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Nonantimicrobial Actions of Macrolides: Overview and Perspectives for Future Development

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***Abstract*——Macrolides are among the most widely prescribed broad spectrum antibacterials, particularly for respiratory infections. It is now recognized that these drugs, in particular azithromycin, also exert time-dependent immunomodulatory actions that contribute to their therapeutic benefit in both infectious and other chronic inflammatory diseases. Their increased chronic use in airway inflammation and, more recently, of azithromycin in COVID-19, however, has led to a rise in bacterial resistance. An additional crucial aspect of chronic airway inflammation, such as chronic obstructive pulmonary disease, as well as other inflammatory disorders, is the loss of epithelial barrier protection against pathogens and pollutants. In recent years, azithromycin has been shown with time to enhance the barrier properties of airway epithelial cells, an action that makes an important contribution to its therapeutic efficacy. In this article, we review the background and evidence for various immunomodulatory and time-dependent actions of macrolides on inflammatory processes and on the epithelium and highlight novel nonantibacterial macrolides that are being studied for immunomodulatory and barrier-strengthening properties to circumvent the risk of bacterial resistance that occurs with macrolide antibacterials. We also briefly review the clinical effects of macrolides in respiratory and other inflammatory diseases associated with epithelial injury and propose that the beneficial epithelial effects of nonantibacterial azithromycin derivatives in chronic inflammation, even given prophylactically, are likely to gain increasing attention in the future.**

***Significance Statement*——Based on its immunomodulatory properties and ability to enhance the protective role of the lung epithelium against pathogens, azithromycin has proven superior to other macrolides in treating chronic respiratory inflammation. A nonantibiotic azithromycin derivative is likely to offer prophylactic benefits against inflammation and epithelial damage of differing causes while preserving the use of macrolides as antibiotics.**

I. Introduction

A. Background and Use of Macrolides

Macrolides are macrocyclic compounds defined by a core structural unit, the lactone ring, containing 12 to 16 carbons. Compounds with 14-, 15-, or 16-membered lactone rings, based on naturally occurring products, include the widely known antibacterial compounds erythromycin, azithromycin (AZM), and clarithromycin. Erythromycin (with a 14-membered ring) was the first macrolide to be isolated and identified from *Saccharopolyspora erythraea* after it exhibited antibacterial properties (Brittain, 1987). Since the discovery of erythromycin in 1952, several other safe semisynthetic derivatives have been made and are now in clinical use.

Slight modifications of the structure of erythromycin (introduction of a methoxy group or a methyl-substituted nitrogen in the lactone ring) improved the stability of the resulting clarithromycin and AZM in an acidic environment (stomach acid), leading to better absorption and increased effectiveness against bacteria. All these macrolides share a common mechanism of antibacterial action, binding to the bacterial 50s ribosomal subunit and inhibiting translation of mRNA, which results in similar activities against Gram positive bacteria and weak activity against Gram negative bacteria. Clarithromycin has superior activity against *Legionella* spp. and subtypes of *Chlamydia pneumoniae*, whereas AZM exerts greater activity against some Gram negative bacteria, including quorum-sensing bacteria such as *Pseudomonas aeruginosa*. The basic nature of AZM facilitates its faster penetration of the outer membrane of Gram negative bacteria, in which it also inhibits the generation of quorum-sensing molecules and biofilm formation (Tateda et al., 2007;

ABBREVIATIONS: Akt, Protein kinase B; ALI, air-liquid interface; ARDS, acute respiratory distress syndrome; AZM, azithromycin; BAL, bronchoalveolar lavage; BM, bone marrow; CAD, chronic airway disease; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; COPD, chronic obstructive pulmonary disease; COVID-19, coronavirus disease 2019; DC, dendritic cell; DEL-1, developmental endothelial locus-1; DPB, diffuse panbronchiolitis; EMT, epithelial-to-mesenchymal transition; ERK, Extracellular signal-regulated kinase; FEV1, forced expiratory volume within 1 second; GPx, glutathione peroxidase; HNE, human neutrophil elastase; IBD, inflammatory bowel disease; ICS, inhaled corticosteroid; ICU, intensive care unit; IL, interleukin; IPF, idiopathic pulmonary fibrosis; LABA, long-acting β-2 agonist; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; MUC5AC, mucin 5AC; MVB, multivesicular body; NF-κB, nuclear factor kappa B; NLRP3, NOD-, LRR-, and pyrin domain-containing 3; NTMB, nontuberculous mycobacteria; p-flux, paracellular flux; PI3K, phosphoinositide 3-kinase; ROS, reactive oxygen species; RSV, respiratory syncytial virus; RV, rhinovirus; SARS-CoV-2, severe acute respiratory distress syndrome - corona virus-2; SASP, senescence-associated secretory phenotype; TEER, transepithelial electrical resistance; Th, T helper; TJ, tight junction; TNFα, tumor necrosis factor α.

Imperi et al., 2014; Parnham et al., 2014; Kruger and Prathapan, 2020). The broad antibacterial activities of macrolides have led to their widespread use for infections of the gastrointestinal tract, ear, eyes, teeth and skin; for sexually transmitted diseases; and particularly for respiratory infections. Many of the macrolide-susceptible microorganisms are respiratory pathogens known to be associated with exacerbations of asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis (CF), and AZM in particular has been widely used to treat respiratory diseases, often beyond its acute use as an antimicrobial agent (Blanchard and Raherison, 2010; Butorac-Petanjek et al., 2010; Brusselle et al., 2013; Uzun et al., 2014; Kiser and Vandivier, 2015; Principi et al., 2015; Gibson et al., 2017; Mayhew et al., 2018; Vermeersch et al., 2019; Bush, 2020; Reijnders et al., 2020). These long-term effects of macrolides in the treatment of respiratory diseases are discussed in more detail below.

It has long been accepted that macrolides in general, as a class of closely related compounds, exhibit a diverse range of properties with confirmed activity in prokaryotic microorganisms, as well as in eukaryotic mammalian cells, with effects on inflammatory and immune cells, mucus secretion, and epithelial cell differentiation (Kanoh and Rubin, 2010; Parnham et al., 2014). In fact, anti-inflammatory and immunomodulatory actions contribute to the therapeutic benefit of macrolides in most of the infectious disorders for which they are approved, as well as leading to their use in other chronic inflammatory conditions that do not have a primarily infectious etiology (Kwiatkowska and Maslinska, 2012; Steel et al., 2012; Parnham et al., 2014; Reijnders et al., 2020; Oliver and Hinks, 2021).

AZM was introduced to the market in 1981 and is the only clinically approved 15-membered macrolide. Subsequently, AZM has over time proven its superiority to other available macrolides as a safe disease-modifying agent for long-term treatment of chronic inflammatory airway diseases (Kruger and Prathapan, 2020). This includes COPD, in which long-term treatment with AZM reduces the exacerbation rate by 30% on an annual basis (Albert et al., 2011; Uzun et al., 2014).

However, the long-term use of AZM and macrolide antibiotics in general is limited by the induction of bacterial resistance, mainly as a result of the induction of the macrolide efflux transporter macrolide efflux protein A [see Gibson et al. (2017) and Vermeersch et al. (2019) for examples]. Therefore, there is an immediate need for new nonantibacterial macrolides that encompass similar effective disease-modifying properties to AZM but which can be used to treat chronic diseases without the risk of bacterial resistance. This is particularly important to maintain AZM as a valuable antibiotic to be used for serious infections that otherwise would be difficult to treat. Thus, even though AZM is now in the GOLD guidelines for the prevention of exacerbations of COPD (https://goldcopd.org/2021-gold-reports), the use of macrolides for long-term treatment of chronic diseases is an off-label use that must be restricted to ensure AZM is available for its approved use as an antibiotic when required (Kruger and Prathapan, 2020).

B. Beyond Antibiosis

The recognition that macrolides possess direct anti-inflammatory and/or immunomodulatory properties, independent of their actions on bacteria, dates back to the 1960s. At this time, several reports by Kudoh and colleagues in Japan appeared demonstrating dramatic decreases in disease activity and mortality among patients with the inflammatory lung disease diffuse pan bronchiolitis (DPB) as a result of treatment with the macrolide antibiotic erythromycin (Kudoh, 2004). DPB is a chronic disease of the airways with diffuse inflammation of the respiratory bronchioles, sinusitis, and chronic productive cough, closely associated with HLA-A11 or HLA-B54, and often exacerbated by *P. aeruginosa* infection in the lungs (Azuma and Kudoh, 2006). Untreated, the disease is fatal in 50% of patients within 5 years of diagnosis. Chronic treatment with erythromycin is effective in 90% of patients with DPB. This observation led to the conclusion that erythromycin can have direct suppressive effects on the immune system and inflammatory cascades in addition to its antimicrobial activity. These findings opened up a whole new research field regarding nonantimicrobial actions of macrolides with the main research focus being the identification of macrolide effects on inflammatory cascades and immune cell function. However, to date, there has been limited success in defining the essential molecular mechanisms underlying these activities (Shinkai et al., 2008; Kanoh and Rubin, 2010; Altenburg et al., 2011a; Reijnders et al., 2020; Steel et al., 2012; Parnham et al., 2014; Zimmermann et al., 2018; Yang, 2020). Yet, based on all these investigations, it is now increasingly appreciated that the beneficial effects of macrolides encompass antibacterial, antiviral, and anti-inflammatory activities; restoration of steroid sensitivity; and in the case of AZM, restoration of epithelial integrity. In this review, we discuss the nonantibiotic effects of macrolides, relevant mechanisms of action, and clinical uses of macrolides beyond treatment of infections, with an emphasis on respiratory tract inflammation and their potential role as respiratory epithelial barrier-enhancing drugs. We also discuss attempts to develop novel anti-inflammatory macrolides without antibacterial actions that have been referred to as “immunolides” (Fecik et al., 2005; Steel et al., 2012), as well as corticoid-macrolide conjugates that have also been termed “sterolides” in a single publication (Tomašković et al., 2013). Finally, we highlight the recent development of “barriolides,”

novel, nonantibacterial macrolides selected for their barrier-protecting actions on epithelial cells.

II. Nonantibiotic Biologic Effects of Macrolides

Macrolide antibiotics exert an almost bewildering number of biologic effects on a variety of mammalian cells, including fibroblasts, epithelial cells, endothelial cells, neutrophils, macrophages, and dendritic cells. Many of the cellular functions affected are highly relevant to host defense and inflammation, particularly in relation to inflammatory diseases of the airways. These anti-inflammatory/immunomodulatory effects of macrolides have been extensively reviewed by different authors (Gemmell, 1993; Labro, 1998, 2000; Wales and Woodhead, 1999; Rubin and Tamaoki, 2000; Culic et al., 2001; Labro and Abdelghaffar, 2001; Tamaoki et al., 2004; Parnham, 2005; Lopez-Boado and Rubin, 2008; Shinkai et al., 2008; Kanoh and Rubin, 2010; Altenburg et al., 2011a,b; Steel et al., 2012; Bartold et al., 2013; Parnham et al., 2014; Zimmermann et al., 2018; Bosnar et al., 2019; Kruger and Prathapan, 2020; Reijnders et al., 2020; Oliver and Hinks, 2021) and are discussed further below.

A. Anti-Inflammatory/Immunomodulatory Effects

After oral administration, macrolides, and particularly AZM, accumulate in a variety of cells and tissues, including fibroblasts, epithelial cells, and white blood cells (Matzneller et al., 2013). The accumulation of AZM is particularly pronounced in phagocytes, achieving intracellular concentrations several-hundred-fold higher than in plasma, suggesting that these cells may act as an endogenous carriers for circulating drug delivery to infected and inflamed sites (Ballow et al., 1998; Matzneller et al., 2013; Parnham et al., 2014). As shown in human neutrophils, macrophages, and epithelial cell lines, AZM accumulates intracellularly much more than other macrolides and is released more slowly from these cells (Bosnar et al., 2005). Uptake appears to be due to a combination of active and passive mechanisms, and as with other cationic amphiphilic drugs, AZM accumulates extensively in lysosomes (Parnham et al., 2014). The actions of AZM on lysosomes are discussed in section *B. Interactions with Lipids* below.

1. Cytokine and Chemokine Release. Among the earliest discoveries on the immunomodulatory actions of macrolides was the demonstration that erythromycin and other macrolides modulate the bacterial lipopolysaccharide (LPS)-induced release of interleukin (IL)-1β, IL-6, IL-10, tumor necrosis factor α (TNFα), and granulocyte-monocyte colony stimulating factor from human monocytes *in vitro* (Bailly et al., 1991; Morikawa et al., 1996). Subsequently, erythromycin was found to inhibit IL-8 production from *P. aeruginosa*–stimulated neutrophils, which provided a possible explanation for inhibition by the macrolide of increased neutrophil counts in bronchoalveolar lavage (BAL) fluid of patients with *P. aeruginosa* infection and also in patients with DPB, since the chemokine IL-8 is an effective chemotactic agent for neutrophils (Oishi et al., 1994). The effects of the different macrolides in the studies by Bailly et al. (1991) varied considerably, from no effect to inhibition or even enhancement, possibly due to the different experimental conditions used. Subsequent studies have demonstrated that, in the majority of white blood cells and tissue cells *in vitro*, macrolides inhibit the inflammagen-induced release of all investigated proinflammatory cytokines while enhancing the release of anti-inflammatory IL-10 from human macrophages (Culic et al., 2001; Vrančić et al., 2012; Bartold et al., 2013). Proinflammatory cytokines are now widely used as reliable markers for the anti-inflammatory effects of macrolides, both in animal models and in patients with inflammatory airway disease (Reijnders et al., 2020). In addition to IL-8 being an important disease-relevant target for macrolides, IL-1β generated by alveolar macrophages also appears to play a crucial role as a target in pulmonary inflammation, as it is able to contribute to an accumulation of neutrophils in the lungs in experimental models of inflammation (Bosnar et al., 2009; Bosnar et al., 2011; Gualdoni et al., 2015). Importantly, AZM, but not clarithromycin or roxithromycin, has been reported to inhibit the LPS-sensing caspase-4 in inflammasomes, which generates IL-1β from LPS-stimulated human monocytes (Gualdoni et al., 2015). Inhibitory effects on proinflammatory cytokine release undoubtedly account, either directly or indirectly, for many beneficial effects of macrolides in inflammatory airway diseases. In this connection, it is worth mentioning that in smokers with emphysema, 8 weeks' treatment with AZM enhanced the BAL fluid concentration of bacterial metabolites, including glycolic acid, indol-3-acetate, and linoleic acid, all of which inhibited LPS-induced cytokine release from alveolar macrophages *ex vivo* (Segal et al., 2017). Some anti-inflammatory effects of AZM may, thus, be mediated by functional modification of the lung microbiome, resulting in generation of anti-inflammatory bacterial metabolites (Dickson and Morris, 2017).

2. Leukocyte Adhesion and Diapedesis. From the outset of studies investigating the anti-inflammatory and immunomodulatory effects of macrolides, it was clear that experimental models and clinical diseases with neutrophil dominance are among the most responsive to this class of drugs. A regular finding in all *in vivo* experimental studies and in patients with inflammatory lung diseases treated with macrolides, including AZM, is the clear inhibition of neutrophil infiltration into inflammatory sites (Parnham et al., 2014; Zimmermann et al., 2018; Reijnders et al.,

2020). It has been consistently demonstrated that AZM will reduce the levels of the important neutrophil chemoattractant IL-8 (CXCL8) in sputum, BAL fluid, or nasal secretions (Simpson et al., 2014; Zimmermann et al., 2018). Similar observations have been made in the serum of healthy volunteers treated with AZM (Culic et al., 2002). The collagen-derived neutrophil chemotactic peptide proline-glycine-proline (PGP) was also shown to be reduced in sputum of patients with COPD treated with AZM for 9–12 months (O'Reilly et al., 2013).

Using leukocytes isolated from sputum obtained from patients with COPD, AZM effectively inhibited the release not only of inflammatory cytokines but also of several chemokines chemotactic for neutrophils, monocytes, and lymphocytes (Marjanovic et al., 2011). Interestingly, in this sputum leukocyte population obtained from patients with COPD, the marked in vitro release of the important neutrophil chemotaxin IL-8 was not inhibited by AZM, and in the serum of patients with COPD, IL-8 concentrations were hardly reduced by AZM treatment (Parnham, 2005). At least with regard to IL-8 release, it is probable that another cellular target for the macrolides may be involved. It is of interest, therefore, that epithelial cells have repeatedly been shown to generate IL-8, and this release is inhibited in vitro by macrolides (Kanoh and Rubin, 2010). Thus, it seems likely that with regard to generation of neutrophil-chemotactic IL-8, epithelial cells are also a potential target for macrolides. On the other hand, epithelial cell lines derived from patients with CF have been reported to be relatively insensitive to inhibition of IL-8 release by AZM (Blau et al., 2007; Saint-Criq et al., 2012). Patients with CF frequently suffer from infection of the airways with *P. aeruginosa*. In normal human bronchial epithelial cells stimulated with *P. aeruginosa*–derived flagellin, clarithromycin exerted extracellular signal-regulated kinase (ERK)- and time-dependent effects on IL-8 release (Shinkai et al., 2007). By itself, the macrolide slightly enhanced IL-8 release after 4 hours and then inhibited IL-8 release after 9–18 hours. In flagellin-stimulated cells, IL-8 release was continually stimulated for the whole 24-hour incubation period, with clarithromycin only inhibiting the release at 4–9 hours and losing its inhibitory effect thereafter, suggesting a possible delayed loss of sensitivity of epithelial cell IL-8 release to macrolides in the presence of *P. aeruginosa*. The role of IL-8 as a neutrophil attractant, thus, appears to differ between inflammatory lung diseases.

It has been known for many years that macrolides inhibit the expression of adhesion molecules on both neutrophils and on endothelial cells, preventing leukocyte adhesion to the endothelium prior to transendothelial migration into the tissue (diapedesis). This has been shown, for instance, for L-selectin and macrophage-1 antigen on neutrophils and for intercellular adhesion moleculate-1 (ICAM-1) on endothelial cells (Culic et al., 2001; Kanoh and Rubin, 2010). It is likely, though, that these actions of macrolides may be indirect subsequent to inhibition of proinflammatory cytokine generation (Bartold et al., 2013). In a recent publication (Maekawa et al., 2020), induced deficiency of the integrin-binding secreted protein developmental endothelial locus-1 (DEL-1) was reported to prevent the inhibitory effect of erythromycin on LPS-induced inflammatory neutrophil infiltration into BAL fluid in mice. Soluble DEL-1 expressed by endothelial cells inhibits the adhesion of neutrophils to the endothelium and suppresses recruitment of neutrophils from the bone marrow (BM); it also reprograms tissue macrophages to an M2 proresolving phenotype, and DEL-1 is downregulated during inflammation (Hajishengallis and Chavakis, 2019). The expression of DEL-1 in human umbilical endothelial cells in vitro is suppressed by IL-17, and this was reversed by treatment with erythromycin (Maekawa et al., 2020). These data indicate an important contribution of endothelial DEL-1 induction to the anti-inflammatory effects of macrolides.

3. Phagocytosis. A major function of both polymorphonuclear leukocytes and the mononuclear phagocytes, such as macrophages and dendritic cells, is their ability to engulf particles, including microbial pathogens, by phagocytosis, degrading and processing them in the resulting phagosomes. Here, particles are broken down by the action of reactive oxygen species (ROS) and degradative enzymes released from neutrophil granules, all of which, as discussed below, can be released extracellularly as well. Although macrolides clearly influence the exocytosis of neutrophil granules, as discussed below, there is little or no evidence of direct effects of these drugs on neutrophil phagocytosis (Pohl et al., 2020). The situation is very different with macrophages. Several macrolides (AZM, erythromycin, clarithromycin, and roxithromycin) were first shown to stimulate the subsequent phagocytosis of latex beads by cells of the J774.1 macrophage cell line after 3 hours of pretreatment with the drugs (Xu et al., 1996). Importantly, the response was determined after a total 7-hour incubation and so was subject to a delay. Phagocytosis is a characteristic of the inflammation-resolving M2 macrophage phenotype, and the effects of macrolides on the generation of different macrophage phenotypes are discussed below in relation to inflammatory cell differentiation and maturation.

4. Reactive Oxygen Species. The generation of ROS by the neutrophil NADPH oxidase is a central microbicidal mechanism, together with phagocytosis, myeloperoxidase, degradative enzyme release, and neutrophil extracellular traps, in the host defense response of the innate immune system (Nguyen et al.,

2017). This generation of ROS and other inflammatory products by neutrophils is enhanced by prior priming of the cells with cytokines or other inflammatory stimuli, acting to enhance cell surface expression of relevant receptors (Nguyen et al., 2017). Highly reactive ROS such as hydroxyl and superoxide radicals, together with peroxides, however, also damage surrounding tissues and promote inflammation. The presence of antioxidant mechanisms, including superoxide dismutase, catalase, peroxiredoxins, and glutathione peroxidase (GPx), is crucial to protect cells from injury and the neutrophils themselves from autolysis (Boukhenouna et al., 2018). Therapies that dampen or ameliorate such ROS release generally exhibit anti-inflammatory activity.

It is of interest therefore, that in addition to their effects on cytokines, discussed above, macrolides have been found to inhibit a variety of activated neutrophil functions *in vitro*, including the oxidative burst, which is also inhibited in macrophages (Culic et al., 2001; Parnham et al., 2014; Reijnders et al., 2020). In pilot studies in patients with either community acquired pneumonia or COPD, however, AZM treatment did not markedly affect the respiratory burst of circulating neutrophils studied *ex vivo* (Parnham et al., 2005; Arnold et al., 2016). This could either be due to reduced sensitivity of inflammation-primed neutrophils to macrolides or to these cells only being inhibited *in situ* at the inflamed site. However, in the study in patients with COPD, a prolonged increase in the GPx activity of circulating neutrophils could be seen after 3 days of AZM treatment, indicating that oxidative defense in these treated cells was enhanced (Parnham et al., 2005).

Clarification of the potential effect of AZM on human neutrophil function came, surprisingly, from a study on healthy human volunteers, intended as a prior control study for the later study in patients with COPD. Using the same 3-day treatment period as in the subsequent study in patients with COPD, AZM in the healthy volunteers initially resulted (in parallel with similar changes in lysosomal enzyme release) in a short-term enhancement of the circulating neutrophil oxidative burst, associated with a decrease in GPx activity, followed by a late decrease in the oxidative burst with restoration of GPx activity after 4 weeks (Culic et al., 2002). Circulating cytokines were inhibited at all time points. It was proposed that acute stimulation of naıve human neutrophils by AZM would promote antibacterial activity and that a later prolonged inhibition would promote protection of surrounding tissue with resolution of inflammation. Interestingly, *in vitro* intracellular killing of *Staphylococcus aureus* by human blood neutrophils has indeed been shown to be enhanced by AZM in a time-dependent manner but without enhancing extracellular H_2O_2 release or cell autolysis (Silvestri et al., 1995). Other time-dependent effects of macrolides will be addressed in subsequent sections of this review.

5. Inhibitory Effects on Inflammatory Enzyme Release. Since AZM and other macrolides accumulate in lysosomes (see section *A. Actions on Lysosomes, Apoptosis, and Autophagy*), it is not surprising that they have been shown to inhibit the release of lysosomal enzymes from phagocytes. Indeed, one of the first experimental demonstrations of the anti-inflammatory effects of AZM was that it inhibited adjuvant arthritis in rats and reduced the concentration of lysosomal enzymes in synovial fluid (Carevic and Djokic, 1988). AZM inhibited neutrophil release of these enzymes from azurophilic granules *in vitro* without affecting enzyme activity. Erythromycin was less potent in both tests. Surprisingly, though, many studies on neutrophils *in vitro*, particularly by the group of Marie-Therese Labro in Paris, showed that, like several other classes of antibiotics, short-term exposure to macrolides stimulated the degranulation of these cells, releasing lysosomal and other degrading enzymes and promoting short-term host antibacterial defense (Culic et al., 2001). The study on AZM treatment of healthy human volunteers, discussed above in relation to ROS, also clarified this anomaly, showing that over the first few days of treatment, neutrophil lysosomal enzyme release was indeed enhanced, but after 4 weeks, release was inhibited (Culic et al., 2002). As for neutrophil ROS release, AZM treatment failed to reduce release of lysosomal enzymes in patients with COPD (Parnham et al., 2005). Thus, similar to the oxidative burst, inhibition of degranulation of neutrophils is time-dependent and modified by pre-existing inflammation.

However, neutrophil secretory granules contain many potent degradative enzymes and bactericidal proteins, such as myeloperoxidase, elastase, cathepsin, collagenase, neuraminidase, heparanase, defensins, and cationic antimicrobial proteins (Culic et al., 2001). Human neutrophil elastase (HNE) is an effective stimulator of mucus secretion by bronchial epithelial cells, which is inhibited by AZM (see section *B. Inhibition of Mucus Secretion*). In a murine ovalbumin-challenge, chronic asthma model, HNE and IL-8 concentrations were both reduced by long-term treatment with AZM, in parallel with inhibition of pulmonary mitogen-activated protein kinase (MAPK)/nuclear factor kappa B (NF-κB) signal pathways and airway inflammation (Kang et al., 2016). HNE activity is also reduced together with levels of IL-8 in nasal secretions, sputum, and BAL fluid obtained from patients with inflammatory airway diseases treated with macrolides (Zimmermann et al., 2018), suggesting that the inhibition of both these and other granule enzymes is associated with inhibition of neutrophil granulocytosis. The release of neutrophil extracellular traps (consisting of fibers of decondensed chromatin bound to

antimicrobial proteins and histones) from neutrophils is also inhibited by AZM in vitro in association with inhibition of degranulation and the oxidative burst, suggesting that the signaling pathways (see section *C. Modulation of Cell Signaling*) for these processes converge (Bystrzycka et al., 2017).

6. Cell Differentiation and Maturation. As stated at the beginning of section *II. Nonantibiotic Biologic Effects of Macrolides*, macrolides affect the functions of a variety of cells involved in inflammatory responses. Although effector functions of neutrophils are modulated, there is no indication that macrolides directly affect the generation or differentiation of these short-lived cells apart from inhibiting the release of neutrophil mobilizing and survival-promoting granulocyte-monocyte and granulocyte colony stimulating factor from inflammatory cells (Parnham et al., 2014; Reijnders et al., 2020). On the other hand, macrolides do modulate the differentiation and maturation of mononuclear phagocytes, endothelial and epithelial cells, and fibroblasts (Kanoh and Rubin, 2010).

Already in 1994, erythromycin was found to stimulate the differentiation of human monocytes and THP-1 cells (a human monocytic cell line derived from an acute monocytic leukemia patient) to macrophages (Keicho et al., 1994), and this has been observed for several macrolides, particularly AZM, in both human and murine cells (Kanoh and Rubin, 2010). Of greater relevance to inflammatory diseases is that macrolides modulate the macrophage phenotype, inhibiting the activation of inflammatory M1 macrophages and facilitating the generation of resolution-promoting M2 macrophages (Parnham et al., 2014). This has been demonstrated in vitro with cells from different sources, including human, and in experimental models of lung inflammation in rodents (Hodge et al., 2006; Murphy et al., 2008; Feola et al., 2010; Vrančić et al., 2012; Shirey et al., 2014). Thus, in zymosan-induced inflammation, AZM treatment enhanced the in vivo generation of M2 macrophages in association with facilitated resolution of the inflammation (Navarro-Xavier et al., 2010). In patients with COPD, AZM given for several months was found to enhance defective phagocytosis of bacteria and the efferocytosis of apoptotic airway epithelial cells by alveolar macrophages (Hodge and Reynolds, 2012). However, although other authors observed deficient phagocytosis of bacteria by alveolar and monocyte-derived macrophages from patients with COPD, they reported that short-term incubation with AZM in vitro had no effect on these cells (Taylor et al., 2010).

These findings emphasize the repeated observation in various experimental systems that the resolving effect of macrolides, including AZM on inflammation, requires time to appear. For instance, the promotion by AZM, but not by a nonsteroidal anti-inflammatory agent, ibuprofen, of the resolution of zymosan-induced inflammation in the peritoneal cavity of mice 72 hours after initiation of inflammation, occurred at a time when M2 macrophage-associated resolution was in progress (Navarro-Xavier et al., 2010). In a murine model of focal brain ischemia, AZM treatment given up to 4.5 hours after injury markedly reduced blood-brain barrier permeability and brain injury in association with polarization of microglia/macrophages to the M2 phenotype in the brain (Amantea et al., 2016). A similar significant increase in the infiltration of M2 macrophages into the inflamed lungs and marked inhibition of infection and inflammation was induced from day 7 of infection by daily treatment with AZM, given from 4 days before infection (Feola et al., 2010). There is thus, strong evidence that the resolving effect of AZM on inflammation is delayed and associated with M2 macrophage appearance.

A further inhibitory action of AZM has been observed on the maturation and activation of dendritic cells (DCs). In human peripheral blood cells differentiated by IL-4 and/or granulocyte-monocyte colony stimulating factor in vitro, AZM generated a unique DC phenotype with enhanced adherence, CD86 costimulatory molecule expression, and increased IL-10 release (Polancec et al., 2012). AZM also modulated LPS-induced maturation and activation of these DCs, generating cells with regulatory properties, exhibiting reduced costimulatory surface molecules, major histocompatibility complex-II molecules, and IL-6 and IL-12 release but enhanced phagocytosis and efferocytosis and IL-10 release and lowered ability to induce a mixed-lymphocyte reaction (Polancec et al., 2012). Preincubation of murine bone marrow–derived DCs with AZM also inhibited expression of costimulatory surface molecules and major histocompatibility complex-II molecules and release of IL-12, indicating inhibition of maturation to effector DCs. When allogeneic T lymphocytes were added to the AZM-treated DCs to induce a mixed-lymphocyte reaction, T-cell proliferation and release of interferon-γ were inhibited and IL-10 release was enhanced, indicating that AZM is able to inhibit effector T-cell proliferation by acting on DCs (Iwamoto et al., 2011). Subsequent in vivo studies in mice revealed that 5-day pretreatment of recipient mice with AZM inhibits the generation of T-cell–mediated graft-versus-host disease, in terms of histopathology and survival, after allogeneic BM transplantation (Iwamoto et al., 2013; Radhakrishnan et al., 2015). In the study by Radhakrishnan et al. (2015), the effect of AZM was associated with reduced antigen-presenting properties of DCs and expression of effector T lymphocytes in parallel with expansion of T regulatory cells. Comparable results on inhibition by AZM of imiquimod-induced psoriatic lesions in mouse skin were also associated with decreased T helper-17 cell-derived cytokines and

DC infiltration of the skin, together with inhibition of costimulatory molecule expression and release of effector cytokines by BM-derived DCs and of interferon-α by plasmacytoid DCs (Huang et al., 2016). Consequently, by actions on DC differentiation and maturation, AZM appears able to inhibit subsequent activation of effector T cells, at least in some types of inflammation [see also Bartold et al. (2013)]. This may also account for the reports that AZM was able to inhibit antibody production in mice receiving *Streptococcus pneumoniae* vaccine (Fernandez et al., 2004) and some T-cell responses in both animals and patients (Ivetić Tkalcević et al., 2012; Steel et al., 2012). Erythromycin has also been shown to inhibit the later phase (after 48 hours) of human lymphocyte proliferation induced by the mitogen concanavalin in vitro, although the mechanism was unclear (Keicho et al., 1993).

Macrolides can also affect the growth and differentiation of tissue cells such as epithelial cells and fibroblasts, which can play active roles in the generation of an inflammatory response. AZM has been reported to promote differentiation of human epithelial cells from the meibomian gland of the eye and those derived from airway epithelium (Liu et al., 2015; Arason et al., 2019). The latter cells are discussed in more detail in section *D. Barrier Integrity* in relation to regulation of barrier function. There is some evidence that macrolides can inhibit myofibroblast differentiation and fibroblast proliferation directly, whereas clarithromycin, but not AZM, may inhibit fibroblast migration (Kanoh and Rubin, 2010; Tsubouchi et al., 2017; Gouzos et al., 2020). To what degree these actions may affect fibrotic changes subsequent to inflammation is unclear. At least AZM has been shown to inhibit experimental bleomycin-induced pulmonary fibrosis in mice (Wuyts et al., 2010). It has also been documented that AZM can inhibit epithelial-to-mesenchymal transition (EMT) in lung epithelium (Banerjee et al., 2012; Pu et al., 2018), further supporting its role as an epithelial barrier–protecting drug. Macrolides are also able to inhibit the release by fibroblasts of vascular endothelial growth factor, and this may contribute to inhibitory effects of clarithromycin on angiogenesis during chronic inflammation (Yatsunami and Hayashi, 2001; Kanoh and Rubin, 2010; Uehara et al., 2016).

7. Animal Models and Inflammation Resolution. Macrolides have been shown to inhibit inflammatory responses in a wide range of experimental animal models, both infective and noninfective, particularly pulmonary and airway inflammatory models (Culic et al., 2001; Lopez-Boado and Rubin, 2008; Amado-Rodriguez et al., 2013; Bartold et al., 2013; Parnham et al., 2014). Actions observed include inhibition of airway inflammation and tissue injury with long-term reduction of lung remodeling and fibrosis (Lee et al., 2015; Kang et al., 2016; Hung et al., 2019), modulation of epithelial ion transport, and inhibition of both mucus production and epithelial permeability. Some of these later effects may be mediated, in part, by inhibition of inflammation.

In infective models, as in infected patients, the inhibitory response to the antibiotics is usually rapid, mainly due to their direct antibacterial activity. However, optimal effects of macrolides on noninfective inflammatory models generally require either pretreatment before administration of the inflammagen and/or therapeutic treatment of several days or weeks (Culic et al., 2001). Anti-inflammatory effects can be observed after acute administration, but only in acute inflammatory models or at high doses in more prolonged inflammatory models. This distinction is illustrated by studies on bacterial LPS given by different routes in mice (Ivetić Tkalcević et al., 2006). Single oral AZM dosing (10–100 mg/kg) inhibited acute inflammation induced by intravenous and intraperitoneal LPS but enhanced more prolonged lung inflammation induced by intranasal LPS. This latter finding is likely to be a reflection of the biphasic, stimulatory, and then inhibitory effect of AZM on inflammatory cells, which was discussed previously and observed in blood neutrophils obtained from healthy human volunteers treated for 3 days with AZM (Culic et al., 2002). In fact, such stimulatory effects of macrolides on neutrophils and macrophages in vivo have also been observed in normal healthy mice and guinea pigs by several authors (Culic et al., 2001; Parnham et al., 2014), indicating that this distinction between effects of the drugs under healthy and inflamed conditions is a general phenomenon.

The fact that the inhibitory effects of AZM and other macrolides on inflammatory conditions are delayed confirms the studies discussed above showing that the anti-inflammatory effects of macrolides are closely associated with the induction of M2 macrophages and the active resolution of inflammation. It would be interesting to investigate whether AZM is also able to induce the generation of the proresolving lipid mediators lipoxin A4 and resolvin D1, which has been observed in pig leukocytes incubated with the veterinary anti-inflammatory macrolide antibiotic tylvalosin (Moges et al., 2018). Assay of such products might represent a potential clinical biomarker for monitoring the resolution of inflammation by macrolides. Moreover, as the expression of the proresolving DEL-1 is enhanced by erythromycin (Maekawa et al., 2020), as discussed above, M2 macrophages, proresolving lipid mediators, and DEL-1, as well as epithelial barrier promotion, would all appear to be involved in the inflammation-resolving actions of macrolides in vivo. The role of macrolides in inflammation is summarized in Fig. 1.

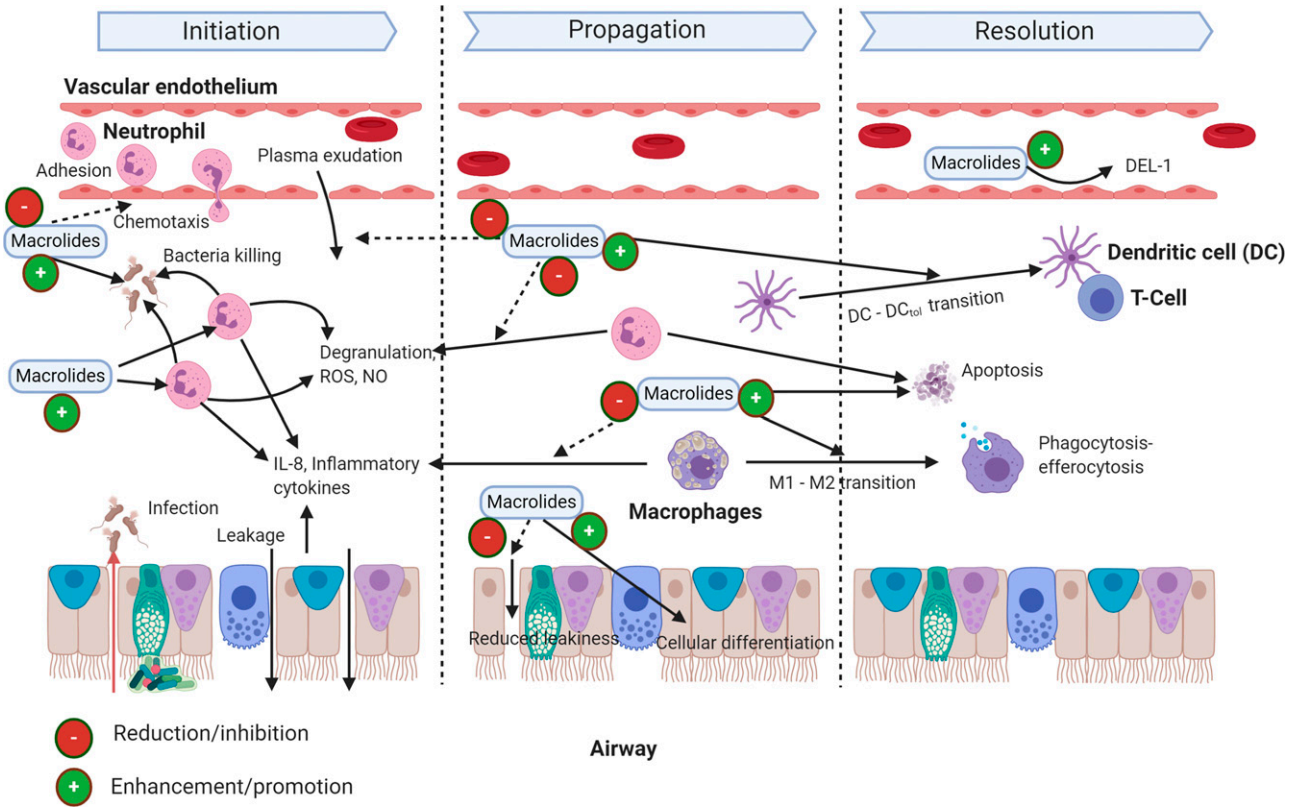

Fig. 1. Functional role of macrolides at different stages of resolution of inflammation. Pathogens such as bacteria and viruses invade through the epithelium into the underlying stroma. Proinflammatory cytokines released from the epithelium and by invading pathogens initially attract neutrophils to initiate an inflammatory response, involving other inflammatory cells such as macrophages and dendritic cells. The presence of macrolides in phagocytic cells in the inflamed or infected tissue ensures a prolonged effect. This is best exemplified by the long half-life of AZM, which has a number of time-dependent disease-modifying effects, including promotion of initial neutrophil bacterial killing, attenuation and subsequent resolution of chronic inflammation, and enhancement of epithelial barrier integrity. The macrolide erythromycin has been shown to inhibit the further diapedesis of neutrophils by upregulating DEL-1 in endothelial cells to help resolve the inflammatory process. DC_{tol}, tolerizing dendritic cell.

B. Inhibition of Mucus Secretion

The ability of macrolides to inhibit mucus secretion from secretory cells in the airways was first shown with erythromycin (Goswami et al., 1990; Tamaoki et al., 1996) and subsequently confirmed both in vitro and in vivo with other macrolides (Tamaoki et al., 2004; Kanoh and Rubin, 2010). The effect of macrolides on mucus secretion seems to be mediated in part by inhibition of the cytokine induction of mucin 5AC (MUC5AC) gene expression. Shimizu et al. (2003) found that clarithromycin and erythromycin decreased mucus secretion in human nasal epithelia in vitro and that MUC5AC messenger RNA was also significantly inhibited. A similar action has recently been reported with the macrolide solithromycin (Kawamoto et al., 2020). AZM (100 mg/mL) has also been shown to decrease induced mucus hypersecretion in NCI-H292 cells by reducing MUC5AC expression by more than 90% at both the mRNA and the protein levels (Imamura et al., 2004). As summarized in a recent review, AZM appears to decrease MUC5AC production by suppressing the phosphorylation of ERK1/2 and c-Jun N-terminal kinase and nuclear translocation of NF-κB (Yang, 2020).

IL-13 is a significant remodeler of the airway epithelium, broadly changing its gene expression patterns and generating hypersecretory MUC5AC-expressing mucus cells, as reviewed by Seibold (2018). In this connection, it has been suggested that the decreased IL-13–induced MUC5AC expression observed after treatment with AZM might be mediated by a reduction in the calcium-activated chloride channel regulator 1, a component of the Th-2 gene signature found in patients with asthma (Mertens et al., 2016).

Abnormal mucus secretion is an integral component of the pathogenesis of a number of common lung diseases such as asthma and COPD (Holgate, 2011; Barnes et al., 2015). Furthermore, abnormal mucus secretion is an important component of CF, although in patients with CF, the clinical significance of the salt and water composition and the rheology of mucus has been debated (Guggino, 1999). The question of what comes first, abnormal transepithelial electrolyte transport, infection, or inflammation in the pathogenesis of CF, has been reviewed recently (Roesch et al., 2018). Interestingly, asthma severity has been linked to genomic markers in chromosomal region 11p.15.5,

which include mucins 5AC and 5B, suggesting that it is a disease with a genetic abnormality in the control of mucus production (Collaborative Study on the Genetics of Asthma, 1997). Moreover, in a subset of patients with asthma, plastic bronchitis, and other less common airway diseases, mucus plugging with atelectasis can produce respiratory failure requiring acute bronchoscopic intervention (Panchabhai et al., 2016).

As mucus hypersecretion has been associated with a worse outcome in asthma and COPD, it is plausible that macrolides might exert some of their beneficial clinical effects through reducing mucus production (Hogg et al., 2007; Martinez-Rivera et al., 2018). However, it should be kept in mind that mucus hypersecretion may also be considered a necessary compensatory defense mechanism, in which case it should not be overly suppressed by treatment with drugs, as this may actually weaken lung defense. Thus, if macrolides are to be used long-term to reduce excessive mucus levels, the safety of this approach is uncertain and needs to be considered alongside the increased risk of fostering the introduction of resistant microorganisms in the lung microbiome, as discussed above (Balsamo et al., 2010).

C. Antisenescence

Senescence is a biologic condition in which cells "retire" from their physiologic function and become replicatively quiescent but remain metabolically active. Senescence goes hand in hand with aging, and senescent cells may produce and secrete proinflammatory cytokines, commonly referred to as the senescence-associated secretory phenotype (SASP), which may, in the long term, result in chronic inflammation and tissue damage (Tominaga, 2015). SASP and inflammasomes are closely linked, as SASP is controlled by inflammasome-mediated IL-1 signaling (Acosta et al., 2013). It is of interest therefore, that Ozsvari et al. (2018) have demonstrated senolytic activity of AZM and roxithromycin. AZM was specifically shown to kill senescence-induced lung fibroblasts without affecting the nonsenescence fibroblasts. This was due to the ability of AZM to redirect the metabolic network in the senescent fibroblasts toward glycolytic pathways, resulting in increased autophagy and cell death (Ozsvari et al., 2018). Consequently, macrolides may also be considered as antiaging drugs.

Chen et al. (2019) have shown that airway epithelial cells in patients with idiopathic pulmonary fibrosis (IPF) acquire an SASP. SASP enhances the fibrotic lesion by inducing myofibroblast formation and excessive collagen production through activation of Wnt/β-catenin signaling, which mediated the expression of the NANOG protein, named after Tír na nÓg (Irish for "Land of the Young"), a transcription factor in embryonic stem cells thought to be a key factor in maintaining pluripotency. In this study, Chen et al. (2019) showed that rapamycin, a well known senolytic drug, was effective in preventing bleomycin-induced fibrosis in mice through eradication of the senescent epithelial cells (Chen et al., 2019). This may indicate that senolytic drugs such as AZM could work through similar processes when halting or reducing exacerbations in patients with IPF. The senescence condition is associated with a cancer stem cell phenotype (Keith et al., 2007). In a recent paper, Fiorillo et al. (2019) demonstrated that doxycycline and vitamin C together with AZM eradicate cancer stem cells. Vitamin C can act as a pro-oxidant, inhibiting glycolysis, which leads to mitochondrial oxidative stress and mitochondrial biogenesis. Doxycycline and AZM inhibit the small and large mitochondrial ribosomal units, respectively (Fiorillo et al., 2019). The authors showed that combination of low doses of doxycycline and AZM inhibits mitochondrial protein translation, resulting in mitochondrial ATP depletion and dysfunctional mitochondria. Since AZM is an established inducer of autophagy, this strategy should also stimulate mitophagy to actively eliminate defective mitochondria. This functional property of AZM may also have implications for aging (Fiorillo et al., 2019).

D. Barrier Integrity

The epithelial barrier found in a variety of organs, such as the skin and the respiratory, urogenital, and gastrointestinal systems, enables the body to withstand hazardous external challenges from infectious agents, chemicals, and toxic materials. By enhancing epithelial barrier integrity, it is possible to reduce or inhibit invasion and damage from external agents. In comparison with their anti-inflammatory or immunomodulatory actions, much less consideration has been given to the barrier-enhancing properties of macrolides.

Paracellular permeability is highly dependent upon intercellular junctions, particularly tight junctions (TJs), comprising various combinations of claudins, occludin, and junctional adhesion molecules, that contribute to selectivity (Anderson and Van Itallie, 2009; Flynn et al., 2009; Kojima et al., 2013). TJs ensure a tight seal in epithelial and endothelial cells, and members of the claudin family determine ion-charge selectivity, which is specific for various tissues, such as "leaky" epithelia of the proximal tubule of the kidney and in the gut versus "tight" epithelia of the skin and airways (Flynn et al., 2009; Gunzel and Yu, 2013). Measuring transepithelial electrical resistance (TEER) and paracellular flux (p-flux), TJ function can be assessed in vitro, and alterations caused by disease or infection can be inferred from TEER and p-flux measurements, with a reduction in TEER and increase in p-flux indicating a leaky, susceptible barrier.

TEER has been used as an indicator to study the epithelial barrier in various models. Indeed, nonantibiotic effects of macrolides have been described using TEER in

various types of epithelial cells (Asgrimsson et al., 2006; Miyagawa et al., 2016; Slater et al., 2016). To date, the most detailed in vitro data on how AZM affects the bronchial epithelial barrier were obtained using epithelial cells cultured in an air-liquid interface (ALI) model. In 2006, it was demonstrated that AZM increases TEER in lung epithelial cells cultured under ALI conditions (Asgrimsson et al., 2006). It was also found that addition of AZM to lung epithelial cells in ALI culture changed the locations and induced processing of the TJ proteins, claudin-1 and claudin-4, occludin, and junctional adhesion molecule A. These effects were reversible and specific to AZM, as no effect was seen when cells were treated with penicillin or erythromycin (Asgrimsson et al., 2006). The fact that AZM enhances the epithelial barrier by affecting the TJs and other adhesion molecules may also be due to its property of inhibiting EMT, as downregulation of adhesion molecules are early events in EMT.

The observations of AZM on the airway epithelia may explain why this macrolide, in particular, is beneficial in patients with chronic lung diseases characterized by epithelial dysfunction (Yuksel and Turkeli, 2017; Aghapour et al., 2018; De Rose et al., 2018) in that, uniquely, AZM increases epithelial integrity, as further validated by Halldorsson et al. (2010). In this study, cells were exposed to *P. aeruginosa*, which subsequently reduced TEER. Virulence factors like rhamnolipids secreted by *P. aeruginosa* are effective at disrupting the epithelial barrier (Zulianello et al., 2006), yet pretreatment of epithelial cells with AZM attenuated these effects and mediated the recovery of the epithelial layer and TJ disruption (Halldorsson et al., 2010). This contrasts with the loss of inhibition of IL-8 release by clarithromycin after a 24-hour incubation with normal human bronchial epithelial cells, stimulated at the same time as macrolide administration with *P. aeruginosa*–derived flagellin, as discussed previously in section *A. Anti-Inflammatory/Immunomodulatory Effects* (Shinkai et al., 2007). This distinction may be due to either differential sensitivity to the two macrolides or, more likely, to the importance of pretreatment with macrolides to induce protective effects in epithelial cells.

Similarly, Slater et al. (2016) showed that AZM effectively increased TEER while reducing p-flux of labeled dextran. In several in vitro studies mimicking infections, epithelia challenged with *P. aeruginosa* led to reductions in TEER and affected TJs, which were able to recover after pretreatment with AZM (Halldorsson et al., 2010; Slater et al., 2016). AZM also mitigated the effects of TNFα-induced insults on gingival epithelium, an action shown to involve upregulation of E-cadherin, an important structural protein involved in epithelial barrier integrity (Miyagawa et al., 2016).

In studies related to effects on epithelial cells, AZM features most prominently, but roxithromycin and several other macrolides have also been reported to have similar effects. For example, roxithromycin had cytoprotective effects on airway epithelia after sulfur-mustard exposure (Gao et al., 2007), and the nonantibiotic erythromycin derivative EM900 suppressed cytokine expression (Tojima et al., 2015; Wakayama et al., 2018) and reduced rhinovirus infection in respiratory epithelial cells (Lusamba Kalonji et al., 2015). Several review articles cover these effects in airway and gingival epithelium in detail (Lopez-Boado and Rubin, 2008; Kanoh and Rubin, 2010; Fujita et al., 2018).

It is unclear whether these are primary or secondary effects, since macrolides affect several proteins that promote epithelial and endothelial barriers. Also, the barrier-protective effects of AZM in airway epithelial cells may be due to its long half-life, accumulation inside cells, and formation of lamellar bodies (Arason et al., 2019). Furthermore, the ability of AZM to repress the expression of proinflammatory cytokines and extracellular matrix enzymes, such as matrix metalloproteinases (MMP) 2 and MMP9, may stabilize the microenvironment and be key to the enhanced barrier protection. Thus, MMP9 has been shown to dysregulate epithelial cell TJs and has been implicated in degeneration of epithelial barriers (Pflugfelder et al., 2005; Vermeer et al., 2009; Nighot et al., 2015). It plays a critical role in cellular and tissue remodeling, including basement membrane breakdown, whether it be as part of the repair processes after injury or under disease conditions, when it is overexpressed (Legrand et al., 1999; Zheng et al., 2002; Guéders et al., 2006; Crosby and Waters, 2010). Of interest here is that elevated MMP9 results in epithelial barrier permeability in both cornea and airways (Vermeer et al., 2009; Mauris et al., 2014; Rajashekhar et al., 2014) and has been shown to inversely correlate with TEER (Slater et al., 2016). Interestingly, treatment of lung transplant patients with AZM leads to a decrease in MMP9 expression, suggesting that part of the benefit of this macrolide in this clinical setting may relate to a reduction in tissue injury (Verleden et al., 2011). This conclusion is supported by another observation that oral administration of clarithromycin in a murine model of aortic aneurysm also resulted in decreases in MMP9 and MMP2, resulting in suppression of aortic rupture (Uchida et al., 2018). Although the mechanism of this effect was not investigated, it appears that the action of clarithromycin on the aortic wall may be indirect by influencing the release of MMPs from proinflammatory macrophages.

Taken together, the findings discussed here provide strong support for a barrier-enhancing effect of macrolides and particularly AZM, mediated predominantly by actions on epithelial cells (Fig. 2).

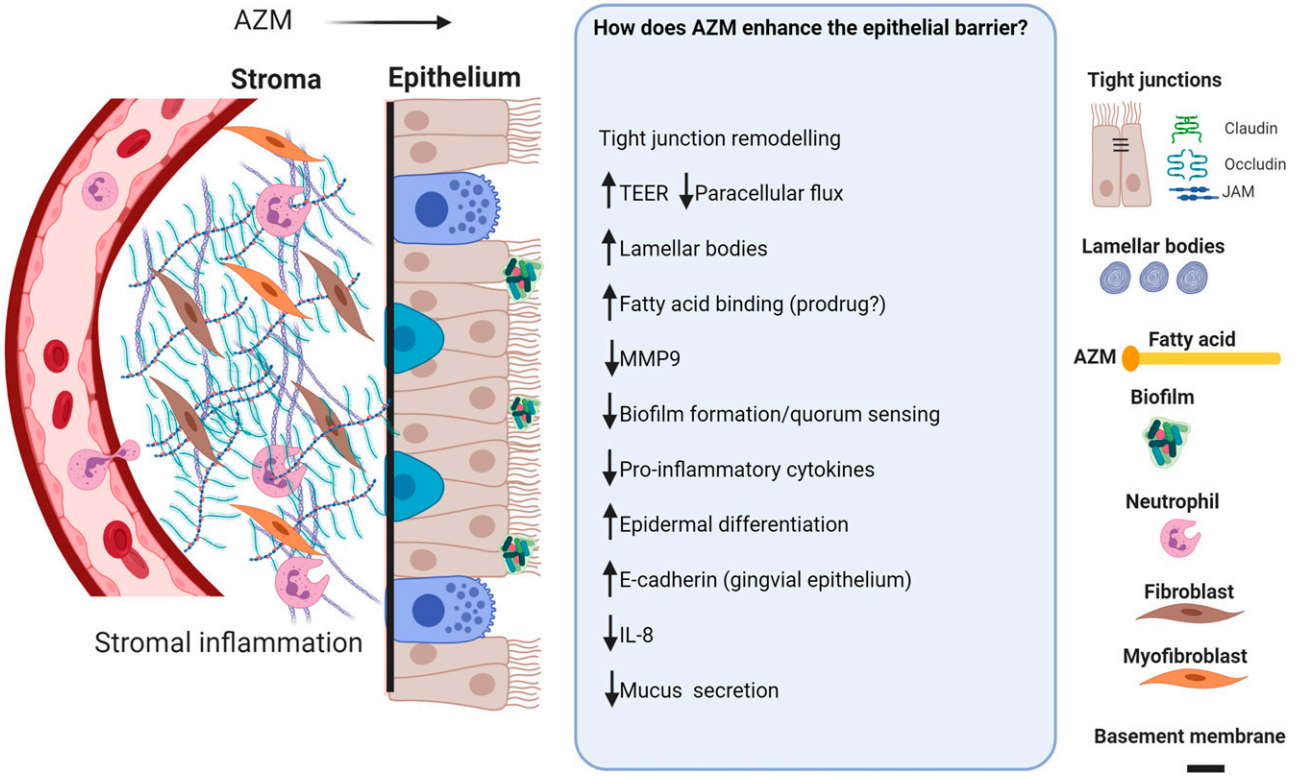

Fig. 2. Enhancement of bronchial epithelial barrier integrity and epithelial-stromal interaction. Upon infectious insult by bacteria/viruses or injury by other toxic agents, such as exposure to smoking and other environmental oxidants like SO_2, the airway epithelial barrier is weakened, facilitating the paracellular entrance of infectious agents into the underlying stroma. Both the epithelium and stromal cells may release proinflammatory cytokines that attract neutrophils and macrophages. Inflammatory stroma may disturb the normal homeostasis, potentially causing fibrotic-like changes that may maintain and/or further weaken the epithelium. AZM enters the infectious area with neutrophils, and its actions (targets of which are indicated in the far-right panel) may be important in restoring normal homeostasis, including resolution of inflammation, reducing fibrosis, and enhancing the airway epithelial barrier.

III. Mechanisms of Action

AZM, in part, is thought to be more effective than other clinically used macrolides, as it accumulates in cells, prolonging its half-life (Carlier et al., 1994; Amsden, 2005). It also has fewer drug-drug interactions than some of the other macrolides (Aronoff et al., 1987). Although the action of macrolides as antibiotics is well understood, the mechanisms of action behind their nonantimicrobial actions are less understood. Studies on the possible mechanism(s) of action of AZM have explored its physical properties and a range of activities, but despite the intense research on the topic, its mode of action as an anti-inflammatory/immunomodulatory drug remains unclear (Sakito et al., 1996; Kurdowska et al., 2001; Shinkai et al., 2006; Verleden et al., 2011). Mechanisms of action of AZM, in particular those on cells of the innate immune system, have been covered in several excellent reviews (Bartold et al., 2013; Parnham et al., 2014; Yang, 2020; Oliver and Hinks, 2021). These papers, however, with the exception of two reviews on macrolides in general (Lopez-Boado and Rubin, 2008; Kanoh and Rubin, 2010), have mostly neglected the possible effects of AZM on the respiratory epithelium. We have summarized what is currently understood about the effects of AZM on both inflammatory and epithelial cells in Figs. 1 and 2, and these are discussed in more detail below.

A. Actions on Lysosomes, Apoptosis, and Autophagy

Lysosomes are organelles containing digestive enzymes necessary for the breakdown and recycling of biomolecules (Ballabio and Bonifacino, 2020). Some of the contents are delivered to the lysosome by fusion with phagosomes. AZM treatment increases phagocytosis by macrophages of apoptotic epithelial cells and neutrophils (so-called efferocytosis) in patients with COPD and seems to involve the phosphatidylserine pathway and the mannose receptor (Hodge et al., 2006, 2008). As highlighted in several reviews, this effect could also be in part due to stabilization of oxidative metabolism and a reduction in lysosomal membrane permeability in macrophages (Parnham et al., 2014; Reijnders et al., 2020).

Macrolides and other cationic amphiphilic drugs that contain a hydrophilic amine group can be protonated in the lysosomal compartment, where they become effectively trapped (Kosol et al., 2012; Kazmi

et al., 2013; Breiden and Sandhoff, 2019). The uptake and accumulation of macrolides alters lysosomal functions through several mechanisms: altered pH, inhibition of lipid catabolism through interference of electrostatic binding of enzymes to lipid substrates, and inhibition of digestive hydrolases including phospholipases A_1 and A_2 (Breiden and Sandhoff, 2019). The interactions of macrolides with lipids are outlined in section *B. Interactions with Lipids*. AZM-induced lysosomal dysfunction was shown to result in lysosomal biogenesis and accumulation in several cancer cell lines, and although alone it did not cause cell death, it potentiated the anticancer action of a proton-pump inhibitor causing cell necrosis (Takeda et al., 2020). AZM lysosomal accumulation has also been shown in fibroblasts isolated from patients with IPF, which has been suggested to be related to AZM-induced apoptosis (Krempaska et al., 2020).

Increased apoptosis of epithelial cells is often associated with chronic airway diseases (CADs) and is implicated in the pathogenesis of both COPD and IPF (Demedts et al., 2006; Le Saux and Chapman, 2018). AZM and clarithromycin both affect apoptosis in a cell type–dependent manner. In IPF, in which increased apoptosis of alveolar type-II cells and decreased apoptosis of fibroblasts are key contributors to the disease, AZM treatment improved survival of patients with IPF (Kawamura et al., 2017). This could, in part, be due to enhanced apoptosis of IPF-isolated fibroblasts in the presence of AZM (Krempaska et al., 2020). Common to the different cell types, macrolide-induced apoptosis appears to involve suppression of the antiapoptotic B-cell lymphoma-extra large protein (Mizunoe et al., 2004; Krempaska et al., 2020). After accumulation in neutrophils, macrolides induce apoptosis of this cell type, subsequently reducing their number and thereby contributing to the resolution of inflammation (see Fig. 1) (Parnham et al., 2014; Reijnders et al., 2020).

Autophagy is a process to degrade and recycle defective or redundant cytoplasmic components that do not originate at the plasma membrane (Galluzzi et al., 2017). Both AZM and clarithromycin have been shown to affect autophagy, albeit with contrasting results, likely reflecting the different cell types used in these experiments. In several cancer cell lines, AZM and clarithromycin have been shown to curb autophagic flux resulting in accumulation of cytoplasmic autolysosomes (Hirasawa et al., 2016; Takeda et al., 2020). Furthermore, in a bleomycin-induced lung fibrosis model, AZM treatment was shown to inhibit autophagy in fibroblasts (Tsubouchi et al., 2017). These authors demonstrated that AZM resulted in crosstalk of proteostasis pathways, leading to a reduction in transforming growth factor-β–induced NADPH oxidase 4 and an increase in proteasome activity and, ultimately, autophagy inhibition. Inhibition of autophagosome clearance by macrophages may explain the increased predisposition of patients with CF, on long-term treatment with AZM, to nontuberculous mycobacteria (NTMB) infection (Renna et al., 2011).

B. Interactions with Lipids

Macrolides interact with a number of lipids and intracellular vacuoles in several cell types (Tyteca et al., 2001; Bosnar et al., 2005; Liu et al., 2014, 2018). Via their cationic groups, AZM and other cationic amphiphilic macrolides bind to negatively charged phospholipids in cell membranes (van Bambeke et al., 1996; Montenez et al., 1999). Consequently, these macrolides can exert several effects on lipids and may be a primary stimulus by which cationic macrolides, such as AZM, initiate subsequent intracellular signaling (see section *C. Modulation of Cell Signaling*) in inflammatory cells (Parnham et al., 2014) and may be a factor in the similarity of the effects of AZM and hydroxychloroquine on human macrophage polarization (Shiratori et al., 2018). In addition, macrolide interactions with phospholipids reduce the fluidity within the cell membrane, resulting in stiffer and more rigid cell membranes that potentially may also contribute to the epithelial barrier–enhancing effects of AZM (see Fig. 1).

A central property of all cationic amphiphilic drugs is their ability to alter phospholipid and sphingolipid metabolism (Breiden and Sandhoff, 2019). This is characterized by intracellular accumulation of phospholipids as a result of phospholipase A inhibition (Halliwell, 1997; Anderson and Borlak, 2006), a phenomenon called phospholipidosis, a well known response to cationic drugs (Patel et al., 2019). Lysosomotropic drug accumulation in lysosomes effectively neutralizes the pH, contributing to phospholipase inhibition. Indeed, studies with LPS-stimulated phagocytic cells show that AZM-induced phospholipidosis is subsequent to membrane binding, leading to interference with cytoplasmic phospholipase A2 signaling (Banjanac et al., 2012; Parnham et al., 2014). Further details of the cell signaling pathways associated with the nonantibiotic actions of macrolides are given in section *C. Modulation of Cell Signaling* below.

AZM pretreatment of airway epithelial cells leads to a dramatic change in cellular gene signatures. Many of these AZM-sensitive genes are enriched in cholesterol and lipid/fatty acid ontology groups (Ribeiro et al., 2009; Arason et al., 2019). These data fit well with the increased phospholipidosis observed as a direct result of macrolide treatment. Arason et al. 2019 and others have related AZM-enhanced intracellular phosphatidylcholine and sphingomyelin levels to a dramatic increase in the number of multivesicular bodies (MVBs) and lamellar bodies, which are traditionally associated with epithelial differentiation and surfactant production (Liu et al., 2014;

Arason et al., 2019; Joelsson et al., 2020b). Of particular interest was the presence of lipid-conjugated AZM species within the MVBs, whereby AZM was bound to saturated fatty acids, particularly palmitate and stearate. The exact purpose of this conjugation remains unknown, but it could help to explain at least some of the additional beneficial effects reported after AZM treatment, such as increased epithelial integrity (Halldorsson et al., 2010; Slater et al., 2016; Arason et al., 2019). Although the potential biologic activities of the lipidated forms of AZM are as yet unknown, it remains plausible that AZM is actually a prodrug, with these lipidated forms contributing to the long-term pharmacodynamic effects observed with AZM treatment. These lipid changes are not limited to in vitro cell studies either, as we have recently shown that lamellar bodies are induced in lung tissue of mice pretreated with AZM followed by challenge with SO_2 exposure (Joelsson et al., 2020a). Pretreatment with AZM offered some protection against the SO_2-induced lung injury, as it reduced the leakage of plasma albumin into the BAL fluid. Although no direct mechanistic involvement has yet been demonstrated, the presence of increased intraepithelial MVBs correlates well with barrier enhancement observed after treatment with AZM.

Clinically significant benefits of macrolide treatment on epithelial cells are not limited to the airways. Liu and coworkers have examined AZM and solithromycin for their potential benefit in dry-eye disease (Liu et al., 2014, 2018). They postulate that the rapid and dose-dependent increase in lipid accumulation in meibomian epithelial cells could be a potential therapeutic mechanism through increased lipid production and, thus, stabilization of tear film production.

C. Modulation of Cell Signaling

Multiple mechanisms are involved in the nonantibiotic actions of macrolides on leukocytes and epithelial and endothelial cells, as exemplified by the diversity of signaling pathways reported (Shinkai et al., 2008; Reijnders et al., 2020; Yang, 2020). Many of the effects on various signaling cascades have been related to lipid interactions and resolution of inflammation (Parnham et al., 2014).

As discussed in an earlier section, AZM has been shown to induce the generation of M2 macrophages. In isolated macrophages from patients with lupus, addition of AZM increased the phagocytic capabilities of macrophages via the protein kinase B/phosphoinositide 3-kinase (Akt/PI3K) pathway (Wang et al., 2018). This also seems to be true in other disease models, whereby Akt and the transcription factor nuclear factor erythroid 2-related factor 2 appear to have an important role in macrolide-induced reduction of inflammation in COPD [see review by Sun et al. (2019)]. Akt is a key signaling molecule in the PI3K pathway mediating activation of the receptor tyrosine kinase receptors. Downstream effectors include mammalian target of rapamycin (mTOR), nitric oxide synthase (NOS), and several cell death regulators.

A recent publication by Gupta et al. (2020) used a genome-wide short hairpin RNA screen to identify targets of macrolides in human cells. Although the authors could not discern between genes involved in antimicrobial and nonantimicrobial activities, they used this screen as a step toward identifying possible mechanisms behind nonantimicrobial effects of macrolides. Not surprisingly, mitochondrial translation genes featured prominently, as well as signaling molecules associated with the anti-inflammatory p38 MAPK pathway (Gupta et al., 2020). Other studies have also reported involvement of p38 MAPK and suppression of the transcription factor NF-κB in response to nonantimicrobial activities of macrolides (Otsu et al., 2011; Li et al., 2013). Furthermore, AZM has been shown to inhibit DNA binding of the NF-κB and activator protein-1 (AP-1) transcription factors in CF cell lines, resulting in reduced expression and secretion of the proinflammatory cytokine IL-8 (Cigana et al., 2006).

The use of nonantimicrobial derivatives such as the erythromycin derivatives EM900, EM703 and the AZM-derivative GS-560660 have been helpful in dissecting the signaling pathways related to nonantimicrobial activity (Otsu et al., 2011; Hodge et al., 2017). Otsu et al. (2011) demonstrated that EM900 suppressed IL-1β–induced IL-8 expression in the A549 alveolar cell line and similarly suppressed MUC5AC expression in HM3-MUC5AC cells. Hodge et al. (2017) demonstrated that GS-560660 inhibits the NLRP3 inflammasome, leading to decreased cleavage and activation of the proinflammatory cytokine IL-1β.

Inflammasomes are organelles found in many cell types, but they are most notably in immune cells such as monocyte/macrophages and neutrophils. They are complex systems that can be activated by both intrinsic and extrinsic factors, resulting in assembly of cytosolic proteins (Broz and Dixit, 2016; Rathinam and Fitzgerald, 2016). The NLRP3 inflammasomes are among the best characterized, although their biologic function has only recently been highlighted. Self-assembly of the NLRP3 complex activates caspase-1, which then activates proinflammatory cytokines such as IL-1β and IL-18 (Mangan et al., 2018). There are a number of papers that show that overexpression/activation of the NLRP3 is detrimental in many acute and chronic inflammatory conditions (Mangan et al., 2018). Xu et al. (2018) have shown using CRISPR/Cas9 gene editing that disruption of NLRP3 in macrophages inhibits the activation of the NLRP3 inflammasome in response to diverse stimuli. This was further shown in vivo, where disruption of NLRP3 attenuates the acute inflammatory response in LPS-

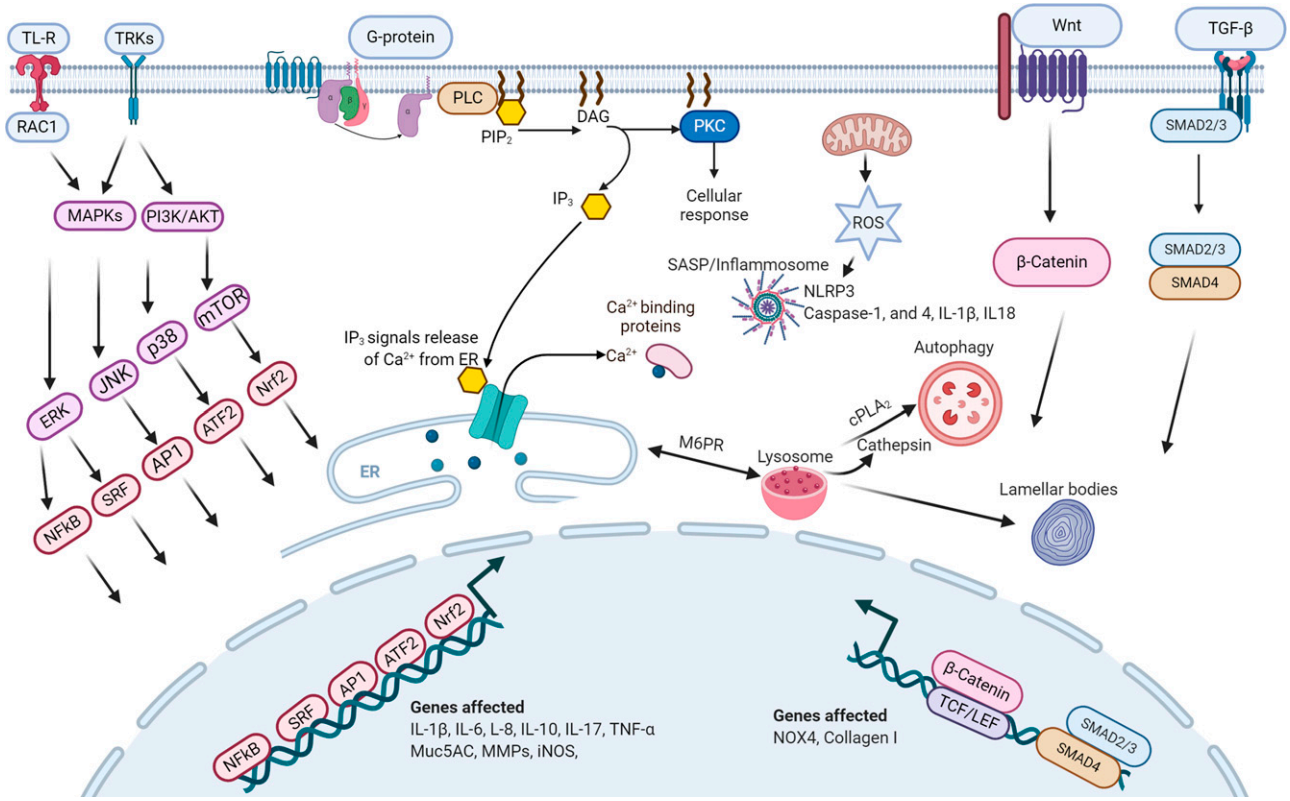

Fig. 3. Macrolides interfere with a number of signaling and cellular processes. Macrolides exert their effects via multiple mechanisms involving a variety of signaling cascades, as shown here. The complexity of this has been simplified, and the majority of pathways mentioned in this review are presented. Macrolides, in particular AZM, are known to target toll-like receptor (TLR) and tyrosine receptor kinase (TRK) pathways, inhibiting activation of their downstream signaling molecules, including ERK, JNK, P38, and mTOR. This, in turn, results in the downregulation of a number of genes, including those involved in inflammation (IL-1β, IL-6, IL-8, IL-17, and TNFα), thus dampening the inflammation response. Conversely, certain proinflammatory and anti-inflammatory cytokines (IL-10) are increased through activation of the transcription factors NF-κB and AP-1. It is also through these pathways that macrolides reduce expression of MUC5AC, MMP (in particular MMP2), MMP9, and induced nitric oxide synthase (iNOS). Macrolides also inhibit fibrosis and EMT by inhibiting signaling pathways related to Wnt and transforming growth factor-β. Macrolide activation of G-protein–coupled receptors, along with TRK, phospholipase C (PLC), and second messengers DAG and IP3, causes the release of calcium from the endoplasmic reticulum (ER). Mobilized calcium leads to a variety of cell type–dependent effects ranging from activation of TRK signaling, stabilizing calcium levels, and affecting ion channels and adhesion molecules, such as E-cadherin and TJs. Macrolides also inhibit ROS generation and can thus reduce cellular damaging cascades generated by ROS-associated activation of NLRP3 that triggers inflammasome formation and SASP. Accumulation of macrolides in lysosomes results in their enhanced stability through binding to lipids and reduction of phospholipase activity, with the subsequent release of enzymes such as cathepsin. Autophagy flux is also blocked by macrolides. AP-1, activator protein-1; ATF2, activating transcription factor-2; DAG, diacylglycerol; IP3, inositol trisphosphate; JNK, c-Jun N-terminal kinases; LEF, lymphoid enhancer factor; mTOR, mammalian target of rapamycin; Nrf2, nuclear factor-erythroid factor 2-related factor 2; PIP2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; RAC-1, ras-related C3 botulinum toxin substrate 1; SMAD2, mothers against DPP homolog 2; SRF, serum response factor.

induced septic shock (Xu et al., 2018). Macrolides such as AZM have been shown to inhibit the damaging effects of NLRP3 inflammasomes. Gualdoni et al. (2015) compared the influence of different macrolides on cytokine induction in human monocytes. They also analyzed the signaling mechanisms involved in inflammasome activation and extended their findings to an in vivo murine sepsis model. Interestingly, AZM, but not clarithromycin or roxithromycin, inhibited IL-1α and IL-1β secretion upon LPS stimulation (Gualdoni et al., 2015). In a recent review, Seys et al. (2019) discuss the potential therapeutic application of AZM in treating neutrophilic asthma by dampening the NLRP3 inflammasome pathway, resulting in decreased IL-1β secretion. The signaling processes affected by macrolides are summarized in Fig. 3.

IV. Nonantibiotic Macrolides

A. Immunomodulators

A variety of synthetic derivatives of 14- and 15-membered macrolides have been investigated as nonantibiotic anti-inflammatory/immunomodulatory agents. Among these, a synthetic derivative of erythromycin, EM900, was one of the first to be reported, and since then, a number of its synthetic derivatives have been described (Sugawara et al., 2011, 2012). These compounds were all optimized on the basis of promotion of differentiation of the THP-1 monocytic cell line to macrophages. A broad number of experimental investigations have been carried out, particularly on EM900, and as shown with antibiotic macrolides, EM900 and its analog EM703 both inhibited the oxidative burst of stimulated human neutrophils (Nozoe et al., 2016). Like erythromycin, EM900 inhibits

expression of proinflammatory cytokines and the MUC5AC gene in the A549 epithelial cell line, inhibits epithelial mucus secretion, and is comparable to clarithromycin as an inhibitor of the generation of the neutrophil-chemotactic cytokine IL-8 from human nasal epithelial cells in vitro (Otsu et al., 2011; Tojima et al., 2015; Wakayama et al., 2018). Moreover, given both before and after in vitro infection of human primary airway epithelial cells with rhinovirus (RV), EM900 inhibited both RV titers and viral RNA, as well as inflammatory cytokine generation by this cell type, apparently by reducing the expression of the intercellular adhesion molecule-1, which acts as a receptor for the RV (Lusamba Kalonji et al., 2015).

Together with two synthetic analogs, EM900 inhibited trinitrobenzene sulfonate–induced rat colitis to a similar degree as sulfasalazine (Sugawara et al., 2012). In contrast to clarithromycin, EM900 also had positive effects on survival of H1N1 influenza virus–infected mice, supposedly by effects on inflammatory macrophage activity (Sugamata et al., 2014). Interestingly, despite lacking antibiotic activity, EM900 also promoted clearance of *S. pneumoniae* after nasal inoculation of mice, apparently by enhancing macrophage recruitment and activation (Iwanaga et al., 2015). Recently, EM900 has been shown by the same group to inhibit airway inflammation and the levels of BAL fluid cytokines induced by polyinosinic-polycytidylic acid exposure in mice, previously sensitized to and challenged with house dust mite, a model of virus-induced asthma exacerbations, as well as in house dust mite–induced airway inflammation in obese mice (Sadamatsu et al., 2020a,b). In both cases, it was suggested that the beneficial effects of EM900 were mediated by actions on macrophages and presumably also on epithelial cells.

Other nonantibiotic, anti-inflammatory/immunomodulatory macrolides (mainly based on AZM or erythromycin structures), together with the test systems by which they were selected, are shown in Table 1.

Most of the initial test systems used involved in vitro stimulation of monocytes or macrophages, although one compound (GS-459755) was initially identified as an inhibitor of epithelial cell mucus production by an action on the epithelial sodium channel (Tarran et al., 2013). As a consequence, those compounds that were investigated further showed anti-inflammatory activity related to inhibition of inflammatory (M1-like) macrophage activity or promotion of an M2-like phenotype. The anti-inflammatory effects

TABLE 1
Nonantibiotic macrolide derivatives reported to exhibit immunomodulatory or anti-inflammatory properties

Company	Product	Indication	Chemistry	Initial Screen[a]	Reference
Synovo	CSY1690	Cancer	AZM conjugate	Macrophage IL-10 release and p38 kinase inhibitor	(Burnet et al., 2015)
Synovo	CSY0073	CF, COPD	AZM derivative	Mouse experimental colitis	(Balloy et al., 2014; Mencarelli et al., 2011)
GSK	Compound 38	COPD	AZM derivative	Murine splenocyte LPS-induced IL-6 production	(Bosnar et al., 2012)
GSK	Macrolide-corticoid conjugates (macronolactones) and macrolide-NSAID conjugates	Asthma	AZM conjugate	Glucocorticoid receptor binding and human PBMC cytokine release	Patent WO2004094448 (Tomašković et al., 2013)
Gilead	GS-560660	COPD	AZM derivative	Phagocytosis of *H. influenzae* by macrophages	(Hodge et al., 2017)
Gilead	GS-459755	CF, COPD	ERY derivative	HNE-induced sodium channel activation in human airway epithelial cells; phagocytosis of *H. influenzae* by macrophages	(Hodge et al., 2017; Tarran et al., 2013)
Taiisho/ Kitasato Inst.	EM900		ERY derivative	Monocyte differentiation to macrophages	(Sugawara et al., 2011)
Ranbaxy		Inflammatory diseases	ERY derivative	Human neutrophil LTB4 release, LPS-induced IL-1β release from human blood monocytes	Patent WO2007054904A3
Zambon	Compounds 1 and 2	Inflammatory, respiratory, and gastrointestinal pathologies	Telithromycin derivative	Mouse TPA-induced contact dermatitis	Patent WO2008/072034, PCT/IB20067054776

ERY, erythromycin; LTB4, leukotriene B4; NSAID, non-steroidal anti-inflammatory drug; PBMC, peripheral blood mononuclear cell; TPA, tetradecanoyl phorbol acetate.
[a]In addition to bacterial screen.

of many of these compounds, also seldomly referred to as immunolides (Fecik et al., 2005), have been reviewed, and they have been proposed as future effective therapeutic agents for neutrophil-dominated diseases, including COPD, bronchiectasis, bronchiolitis obliterans syndrome, and CF (Erakovic Haber et al., 2014). A further group of compounds, the macrolactonides, conjugates of a macrolide with a steroid, initially called sterolides (Mercep et al., 2004), were synthesized for aerosol administration in asthma but were not developed to the clinic (Tomašković et al., 2013). More recently, a series of novel macrolide compounds was found to be active on a screen for inhibition of RV infectivity of primary bronchial epithelial cells and proposed to represent a novel class of anti-inflammatory, antibacterial, and antiviral candidate compounds (Porter et al., 2016).

B. Barriolides

Barriolides are 15-membered macrolides optimized for epithelial barrier modulation and enhancement of epithelial integrity. Their lack of antibacterial potency importantly pertains to NTMB/*Mycobacterium avium* complex as well as relevant extracellular bacteria.

The epithelial lining of the airways has received increasing attention over the last decade as a crucial anatomic structure for maintaining respiratory health. This is evident simply by entering "airway epithelial integrity" into search engines. Asthma and allergy research papers have described the airway epithelium in terms of its barrier function for decades (Holgate, 2011; Gon and Hashimoto, 2018; Heijink et al., 2020). Cigarette smoke–induced airway epithelial damage is also well described (Goldie et al., 1988; Heijink et al., 2012; Nyunoya et al., 2014; Amatngalim et al., 2016; Aghapour et al., 2018; Calven et al., 2020). This leads to the loss of barrier integrity and contributes to the high incidence of bronchial infections among patients with COPD, producing exacerbations, hospitalization, and mortality. In addition, a number of studies have shown that mechanical ventilation in the intensive care unit (ICU) induces significant changes in airway epithelia, indicating that this common therapeutic procedure disrupts the airway epithelial barrier, increasing susceptibility to life-threatening complications such as ventilator-induced pneumonia (Jacob and Gaver, 2012; Joelsson et al., 2019, 2020b).

Taken together, the clinical importance of airway epithelial integrity is unquestioned, generating a crucial barrier that protects the lungs against pollution, infective particles, and disruptive mechanical forces. One well described consequence of barrier failure is an unhindered access of external particles to various cells of the immune system, producing an inappropriate inflammatory response, the hallmark of many lung diseases (Knight and Holgate, 2003; Wittekindt, 2017).

Despite this widespread clinical and scientific documentation of airway epithelial barrier dysfunction, apart from drugs that inhibit inflammatory responses, selective pharmacological approaches to therapeutically strengthen the barrier, to our knowledge, have not been published (Knight and Holgate, 2003). A gap exists between the basic science knowledge and the significant health consequences of airway epithelial barrier failure observed by clinicians. In this regard, clinical trials on AZM in CF caught the attention of our laboratory some 20 years ago. Particularly interesting was the finding that patients' clinical status improved, despite sputum cultures continuing to show *P. aeruginosa*, after long-term treatment with AZM (Saiman et al., 2003). As discussed above in relation to barrier function (section *D. Barrier Integrity*), we followed up this clinical finding to show that AZM increased TEER human airway epithelia in vitro significantly compared with control (Asgrimsson et al., 2006). This was confirmed in airway epithelia challenged with live *P. aeruginosa* or with media from culturing *P. aeruginosa* alone, in that AZM improved epithelial integrity compared with controls (Halldorsson et al., 2010).

These important observations all support the relevance of barriolides, the lead compound of which, EP395, entered early clinical development in 2021 (Gardarsson et al., 2017).

V. Clinical Effects of Macrolides beyond Antibiosis

The three most common CADs are asthma, bronchiectasis, and COPD (Gibson et al., 2013). These diseases are characterized by acute exacerbations or worsening of the diseases, usually resulting from a bacterial or viral infection, although sometimes the etiology is unknown. Macrolides, particularly AZM, have been used to treat these diseases on the basis of actions beyond their antibiotic actions, and the evidence supporting their use in these clinical settings is discussed below.

A. Chronic Obstructive Pulmonary Disease

COPD is a major global health problem associated with chronic inflammation of the peripheral airways and lung parenchyma caused by exposure to environmental oxidants such as air pollution and tobacco smoke (Hogg et al., 2004). In contrast to asthma, an important problem in the treatment of COPD is the lack of convincing clinical benefit in the majority of patients after administration of steroids (Ernst et al., 2015), and there is an increasing awareness that inhaled corticosteroids (ICS) can increase the risk of pneumonia in patients with COPD (Ferguson et al., 2008; Suissa et al., 2013; Mathioudakis et al., 2020). It is of interest, therefore, that a novel macrolide,

solithromycin, restored the sensitivity to the actions of steroids of peripheral blood mononuclear cells obtained from patients with COPD (Kobayashi et al., 2013). The authors of this paper suggested that solithromycin (and by implication other macrolides) may be a novel approach to treating COPD, particularly in those patients who are insensitive to steroids. In support of this conclusion are clinical observations from a number of studies that have reported that long-term treatment with macrolides is able to reduce exacerbations of COPD (Milstone, 2008; He et al., 2010; Albert et al., 2011; Pomares et al., 2011). It has been proposed that maintenance treatment with AZM should be considered in patients with COPD who have a frequent exacerbator phenotype and who are refractory to standard of care (Uzun et al., 2014). This conclusion is supported by a meta-analysis demonstrating that prophylactic use of macrolides is an effective approach to reduce exacerbations in patients with COPD (Donath et al., 2013). Indeed, this evidence has led to the inclusion of AZM, in particular, in the GOLD guidelines for the management of COPD as an add-on therapy to standard of care for the treatment of patients with COPD (https://goldcopd.org/2021-gold-reports).

One of the concerns, however, about the long-term use of AZM in patients with COPD is the increased risk of developing bacterial resistance (Huckle et al., 2018). If there is a need to use AZM to treat lung infections caused by *M. avium*, for example, which is normally sensitive to macrolides, these infections, in the case of resistance, would no longer respond to treatment. In this regard, it is of interest that a recent follow-up of the Influence of Macrolides on Exacerbation Frequency in COPD Patients (COLUMBUS) trial reported that the acquisition rate of macrolide-resistant bacterial genes in patients with COPD receiving AZM treatment of 1 year was limited, although the relative abundance of these genes did increase significantly over time when compared with patients treated with placebo (Djamin et al., 2020). Given that the long-term use of AZM in the maintenance treatment of COPD is based on anti-inflammatory and immunomodulatory actions rather than the antibacterial effects of macrolides, this highlights the need to find safe and effective macrolides lacking antimicrobial activity for the maintenance treatment of both COPD and asthma. Clinically, AZM and clarithromycin are often used to treat acute exacerbations of patients with COPD presenting with infections of *Haemophilus influenzae*, *Moraxella catarrhalis*, and *S. pneumoniae* (Butorac-Petanjek et al., 2010; Uzun et al., 2014; Wong and Herath, 2014; Kiser and Vandivier, 2015; Mayhew et al., 2018).

To date, it has not been possible to precisely define clinical biomarkers for the different causes of exacerbations of COPD (Mathioudakis et al., 2020), suggesting that better characterization of these different etiologies might facilitate biomarker discovery. Another possibility would be to search for markers of epithelial damage, more likely to represent basic structural defects that precede the inflammatory cascade, and which subsequently affect various inflammatory biomarkers downstream. In this regard, the effects of AZM on the expression of epithelial genes and epidermal differentiation are worth exploring further (Arason et al., 2019), especially in the light of the effects of smoking and other forms of oxidant pollution on the epithelium, the main cause of COPD.

B. Asthma

Asthma is a common respiratory disease affecting millions of people worldwide. Despite the introduction of effective therapies over the last two decades, particularly ICS and long-acting β-2 agonists (LABAs), often in the form of fixed-dose inhalers (Cazzola et al., 2020), there remains a significant unmet need in the treatment of this disease. In particular, therapies are needed that can be used safely to reduce exacerbations that can occur despite the use of ICS and LABAs (Gibson et al., 2017). There is also a major problem with adherence to inhaled medicines, which often contributes to suboptimal treatment of many patients with asthma (Lavorini et al., 2019). However, only a few orally active drugs are approved for the treatment of asthma—the leukotriene receptor antagonist montelukast (which is not considered as a first-line therapy in most countries) and the xanthines, such as theophylline and doxophylline, which are only recommended on top of standard of care—and these often have problematic unwanted side effects (Matera et al., 2017).

It is of interest, therefore, that oral AZM can have clinical benefit in patients with persistent asthma, the rationale for this approach having been drawn from the success of macrolide therapy in DPB and CF (Kudoh, 2004; Saiman, 2004; Saiman and Schechter, 2020). The AZM studies in asthma have been reviewed elsewhere (Richeldi et al., 2005; Reiter et al., 2013; Kew et al., 2015; Tong et al., 2015) and demonstrated improvement in a number of clinical endpoints when AZM was used in the long-term management of asthma, although some studies were not powered sufficiently to demonstrate any activity against exacerbations (Gibson et al., 2017). However, a more recent study has shown that 48 weeks' AZM treatment of adults with persistent symptomatic asthma resulted in fewer asthma exacerbations and improved quality of life. This randomized, double blind, placebo-controlled study concluded that AZM (the Effect of Azithromycin on Asthma Exacerbations and Quality of Life in Adults with Persistent Uncontrolled Asthma (AMAZES) trial) should be considered as a useful add-on therapy for patients with persistent asthma already receiving treatment with high-dose ICS and LABA (Gibson et al., 2017).

An earlier study [the AZIthromycin in Severe ASThma Study (AZISAST) trial] had reported that 250 mg AZM administered three times per week for 26 weeks to 109 adults with asthma did not produce an overall reduction in the number of exacerbations, although a subgroup analysis showed a positive effect in patients with noneosinophilic asthma (Brusselle et al., 2013). In addition, clarithromycin has been reported to reduce the need for corticosteroids in patients with corticosteroid-dependent asthma (Garey et al., 2000; Gotfried et al., 2004).

C. Diffuse Panbronchiolitis

Diffuse panbronchiolitis (DPB), a CAD mostly restricted to East Asia, and particularly Japan, is characterized by extensive bronchial infections and inflammation and has been carefully reviewed elsewhere (Poletti et al., 2006). Despite traditional treatment with mucolytic agents, steroids, and traditional antibiotics against *H. influenzae* and *P. aeruginosa*, the most common pathogens found in sputum cultures, mortality remained high among patients with DPB until the late 1980s. Around that time, increasing anecdotal clinical experience suggested that erythromycin significantly improved survival. A general practitioner in Japan (Dr. Miyasawa) gave erythromycin to his patients with DPB speculatively, based on use in asthma, and he saw unbelievably dramatic results. One of these patients was presented to Prof. Shoji Kudoh in Tokyo, who conducted a small study with equally dramatic results, and Kudoh then proposed a proper randomized controlled trial. However, by the time the study had been approved, it was difficult to recruit subjects in Japan because, by then, erythromycin was almost uniformly being used as therapy for DPB. The first double blind randomized controlled trial was subsequently reported by M. Yamamoto in 1991 (Yamaya et al., 2017). Following on from the early studies, erythromycin was found to decrease IL-1β, IL-8, and neutrophils in BAL fluid from patients with DPB, indicating that anti-inflammatory or immunomodulatory mechanisms produced the positive clinical results (Sakito et al., 1996). Other studies found that macrolides decrease sputum volume, proinflammatory cytokines, MMPs, neutrophil activation, and lung infiltration (Kudoh, 2004; Kobayashi et al., 2013). The early introduction of macrolide therapy for DPB was mostly based on clinical experience and small, nonrandomized trials, an unusual sequence of events given the successful decrease in mortality observed. A recent systematic review of macrolide treatment of DPB in Japan, Korea, and China reports that the incidence of DPB is declining, at least partially because of the use of macrolides (Lin et al., 2015). A similar result was obtained in a review in Japan (Kono et al., 2012).

D. Bronchiectasis

Bronchiectasis is a condition in which lung injury from any cause leads to damage to the bronchi with widening and thickening, resulting in buildup of bacteria and mucus, with frequent infections and airway blockage characterized by chronic cough and wheezing. Given the clinical similarities between different types of bronchiectasis, regardless of cause, the positive effects of macrolides on patients with DPB led to multiple clinical trials in CF and non-CF bronchiectasis (Cramer et al., 2017). A randomized clinical trial published in 2003 showed that AZM (250 or 500 mg) three times a week for 6 months improved lung function in patients with CF older than 6 years who were infected with *P. aeruginosa* and had FEV1 >30% of predicted at baseline. Significant improvements were found in FEV1, weight, and exacerbation rates (Saiman et al., 2003). Additional studies extended treatment to 12 months and found that cough and exacerbation rates continued to improve, but FEV1 appeared to have reached a steady state or plateau at 6 months of treatment (Clement et al., 2006; Saiman et al., 2010). The role of AZM in non-CF bronchiectasis is well established from several randomized controlled trials (Wong et al., 2012; Altenburg et al., 2013; Valery et al., 2013). A recent systematic review of 15 randomized controlled trials of macrolides in bronchiectasis, six of which were in adults, and four in children used AZM, concluded that long-term macrolide antibiotic therapy may suppress bacterial infection and reduce inflammation. As a result, there are fewer exacerbations, fewer symptoms, improved lung function, and improved quality of life, although increased bacterial resistance is a concern (Kelly et al., 2018).

E. Cystic Fibrosis

CF is a genetic disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) protein, an ABC transporter class ion channel protein that conducts chloride and bicarbonate ions and also regulates chloride transport across epithelial cell membranes. The disease is characterized by excessive mucus production, coughing, and increased infections. The multicentre, randomized clinical trial from 2003 referred to above showed that AZM improved clinically relevant endpoints (FEV1, exacerbation risk) in patients with CF older than 6 years, with an FEV1 > 30% of predicted at baseline, and infected with *P. aeruginosa* (Saiman et al., 2003). A more recent study in children 6 months to 18 years of age showed a 44% decreased risk of pulmonary exacerbations during 18 months of AZM treatment (Mayer-Hamblett et al., 2018). Maintenance therapy with AZM in chronically infected patients with CF is now a recommendation in clinical guidelines

(Castellani et al., 2018) despite the potential of inducing antimicrobial resistance.

The somewhat surprising success of AZM in treating chronically infected patients with CF, especially those with bacterial strains that are not sensitive to AZM, led researchers to speculate that its positive effects might be due to, among others, direct effects on the dysfunctional airway epithelium in patients with CF. To explore this notion, TEER of airway epithelia in vitro was measured and found to be increased significantly after treatment with AZM, suggesting the possibility that AZM might enhance airway epithelial barrier integrity (Asgrimsson et al., 2006).

In CF, it is intriguing that an antibiotic made its way into clinical guidelines not solely based on its antimicrobial activity. Suggested mechanisms of action explaining the beneficial effects of AZM include the anti-inflammatory and immunomodulatory effects reviewed here and elsewhere (Amsden, 2005; Parnham et al., 2014; Bush, 2020; Reijnders et al., 2020) in addition to modest antipseudomonal effects (Saiman et al., 2002). However, effects of AZM on the biophysical properties of airway epithelia have not been studied as extensively, although the possibility of direct effects on the CFTR chloride channel have been suggested (Altschuler, 1998). This notion was further expanded by LeSimple and coworkers, suggesting that CFTR might be important to maintain airway epithelial integrity in general by affecting TJ expression. They found that CFTR trafficking to the apical membrane was required for the function of TJ proteins in an airway epithelial model, as measured by TEER and protein expression (Baldursson, 2010; LeSimple et al., 2010). Other studies also indicated that AZM increased TEER in airway epithelia in vitro by affecting the expression of TJ proteins and that these effects fended off *P. aeruginosa* attacks on the epithelium (Asgrimsson et al., 2006; Halldorsson et al., 2010). Recently, the limitations of AZM in the treatment of CF, particularly with regard to the risk of bacterial resistance, have been emphasized, and an appeal has been made to develop nonantimicrobial derivatives for pediatric inflammatory airways diseases (Bush, 2020).

F. Idiopathic Pulmonary Fibrosis

IPF is a common, progressive fibrotic lung disease of unknown cause associated with interstitial pneumonia, lung scarring, and declining lung function. It has a poor prognosis. A retrospective analysis of hospitalized patients with acute exacerbation of IPF revealed that treatment with AZM (500 mg/d for 5 days, $n = 38$) was significantly more effective than fluoroquinolones ($n = 47$) in reducing 60-day mortality (Kawamura et al., 2017). The authors suggested that the additional immunomodulatory actions of the macrolide were responsible for this difference. More recently, prophylactic treatment with AZM (250 mg/kg, three times per week) for up to 12 months has been reported to significantly reduce hospitalization rate (Macaluso et al., 2019). However, no reports have appeared suggesting that AZM or other macrolides provide any meaningful clinical improvement in the underlying disease or its progression.

In a murine bleomycin-induced pulmonary fibrosis model of IPF, AZM given at an early stage was reported to reduce both fibrosis and lung function limitation (Wuyts et al., 2010). In parallel, AZM inhibited both neutrophil function (innate immunity) and modified T-helper cell cytokine generation, once again supporting an immunomodulatory effect. In a subsequent short communication, the same authors failed to find an effect of AZM when administered after day 21 (Willems et al., 2012). Epithelial regenerative capabilities are nearly exhausted in advanced IPF disease, and epithelial tissue is largely replaced by fibrotic scar tissue, so it is unlikely that, at this stage, the epithelial-enhancing properties of AZM play a role.

Interestingly, however, AZM has recently been reported to inhibit collagen generation by primary human IPF fibroblasts, not by healthy human fibroblasts, suggesting an alternative target for the macrolide in this disease (Krempaska et al., 2020).

G. Other Diseases

1. Airway Viral Infections and Acute Respiratory Distress Syndrome. Although macrolides have a variety of antiviral effects, in experimental studies, clinical data are less clear. For many of the more common and serious respiratory viruses, such as respiratory syncytial virus (RSV), rhinovirus, influenza virus, and other less common viruses (Min and Jang, 2012), as well as against SARS-CoV-2 virus (Sultana et al., 2020; Oliver and Hinks, 2021), clinical results have been contradictory, causing uncertainty about their potential therapeutic use. Optimal treatment strategies and clinical evidence for their efficacy are considered weak at best.

For example, when given acutely for treatment of RSV-related bronchiolitis in infants at the point of hospital admission, AZM treatment did not improve prognosis of the RSV-infected infants compared with controls (Pinto et al., 2012). However, some patients in the same cohort show improved biomarkers and longer-term outcomes, such as time to next wheezing episode (Kneyber et al., 2008; Kong et al., 2020). It is interesting then, that AZM was effective in children in Niger in reducing viral titers of SARS-CoV-2 on repeated prophylactic treatment (Doan et al., 2020). In adults, no reliable evidence exists for any such antiviral effects of AZM in new SARS-CoV-2 infections, although a few trials are underway (Oliver and Hinks, 2021).

Although the lack of efficacy of AZM as an acute treatment of COPD-related exacerbations has not been formally confirmed, retrospective analysis as well as randomized trials have confirmed its obvious efficacy as continuous maintenance therapy in reducing frequency and severity of exacerbations in various CADs, in which many of the exacerbations are of viral cause (Oliver and Hinks, 2021). Furthermore, viral exacerbations tend to be milder in AZM-pretreated patients compared with controls.

Clarification of the heterogeneity in trial structure of available studies on the efficacy of macrolides in viral respiratory infections, in both children and adults, may help to determine how these drugs can be used effectively against various airborne viruses, as well as distinguishing between ineffective and effective dosing strategies for the future.

The airway mucosal inflammatory infiltrate in most viral airway infections is predominantly neutrophilic in the first 3 days of inflammation, but during the peak of symptoms, it becomes lymphocytic. Since the immunomodulatory properties of macrolides target mainly macrophages and granulocytes (eosinophils and neutrophils), and viral drug sensitivity is maximal during early replication, after internalization, it follows that macrolide therapy appears most effective when administered early in the course of the infection process or even a few weeks prior to likely exposure. Such a dosing regimen is likely to reduce further the chances of exacerbations and possible respiratory failure.

ARDS is a common, severe outcome in patients entering the ICU with either viral or bacterial infections. Antibiotics, including macrolides, in addition to nonpharmacological measures are among the first-line treatments for this condition. Recent studies have sought to assess the therapeutic benefit of macrolide therapy in terms of outcome. In a secondary analysis of data from the Acute Respiratory Distress Syndrome Network Lisofylline and Respiratory Management of Acute Lung Injury Trial, macrolide treatment (mostly erythromycin and AZM) of 47 patients was found, after adjusting for covariate confounding, to have significantly reduced both 180-day mortality and time to discontinuation of mechanical ventilation (Walkey and Wiener, 2012). Importantly, fluoroquinolone and cephalosporin antibiotics were ineffective, pointing to an additional benefit of macrolide therapy. This is supported by the results of the retrospective study discussed earlier on patients with acute exacerbations of IPF, showing that AZM was significantly more effective than fluoroquinolones in reducing 60-day mortality (Kawamura et al., 2017). In a later, large, multicenter, prospective observational ICU study, 158 of 873 patients with ARDS were treated with macrolides (97% erythromycin), and again, the treatment significantly reduced mortality, in this case assessed over a 30-day period (Simonis et al., 2018). A further single-center retrospective cohort evaluation identified 62 patients with moderate or severe ARDS who had received AZM. Treatment with this macrolide significantly reduced both 90-day mortality and time to successful discontinuation of mechanical ventilation (Kawamura et al., 2018). Interestingly, 28-day mortality was not significantly reduced, suggesting that the longer-term effects of AZM were relevant for this condition. It is also relevant to point out that clarithromycin has also been found to reduce 90-day mortality and hospitalization costs in patients with sepsis, in whom ARDS is a common sequela (Tsaganos et al., 2016).

Lessons on the macrolide treatment of ARDS can be inferred from reports on the use of AZM in the treatment of COPD, RSV, and various coronavirus infections, such as Middle East respiratory syndrome and COVID-19, in which ARDS and ventilator-induced injury is less likely to develop (Hinks et al., 2020; Pani et al., 2020; Oliver and Hinks, 2021).

A further potentially beneficial indirect viral-inhibitory effect of AZM against influenza virus A-H1N1 in epithelial cells was recently reported showing that virus internalization was reduced by AZM compared with controls, although surface adhesion was not affected. Also, progeny virus replication was remarkably inhibited by treating viruses with AZM before infection, although AZM administration after infection did not affect this process. The same study reported superior outcomes in AZM-treated mice compared with placebo in reducing viral load and relieving hypothermia in response to influenza infection when administered intranasally (Tran et al., 2019).

Therefore, it seems that the most promising regimen for AZM would be as daily doses, either prophylactically during viral pandemics or as maintenance therapy in patients with CAD. Ironically, such an application of AZM or other macrolide antibiotic also happens to be the most unappealing strategy from the standpoint of bacterial resistance development. As with the canaries in the coal mines in the 19th century, the morbidity and mortality related to macrolide-resistant NTMB among vulnerable patients with end-stage airway disease has raised awareness in the professional community and the general public of the risks of macrolide overprescription. The current chronic overuse of macrolides off-label, AZM in particular, is likely to worsen in the light of recent suggestions of potential benefit of AZM treatment in patients with COVID-19, although the place of this macrolide in the COVID-19 armamentarium is far from clear (Sultana et al., 2020; Oliver and Hinks, 2021). Nevertheless, the heightened interest in AZM during the COVID-19 pandemic further highlights the need for a nonantibiotic substitute for AZM that possesses the

immunomodulatory and antiviral properties of this drug and its capacity to enhance epithelial barrier integrity. Such drug therapy will further minimize viral epithelial impact and promote regeneration and resolution of inflammation (Bush, 2020) (Fig. 4).

2. Pediatric Uses. In pediatric medicine, the longest tradition for macrolide use, mainly erythromycin, in airway disease is in the treatment of asthma. In recent years, though, AZM has taken the place of erythromycin in CF therapy. AZM has been recommended for off-label maintenance treatment of severe non-Th-2 (neutrophilic) corticosteroid-resistant childhood asthma (Sugawara et al., 2012).

Approved pediatric macrolide antibiotic formulations are limited and perhaps harder to prescribe in many regions than β-lactams. There are indications, as reported for China, The Netherlands, and some parts of the United States, that overuse of macrolides as antibiotics is greater for children than for adults, possibly because of limited possibilities for controlled analysis of continuous use (Fleming-Dutra et al., 2018; Bandell et al., 2019; Wang et al., 2020).

Although perhaps not recognized by pediatricians, macrolide resistance in *Mycoplasma pneumoniae* is increasing in children (Li et al., 2009), and it is crucial that macrolide-sensitive bacterial pathogens such as *Ureaplasma ureolyticum* do not develop resistance in children. This can only be sustained if macrolide antibiotics are conserved for short-term use, both in hospitals and in the community. A recent review article on the current situation in pediatrics clearly presents the situation and suggests how to act preemptively in the future (Bush, 2020).

3. Inflammatory Skin Diseases. Macrolides, particularly erythromycin, are widely used in topical formulations for the treatment of skin infections, including acne. This is also true for rosacea, which has an unclear etiology, for which AZM has also shown efficacy, probably related to its anti-inflammatory actions (Bakar et al., 2007; Alzolibani and Zedan, 2012). The evidence is also strong for macrolide efficacy in various dermatitis syndromes ranging from atopic dermatitis to psoriatic skin lesions, the latter on oral macrolide administration (Alzolibani and Zedan, 2012).

As macrolides can be applied topically without provoking generalized resistance in host flora, the risk of generating macrolide-resistant bacteria is much less than with other oral macrolide–sensitive inflammatory disorders of soft tissues. Moreover, novel macrolides with anti-inflammatory properties are being developed for topical administration in skin disorders (Rodriguez-Cerdeira et al., 2012). Such topical nonantibiotic macrolides could markedly expand the therapeutic indications for macrolides, with aerosols and gut-targeted formulations for treatment of other types of inflammatory disorders.

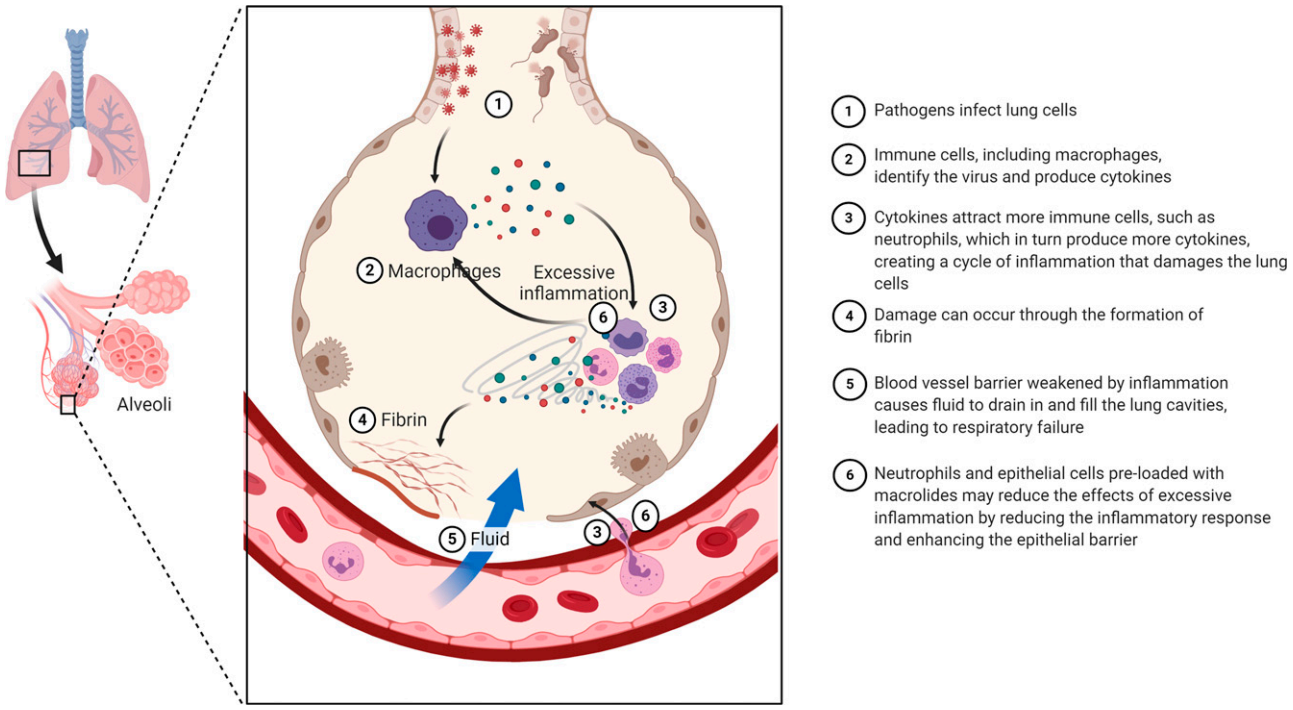

Fig. 4. Potential actions of AZM in ameliorating the damaging effects of excessive inflammation. Pathogen infection of the respiratory system may, in the worst-case scenario, result in hypercytokinemia that involves macrophage-neutrophil interactions with increased secretion of proinflammatory cytokines that exacerbate the inflammation. Subsequently, this may seriously disturb homeostasis and induce weakening of the epithelium and result in organ failure. AZM treatment attenuates these effects by reducing the expression and secretion of proinflammatory cytokines and causing macrophage polarization, with subsequent promotion of epithelial barrier function.

4. Inflammatory Bowel Diseases. The evidence for local anti-inflammatory effects of macrolides in populations suffering from IBD is hard to find. Preclinical evidence exists suggesting potential use of macrolides as immunomodulators in gut inflammation (Anderson et al., 2019). Intriguingly, it has been shown with the murine dextran sulfate sodium colitis model that single early-life treatment with antibiotics, including the macrolide tylosin, can disturb the gut microbial environment sufficiently to exacerbate subsequent IBD later in life (Ozkul et al., 2020).

Although people suffering from chronic inflammatory skin disease, as well as airway diseases, tend to be elderly, IBD tends to arise in otherwise healthy young adults, adolescents, and even children. Since this population is less likely to be treated with antibiotics, data on clinical effects of macrolides are fewer than in older people with COPD.

Consequently, it seems likely that, as with respiratory and skin disorders, long-term macrolide use in young adults would reveal disease remission in IBD with continuous therapy. In fact, several studies have been carried out with clarithromycin and combined metronidazole/AZM treatment as antibiotics for several months, showing that partial remission of IBD can be achieved. It is suggested that both antibacterial and immunomodulatory actions of the macrolides contribute to these beneficial effects in this therapeutic setting (Ledder and Turner, 2018).

Since most of the macrolide-sensitive inflammatory diseases seem to center around disorders displaying acute epithelitis and compromised barrier integrity, the epithelial-protective activities and immunomodulatory effects of the 15-membered macrolactones (barriolides) may not only provide the basis for addressing granulocytic inflammatory cells and innate inflammatory cascades directly but also facilitate barrier-enhancement and maintain barrier function and epithelial integrity in the longer-term, contributing to disease remission. This element of the tissue-specific properties of different macrolides may be relevant for many epithelial inflammatory disorders of a variety of organ systems.

VI. Perspectives: Macrolides as Barrier Protectors

In this review, we have briefly outlined the nonantibiotic effects of macrolides and the relevant mechanisms of action and clinical uses of macrolides. We have also discussed attempts to develop novel anti-inflammatory macrolides without antibacterial actions and then highlighted the recent development of barriolides, nonantibacterial macrolides optimized for barrier-protecting actions on epithelial cells.

One of our primary conclusions is that the bulk of the evidence for the clinical efficacy of macrolide antibiotics as disease-modifying agents in noninfectious inflammatory diseases rests mainly on three members—namely, erythromycin, clarithromycin, and AZM. Relevant data also exist for the clinical use of roxithromycin in this regard, although its use is much more limited geographically, and the volume of data is less than that for the other three.

The latest macrolide antibiotic to reach the market is solithromycin, a 14-membered fluoroketolide known to have immunomodulatory effects on macrophages, but its clinical use has been limited to short-term antibacterial therapy due to long-term toxicity issues (which led to nonapproval by the Food and Drug Administration) when tested for anti-inflammatory efficacy in patients with COPD (Fernandez et al., 2004; Kocsis and Szabo, 2017).

Secondly, another broad observation is that despite being the last of the three to be introduced to clinical practice, with initial market registration in 1981, AZM over the last four decades has become the macrolide of choice to prescribe to patients for off-label use for nonantimicrobial actions when administered chronically to patients with a range of diseases. This is due to its favorable safety and obvious efficacy. This, for example, is evidenced by the fact that AZM is the most widely used macrolide for the off-label treatment of DPB, CF, bronchiolitis obliterans syndrome non-CFBE, and therapy-resistant asthma and is in the GOLD clinical guidelines for the treatment of COPD (GOLD guidelines) (https://goldcopd.org/2021-gold-reports).

Although, in the light of history, erythromycin and clarithromycin have clear efficacy compared with no treatment and have been in longer clinical use than AZM, it seems as though AZM, more than its closest 14-membered relatives, addresses the underlying causative factors in a broader and more profound way, as well as treating complications of the macrolide-sensitive noninfectious airway diseases (Cameron et al., 2012; Kelly et al., 2018; Bush, 2020; Reijnders et al., 2020; Yang, 2020).

Whether this distinction is due to more favorable pharmacokinetic or pharmacodynamic properties of the 15-membered, compared with the 14-membered, macrocyclic backbone is yet to be determined, and it is likely to depend on a variety of pharmacological factors.

Since all the commercially available macrolides are primarily antibiotics, the need for a nonantibiotic macrolide is in large part due to the loss of macrolide sensitivity in otherwise hard-to-treat infectious diseases, arising from frequent off-label overprescriptions, particularly since macrolides are often administered chronically. This applies to infections caused by intracellular pathogens like *Chlamydia* species, *Legionella*, *Ureaplasma,* and *Mycoplasma*

and other clinically relevant macrolide-sensitive microorganisms such as gonococci and pneumococci. For both physicians and a particular group of patients with advanced CAD-like, including CF-related, bronchiectasis, the spread of macrolide resistance among atypical or opportunistic pathogens, like the NTMB species or *M. avium* complex, is of grave concern (Kelly et al., 2018).

The development of nonantimicrobial macrolides has been driven by the recommended off-label chronic use of macrolides as maintenance treatment in a range of airway diseases and nonairway diseases as discussed above. Although reliable data for 2020 are not available yet, it is to be expected that the trend toward overprescription of AZM, as well as that of hydroxychloroquine, will have been temporarily accelerated after the controversial publication by Gautret et al. (2020) in mid-March 2020 showing lowered nasopharyngeal SARS-CoV-2 viral titers with the combination of both drugs.

Should this turn out to be the case, it will then be vital to monitor whether this trend is reversed with increased awareness among physicians of multiresistant bacteria arising from unwarranted antibiotic use. It is also worth noting that, at the time of writing this review, based on available clinical data, there is no clear evidence of a benefit of treatment of COVID-19 patients with AZM (Sultana et al., 2020).

Numerous novel investigational macrolides have been described, tracing back to the late 1970s, which have been tested and compared with the clinically relevant macrolides in preclinical models (see Table 1).

Many of these investigational compounds were intended for use as nonantibiotic derivatives for long-term therapy of chronic inflammatory diseases, without provoking bacterial resistance to macrolactones. Interestingly, none of them seem to have reached clinical development before the programs were terminated, and none appear to have been developed further.

When the modified macrolide space is analyzed, many of the published nonantibiotic macrolides have closely followed erythromycin in their design, with anti-inflammatory effects being used to select the molecules. Some 15-membered derivatives exhibiting anti-inflammatory effects in preclinical models have also been described (Erakovic Haber et al., 2014; Burnet et al., 2015). Some developers have also described 12-membered macrolactones with immunomodulatory properties and anti-inflammatory effects as potential therapeutic agents, but to date, there are no reported clinical trials with such drugs (Sugawara et al., 2011, 2012, 2016).

On the grounds of its well established immunomodulatory properties, reviewed above, erythromycin derivatives have been referred to as immunolides because of their adherence to the erythromycin-based, 14-membered standard for clinical efficacy (Fecik et al., 2005). The term sterolides has occasionally been used to denote steroid-conjugated compounds with additional immunomodulatory effects compared with erythromycin and AZM (Mercep et al., 2004). The fluoroketolides such as solithromycin, have been discussed above, but nonantibiotic fluoroketolides have been described as well (Janas and Przybylski, 2019). The recent introduction of the class of 15-membered AZM derivatives called barriolides, selected on the basis of modulating barrier enhancement, is a promising development, with the lead compound, EP395, about to enter clinical trials in 2021.

It seems as if all the clinically used macrolide antibiotics share a common pattern of immunomodulatory properties with anti-inflammatory efficacy addressing many aspects of inflammatory pathology. Although the epithelial protecting activity of AZM in vitro has distinguished it from erythromycin since 2006 (Asgrimsson et al., 2006), it is only recently that epithelial integrity has fully emerged and been recognized as a fundamental factor in diseases of epithelial inflammation and dysfunction (Knight and Holgate, 2003; Wittekindt, 2017).

Challenges to the airway epithelium are gaining increasing awareness with the addition of SARS-CoV-2 to the environmental factors that have been the main drivers of COPD on a global scale for over a decade. It is thus, tempting to infer that the excess efficacy of the 15-membered macrolide structure may be related to its ability to enhance epithelial cell integrity in addition to modifying inflammation, both actions leading to improved health and prognosis compared with erythromycin and its closest derivatives.

The overlapping spectrum of immunomodulatory properties of macrolides on granulocytes, macrophages, and other cells will remain important for patients with macrolide-sensitive airway diseases, or even in postviral and ICU-related airway inflammatory syndromes. Nevertheless, it seems as though the clear proepithelial activity of the nitrogen-containing 15-membered lactone ring, were it not in an antimicrobial molecule, could facilitate therapeutic use beyond that of the 14-membered relatives. Such an action could potentially lead to prophylactic therapy prior to looming airway exposure to viruses, pollen, or toxic particles (Bush, 2020).

Such a set of therapeutic characteristics in a single macrolide might be applicable not only as maintenance therapy for patients with CAD and other vulnerable populations but also as a seasonal or otherwise temporary prophylaxis for healthy individuals at increased risk from exposure to airborne pollution, allergens, or infectious agents.

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