelial cell layer, leading to the release of more CXCL8 (**8**). Moreover, this activation leads to the release of matrix metalloproteinase (MMP) 8 and 9 (**9**), which denature and proteolytically cleave collagen to fragments 30–100 amino acids in length. These collagen fragments are then further cleaved into PGP by prolyl endopeptidase (PE) (**10**). Subsequently, this tripeptide attracts neutrophils to the lung (**11**) and induces firm adhesion by promoting activation of Mac-1 (**12**), which leads to neutrophil transmigration. In the lung tissue, N-ac-PGP exposure activates the neutrophil to release CXCL8 and MMP8 and MMP9 (**13**), which results in a self-sustaining cycle of neutrophil infiltration and collagen destruction.*

CXCR2 rat basophilic leukemia (RBL) cells, it was shown that the binding of [^{125}I]-CXCL8 was suppressed in the presence of unlabeled CXCL8 or N-ac-PGP, leading to the conclusion that N-ac-PGP binds to CXCR1 or CXCR2 [35]. These results were recently repeated by Jackson *et al*. [56], in a study showing that all four chiral isomers of N-ac-PGP bind to the CXCR1 and CXCR2 [56]. However, in collaboration with De Kruijf and colleagues it was proposed that N-ac-PGP may interact indirectly with CXCR1 and CXCR2 via the release of chemokines, known to bind these receptors, or through activation of other receptors on the neutrophil to induce chemotaxis, since the peptide was unable to activate G-protein dependent and G protein-independent signaling and unsuccessful in displacing the radioligands [^{125}I]-CXCL8 and [^{3}H]-SB265610 from CXCR1 and CXCR2 expressing human embryonic kidney (HEK) 293T cells or neutrophils [57]. To investigate if the chemotactic effect of N-ac-PGP is cell type dependent, a chemotaxis assay using murine pre-B lymphoma cells (L1.2 B cells) transiently expressing CXCR2 was performed (**chapter 5**). These L1.2 cells did not show any chemotactic response upon exposure to N-ac-PGP, while CXCL8 did. These data indicate that N-ac-PGP is able to activate signaling in neutrophils, which is not apparent in HEK293T or L1.2 B cells. The effect of N-ac-PGP appears therefore cell type dependent and the results of CXCR signaling studies performed on HEK293T cells, L1.2 B cells or neutrophils may therefore differ in outcome. In literature, Jackson *et al*. argued that de Kruijf *et al*.'s inability to repeat the previous findings that N-ac-PGP can compete with CXCL8 for binding to CXCR1/2 in a radioreceptor assay were due to differences in the methodology of the radioreceptor assays performed; Weathington *et al*. and Jackson *et al*. preincubated with N-ac-PGP before adding [^{125}I]-CXCL8 [35, 56] whereas De Kruijf *et al*. added the N-ac-PGP and the [^{125}I]-CXCL8 simultaneously to the cells [57]. Jackson *et al*. reasoned that preincubation with N-ac-PGP is the best approach, as N-ac-PGP is at least 1000 fold inferior to CXCL8 in its capacity to bind CXCR1/2 [56], most likely since N-ac-PGP contacts a single site of the receptor whereas CXCL8 contacts multiple sites [35]. Interestingly, a recently published study by Kim *et al*. further supports the idea that N-ac-PGP probably acts via direct interaction with CXCR1/2. In this study, the protective role of N-ac-PGP in murine models for sepsis was investigated. There it was shown that FITC-labeled N-ac-PGP binds directly to CXCR2-expressing RBL-2H3 cells and that this binding is prevented by an excess unlabelled N-ac-PGP [58].

In addition, *in vivo* mouse data indicate a role for CXCR2 in N-ac-PGP- induced neutrophilic migration. In **chapter 6** it was described that oropharyngeal aspiration of N-ac-PGP resulted in a neutrophil influx in lung tissue and BAL fluid in an inflammatory mouse model. Treatment of mice with specific CXCR2 antagonists significantly reduced neutrophil recruitment to both N-ac-PGP and CXCL1, indicating that the N-ac-PGP-induced neutrophil influx may be

mediated via a direct interaction with CXCR2 (**chapter 6**). These results are in accordance with previous studies with $CXCR2^{-/-}$ mice [35].

Taking together, in vitro we have shown that PGP-induced CXCL8 is not responsible for the chemotactic response of neutrophil, and from the results currently available in literature it appears probable that N-ac-PGP may act via direct interaction with CXCR1/2.

Possible therapeutic targets in neutrophilic inflammatory disease

This thesis opens new venues to investigate different therapeutic targets in chronic inflammatory disease were neutrophils play an important role. In the following section, several strategies are discussed to beneficially affect disease progression.

Neutrophil recruitment and transmigration

Since cell adhesion molecules play an important role in cell recruitment, these receptors have attracted the attention of the pharmaceutical industry as potential therapeutic targets. Although the development has been centered around selectins and integrin VLA-4 ($\alpha_4\beta_1$) as therapeutics for COPD [59] and CD [60], *in vitro* and *in vivo* studies have implicated the β_2-integrins Mac-1 and LFA-1 as interesting targets. The following section will discuss some examples of new coumarine-derived drugs, platelet inhibitors, angiotensin II receptor antagonists and CXCR2 antagonists, which all target β_2-integrins.

Bucolo *et al.* described an antagonist of Mac-1 and LFA-1, the coumarine-derived drug BOL-303225-A as a possible new anti-inflammatory drug in diabetic retinopathy. In *in vitro* adhesion assays BOL-303225-A showed specific binding to Mac-1 and LFA-1, which were expressed by U937 (a human leukemic monocyte lymphoma cell line) and Jurkat (human T cell lymphoblast-like cell line) cells respectively. Moreover, the pharmacokinetic profile of BOL-303225-A indicated rapid adsorption following oral administration in male Sprague-Dawley rats. This pharmacokinetic profile was measured in a retinal ischaemia-reperfusion injury model where the intraocular pressure of a cannulated eye was raised by elevating the saline reservoir and subsequent cannula removal led to the reperfusion of the retina vessels and neutrophil infiltration in the retina. A significant reduction of MPO levels was observed in the retina of drug-treated rats after retinal ischaemia-reperfusion injury [61], leading to the conclusion that BOL-303225-A is a potent inhibitor of neutrophilic migration mediated via Mac-1 and LFA-1.

Other antagonists that showed inhibition of neutrophil adhesion via Mac-1 are the platelet inhibitor dipyridamole and the angiotensin II receptor antagonist candesartan. Hallevi *at al.* studied the effects of these drugs on the adhesion of neutrophils from stroke patients to endothelial cells. Dipyridamole and the combination of dipyridamole and candesartan inhibited significantly the adhesion of neutrophils from ischemic stroke patients as compared

to controls with a prominent additive effect. No inhibition was seen in the control groups. These drugs also reduced significantly the expression of Mac-1 [62].
Although both drugs are not designed as direct Mac-1 antagonists, these drugs significantly reduced the expression of Mac-1. Hallevi *et al*. hypothesized for dipyridamole that the downregulation of Mac-1 may be related to an increase in intracellular cAMP level which has an inhibitory effect on superoxide production and adhesion capacity of neutrophils. Candesartan had a less pronounced effect on Mac-1 expression and it was postulated that the inhibitory effect may be related to reduction in the avidity of Mac-1 without a significant quantitative change in its expression [62].
Of interest is the CXCR2 antagonist SB-656933 that besides blocking the CXCR2 receptor also inhibited neutrophil CD11b upregulation and shape change induced by CXCL1 in COPD patients, indicating that CXCR2 antagonism is a promising therapeutic approach in the treatment of COPD [63].

Proteolytic enzymes
In COPD and CD, tissue damage is a hallmark of disease. In both chronic diseases, transmigrated neutrophils release and produce different mediators, including chemokines, serine proteases, myeloperoxidase (MPO), reactive oxygen species (ROS) and MMPs [1, 3, 64-66], which leads to tissue breakdown, increased neutrophil recruitment and activation and further inflammation.
In this thesis, the roles of neutrophil-derived MMP8, MMP9 and PE were investigated in the breakdown of collagen into chemotactic tripeptide N-ac-PGP. MMPs, and more specifically MMP9 [67], are considered to be valuable drug targets in the therapy of COPD [68]. Moreover, therapies against specific pro-inflammatory MMPs are highly promising in the treatment of IBD [69]. However, no compound developed as an MMP inhibitor has been licensed for clinical use thus far [70]. A reason for this may be that inhibiting MMPs can be precarious, since these proteases are crucial in normal physiology [25]. However, from this thesis it can be implicated that specifically inhibiting MMP8 and MMP9 may have an additional advantage; in addition to suppressing the accelerated extracellular matrix turnover, the generation of chemotactic N-ac-PGP can be counteracted. One way to tackle the problem of inhibiting MMP effects too strongly is to deliver low, but effective, dosages via the airways to avoid serious systemic side effects.
Since PE is relatively new in the pathogenesis of COPD and CD, no PE inhibitors have been developed for these diseases. However, PE inhibitors have been tested as interesting drug targets for memory impairment [71] and the treatment of Alzheimer's [72]. Interestingly, valproic acid, a drug normally used in the therapy against epilepsy [73], may also be used as a PE inhibitor in COPD. It was found that cigarette smoke-induced neutrophil migration in the

BAL fluid was significantly decreased in mice after treatment with valproic acid [74]. However, since these PE inhibitors pass the blood-brain-barrier, it is very likely that neurological side effects will occur, making these inhibitors no first-choice therapy to use in COPD or CD. The development of new PE-inhibitors that do not pass the blood-brain- barrier and can be locally applied in the airways and intestine, might be of future therapeutic value.

Collagen fragment N-ac-PGP

From this thesis, it can be proposed that N-ac-PGP may be a very interesting drug target. In terms of therapeutic manipulation of N-ac-PGP in a clinical setting there would appear to be several strategies [75]. In addition to preventing the generation of PGP through the inhibition of MMPs and PE (as described above), the complementary peptide to PGP, arginine-threonine-arginine (RTR), can be regarded as an interesting lead compound. RTR has been tested in murine models and it was found that RTR blocks the chemotactic activity of N-ac-PGP but also of CXCL8 and inhibits N-ac-PGP- and LPS-induced emphysema and cigarette smoke-induced neutrophil recruitment [76, 77]. Alternatively, antibodies specific to N-ac-PGP could prove to be of therapeutic benefit in COPD [35]. Very recent data from Koelink *et al.* strongly indicates that inhibiting PGP may be beneficial in the treatment of IBD as neutralization of PGP by using RTR (arginine-threonine-arginine) or an anti-PGP antibody significantly reduces intestinal inflammation in a murine IBD model [54].

Since CXCR2 is important in N-ac-PGP signaling, it seems rational that blocking this receptor could prove beneficial, as shown in **chapter 6**. Moreover, CXCR2 antagonists reduce neutrophilic inflammation in the lungs of rats and mice exposed to cigarette smoke [78, 79]. Several small molecule inhibitors of CXCR2 are currently in clinical development for treatment of COPD and CF [80-82]. CXCR2 antagonism may also be beneficial in the therapy of CD, since CXCR2 antagonist SB225002 reduced the 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis in mice, suggesting that the use of SB225002 is a potential therapeutic approach for the treatment of IBD and other related inflammatory disorders [83].

Recently, it was published that the four chiral forms of N-ac-PGP have different properties; LL-N-ac-PGP has the highest activity, whereas DL-N-ac-PGP had the most modest loss of activity and LD-N-ac-PGP had a more pronounced loss of activity. However, DD-N-ac-PGP blocks the chemotactic activity of LL-N-ac-PGP and CXCL8 by binding to CXCR1 and/or CXCR2, making this isomer a potential therapeutic target [56].

Conclusion

In summary, this thesis provides evidence for the self-sustaining role of neutrophils in the inflammatory state in the pathogenesis of COPD and CD. In active disease, neutrophils release proteolytic enzymes that breakdown collagen. One of the collagen fragments can be neutrophilic chemoattractant N-ac-PGP, which leads to enhanced neutrophil inflammation and further inflammation. In COPD, cigarette smoking initiates a vicious circle of events leading to collagen breakdown, PGP generation and PGP-induced neutrophilic transmigration (fig. 1).

This PGP generation can also play a significant role in the pathogenesis of CD, since neutrophils from CD patients generate higher amounts of PGP from whole collagen under basal conditions than neutrophils from healthy controls. It was proposed that intestinal damage initiates a vicious circle of events leading to collagen breakdown, PGP generation and PGP-induced neutrophilic transmigration (fig. 2).

Since N-ac-PGP likely plays a significant role in both chronic diseases, N-ac-PGP is a very interesting drug target. Medication such as CXCR2 antagonists or MMP and PE inhibitors could be provided to tackle the sustained neutrophilic inflammation.

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Nederlandse samenvatting

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Introductie

In de Westerse samenleving zal het aantal chronische aandoeningen de komende decennia sterk stijgen door de vergrijzing van de bevolking en de toenemende welvaart dat gepaard gaat met slechte eetgewoonten en overdreven hygiëne dat mogelijk het immuunsysteem verzwakt. De toename van chronische ziekten levert zowel een druk op voor de maatschappij als geheel, maar ook voor patiënten met deze ziekten en hun naasten. Er zijn op dit moment therapieën die enkel symptomen bestrijden, maar die de chronische ziekten als zodanig niet genezen. Het is dan ook van belang dat er onderzoek gedaan wordt naar deze ziekten om curatieve therapieën te ontwikkelen. Voorbeelden van chronische ziekten zijn COPD (Eng: chronic obstructive pulmonary disease, Ned: chronisch obstructieve longziekten) en de ziekte van Crohn (Eng: Crohn's disease). Met dit proefschrift wordt bijgedragen aan de opheldering van de pathogenese van COPD en de ziekte van Crohn waarbij met name de rol van neutrofielen (een witte bloedcel) is onderzocht in de chronische ontstekingsprocessen.

De pathogenese van COPD en de ziekte van Crohn

COPD is één van de meest voorkomende doodsoorzaken in de wereld. Volgens de richtlijnen van het Global Initiative for Chronic Obstrucive Lung Disease (GOLD) wordt COPD omschreven als een ziektetoestand die gekarakteriseerd wordt door een beperking van de luchtstroom die niet volledig omkeerbaar is; deze luchtstroombeperking is gewoonlijk progressief van aard en gaat gepaard met een abnormale ontstekingsreactie van de longen op schadelijke deeltjes of gassen. COPD is een verzamelnaam voor de longaandoeningen chronische bronchitis en longemfyseem. De chronische bronchitis kenmerkt zich door een chronische ontsteking van het slijmvlies in de luchtwegen waarbij dagelijks slijm opgehoest wordt. Longemfyseem uit zich in het verlies van longweefsel doordat longblaasjes stuk gaan. In de longblaasjes vindt de gasuitwisseling plaats en bij emfyseem is de opname van zuurstof en afgifte van koolzuur dan ook minder goed mogelijk.

Roken is de belangrijkste oorzaak voor het ontstaan van COPD: 90% van alle COPD patienten rookt of heeft in het verleden gerookt. Er zijn echter ook andere oorzaken, namelijk luchtvervuiling, erfelijkheid en langdurig contact met giftige stoffen of stoffige lucht.

Hoewel het niet exact bekend is hoe COPD ontstaat, zijn er grofweg drie processen die een rol spelen in de pathogenese van COPD: een abnormale ontstekingsreactie op stimuli, een disbalans tussen reactieve zuurstofradicalen en anti-oxidanten en een disbalans tussen proteases en anti-proteases. Momenteel is er geen middel dat COPD patienten kan genezen. Wel kunnen symptomen bestreden worden met behulp van luchtwegverwijders en

ontstekingsremmers (corticosteroïden). Stoppen met roken is op dit moment de meest effectieve manier om de verergering van symptomen tegen te gaan. Helaas leidt het stoppen met roken niet tot volledig herstel van de longblaasjes of het verminderen van de overmatige slijmproductie. Het is dan ook van groot belang dat wetenschappelijk onderzoek leidt tot de ontwikkeling van nieuwe middelen die de luchtstroombeperking omkeerbaar maken. Mogelijke middelen zijn middelen die ingrijpen op één van bovengenoemde processen die een rol spelen in de pathogenese van COPD.

De ziekte van Crohn is één van de twee hoofdvormen van IBD (Eng: inflammatory bowel disease, Ned: inflammatoire darmziekten). De ziekte van Crohn wordt gekenmerkt door ontstekingen van de slijmvliezen van het maag-darmkanaal. Deze ontstekingen kunnen overal voorkomen, maar vooral van de overgang van de dunne naar de dikke darm. Deze ontstekingen kunnen een grillig verloop vertonen; hoewel er tijdens remissie weinig klachten zijn en behandeling soms nauwelijks nodig is, is er tijdens exacerbatie sprake van ernstige ontstekingen die zich uitbreiden naar andere delen van de darm en is intensieve behandeling nodig.

Ook van deze chronische ziekte is de exacte oorszaak onbekend, maar een complex geheel van erfelijkheidsfactoren, omgevingsfactoren en immunologische factoren speelt een belangrijke rol. De hypothese is dat beschadigingen van het darmweefsel veroorzaakt worden door een ontregelde immunologische reactie op pathogene en commensale bacteriën, hun producten en voedsel componenten in de darm. Veranderingen in de opbouw van het darmweefsel spelen mogelijk een grote rol in de pathogenese: patiënten hebben naast een aangetast darmepitheel ook een veranderde expressie van pattern-recognition receptors (PPRs), zoals Toll-like receptors (TLRs) en nucleotide-binding oligomerization domains containing proteins (NODs), op het darmepitheel. Deze receptoren herkennen constante molecuulpatronen van bacteriën, schimmels en virussen. De veranderingen in de expressie van de PPRs kunnen in het beginstadium van de ziekte leiden tot een inadequate reactie op bacteriën en andere ziekteverwekkers doordat het immuunsysteem deze ziekteverwekkers niet als zodanig herkent. Ook is het mogelijk dat het immuunsysteem een overdreven reactie heeft op voor gezonde mensen onschuldige stoffen in de darmen. Beide reacties leiden uiteindelijk tot een sterk ontregelde immunologische reactie en ernstige weefselschade.

In zowel COPD als de ziekte van Crohn speelt de neutrofiel een belangrijke rol in de aanhoudende ontsteking. Tijdens de acute fase in beide ziekten worden grote hoeveelheden neutrofielen gevonden in respectievelijk de longen en de darmen. Deze neutrofielen migreren vanuit het bloed het long- of darmweefsel in waarna deze cellen na activatie verschillende mediatoren vrijzetten die in hoge concentraties schadelijk zijn voor het weefsel. Voorbeelden van deze mediatoren zijn de enzymen matrix metalloproteases (MMPs) en

elastase, die verantwoordelijk zijn voor afbraak van het weefsel. Ook zet de neutrofiel CXCL8 (interleukine-8) vrij. CXCL8 is een chemoattractant en trekt meer neutrofielen vanuit het bloed aan en dit leidt tot nog meer neutrofielen in het weefsel die schade kunnen veroorzaken. Zowel long- als darmweefsel bestaan voor een groot gedeelte uit collageen en proteases kunnen dit collageen afbreken tot kleine tripeptides zoals N-acetyl Proline-Glycine-Proline (N-ac-PGP) en Proline-Glycine-Proline (PGP). Deze collageen fragmenten zijn specifiek via CXCR1 en CXCR2 chemotactisch voor neutrofielen. De productie van de PGP-fragmenten leidt er in theorie dan ook toe dat meer neutrofielen vanuit het bloed het weefsel binnendringen. Momenteel is er in COPD al mogelijk een rol voor N-ac-PGP. Dit tripeptide wordt gevonden in bronchoalveolaire longvloeistof (lavagevloeistof), sputum en serum van COPD patiënten, terwijl N-ac-PGP niet gemeten kan worden in astmapatiënten of gezonde mensen. Dit lijkt erop dat N-ac-PGP mogelijk naast een ontstekingsmolecuul en chemoattractant ook een biomarker voor COPD kan zijn. Ook lijkt er een rol voor N-ac-PGP te zijn in inflammatoire darmziekten: in een IBD-muizenmodel werd N-ac-PGP gemeten in de darmen van muizen. Deze N-ac-PGP kon geneutralizeerd worden met RTR (een PGP-bindend molecuul dat bestaat uit arginine-threonine-arginine) en een antilichaam tegen N-ac-PGP, waarna de ernst van de ontstekingsreactie in de darmen van de muizen verminderd werd.

Belangrijkste bevindingen en conclusies van dit proefschrift.

Met de studies beschreven in dit proefschrift is bijgedragen aan de ontrafeling van de rol van neutrofielen in de pathogenese van COPD en de ziekte van Crohn. Hiertoe zijn experimenten gedaan met menselijke neutrofielen om de rol van deze cellen te bestuderen in deze chronische ziekten: er zijn *in vitro* modellen opgezet om zowel het migratieproces van de neutrofiel als de activatie van de gemigreerde neutrofiel te bestuderen. Daarnaast is er gebruik gemaakt van een diermodel om het effect van CXCR2 te bestuderen op de migratie van neutrofielen onder invloed van N-ac-PGP. Hier volgt een samenvatting van de belangrijkste bevindingen en conclusies van deze *in vitro* en *in vivo* experimenten.

1. **Chemokines en chemokinereceptoren kunnen een belangrijke target zijn in de ontwikkeling van nieuwe therapieën voor COPD en de ziekte van Crohn.**

Immuuncellen bewegen zich van de ene naar de andere plaats in het lichaam onder invloed van chemische stoffen (chemokines) in een proces dat chemotaxie heet. De migratie van deze cellen wordt geregeld door verschillende chemokines en chemokinereceptoren. In hoofdstuk 2 zijn de belangrijkste chemokines en bijbehorende receptoren besproken voor COPD en de ziekte van Crohn. Uit dit hoofdstuk kan geconcludeerd worden dat interventie in

de migratie van verschillende typen immuuncellen mogelijk tot een nieuwe therapie kan leiden.

2. Roken start een cascade dat leidt tot een vicieuze circel van collageenafbraak, PGP productie en verdere neutrofiel influx.

Aangezien roken de belangrijkste oorzaak is van COPD is er in dit proefschrift gekeken naar de effecten van sigarettenrook op de verschillende functies van de neutrofiel. De directe effecten van rook op migratie en activatie van neutrofielen zijn beschreven in de hoofdstukken 3 en 7. Tijdens de degranulatie worden ondermeer MMPs vrijgezet door de neutrofiel wat leidt tot collageenafbraak en de vorming van N-ac-PGP. Dit tripeptide heeft ook directe effecten op zowel migratie als activatie van de neutrofiel, welke beschreven zijn in hoofdstuk 4, 5 en 6. Deze hele hypothese is samengevat in figuur 1 van hoofdstuk 9 (pg. 176). Hieronder worden de belangrijkste bevindingen van de genoemde hoofdstukken belicht.

Sigarettenrook heeft een directe invloed op de migratie van neutrofielen: de activatie van β_2-integrine Mac-1 op de neutrofiel onder invloed van sigarettenrook leidt tot verhoogde bindingscapaciteit aan fibrinogeen en transmigratie tussen endotheelcellen.

In de lavagevloeistof en het sputum van COPD patiënten worden hoge aantallen neutrofielen gevonden. Ook in rokers zonder longaandoeningen worden verhoogde hoeveelheden neutrofielen gevonden in vergelijking met niet-rokers. Dit getal is echter lager in rokers dan in COPD patiënten. Neutrofielen gebruiken bij de migratie vanuit het bloedvat specifieke adhesie- en chemokinereceptoren. In hoofdstuk 3 is er gekeken naar het directe effect van sigarettenrook op de migratie van neutrofielen.

Conclusie: Uit deze studie kan geconcludeerd worden dat neutrofielen migreren onder invloed van sigarettenrook naar het weefsel: in de longen van muizen die blootgesteld werden aan rook gedurende 5 dagen werden neutrofielen gevonden. Naast dit *in vivo* experiment werd ook in een *in vitro* transwell migratie systeem bevestigd dat neutrofielen migreren onder invloed van sigarettenrook. Uit de adhesie- en transmigratie-experimenten beschreven in hoofdstuk 3 kan daarnaast geconcludeerd worden dat neutrofielen migreren door een endotheellaag doordat sigarettenrook de β_2-integrine Mac-1 activeert: deze Mac-1 activatie leidt tot een sterkere binding aan ICAM-1 van het endotheel, waarna de neutrofiel door de endotheellaag kan extravaseren.

Sigarettenrook activeert de neutrofiel tot degranulatie en tot de productie van ontstekingsmediatoren.

In een vroeg stadium van de ontsteking migreren neutrofielen onder invloed van een CXCL8 gradient vanuit het capillair het weefsel in. Aangekomen bij de ontstekingshaard raken de neutrofielen geactiveerd, produceren intracellulair CXCL8 en zetten CXCL8 vrij. Hierdoor transmigreren meer neutrofielen via CXCR1 en CXCR2. Daarnaast zetten ze proteases vrij middels degranulatie. In COPD worden hoge MMP concentraties gemeten die gecorreleerd zijn aan de afbraak longblaasjes en luchtstroombeperking. In hoofdstuk 7 is er onderzoek gedaan naar de effecten van sigarettenrook op neutrofiel activatie en degranulatie.

Conclusie: Uit de resultaten van hoofdstuk 7 kan geconcludeerd worden dat sigarettenrook de neutrofiel kan activeren: activatie leidt tot CXCL8 en MMP8 en MMP9 vrijzetting. In theorie zal de CXCL8 vrijzetting leiden tot een verdere neutrofielinflux en de MMP-vrijzetting zal leiden tot collageenafbraak en de vorming van N-ac-PGP.

Neutrofielen breken collageen af tot N-ac-PGP onder invloed van sigarettenrook.

In hoofdstuk 7 is tevens gekeken naar de invloed van sigarettenrook op collageenafbraak. Hiertoe werden sigarettenrook-geactiveerde neutrofielen geïncubeerd met collageen. Met dit experiment is onderzocht of de neutrofiel collageen kan afbreken in specifieke N-ac-PGP fragmenten.

Conclusie: De neutrofielen zetten onder invloed van sigarettenrook MMP8 en MMP9 vrij. Deze twee proteases knippen collageen in kleinere stukken van 30 tot 100 aminozuren. Hierna worden specifieke N-ac-PGP fragmenten geknipt uit deze stukken collageen door prolylendopeptidase (PE). Hoewel sigarettenrook invloed had op MMP8 en MMP9 vrijzetting, had het geen invloed op de activatie of vrijzetting van intracellulair PE. Naast de neutrofiel kunnen echter ook andere cellen bijdrage aan verder weefselschade door PE vrij te zetten aangezien PE werd gevonden in macrophagen en epitheel cellen. PE speelt mogelijk een grote rol in COPD: er werd in COPD longweefsel veel meer PE gevonden dan in controle longweefsel.

Collageenfragment N-ac-PGP houdt zijn eigen productie en aan de daarmee de aanhoudende ontsteking aan de gang.

Al sinds de jaren 70 van de twintigste eeuw is bekend dat collageenfragmenten chemotactisch kunnen zijn. Hoewel al langer bekend is dat N-ac-PGP specifiek chemotactisch is voor neutrofielen, zijn andere pro-inflammatoire effecten nog niet beschreven. In hoofdstuk 4, 5, 6 en 7 is gekeken naar de effecten van N-ac-PGP op neutrofiel activatie, degranulatie en transmigratie.

Conclusie:

In hoofdstuk 5 en 6 is bevestigd dat N-ac-PGP chemotactisch is voor neutrofielen *in vitro* en *in vivo.* Tevens kan uit de resultaten van hoofdstuk 5 worden geconcludeerd dat neutrofielen CXCL8 produceren en vrijzetten onder invloed van N-ac-PGP. Deze CXCL8 vrijzetting is echter niet verantwoordelijk voor het geobserveerde chemotactische effect van N-ac-PGP: neutrofielen gepreïncubeerd met antilichamen tegen CXCL8 (α-CXCL8) lieten een chemotactisch effect zien onder invloed van N-ac-PGP, terwijl het effect van CXCL8 volledig geremd werd. Tevens werd dit chemotactische effect gedemonstreerd in hoofdstuk 4 in een fysiologisch relevantere omgeving: neutrofielen migreerden onder invloed van basolateraal N-ac-PGP door een endotheellaag heen. Dit gaat mogelijk via de activatie van Mac-1 aangezien N-ac-PGP incubatie leidt tot een conformatieverandering van Mac-1 en een sterkere binding tussen Mac-1 op de neutrofiel en fibrinogeen.

N-ac-PGP is mogelijk veranwoordelijk voor zijn eigen productie: activatie van de neutrofiel door N-ac-PGP resulteerde in MMP8- en MMP9-vrijzetting, welke cruciaal zijn in de N-ac-PGP productie uit collageen. Ook N-ac-PGP had net als sigarettenrook geen invloed op de activatie of vrijzetting van intracellulair PE.

3. In de ziekte van Crohn leidt darmschade mogelijk tot een vicieuze circel van collageenafbraak, PGP productie en transmigratie van neutrofielen.

In hoofdstuk 8 is gekeken naar het migratieproces van neutrofielen in de ziekte van Crohn. Hiertoe werden de verschillende componenten in dit proces, zoals CXCL8 en N-ac-PGP, bestudeerd.

Conclusie: De neutrofielen van patiënten met de ziekte van Crohn zijn mogelijk verschillend van gezonde mensen aangezien een verhoogde basale migratiecapacitieit werd gemeten. Tevens waren neutrofielen van Crohn patiënten in staat collageen beter af te breken in vergelijking met neutrofielen van gezonde mensen. CXCR1 en CXCR2 op neutrofielen van Crohn patiënten werden verhoogd tot expressie gebracht.

Uit hoofdstuk 8 kan ook geconcludeerd worden dat de pathogenese van de ziekte van Crohn mogelijk het gevolg is van een verzwakt respons op pathogene en commensale bacteriën, hun producten en voedsel componenten in de darm. In gezonde individuen produceerden de perifere neutrofielen meer CXCL8 dan de perifere neutrofielen van Crohn patienten. Verlaagde CXCL8 concentraties werden ook gemeten in homogenaten van niet-ontstoken weefsel van patiënten in vergelijking met CXCL8 concentraties in controle patiënten met darmkanker. In ontstoken weefsel waren deze CXCL8 concentraties weer sterk verhoogd. Dit lijkt erop dat de gradient bij gezonde individuen en mensen met exacerbatie groter is dan in de gradient bij patiënten in remissie. Aangezien neutrofielen van patiënten meer N-ac-PGP uit collageen kunnen produceren dan gezonde neutrofielen is het zeer goed mogelijk

dat dit tripeptide een rol heeft in de pathogenese van de ziekte van Crohn. Deze hele hypothese is samengevat in figuur 2 van hoofdstuk 9 (pg. 182).

4. De neutrofielinflux onder invloed van N-ac-PGP kan geremd worden door het gebruik van CXCR2 antagonisten.

In hoofdstuk 6 is het effect van twee verschillende CXCR2 antagonisten bestudeerd in een muismodel. In dit muismodel werd de neutrofielinflux gemeten na locale toediening van N-ac-PGP in de long. Met dit experiment werd onderzocht of CXCR2 activatie is betrokken bij een door N-ac-PGP geïniteerde neutrofiel migratie en activatie in de muizenlong.

Conclusie: De door N-ac-PGP geïniteerde neutrofiel migratie in het longspoelvloeistof en longweefsel van de muis kan worden geremd door de twee verschillende CXCR2-antagonisten SB225002 en SB332235. Het CXCL1 dat vrijgezet wordt na N-ac-PGP toediening is echter onafhankelijk van CXCR2 activatie. Tevens was N-ac-PGP in vergelijking met CXCL1 beter in staat om neutrofielen aan te trekken naar het longweefsel dan naar het longspoelvloeistof. Uit de resultaten van dit hoofdstuk kan geconcludeerd worden dat de neutrofielmigratie onder invloed van N-ac-PGP wordt veroorzaakt door een interactie met CXCR2.

Eindconclusie

Met de studies beschreven in dit proefschrift is bijgedragen aan de ontrafeling van de rol van neutrofielen in de pathogenese van COPD en de ziekte van Crohn en is er bewijs geleverd voor een mogelijke zelfvoorzienende rol van neutrofielen in de aanhoudende ontsteking zoals gevonden wordt bij patiënten met COPD of de ziekte van Crohn. Gedurende actieve periodes in deze ziekten zetten neutrofielen verschillende mediatoren vrij die leiden tot verdere ontsteking en weefselschade. In COPD start sigarettenrook mogelijk een cascade dat leidt tot een vicieuze circel van collageenafbraak, PGP productie en verdere neutrofiel influx. Ook in de ziekte van Crohn speelt PGP mogelijk een rol in de pathogenese aangezien neutrofielen van deze patiënten basaal meer collageen kunnen knippen tot PGP fragmenten in vergelijking tot neutrofielen van gezonde mensen. Mogelijk leidt darmschade tot een vicieuze circel van collageenafbraak, PGP productie en transmigratie van neutrofielen.

Aangezien PGP een rol lijkt te spelen in de pathogenese van beide ziekten is PGP mogelijk een interessante target. Vervolgonderzoek zal nodig zijn om bijvoorbeeld CXCR2-, MMP- en PE-antagonisten of PGP-remmers te testen als therapie bij deze ziekten.

Dankwoord

Dankwoord

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Curriculum Vitae & Bibliography

Curriculum Vitae

The author of this thesis, Saskia Adriana Overbeek, was born on December 4, 1981 in Utrecht, the Netherlands. In 2000 she graduated from secondary school at the Niftarlake College in Maarssen. In September of the same year she started the study Pharmacy at the Utrecht University, from which she graduated as a pharmacist in August 2006. During this study she performed a six month research project about the immunoglobulin light chain receptor under the supervision of Dr. F.A.M. Redegeld at the department of Pharmacology within the Utrecht University. After graduation she was offered a post as a PhD student within the same department supervised by Prof Dr G. Folkerts, Prof Dr F.P.Nijkamp, Dr A.D. Kraneveld and Dr P.A.J. Henricks and in September 2006 she started the research described in this thesis. During this PhD project she also worked at the Prof Dr J.E. Blalock lab within the Division of Pulmonary, Allergy and Critical Care Medicine at the University of Alabama in Birmingham, USA for one month. Currently, the author is employed as a postdoctoral research associate in the Infectious Diseases and Immunity Section within the Department of Medicine at the Imperial College London. There she works under the supervision of Prof Dr D.M. Altmann and Dr R.J. Boyton on the T cell immunity to *Burkholderia*, the causative agent in melioidosis, which is the major cause of sepsis in South East Asia.

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* Contributed equally to this work.

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