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# Treating neutrophilic inflammation in COPD by targeting ALX/FPR2 resolution pathways.

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**Abstract**

Neutrophilic inflammation persists in COPD despite best current therapies and it is particularly resistant to inhaled glucocorticosteroids. Persistent neutrophil activation not only contributes to matrix breakdown, but can maintain inflammation through the release of endogenous damage associated molecule patterns (DAMPs). Inhibiting excessive neutrophilic inflammation is challenging as many pathogen recognition receptors can initiate migration and the targeting of downstream signaling molecules may compromise essential host defense mechanisms. Here, we discuss new strategies to combat this inflammation in COPD by focusing on the anti-inflammatory role of ALX/FPR2 receptors. ALX/FPR2 is a promiscuous G-protein coupled receptor (GPCR) responding to lipid and peptide agonists that can either switch on acute inflammation or promote resolution of inflammation. We highlight this receptor as an emerging target in the pathogenesis of COPD because known ALX/FPR2 endogenous agonists are enriched in COPD. Serum Amyloid A (SAA) has recently been discovered to be abundantly expressed in COPD and is a potent ALX/FPR2 agonist that unlike almost all other inflammatory chemoattractants, is induced by glucocorticosteroids. SAA not only initiates lung inflammation via ALX/FPR2 but can allosterically modify this receptor so that it no longer transduces pro-resolving signals from endogenous lipoxins that would otherwise promote tissue healing. We propose that there is an imbalance in endogenous and microbial ALX/FPR2 receptor agonists in the inflamed COPD lung environment that oppose protective anti-inflammatory and pro-resolution pathways. These insights open the possibility of targeting ALX/FPR2 receptors using synthetic agonists to resolve persistent neutrophilic inflammation without compromising essential host defense mechanisms.

**Key words: airway inflammation, COPD, neutrophils, resolution**

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## 1. Neutrophilic inflammation in COPD and AECOPD

Chronic Obstructive Pulmonary Disease (COPD) is estimated to affect 5% of the global adult population and is predicted to become the third leading cause of death by 2030 (Jemal, Ward, Hao, & Thun, 2005). This is primarily as a consequence of long term tobacco smoking in an increasingly ageing population (Mannino & Buist, 2007), however exposure to indoor biomass fuels is also related to higher rates of COPD among women in developing countries (Lopez, Mathers, Ezzati, Jamison, & Murray, 2006). COPD is characterized by chronic airway inflammation involving both innate and adaptive cells that accumulate with disease progression (Hogg, 2004; Hogg, et al., 2004; Saetta, et al., 1999), which is very persistent as smoking cessation fails to fully resolve this inflammatory profile (Willemse, et al., 2005). The accumulation of inflammatory cells contribute to key pathological processes in COPD including small airway narrowing, destruction of alveolar walls (emphysema) and mucous hypersecretion (reviewed in (Barnes, 2008)).

The establishment of chronic inflammation in COPD is multi-factorial; however the presence of pathogenic microbes in the airways is likely to be a major cause. It is estimated that about 50% of COPD patients are chronically colonized with potentially pathogenic microorganisms including *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis* (Monso, et al., 1995; Pela, et al., 1998). The presence of microbial pathogens in the lower airways is related to defective innate and cellular immunity as a consequence of chronic cigarette smoke exposure (reviewed in (Stampfli & Anderson, 2009)). There is a direct relationship between colonization and the degree of airway inflammation. Colonized COPD patients display worse health status and increased neutrophilic inflammation, as reflected in increased levels of interleukin-8 (CXCL8), leukotriene $B_4$ ($LTB_4$), and neutrophil elastase (Banerjee, Khair, &

Honeybourne, 2004). Colonization is also associated with increased airway shedding of microbial products such as endotoxin (S. Sethi, Maloney, J., Grove,L., Wrona, C., Berenson, C.S., 2006) that may perpetuate ongoing inflammation, neutrophil activation and subsequent tissue damage through persistent activation of pathogen recognition receptors (PRRs).

In addition, neutrophilic inflammation is particularly elevated during acute exacerbations of COPD (AECOPD) that are mainly triggered by acquisition of a new respiratory pathogen (Papi, et al., 2006). AECOPD are responsible for an increased risk of mortality, particularly when patients experience recurrent severe exacerbations that require hospitalization (Soler-Cataluna, et al., 2005). They result in a more rapid decline in lung function (Donaldson, Seemungal, Bhowmik, & Wedzicha, 2002), impaired health related quality of life (Donaldson, Wilkinson, Hurst, Perera, & Wedzicha, 2005) and have a major impact on health care expenditure (Sullivan, Ramsey, & Lee, 2000). Viral infections are common, accounting for approximately 50% of AECOPD and a causal relationship has been recently described (Hutchinson, et al., 2007; Mallia, et al., 2011). Bacterial infections are also a common cause of purulent AECOPDs that are associated with a marked increase in neutrophilic inflammation (Gompertz, O'Brien, Bayley, Hill, & Stockley, 2001), where clinical severity tracks with the degree of airway and systemic inflammation (S. Sethi, et al., 2008).

## 2. Causes of excessive neutrophilic activation/degranulation in COPD

### 2.1 Role of colonization and AECOPD

Limited exocytosis of proteinases in primary azurophilic granules is normally required for efficient intracellular killing of microorganisms in the phagolysosome (Belaaouaj, et al., 1998).

The majority of neutrophil elastase is expressed on the activated neutrophil surface as a mechanism to facilitate egress from the vasculature and limit damage to surrounding tissue (Owen, Campbell, Sannes, Boukedes, & Campbell, 1995). Anti-proteinases such as α1-antitrypsin (α1-AT), secretory leukoprotease inhibitor (SLPI) and tissue inhibitor of metalloproteinases (TIMPs) are also in excess to provide an anti-proteinase screen to prevent deleterious effects. However, persistent neutrophil activation can lead to excessive proteinase release that can cause host tissue damage (Taggart, Greene, Carroll, O'Neill, & McElvaney, 2005). Inappropriate neutrophil activation leads to neutrophil elastase activity that increases with COPD severity in the presence of inhaled glucocorticosteroids (GCs) (Vlahos, Wark, Anderson, & Bozinovski, 2012). The excessive release of this proteinase into the extracellular milieu of COPD airways is likely to be mediated by multiple mechanisms. Colonizing pathogens such as *Haemphilus influenzae* can directly cause neutrophil necrosis and release of azurophilic granular content (Naylor, et al., 2007). Rhinovirus infection can also induce release of neutrophil elastase that causes degradation of antimicrobial peptides, which may increase susceptibility to secondary bacterial infection in COPD (Mallia, et al., 2012).

### 2.2 Role of defective macrophage clearance

A critical component in the resolution of inflammation is the clearance of apoptotic neutrophils. Inflammatory monocytes accumulate in damaged tissue and differentiate into resolving macrophages that release anti-inflammatory mediators such as IL-10 and promote non-phlogistic phagocytosis of apoptotic cells and debris. This process is perturbed by excessive oxidative stress in COPD as cigarette smoke impairs non-phlogistic efferocytosis of dying cells (Hodge, et al., 2007) and phagocytosis of *Haemphilus influenzae* (Marti-Lliteras, et al., 2009). In addition,

non-eosinophilic asthmatics that are characterized by persistent airway neutrophilia also display impaired macrophage efferocytosis (Simpson, et al., 2013). The defect appears to be bacterial specific as the removal of inert particles such as latex beads are not compromised (Taylor, et al., 2009). Oxidants in cigarette smoke inhibit actin polymerization and cytoskeletal rearrangement, which is normally required for efficient efferocytosis (Minematsu, Blumental-Perry, & Shapiro, 2011). Macrophage pseudopodia also become heavily carbonylated (Bozinovski, et al., 2011), and protein carbonylation is known to cause significant cytoskeletal instability (Banan, Zhang, Losurdo, & Keshavarzian, 2000; Smerjac & Bizzozero, 2008). In addition, macrophages interact with carbonyl-adduct modified extracellular matrix proteins, which impair their ability to clear apoptotic neutrophils (Kirkham, Spooner, Rahman, & Rossi, 2004). Important secreted proteins including the anti-protease α1-AT and the anti-microbial surfactant protein A (SPA) are also known to be carbonylated (Starosta & Griese, 2006), and this modification in SPA is related to reduced macrophage phagocytosis (Mikerov, et al., 2008).

### 2.3 Persistent neutrophilic activation sustains inflammation

The reduced capacity to clear exhausted neutrophils will lead to release of degranulation products from necrotic bodies. Neutrophil elastase degrades extracellular matrix components including elastin, collagens I-IV and fibrinogen and the degree of elastase localized to lung elastic fibers correlates with the degree of emphysema (Damiano, et al., 1986). Elevated neutrophil elastase levels can also directly contribute to ongoing inflammation by activating TLR4 signaling that promotes CXCL8 expression in bronchial epithelial cells (Kuwahara, et al., 2006; Walsh, et al., 2001). In addition, neutrophil elastase resides at the apex of a signaling cascade that can promote mucin production via epidermal Growth factor receptor (EGFR)

transactivation (Shao & Nadel, 2005). Furthermore, increased EGFR transactivation augments inflammatory responses initiated by rhinovirus infection in bronchial epithelial cells (Liu, Gualano, Hibbs, Anderson, & Bozinovski, 2008).

The extent of protein oxidation is also proportional to the number of airway neutrophils in chronic lung disease (Starosta & Griese, 2006). This is consistent with neutrophils becoming an important cellular source of reactive oxygen species. Neutrophil-derived myeloperoxidase metabolizes hydrogen peroxide in the presence of chloride ions to generate hypochlorous acid, which is a strong oxidant. Therefore, the need to control neutrophil activation extends beyond limiting matrix degradation, as excessive degranulation can itself maintain airway inflammation and oxidative stress (as summarized in Figure 1).

## 3. Prominent signaling pathways activated in COPD

Due to their pathogen sensing capacity, PRRs regulate inflammation in response to chronic airway colonization and AECOPD. The recognition of bacterial and viral products by PRRs expressed on resident and recruited cells will modulate the degree of neutrophilic inflammation. The Toll-Like Receptor (TLR) family including TLR2 and TLR4 are capable of initiating neutrophilic inflammation in response to cigarette smoke by sensing oxidants (Paul-Clark, et al., 2009) independently of classic lipopolysaccharide (LPS) interactions (Doz, et al., 2008). The activation of TLRs initiates a signaling cascade that culminates in the activation of MAP kinases and NFκB, which in turn promotes expression of pro-inflammatory genes (Akira, Uematsu, & Takeuchi, 2006). Paradoxically, there is also increasing evidence that cigarette smoke is immunosuppressive via its perturbation of TLR signaling (Laan, Bozinovski, & Anderson,

2004). TLRs also recognize damage associated molecular patterns (DAMPs), which are endogenous proteins released from the injured cell during necrosis and contribute to sterile inflammation. DAMPs that are elevated in COPD airways include uric acid (Yigla, Berkovich, & Nagler, 2007), which can activate TLR2/4 and also engage the inflammasome during lung injury to promote inflammation (Gasse, et al., 2009). High-mobility group box-1 (HMGB1) is a DNA binding protein that is elevated in COPD airways and is negatively correlated with lung function (Ferhani, et al., 2010). HMGB1 synergizes with microbial products like LPS and endogenous cytokines to enhance TLR signaling (Sha, Zmijewski, Xu, & Abraham, 2008; Youn, Oh, Kim, Choi, & Shin, 2008) and can also promote airway inflammation through activation of the receptor for advanced glycosylation end products (RAGE) (Ferhani, et al., 2010). There is also a soluble form of RAGE (sRAGE) that can antagonize ligand mediated RAGE signaling. sRAGE has been shown to be reduced in COPD, which may contribute to increased neutrophilic inflammation (Sukkar, et al., 2012).

Hyaluronan is also a component of the extracellular matrix that becomes degraded in COPD and is associated with the degree of inflammation (Dentener, Vernooy, Hendriks, & Wouters, 2005). Hyaluronan degradation fragments can activate macrophage-mediated inflammatory responses in a TLR2/4 dependent manner (D. Jiang, et al., 2005). The calcium binding, cytoplasmic S100A8/9 protein complex is also an endogenous TLR4 ligand that is elevated in COPD (Merkel, Rist, Seither, Weith, & Lenter, 2005). S100A8 is abundantly secreted into the airways in a GC refractory manner during acute inflammation, and neutralizing its activity partially blocks the recruitment of airway neutrophils (Bozinovski, et al., 2005). S100A8/9 can also ligate with RAGE, where it has been shown to promote tumor growth (Ghavami, et al., 2008). The

enriched environment for host derived DAMPs in COPD airways is reflective of damaging inflammation and deficient reparative processes that may overwhelm endogenous mechanisms normally required to resolve inflammation.

Another important class of PRRs are the cytosol localized RIG-I-Like Receptor (RLR) family including RIG-I and MDA, which engage similar signaling intermediates to TLR in order to promote the production of pro-inflammatory cytokines and type I interferons in response to viral and bacterial nucleic acids in the cytoplasm. RLRs contain caspase activation and recruitment domains (CARDs), which function as protein–protein interaction motifs. Binding of RLR to RNA that is either viral in origin or generated by RNA polymerase III from microbial DNA templates causes a conformational change (F. Jiang, et al., 2011). This enables its CARD domains to initiate signaling that culminate in the activation of MAPKs and the transcription factors IRF3, IRF7, and NFκB via formation of large prion-like aggregates (Hou, et al., 2011). The convergence on MAPK and NFκB by TLRs, RLR and RAGE represent potential therapeutic options to combat inflammation in a microenvironment containing multiple PRR ligands.

The NOD-Like Receptor (NLR) family represents another class of cytosolic PRRs that can promote neutrophilic inflammation. NRLs contain a central nucleotide-binding oligomerization (NOD) domain, extracellular leucine-rich-repeat motifs for ligand sensing and cytoplasmic signaling domains such as CARD, Pyrin domains (PYD) or baculovirus inhibitor repeats (BIR) (Fritz, Ferrero, Philpott, & Girardin, 2006). NRLs promote inflammation through signaling to MAPK/NFκB and can also initiate an alternative 'NLRP3 inflammasome' signaling process involving secretion of IL-1β and IL-18, as reviewed in (Birrell & Eltom, 2011). NOD1 and

NOD2 detect conserved cell wall peptidoglycan components of gram negative and gram positive bacteria and interact with the CARD containing serine/threonine kinase RIP2 to activate MAPK and NFκB signaling (Kobayashi, et al., 2002; Park, et al., 2007). The NLRP3 inflammasome responds to a broader array of ligands including DAMPs (ATP, uric acid metabolites and hyaluronan), microbial molecules / toxins and cytoplasmic DNA that appears during tissue injury. Upon ligand activation, a complex consisting of the adapter molecule ASC (Apoptosis associated Speck-like protein containing a CARD), which is a critical NLRP3 inflammasome component recruits and activates caspase-1 (Mariathasan, et al., 2004). Activated caspase-1 not only regulates cell death, but also generates mature IL-1β and IL-18 by cleaving their pro-forms produced during the initial phase of inflammation (Martinon, Burns, & Tschopp, 2002).

The NLRP3 inflammasome is implicated in COPD as elevated IL-1β levels are associated with neutrophil markers in COPD airways (Ekberg-Jansson, et al., 2001) and targeting caspase-1 reduces IL-1β and airway inflammation in cigarette smoke models (Churg, Zhou, Wang, Wang, & Wright, 2009). In addition, increased levels of IL-1β and IL-18 are observed in induced sputum of COPD patients (Pauwels, et al., 2011; Rovina, et al., 2009). These studies also suggest that IL-1α should also be considered in the pathogenesis of COPD as a blocking Il-1α effectively reduced lung neutrophilia (Pauwels, et al., 2011). The significance of pathways that converge on IL-1R in AECOPD remains undetermined, but will likely contribute to airway inflammation as the NLRP3 inflammasome is activated in the lungs following *Haemophilus influenza* and Influenza A virus infection (Allen, et al., 2009; Wieland, Florquin, & van der Poll, 2007).

In addition, the P2X7 inflammasome pathway has been shown to contribute to inflammation in COPD as a selective P2X7 receptor antagonist attenuated airway neutrophilia in a cigarette smoke model (Eltom, et al., 2011). Another emerging paradigm is the initiation of immunological autophagy mechanisms. Although autophagic markers are elevated in COPD, cigarette smoking may be impairing autophagic processing (reviewed in (Ryter, Nakahira, Haspel, & Choi, 2012). The implications of this are intriguing as autophagy can negatively regulate the inflammasome by removing endogenous irritants, and inversely positively regulate inflammation by delivering alarmins such as IL-1β to the outside of the cells. In addition, autophagic processes are increasingly recognized for their ability to degrade microorganisms that invade intracellularly (Levine, Mizushima, & Virgin, 2011), and perturbation of this process may have important implications in AECOPD.

## 4. Current and emerging therapies for COPD and AECOPD

### 4.1 Current therapies

Neutrophilic inflammation in COPD remains a challenging area of unmet medical need as the targeting of excessive inflammation must be achieved without compromising essential host defense mechanisms required for the clearance of infection. Antibiotics are used in AECOPDs as treatment success rates are higher in patients with features of an infectious exacerbation (Anthonisen, et al., 1987). Long-term azithromycin therapy in COPD has also been shown to reduce exacerbation frequency (Albert, et al., 2011). Macrolides also display immunomodulatory actions including inhibition of MAPK and NFκB activity required for neutrophil chemokine production (Kanoh & Rubin, 2010). However, since macrolides can prolong the QTc interval, long term azythmycin is not recommended for COPD patients with baseline cardiovascular

disease and development of antibiotic resistance remains an issue of concern. Non-antimicrobial macrolides that retain immunomodulatory properties without inducing resistance are currently in development (Kanoh & Rubin, 2010).

Systemic GCs are clinically used during AECOPD as they have been shown to reduce treatment failure in the short-term (30-90 days, defined as death from any cause or the need for intubation and mechanical ventilation, readmission to the hospital for COPD, or intensification of drug therapy). However, no difference was seen at six months post exacerbation (Niewoehner, et al., 1999). GCs also modestly improve post bronchodilator lung function during AECOPD and reduce length of hospital stay (Davies, Angus, & Calverley, 1999). At the molecular level, COPD is viewed as a GC-resistant disease due to changes in the GC machinery that coordinate its anti-inflammatory actions (reviewed in (Barnes & Adcock, 2009)). Histone deacetylase 2 (HDAC2) is normally recruited by the activated GC receptor (GR) and promotes NFκB transrepression, as the deacetylated GR complex binds to and inactivates NFκB. HDAC2 expression and activity in patients with COPD is reduced as a consequence of oxidative stress (Ito, et al., 2005). Therapies that restore HDAC2 expression in COPD include low dose theophylline. This is achieved at therapeutic concentrations below that required for inhibition of Phosphodiesterase (PDE) 4, and mechanistically involves restoration of HDAC2 activity by preventing tyrosine nitration of the enzyme. A recent clinical trial has shown that low dose theophylline did increase HDAC2 activity, which was associated with improved lung function and GC activity in COPD (Ford, et al., 2010).

Another class of anti-inflammatory agent used in COPD are PDE 4 inhibitors, which are predominantly expressed in inflammatory cells including neutrophils. Its actions relate to inhibiting the degradative actions of PDE4 on the secondary messenger, cAMP. In a preclinical model, the PDE4 inhibitor roflumilast, reduced lung inflammation and emphysema in mice chronically exposed to cigarette smoke (Martorana, Beume, Lucattelli, Wollin, & Lungarella, 2005). In COPD patients, roflumilast administered over four weeks also reduced percentage of sputum neutrophils and eosinophils and improved lung function over this short duration (Grootendorst, et al., 2007). Longer term trials also show an improvement in lung function even with concomitant use of long-acting β2-agonists and a reduction in annual rate of exacerbations (Bateman, et al., 2011). Roflumilast appears to reduce exacerbations more effectively in a subset of COPD patients on inhaled GCs with chronic bronchitis, although adverse effects relating to the use of roflumilast were elevated (Rennard, Calverley, Goehring, Bredenbroker, & Martinez, 2011). Side-effects associated with roflumilast including nausea, headache, diarrhoea and weight loss were usually not severe, but do increase patient withdrawal across the studies. Since AECOPDs are associated with an increase in airway and systemic inflammation, it remains to be determined whether the maximal tolerated dose will be effective in controlling enhanced inflammation in this setting.

### 4.2 Emerging therapies

Since the majority of PRRs that recognize microbial products and DAMPs elevated in COPD airways converge on MAPK and NFκB signaling to promote inflammatory gene expression, there is considerable interest in therapeutically targeting these signaling mediators. There are multiple strategies in development for the inhibition of NFκB. A central step in this pathway is

the liberation of cytoplasmic NFκB from Inhibitor of NFκB (IκB). Upon PRR activation, IκB is phosphorylated by Inhibitor of NFκB Kinase 2 (IKK2), which promotes IκB degradation through the ubiquitin pathway, thereby liberating NFκB to enter the nucleus and initiate inflammatory gene expression. Inhibiting IKK2 in an acute model reduced NFκB activity and airway neutrophilia in response to LPS (Birrell, et al., 2006) and there are several IKK inhibitors with promising pharmacokinetic characteristics (Suzuki, et al., 2011). Of the three MAPK members (ERK1/2, JNK and p38), there has been interest in the use of small molecule inhibitors of the p38 pathway as levels of the phosphorylated form of p38 are increased in COPD (Renda, et al., 2008). Inhibitors of p38 may be beneficial in AECOPD as they reduce neutrophil accumulation in response to acute LPS challenge (Nick, et al., 2000), however there is a need to examine p38 activity in COPD patients when they are exacerbating. Inhibitors of p38 also reduce leukocyte airway infiltration in response to cigarette smoke exposure (Medicherla, et al., 2008). ERK1/2 and JNK can also contribute to regulating airway inflammation, although their importance in proliferation, differentiation and apoptosis may require development of targeting strategies that selectively inhibit cell-type specific isoforms. The clinical applicability of NFκB and MAPK inhibitors may also be confounded by potential adverse events including prolonged immunosuppression and drug toxicity, which may be reduced by direct delivery into the lung. Another strategy in limiting excessive neutrophilic inflammation is to directly target the cytokine networks that are prominent in COPD such as GMCSF (Vlahos, Bozinovski, Hamilton, & Anderson, 2006) and IL-17A (Chang, et al., 2011; Di Stefano, et al., 2009; Prause, et al., 2009), as this approach may preserve broader PRR mediated host defense mechanisms.

There are also a suite of chemokine receptor antagonists that are being evaluated in chronic lung disease (reviewed in (Chapman, et al., 2009)). The significance of these systems is reflected by inbuilt redundancy to ensure efficient host immunity to infection, and it is this redundancy that contributes to the complexity of therapeutically targeting this network. The CXCR family of G coupled protein receptors (GPCR) bind to endogenous CXCL chemokines that are elevated in AECOPD (Qiu, et al., 2003) and cigarette smoke exposure models (Stevenson, et al., 2005) including IL8 (CXCL8), ENA78 (CXCL5), GCP-2 (CXCL6) and GRO isoforms (CXCL1-3) . CXCR2 is the cognate receptor for this family of chemokines, whereas CXCR1 is preferentially activated by CXCL8 and GCP-2 (Wolf, et al., 1998). In a preclinical mouse model, CXCR2 antagonism reduced lung neutrophilia in response to cigarette smoke challenge (Thatcher, et al., 2005). In another proof-of-principle study, the alternate CXCR2 antagonist (SCH527123) was shown to inhibit ozone-induced neutrophil recruitment in healthy humans (Holz, et al., 2010). However, a general challenge in targeting chemokines in AECOPD is that in addition to the CXCL chemokine network, there is also a signal from microbial products and other ligands that promote chemotaxis independently of the CXCR family. There is also a hierarchy of chemoattractants, where end target ligands such as bacterial products tend to prevail over intermediate chemokines such as CXCL8 via differential signaling by PTEN and p38 MAPK (Heit, et al., 2008).

N-formyl peptides released from bacteria display chemotactic activity for phagocytes (Schiffmann, et al., 1975). In addition, N-formyl peptides are released from degenerating mitochondria, which contribute to the migration of phagocytes to sites of tissue damage (Chiang, et al., 2006). These microbial and endogenous ligands bind to the N-formyl peptide receptor

(FPR) family, of which there are three human members (FPR1, ALX/FPR2 and FPR3). N-formyl peptides such as N-Formyl-Met-Leu-Phe (fMLF) of *E.coli* origin preferentially bind to FPR1 with high affinity, whereas mitochondrial derived N-formyl peptides display similar affinity for FPR1 and FPR2 (Rabiet, Huet, & Boulay, 2005). FPRs demonstrate wide tissue distribution, although FPR1 and ALX/FPR2 are prominent in myeloid cells including neutrophils and monocytes (Murphy, et al., 1992), whereas FPR3 becomes the dominant FPR in mature dendritic cells (Migeotte, et al., 2005). FPR1 appears to be an important receptor in mouse models of cigarette smoke-induced emphysema, as genetic ablation of this receptor confers protection (Cardini, et al., 2012). ALX/FPR2 is also expressed in the epithelium of COPD airways (Bozinovski, et al., 2012) and has been shown to be elevated in response to epithelial injury via COX-2 dependent mechanisms (Bonnans, Fukunaga, Levy, & Levy, 2006). FPRs are members of the GPCR superfamily characterized by seven putative TMs with an N terminus on the extracellular side and a C terminus on the intracellular side of the membrane. There are highly conserved sequences among the human and murine receptors that suggest an essential role for these regions in ligand recognition and functional G protein coupling signaling (Chiang, et al., 2006). GPCRs can initiate multiple signaling intermediates including PKC, PKA, MAPK and PI3K, which in turn initiate expression of adhesion molecules/pro-inflammatory mediators via NFκB and also directly drive neutrophil transmigration and diapedesis.

## 5. Role of SAA and ALX/FPR2 in neutrophilic inflammation

ALX/FPR2 displays diverse ligand affinities, with over 30 peptides described to date and is unique in that it is the only FPR member to bind to the eicosanoid Lipoxin$A_4$ ($LXA_4$) (Ye, et al., 2009). As ALX/FPR2 ligands can promote opposing biological actions, their relative abundance

may contribute to the intensity and resolution of airway inflammation in COPD. Specifically, this receptor complex is known to play a central role in resolution processes that are required for the termination of acute inflammation via its interactions with endogenous pro-resolving lipid mediators (reviewed in (Levy, Vachier, & Serhan, 2012)). High affinity ligands for ALX/FPR2 that promote inflammation include mitochondrial N-formylated hexapeptides derived from NADH dehydrogenase and cytochrome *c* oxidase subunits (Rabiet, et al., 2005). These formylated peptides released from damaged cells can act as endogenous signaling DAMPs via FPR1 and ALX/FPR2 (Rabiet, et al., 2005). Oxidative stress appears to be a major stimulus that can cause tissue damage in COPD relating to the increased presence of apoptotic and necrotic lung and airway epithelial cells (Hodge, Hodge, Holmes, & Reynolds, 2005; Wickenden, et al., 2003). Although it is not known whether these ligands are elevated in COPD, mitochondrial dysfunction and increased cytochrome *c* oxidase levels are seen in the skeletal muscle of COPD patients (Puente-Maestu, Perez-Parra, Godoy, Moreno, Tejedor, Gonzalez-Aragoneses, et al., 2009; Puente-Maestu, Perez-Parra, Godoy, Moreno, Tejedor, Torres, et al., 2009; Rabinovich, et al., 2007).

The anti-microbial peptide, LL-37 (or hCAP-18) has also been shown to be elevated in stable COPD (Xiao, Hsu, Ishizaka, Kirikae, & Moss, 2005) and is further elevated during bacterial AECOPDs (Parameswaran, Sethi, & Murphy, 2011). LL-37 is a breakdown product of cathelicidin that is expressed by epithelial cells and is a component of the leukocyte granule pool. In addition to microbial killing through pore formation, LL-37 is also a chemoattractant for neutrophils, monocytes and lymphocytes via its actions on ALX/FPR2 (De, et al., 2000). The relative contribution of LL-37 to neutrophilic inflammation in COPD has yet to be determined.

Another known agonist of ALX/FPR2 that is elevated in COPD is the soluble form of the urokinase-type plasminogen activator receptor (uPAR) (Xiao, et al., 2005). uPAR is a high affinity receptor for the serine protease urokinase–type plasiminogen activator (uPA), which when bound to uPAR cleaves and releases the soluble form of the receptor. The cleaved form of uPAR ($D2D3_{88-274}$) induces cell migration via ALX/FPR2 dependent mechanisms (Resnati, et al., 2002).

Another ALX/FPR2 ligand that is increased in COPD and AECOPD is Serum Amyloid A (SAA). SAA is a systemic biomarker that proved to be a more sensitive marker for AECOPD severity than CRP alone or in combination with dyspnea (Bozinovski, et al., 2008). SAA is also elevated in the lungs of COPD patients, as assessed immunohistochemically in lung resection tissue (Bozinovski, et al., 2012). SAA transcript levels were also positively associated with neutrophil numbers in COPD lung tissue (Anthony, et al., 2013) and secreted SAA levels in the BAL fluid positively related to the neutrophil activation marker, neutrophil elastase (Bozinovski, et al., 2012). The close association between SAA and neutrophils is consistent with the functional activities of this innate immune regulator. SAA delays apoptosis of neutrophils via concurrent activation of Erk and Akt signaling in a manner that is opposed by the ALX/FPR2 ligand, 15-epi-$LXA_4$ (El Kebir, et al., 2007). In addition, SAA is a potent chemotactic factor that mediates phagocyte migration via ALX/FPR2 (Su, et al., 1999). SAA also promotes expression of CXCL8 and other inflammatory mediators via activation of the ALX/FPR2 receptor under *in vitro* (He, Sang, & Ye, 2003) and *in vivo* conditions (Bozinovski, et al., 2012).

SAA represents a family of acute phase proteins classically induced by the liver in response to inflammatory mediators such as IL-6 and IL-1β. There are four genes for SAA, where SAA1 and SAA2 display a high level of sequence homology and are predominantly produced during inflammation. SAA3 is a pseudogene in humans and SAA4 is constitutively expressed at low levels. Systemically, SAA associates with HDLs (Coetzee, et al., 1986), which plays an important physiological function in mobilizing and recycling macrophage cholesterol during tissue injury and does not display the same inflammatory activity as lipid free SAA (reviewed in (Kisilevsky & Manley, 2012)). Circulating SAA has also been characterized as a marker for adverse cardiovascular events, where systemic inflammation may contribute to atherosclerotic plaque destabilization and endothelial dysfunction (Filep & El Kebir, 2008; Johnson, et al., 2004). Persistent innate and adaptive inflammatory responses within vascular lesions can contribute to generation of vulnerable plaques that occlude the local artery (Kullo, Edwards, & Schwartz, 1998) and epidemiologically, reduced lung function is a major risk factor for CVD (Hole, et al., 1996).

SAA is also produced by organs other than the liver and SAA transcript is increased in the lungs in response to cigarette smoke, LPS and Influenza virus infection (Bozinovski, et al., 2012). Additionally, SAA staining in COPD lungs was particularly intense in the submucosa and in regions where $CD68^{+}$ macrophages were localized. *In vitro* stimulation of THP-1 macrophages with the TLR4 agonist, LPS promoted expression of SAA. Strikingly, when macrophages were treated with the GC dexamethasone, there was a synergistic increase in SAA expression (Bozinovski, et al., 2012). This response is due to the presence of a GC responsive element (GRE) in the promoter region of SAA (Thorn, Lu, & Whitehead, 2003; Thorn & Whitehead,

2002) and the synergism between LPS and GCs suggest that activation of inflammatory transcription factors such as NFκB and the engagement of the GRE region are required for maximal expression. SAA synthesized in the local lung microenvironment may also generate HDL free aggregates, which unlike HDL bound SAA promote inflammatory cytokine production through activation of PRRs such as ALX/FPR2. Furthermore, extracellular matrix products such as heparin sulfate fragments have recently been shown to promote dissociation of SAA bound to HDLs under acidic conditions (Noborn, Ancsin, Ubhayasekera, Kisilevsky, & Li, 2012). Intriguingly, cigarette smoke exposure causes increased shedding and fragmentation of heparin sulfate (Yao, et al., 2010), which may further facilitate the formation of active SAA aggregates in the lung. The close proximity of ALX/FPR2, SAA and extracellular matrix proteoglycans in the submucosa of COPD lung favor this interaction and its actions are summarized in Figure 2. Although a number of SAA receptors have now been described including ALX/FPR2 (Su, et al., 1999), TLR2 (Cheng, He, Tian, Ye, & Ye, 2008), CD36 (Baranova, et al., 2010) and scavenger receptor class B type I (Cai, de Beer, de Beer, & van der Westhuyzen, 2005), mucosal epithelial inflammatory responses were predominately mediated via ALX/FPR2 (Bozinovski, et al., 2012).

## 6. SAA blocks resolution of inflammation mediated by ALX/FPR2

The temporal progression of acute inflammatory responses is normally counterbalanced by a secondary phase that includes i) induction of anti-inflammatory mediators that stop neutrophil infiltration and ii) active resolution processes that promote clearance of tissue inflammation and invading microbes with restoration of tissue integrity and function. Eicosanoids, such as lipoxins, and new families of endogenous mediators, termed resolvins and protectins are integral to the control of local inflammatory responses (reviewed in (Dufton & Perretti, 2010; Levy, et al.,

2012; Serhan, Chiang, & Van Dyke, 2008)). The resolution of inflammation involves a class switch of eicosanoid production from chemoattractants such as the leukotriene $LTB_4$ to pro-resolving mediators such as lipoxins (Levy, Clish, Schmidt, Gronert, & Serhan, 2001). There are two major routes to lipoxin biosynthesis involving cell-cell interactions (reviewed in (Chiang, et al., 2006; Serhan, 2005)). In the lung, mucosal epithelial surfaces can convert arachidonic acid to 15S-hydroxyleicosatetraenoic acid (15S-HETE) by the enzyme 15-lipoxygenase. This metabolite is then taken up by neutrophils and converted to $LXA_4$ and $LXB_4$, a positional isomer of $LXA_4$ by the enzyme 5-lipoxygenase (Serhan, 1997). In addition to ALX/FPR2, others receptors have been identified for $LXA_4$, including the intracellular aryl hydrocarbon receptor (AhR) (Schaldach, Riby, & Bjeldanes, 1999) and CysLT1 receptor (Gronert, Martinsson-Niskanen, Ravasi, Chiang, & Serhan, 2001). As these receptors are not reported to be SAA receptors, they are unlikely to directly counteract the pro-inflammatory actions of SAA.

$LXA_4$ blocks leukocyte diapedesis and initiates resolution of inflammation by targeting multiple cell types including leukocytes, mucosal epithelial and vascular cells (reviewed in (Serhan, 2005)). The neutrophil represents a prime target as $LXA_4$ suppresses tranendothelial (Papayianni, Serhan, & Brady, 1996) and transepithelial (Colgan, Serhan, Parkos, Delp-Archer, & Madara, 1993) migration, superoxide anion generation (Levy, et al., 1999) and azurophilic deganulation (Soyombo, Spur, & Lee, 1994). Its pro-resolving actions target alternative cell types including mucosal epithelial cells via suppression of cytokine release (Bonnans, et al., 2006), stimulation of intracellular calcium levels (Bonnans, Mainprice, Chanez, Bousquet, & Urbach, 2003) and wound healing proliferation following acid injury (Bonnans, et al., 2006). Furthermore, prostacyclin production from endothelial cells is increased in response to $LXA_4$ (Brezinski,

Gimbrone, Nicolaou, & Serhan, 1989). ALX/FPR2 activation can also coordinate neutrophil apoptosis, whereby SAA, in contrast to $LXA_4$ (El Kebir, et al., 2007) and AnnexinA1 (Perretti & D'Acquisto, 2009), provides a pro-survival signal (El Kebir, et al., 2007). In addition, secondary to the immunomodulatory actions of lipoxins on neutrophils, these counter-regulatory lipid mediators enhance macrophage function by stimulating nonphlogistic phagocytosis (El Kebir, et al., 2009; Godson, et al., 2000).

Since ALX/FPR2 can initiate counter-regulatory signaling during lung inflammation (Levy, et al., 2002), the relative expression of ALX/FPR2 agonists in the lung microenvironment can control the degree and persistence of inflammation (summarized in Figure 3). An imbalance in lipoxygenase-derived eicosanoid biosynthesis involving reduced $LXA_4$ relative to cysteinyl leukotrienes has been implicated in the persistence of inflammation in severe asthma (Levy, et al., 2005; Planaguma, et al., 2008). To investigate this in COPD, the circulating levels of the ALX/FPR2 receptor ligands, SAA and $LXA_4$ were measured when the patients were clinically stable and during AECOPD. Here, SAA was disproportionally increased relative to $LXA_4$ during an AECOPD (Bozinovski, et al., 2012). Hence, the persistence of inflammation may reflect an imbalance between inflammatory and pro-resolving mediators that target the ALX/FPR2 receptor. There are several plausible mechanisms that may explain the versatility of ALX/FPR2 receptors and its ability to modulate cell responsiveness in a ligand-biased fashion. This includes differential conformational ligand activation of specific receptor domains, the recruitment of ligand-specific signaling pathways or the formation of homologous and/or heterologous receptor dimers. Conformation is the most likely explanation because $LXA_4$ has been shown to activate ALX/FPR2 by interacting with extracellular loop III and the associated transmembrane region

(Chiang, Fierro, Gronert, & Serhan, 2000), whereas SAA initiates extracellular loops I and II dependent signaling (Bena, Brancaleone, Wang, Perretti, & Flower, 2012).

## 7. Concluding remarks

In summary, we have provided a comprehensive overview of the mechanisms that can cause persistent neutrophilic inflammation in COPD and AECOPD and the consequences of unregulated neutrophil activation in the airways. We have also provided a summary of the current and emerging therapies aimed at controlling excessive neutrophilic inflammation in this debilitating disease. Since there is a diverse suite of microbial and endogenous ligands that act on many PRRs to initiate airway inflammation, a targeted approach that neutralizes the actions of specific chemokines and/or chemokine receptors may have limited efficacy in blocking neutrophil migration in COPD. Furthermore, as PRR signaling intermediates are highly conserved and are shared amongst many pathogen sensing receptors, their inhibition may lead to destabilization of essential host defense mechanisms. COPD is also a heterogeneous disease and the success of new therapies will be dependent on distinguishing disease phenotypes in order to better identify populations that are more likely to respond. Here, we have focused on the promotion of resolution pathways via engagement of the ALX/FPR2 receptor as an alternative strategy to dampen neutrophilic inflammation in COPD and AECOPD.

ALX/FPR2 is the first receptor described to bind both peptide and lipid ligands and the nature of this dual recognition of structurally distinct ligands continues to be an area of considerable interest. Furthermore, as this GPCR interacts with microbial ligands and endogenous DAMPs that are known to be elevated in COPD airways, ALX/FPR2 represents a novel target for

therapeutic intervention in COPD and AECOPD. The role of SAA is discussed in detail as this important ancient and innate molecule is a potent ALX/FPR2 receptor ligand that i) promotes neutrophilic airway inflammation, ii) is elevated in COPD airways and iii) is increased in response to steroid treatment. The overarching hypothesis is that activation of the ALX/FPR2 receptor in a lung environment that is enriched for pro-inflammatory microbial and endogenous ligands overwhelm protective anti-inflammatory and pro-resolution ALX/FPR2 pathways. Addressing this imbalance via the use of synthetic agonists that mimic the natural resolving properties of lipoxins offers an alternative strategy to reducing damaging neutrophilic inflammation in COPD.

## 8. Conflict of Interest Statement

Dr. Levy is a co-inventor on patents on lipoxins in airway disease that are assigned to Brigham and Women's Hospital and licensed for clinical development. The remaining authors have no competing interests to disclose.

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**Figure Legends**

**Figure 1: Mechanism for self-sustaining neutrophilic inflammation in COPD.** Chronic smoke exposure in COPD leads to bacterial colonization and is further confounded by recurrent respiratory infections or exacerbations. Cigarette smoke constituents and microbial products activate the respiratory mucosa and resident macrophages leading to the increased expression of inflammatory cytokines such as CXCL8 (IL-8) and Serum Amyloid A (SAA), which are potent chemoattractants for neutrophils. Secondary to neutrophil recruitment is the generation of monocyte derived macrophages under the influence of locally derived mediators that normally resolve inflammation by clearing exhausted neutrophils and damaged tissue. As oxidative stress in COPD impairs the phagocytotic function of macrophages, the clearance of neutrophils is compromised. Exhausted neutrophils inappropriately release their cytosolic and granular content including cytosolic DAMPS such as S100A8 and degranulation products such as neutrophil elastase, which act on pathogen recognition receptors to further activate inflammatory pathways. In addition, proteolytic degranulation products cleave extracellular matrix proteins including elastin, whose fragments are also chemotactic for leukocytes. Collectively, these endogenously derived products can maintain neutrophilic inflammation that is not counteracted by current anti-inflammatory agents such as glucocorticosteroids.

**Figure 2. Schematic representation of SAA signaling in COPD.** Microbial products synergistically increase SAA production by macrophages in the presence of synthetic steroids during an infective exacerbation of COPD (AECOPD), which interact with mucosal epithelial ALX/FPR2 receptors. In the presence of extracellular matrix products such as heparan sulfate, SAA forms active aggregates in the submucosa that signal to pathogen recognition receptors. In addition to being a direct chemoattractant, SAA increases expression of neutrophil chemokines

(CXCL1/2/8) from epithelial cells and promotes survival of neutrophils. Both events are normally opposed by natural pro-resolving Lipoxins (LXs) via allosteric interaction with ALX/FPR2 receptors, which are produced through cell-cell interactions at the mucosal surface. In COPD and AECOPD, increased airway SAA expression relative to LXs may promote persistent neutrophilic inflammation by overwhelming ALX/FPR2 receptors.

**Figure 3. COPD airways are enriched for pro-inflammatory ALX/FPR2 receptor ligands.** Bacterial colonization, infective exacerbations and chronic tissue damage result in the enrichment of pro-inflammatory ALX/FPR2 ligands in the airways of COPD patients. They include endogenous host defense molecules such as SAA, LL-37 and uPAR, markers of tissue damage such as mitochondrial N-formylated hexapeptides and N-formylated bacterial peptides. The ALX/FPR2 receptor is expressed on neutrophils, where activation of this receptor with pro-inflammatory ligands promotes migration and survival and its activation on mucosal epithelial cells promotes increased expression of pro-inflammatory mediators. As a consequence of increased and sustained ALX/FPR2 ligand release, they may also overwhelm and counteract the pro-resolving and anti-inflammatory actions of Lipoxin$A_4$ and AnnexinA1 that normally switch off inflammation. In addition, lipoxins can promote increased phagocytosis by monocytes/ macrophages, which may be counteracted by the pro-inflammatory ALX/FPR2 ligands present in COPD airways. This imbalance in ALX/FPR2 receptor ligands may contribute to the persistent and damaging neutrophilic inflammation seen in COPD.

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**Figure 1**

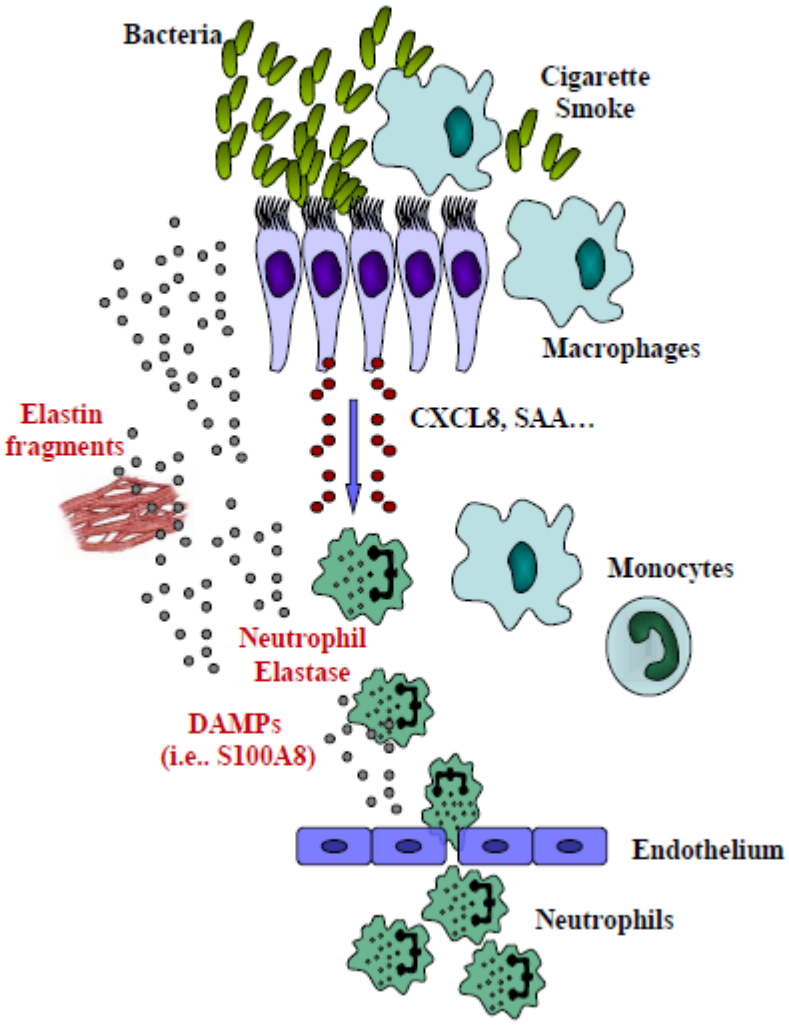


**Figure 2**

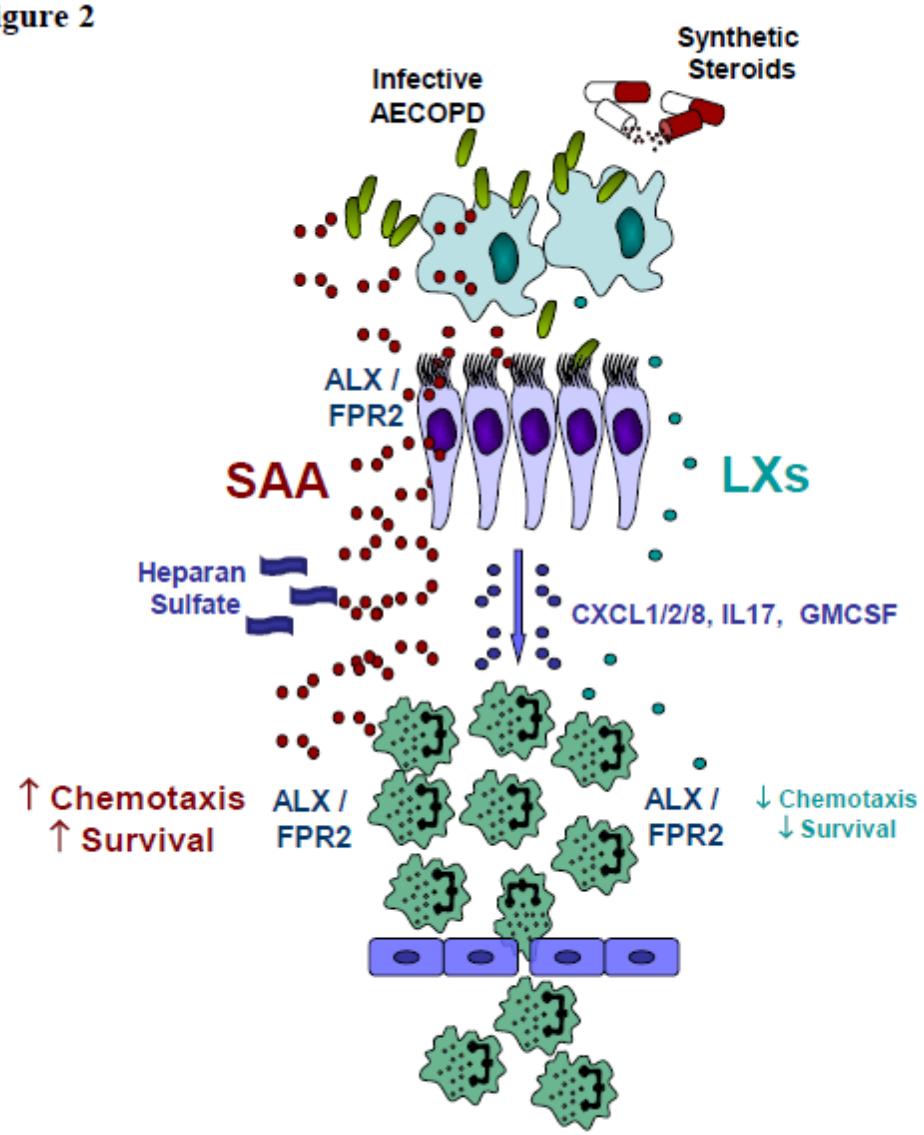
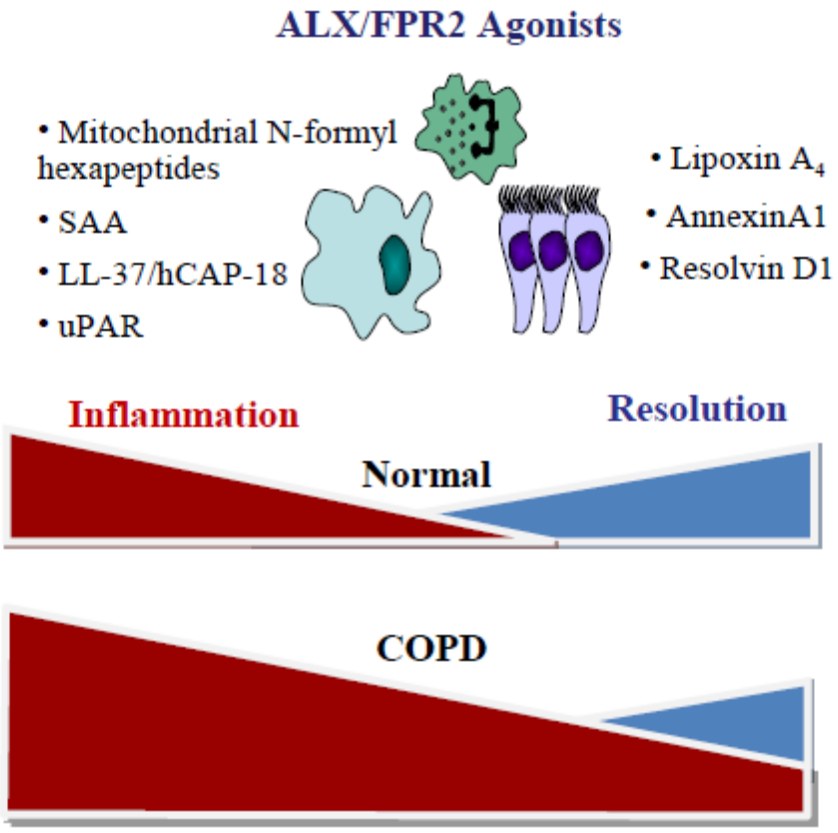


**Figure 3**



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