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CD38 in the pathogenesis of allergic airway disease: potential therapeutic targets

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Abstract

CD38 is an ectoenzyme that catalyzes the conversion of β -nicotinamide adenine dinucleotide (β -NAD) to cyclic adenosine diphosphoribose (cADPR) and adenosine diphosphoribose (ADPR) and NADP to nicotinic acid adenine dinucleotide phosphate (NAADP) and adenosine diphosphoribose-2'-phosphate (ADPR-P). The metabolites of NAD and NADP have roles in calcium signaling in different cell types including airway smooth muscle (ASM) cells. In ASM cells, inflammatory cytokines augment CD38 expression and to a greater magnitude in cells from asthmatics, indicating a greater capacity for the generation of cADPR and ADPR in ASM from asthmatics. CD38 deficient mice develop attenuated airway responsiveness to inhaled methacholine following allergen sensitization and challenge compared to wild-type mice indicating its potential role in asthma. Regulation of CD38 expression in ASM cells is achieved by mitogen activated protein kinases, specific isoforms of PI3 kinases, the transcription factors NF- κ B and AP-1, and post-transcriptionally by microRNAs. This review will focus on the role of CD38 in intracellular calcium regulation in ASM, contribution to airway inflammation and airway hyperresponsiveness in mouse models of allergic airway inflammation, the transcriptional and post-transcriptional mechanisms of regulation of expression, and outline approaches to inhibit its expression and activity.

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Keywords

Airway smooth muscle; asthma; CD38; microRNAs; calcium regulation

1. Introduction

Asthma is a chronic inflammatory disease of the lung affecting over 300 million people globally. Despite several decades of research on asthma pathophysiology and pharmacology, efficacious anti-asthma medications continue to be an unmet medical need as more than half of asthmatics are inadequately controlled. Fulfilling this need involves, in part, identifying novel therapeutic targets for asthma. Allergen exposure in susceptible individuals leads to airway inflammation characterized by release of mediators such as cytokines and chemokines. These molecules regulate the expression of a variety of genes in the resident airway cells including airway smooth muscle (ASM) cells leading to important phenotypic changes, including airway remodeling and heightened bronchoconstriction.

Different cell types mediate the structural and functional changes underlying the asthmatic phenotype. For example, immune cells account for inflammatory response to allergen and smooth muscle cells contribute towards hyperresponsiveness and bronchoconstriction. The ASM is also capable of modulating local inflammatory response via production and release of chemokines and cytokines. Interestingly, we and others have demonstrated that CD38, a plasma membrane bound multifunctional enzyme, is expressed on immune and ASM cells, and plays roles in the orchestration of immune responses, regulation of ASM contraction and airway hyperresponsiveness. Therefore, CD38 is an attractive therapeutic target in asthma (Figure 1). This review summarizes the data highlighting the role of CD38 in asthma pathogenesis with an emphasis on approaches to therapeutically target CD38 in asthma.

2. Receptor and Enzymatic functions of mammalian CD38

The protein encoded by CD38 is a type II transmembrane glycoprotein with a molecular weight of ~45kDa (Ferrero, et al., 2000; Lee, 2006; Zocchi, et al., 1993). CD38 expression is ubiquitous and has been described in smooth muscle cells (Deshpande, et al., 2003; White, et al., 2000), pancreatic cells (Okamoto, et al., 1997), astrocytes (Banerjee, et al., 2008; Kou, et al., 2009; Mamik, et al., 2011), and in B lymphocytes (Deaglio, et al., 2003; Malavasi, et al., 2011; Morabito, et al., 2002). As a functional molecule, CD38 is a dimer and the catalytic site is contained within the central part of the molecule (Munshi, et al., 2000; Zhao, et al., 2012; Zhao, et al., 2015). In addition, CD38 appears to be localized within lipid microdomains of the plasma membrane (Lund, et al., 2006) where it associates with several other proteins to form a complex. Among the proteins that CD38 forms a complex in B lymphocytes are CD19/CD81 (Song, et al., 2016), CXCR4, a chemokine receptor (Majid, et al., 2011), and CD49d, an adhesion molecule (Zucchetto, et al., 2012).

It is interesting to note that the mammalian CD38 has similarity (~35% amino acid identity) to a soluble form of the enzyme adenosine diphospho-ribosyl cyclase (ADP-ribosyl cyclase) in the ovotestis of the mollusk *Aplysia californica* (Lee & Aarhus, 1991; States, et al., 1992). ADP-ribosyl cyclase is capable of cyclizing NAD⁺ to form cyclic ADP ribose (cADPR)

(Lee & Aarhus, 1991). cADPR has been shown to release calcium from intracellular stores that are distinct from that released by IP₃ in sea urchin eggs (Dargie, et al., 1990), porcine airway smooth muscle cells (Prakash, et al., 1998) and Ascidian oocytes (Albrieux, et al., 1998). The mammalian CD38 possesses ADP ribosyl cyclase activity that converts NAD⁺ to cADPR as well as cADPR hydrolase activity that converts cADPR to ADPR (Howard, et al., 1993; Zocchi, et al., 1993). It is important to note that CD38 is predominantly a NAD glycohydrolase enzyme and generates ADPR directly from NAD (Schuber & Lund, 2004). Therefore, ADPR production largely comes from direct conversion of NAD by CD38. Nevertheless, cADPR has been described as a second messenger molecule capable of releasing calcium from the intracellular stores in a variety of cells (Lee, 2011). cADPR hydrolase activity of CD38 converts cADPR to ADPR thereby controlling the calcium-mobilizing activity of cADPR. Other reports have also identified an enzymatic activity of CD38 that is capable of converting NADP⁺ to NAADP⁺ via a base-exchange reaction in a pH-dependent fashion (Aarhus, et al., 1995; Chini, et al., 2002; Lee, 1999). In airway smooth muscle cells, cADPR releases calcium from the sarcoplasmic reticulum via activation of the ryanodine receptor channels (Prakash, et al., 1998; White, et al., 2003). NAADP signaling has also been shown to play a role in muscarinic receptor-induced contraction of guinea pig trachea (Aley, et al., 2013) (Figure 2). A byproduct of NAD⁺ metabolism by CD38 is nicotinamide and CD38 is a critical enzyme involved in NAD⁺ homeostasis and cellular energy metabolism (Al-Abady, et al., 2013; Barbosa, et al., 2007; Camacho-Pereira, et al., 2016; Chini, 2009; Young, et al., 2006). The receptor functions of CD38 appear to be distinct from the enzymatic functions that generate calcium-mobilizing second messenger molecules (Lund, 2006).

The best-characterized mammalian ADP-ribosyl cyclase enzyme is CD38. Thus, CD38 is considered to be critical for calcium-dependent biological processes *in vivo*. Early reports identified CD38 in the activation and proliferation of T lymphocytes (Lund, 2006). Prior investigations in neutrophils revealed that cADPR produced by CD38 is required for neutrophil chemotaxis and bacterial clearance, providing a critical role for this molecule in inflammation and innate immunity (Lund, 2006). In this review, we will outline the contribution of CD38 to intracellular calcium regulation of airway smooth muscle, its role in airway inflammation and airway hyperresponsiveness in the context of allergic airway disease, the regulation of CD38 expression and specifically the role of MAP kinases and transcription factors involved, and post-transcriptional regulation of expression by microRNAs (miRNAs). We will identify therapeutic targets that can result in modulation of CD38 expression in an attempt to provide a potential translational relevance to the small molecule inhibitors of CD38 and cADPR antagonists in the mitigation of airway hyperresponsiveness in diseases such as asthma.

3. CD38/cADPR in the regulation of intracellular calcium in airway smooth muscle

Our laboratory undertook studies to delineate the contribution of the two major pathways of SR calcium release, namely that mediated by activation of the IP₃-receptor channels and that mediated by activation of the ryanodine receptor channels (Deshpande, et al., 2003; Kannan,

et al., 1997; Kotlikoff, et al., 2004; Prakash, et al., 1998). The involvement of CD38-derived cADPR in the regulation of intracellular calcium responses to agonists has been supported by studies in our laboratory (Figure 2). In earlier studies, we showed that exposure of ASM cells to acetylcholine elicited oscillations in intracellular calcium (Pabelick, et al., 1999; Prakash, Kannan, et al., 1997a, 1997b; Prakash, et al., 2000; Prakash, van der Heijden, et al., 1997). While the peak to trough amplitude of the intracellular calcium oscillations was relatively constant in any region of the cell as measured by real-time confocal microscopy, the frequency of the oscillations increased with increasing concentration of the agonist. Furthermore, the velocity of propagation of the oscillations from one region to another region of the cell also increased with increasing agonist concentration. The spatial and temporal integration of the intracellular calcium oscillations resulted in a mean calcium response to an agonist whose magnitude increased with the concentration of the agonist. During ongoing intracellular calcium oscillations, addition of 8-amino-cADPR, a cADPR antagonist (Walseth & Lee, 1993) resulted in slowing of the frequency of oscillations, whereas addition of cADPR (to permeabilized cells) caused an increase in the frequency of the oscillations (Prakash, et al., 1998). This slowing and the increase in the frequency of intracellular calcium oscillations would be reflected respectively in a reduction and increase of the mean calcium response. In permeabilized ASM cells, addition of cADPR causes a calcium transient. This calcium transient is absent in cells preincubated with ryanodine or ruthenium red which inhibit calcium release from the sarcoplasmic reticulum through ryanodine receptor channels, but present in the presence of heparin which inhibits calcium release through IP₃-receptor channels (Prakash, et al., 1998). Studies from other investigators showed that addition of cADPR increased the amplitude and frequency of spontaneous transient inward currents, providing evidence for spontaneous calcium release by cADPR (Y. X. Wang, et al., 2004). While these results indicate that cADPR mediates calcium release and regulates the frequency of intracellular calcium oscillations, the definitive proof that CD38-derived cADPR contributes to regulation of intracellular calcium came from other studies in our laboratory. The magnitude of the intracellular calcium responses to agonists in airway smooth muscle cells obtained from CD38 knockout mice were significantly attenuated compared to responses in cells obtained from wild-type mice (Deshpande, et al., 2005).

The mechanisms by which cADPR causes calcium release in ASM cells have been explored. In airway myocytes obtained from CD38 knockout mice, the calcium response elicited by the addition of acetylcholine or endothelin-1 is significantly attenuated as compared to responses elicited in myocytes obtained from wild-type mice (Deshpande, et al., 2005). Furthermore, pre-incubation of myocytes from wild-type mice with ryanodine causes significant attenuation of calcium responses to acetylcholine, while having no significant effect on the calcium response in myocytes obtained from CD38 knockout mice (Deshpande, et al., 2005). It is also interesting to note that in porcine ASM cells, the calcium response elicited by acetylcholine or endothelin-1, but not by histamine, is significantly attenuated in the presence of 8-Br-cADPR, a competitive membrane-permeant antagonist of cADPR (White, et al., 2003). Histamine-induced calcium response is not sensitive to inhibition by ryanodine as the response to acetylcholine or endothelin-1. These observations strongly indicate that the agonist-selectivity of cADPR-mediated calcium release is due to

recruitment of ryanodine-receptor mediated calcium release by some agonists but not by all agonists in ASM (White, et al., 2003). During ongoing intracellular calcium oscillations initiated by the addition of acetylcholine, exposure to cADPR increases the frequency of these oscillations. Other studies have shown that cADPR-mediated calcium release from the sarcoplasmic reticulum may involve binding to accessory proteins such as FK506-binding protein (Y. X. Wang, et al., 2004; X. Zhang, et al., 2009). In the pancreatic acinar cells, addition of cADPR causes the dissociation of FK506-binding protein from the ryanodine receptors (Ozawa, 2004, 2006). A similar mechanism of binding of cADPR to FKBP-12.6 leading to its dissociation from ryanodine receptor channels has been proposed for arterial and tracheal smooth muscle cells (Tang, et al., 2002; Y. X. Wang, et al., 2004). Further evidence that cADPR-mediated calcium response involves binding to FK506-binding protein is supported by a lack of calcium response in ASM cells obtained from FKBP12.6 knockout mice (Y. X. Wang, et al., 2004). It should be emphasized that dissociation of FKBP-12.6 from ryanodine receptor channels by cADPR as the mechanism underlying the activation of the channels has been challenged by other investigations (Copello, et al., 2001). Alternately, cADPR/FKBP-12.6 complex can bind to ryanodine receptors to cause its activation. It should also be noted that other accessory proteins have been suggested in the actions of cADPR in different cell types (Venturi, et al., 2012). In summary, experimental evidence strongly indicate that cADPR-mediated calcium release from the intracellular stores involves interaction with the ryanodine receptor channels directly or indirectly via involving accessory proteins such as FK506-binding protein (Figure 2).

4. Calcium Regulation in Airway Smooth Muscle by Inflammatory Cytokines

Airflow limitation that occurs in inflammatory diseases such as asthma arises from heightened contraction of ASM. Contraction of ASM is mediated by spasmogens that act via G-protein-coupled receptors and include acetylcholine released from post-ganglionic parasympathetic nerve terminals within the airways, and histamine, leukotrienes and prostaglandins that are released from mast cells. Regulation of smooth muscle contraction is achieved by phosphorylation of myosin light chain (MLC) by the MLC kinase (MLCK) (Chiba, et al., 2010; Kitazawa, Gaylinn, et al., 1991; Kitazawa, Masuo, et al., 1991; Shirazi, et al., 1994). Dephosphorylation of MLC is brought forth by myosin phosphatase that terminates the contractile process.

Phosphorylation of myosin phosphatase by Rho-kinase, a serine/threonine kinase, inactivates myosin phosphatase, resulting in contraction of smooth muscle (Somlyo & Somlyo, 2003). An increase in the intracellular calcium concentration activates MLCK and results in a calcium-dependent smooth muscle contraction (Figure 2). On the other hand, the inactivation of myosin phosphatase by Rho-Kinase results from an increase in sensitivity to calcium and this process is termed calcium sensitization. A variety of mechanisms have been proposed to account for the rise in intracellular calcium following stimulation of ASM by agonists that act via G-protein-coupled receptors. In ASM, an important and significant source of rise in intracellular calcium concentration by spasmogens is calcium release from intracellular stores such as the sarcoplasmic reticulum (SR). Thus, mechanisms that regulate calcium release from the SR can have a significant impact on contractility of airway smooth muscle.

We investigated the regulation of CD38/cADPR signaling in the intracellular calcium responses to agonists by inflammatory cytokines (Deshpande, et al., 2004; Deshpande, et al., 2003). Human ASM cells were isolated from lungs of healthy subjects and asthmatics (de-identified lungs from donors and with Institutional approval). Cells were grown to confluence and growth-arrested in serum-free medium for 24–48 hr. Exposure of human airway smooth muscle cells to inflammatory cytokines such as IL-13 and TNF- α for 24–48 hr caused an increased expression of CD38 and NAD⁺-catalyzed cADPR generation. The intracellular calcium responses to agonists such as acetylcholine, bradykinin and ET-1 were significantly augmented in cells treated with the cytokines compared to responses in vehicle-treated cells. Furthermore, pre-incubation of cells with 8-bromo-cADPR, a cell permeant competitive cADPR antagonist (Sethi, et al., 1997; Walseth & Lee, 1993), resulted in a significant reduction in the intracellular calcium responses to agonists and the responses seen in the cells were comparable to those seen in cells treated with the vehicle. Knockdown of CD38 expression by siRNA or following transfection with anti-sense oligonucleotides also caused a reduction in the intracellular responses to agonists in cytokine-treated cells (Kang, et al., 2005). These results clearly showed that the augmented intracellular calcium responses to agonists following cytokine exposure can be attributed to the increased CD38 expression and cADPR-mediated calcium release, and CD38 expression and cADPR-mediated calcium responses to agonists are expected to be greater in an inflammatory milieu such as in the lungs of asthmatics. In this context, we compared the induction of CD38 expression by TNF- α in ASM cells obtained from asthmatics and non-asthmatics (Jude, et al., 2010). Exposure to TNF- α resulted in a concentration-dependent induction of CD38 expression and ADP-ribosyl cyclase activity in ASM cells from both asthmatics and non-asthmatics. However, the magnitude of CD38 induction was significantly greater in cells from asthmatics than in cells from non-asthmatics, suggesting a greater capacity for cADPR-mediated calcium response to agonists. This differential induction of CD38 expression is due to increased transcriptional regulation involving ERK and p38 MAPK activation and is independent of changes in NF- κ B or AP-1 activation. The findings suggest a potential role for CD38 in the pathophysiology of asthma (Table 1).

Airway inflammatory diseases such as asthma are characterized by structural changes in the lungs collectively called as “airway remodeling” and phenotype modulation of airway cells (Prakash, 2013). Airway remodeling includes excessive accumulation of smooth muscle mass, sub-epithelial fibrosis, deposition of extracellular matrix including collagen and fibronectin, mucus cell hyperplasia and thickening of airway wall. Airway cells including ASM cells undergo phenotypic changes due to inflammation. ASM cells are “contractile” in nature and assume a “synthetic or proliferative” phenotype during airway inflammation thereby contributing to ASM remodeling. Considering the up-regulation of CD38-cADPR in ASM by inflammatory cytokines, it is plausible that this pathway plays a critical role in the regulation of ASM growth, a hallmark feature of airway remodeling. Future studies are needed to establish the role of CD38-cADPR pathway in ASM cell proliferation. Interestingly, lack of CD38 in coronary arterial smooth muscle cells induced a proliferative phenotype (Xu, et al., 2015; Y. Zhang, et al., 2014). This phenotype switch is accompanied by increased expression of vimentin in smooth muscle cells, collagen deposition around the arterial wall, and decreased expression of contractile phenotype markers such as calponin,

SM22 α and α -Smooth muscle actin. Stimuli that enhance atherosclerosis in arteries enhance phenotype switch induced by CD38 deficiency suggesting a potential role of CD38-cADPR in the regulation of smooth muscle proliferation and development of atherosclerosis. Furthermore, CD38 or cADPR mediated signaling pathway has been implicated in the regulation of differentiation of stem cell or development of tumors (Nipp, et al., 2014; Wei, et al., 2012). Chronic lymphocytic leukemia is a good example to demonstrate the role of CD38 in human proliferative disease (Vaisitti, et al., 2015). Airway remodeling in asthma comprises a similar cellular phenotype modulation, and based on the findings described above it is reasonable to hypothesize that CD38-cADPR signaling pathway plays a role in airway remodeling.

Studies have demonstrated synthetic functions of ASM cells in which ASM cells secrete inflammatory cytokines and chemokines and play an immunomodulatory role in asthma (Tliba & Panettieri, 2009). Tliba et al., demonstrated that CD38-cADPR-mediated signaling contributes to transcriptional regulation of inflammatory genes in ASM cells (Jain, et al., 2008; Tliba, et al., 2004). Further, interferon (IFN)- β is an additional signaling molecule that mediates TNF α -induced up-regulation of CD38 expression and thereby contributing to immunomodulatory function of ASM cells.

5. Contribution of CD38 to Airway Hyperresponsiveness in Mouse Models of Allergic Airway Disease

Our initial studies were directed at determining whether the CD38 knockout mice have an airway phenotype that is distinct from that of the wild-type mice. The changes in resistance to airflow and dynamic compliance following inhaled methacholine challenge were significantly attenuated in the CD38 knockout mice compared to wild-type mice (Deshpande, et al., 2005). We then investigated the methacholine responsiveness of CD38 knockout and wild-type mice exposed to the asthma-relevant cytokines TNF- α and IL-13. CD38 knockout mice developed significantly lower airway responsiveness to inhaled methacholine following IL-13 challenge than wild-type control mice. Airway inflammation in response to IL-13 was similarly robust in both mice, but airway reactivity to inhaled methacholine was significantly lower in the CD38 knockout mice than in wild-type controls. Using tracheal ring preparations from these mice, it was evident that CD38 within ASM mediated the IL-13-associated increase in isometric force generation to carbachol stimulation. Airway smooth muscle relaxation to the β -adrenergic receptor agonist isoproterenol was similar between the two mice (Guedes, et al., 2006). A single intranasal challenge with TNF- α caused airway hyperresponsiveness to methacholine in wild-type but not in CD38 knockout mice without major changes in airway inflammation. More prolonged exposure to TNF- α caused similarly robust airway inflammation in the mice, but the differences in airway phenotype were no longer present (Guedes, et al., 2008). Together, these results provide evidence that CD38 expressed in ASM cells plays a significant role in airway hyperresponsiveness to two asthma-relevant cytokines.

Earlier studies from Lund's laboratory indicated that CD38 knockout mice developed attenuated T cell-dependent humoral immune response to protein antigens (Cockayne, et al.,

1998). The underlying mechanisms for this attenuated allergen-induced antibody response were complex and include defective priming of T cells by *Cd38*^{-/-} dendritic cells and inefficient migration of *Cd38*^{-/-} dendritic cells from sites of inflammation to the draining lymph nodes (Partida-Sanchez, et al., 2007). Therefore, we hypothesized that allergen sensitization and challenge would result in an attenuated asthmatic phenotype in the CD38 knockout mice (Guedes, Jude, et al., 2015). We compared the airway responsiveness of CD38 knockout and wild-type mice following intranasal sensitization and intranasal challenge with fungal antigens as well as following intraperitoneal sensitization followed by intranasal challenge with ovalbumin antigen. Regardless of the route of allergen sensitization, the CD38 knockout mice developed significantly attenuated methacholine responsiveness compared to wild-type mice. However, the airway inflammatory response characterized by the number and types of inflammatory cells in the bronchoalveolar lavage (BAL) fluid was comparable in the two groups of mice. The changes in BAL fluid content of IL-5 and IL-13 were similar between CD38 knockout and wild-type mice in both allergen models. On the other hand, BAL fluid eotaxin-2 concentration increased following challenge with either allergen but the increase was lower in the CD38 knockout mice following fungal allergen challenge but not following ovalbumin. These findings indicated that CD38 expression is critical for the development of heightened airway hyperresponsiveness following allergen sensitization and challenge, although the magnitude of airway inflammation may not be dependent on CD38 expression. To explore this mechanism further, we developed bone marrow chimeras to assess if reciprocal transfer of *Cd38*^{+/+} or *Cd38*^{-/-} bone marrow-derived cells would restore the airway phenotype in the hosts under naïve conditions and following ovalbumin sensitization and challenge. Reciprocal bone marrow transfer did not affect the airway phenotype under naïve conditions. Following ovalbumin challenge, bone marrow transfer from either source partially reversed the airway phenotype in CD38 knockout mice whereas the airway constriction observed in WT hosts was unaffected regardless of whether the bone marrow cells were derived from WT or CD38 deficient animals (Guedes, Jude, et al., 2015). These results thus confirm that loss of CD38 in hematopoietic cells is not sufficient to prevent airway hyperresponsiveness to allergen sensitization and challenge and that airway inflammation is not the main determinant of airway hyperresponsiveness in these models. Jain et al. used *Aspergillus fumigatus* sensitization and challenge model and demonstrated that both wild-type and CD38 knockout mice developed a robust inflammatory response, although AHR to methacholine was attenuated in CD38 knockout mice compared to that in wild-type mice (Jain, et al., 2008). Furthermore, tracheal rings obtained from wild-type mice treated with TNF- α demonstrated enhanced contractile responses to carbachol and badykinin suggesting airway hypercontractility. Cytokine-induced airway hyperresponsiveness was completely abrogated in tracheal rings obtained from CD38 knockout mice.

Altogether, the above findings in the cytokine and allergen models are consistent with the role of CD38 in ASM calcium responses and contractility to agonists and provide evidence that this mechanism is the main driver for CD38-mediated airway hyperresponsiveness.

6. Regulation of CD38 Expression in Human Airway Smooth Muscle

The *cd38* gene is localized on chromosome 4 in human and chromosome 5 in the mouse (Ferrero, et al., 2000). The >80 kb length gene encoding CD38 has 8 exons and binding sites for several transcription factors. In human myeloid cells, CD38 expression is induced by retinoic acid binding to the retinoic acid response element located within the first intron (Mehta & Cheema, 1999). Response elements for transcription factors AP-1 and NF- κ B in the *cd38* gene have been described in erythropoietic cells (Sun, et al., 2006). In addition, glucocorticoid response elements (GREs) localized within the promoter region of the *cd38* gene have been identified (Tirumurugan, et al., 2008). In human ASM cells transfected with the 3 kb human *cd38* promoter, exposure to TNF- α resulted in a two-fold activation of the promoter. However, there was no induction of promoter activity in TNF- α stimulated human ASM cells transfected with a 1.8 kb *cd38* promoter that lacked the NF- κ B binding site, or promoter constructs lacking NF- κ B and/or AP-1 sites, or in the presence of dexamethasone. These findings therefore confirmed the functional importance of NF- κ B, AP-1 and GREs in the *cd38* promoter in the transcriptional regulation of CD38 expression (Tirumurugan, et al., 2008).

We and other investigators have reported the regulation of CD38 expression by cytokines involved in asthma pathogenesis (Table 1). The cytokines such as TNF- α and IL-1 β signal via NF- κ B and regulate CD38 expression. Glucocorticoids are most commonly used as anti-asthma medications and Tliba et al. investigated the effect of glucocorticoids on cytokine-induced up-regulation of CD38 expression (Tliba, et al., 2006; Tliba, et al., 2004). Glucocorticoid treatment inhibits CD38 expression induced by TNF- α alone or in combination with IL-1 β or IL-13. Interestingly, CD38 expression in human ASM cells in response to a combination of TNF- α and IFN- γ was insensitive to glucocorticoid. Glucocorticoid effect on CD38 expression potentially involves binding of glucocorticoid to GRE in the promoter region of *cd38* gene. TNF- α and IFN- γ together inhibit glucocorticoid-induced glucocorticoid receptor- α -DNA binding activity. These studies collectively demonstrate that the regulation of CD38 expression in airways during inflammation is complex and involves a combination of positive and negative regulators (Table 1).

Recent investigations have centered on post-transcriptional mechanisms of regulation of CD38 expression in human ASM. In this context, we have investigated the role of microRNAs (miRNAs) in such regulation (Deshpande, et al., 2015; Dileepan, et al., 2014; Guedes, Deshpande, et al., 2015; Jude, et al., 2012). miRNAs are small non-coding RNAs that are involved in the regulation of gene expression by binding to the 3'-Untranslated Region (3'UTR) of target mRNAs (Bartel, 2004; Ha & Kim, 2014; He & Hannon, 2004). This binding results in the degradation of mRNA and/or translational repression. Furthermore, a single miRNA is capable of regulating several genes. Altered miRNA expression has been implicated in airway inflammation (Chiba, et al., 2009; Dileepan, et al., 2014; Jude, et al., 2012; Kuhn, et al., 2010). We have identified miR-140-3p and miR-708 in the regulation of CD38 expression as well as many chemokine genes in ASM (Dileepan, et al., 2014). Target prediction algorithms have identified multiple binding sites for several miRNAs in *CD38* 3'UTR (Table 1). However, expression levels measured by qRT-PCR

revealed miR-140-3p and miR-708 as likely candidates for post-transcriptional regulation of expression. We investigated the mechanisms by which these miRNAs regulate CD38 expression in human ASM cells. Over-expression of miR-708 by transfection increased the expression of PTEN, a phosphatase that terminates PI3K signaling (Dileepan, et al., 2014). This increased PTEN expression was also associated with a decrease in AKT phosphorylation. miR-708 transfection of cells also resulted in enhanced expression of MKP-1, a phosphatase that terminates signaling through MAPKs. This increased MKP-1 expression was associated with decreased JNK MAPK phosphorylation. Prior studies have demonstrated that the PI3K/AKT signaling pathway has a major role in the proliferation of ASM cells from asthmatics (Burgess, et al., 2008). Dual luciferase reporter assays in a heterologous cell line also revealed binding of miR-708 to *cd38* 3'UTR. These results indicated that miR-708 binds to 3'UTR to regulate CD38 expression and indirectly regulates airway inflammation, ASM contractility and cell proliferation by regulating JNK MAP kinase activation and PI3K/AKT signaling.

Over-expression of miR-140-3p by transfection in human ASM cells also decreased CD38 expression and the CD38-associated enzyme activities with no changes in CD38 mRNA stability (Jude, et al., 2012). Dual luciferase reporter assays revealed binding of miR-140-3p to 3'UTR to regulate CD38 expression. Transfection of cells with the miR-140-3p mimic resulted in decreased activation of p38 MAPK as well as NF- κ B. It should be emphasized that MAPK and PI3K signaling as well as NF- κ B activation are required for expression of several pro-inflammatory genes. These findings indicated that there may be common as well as distinct mechanisms by which the two miRNAs regulate CD38 expression in human ASM cells. Inhibition of JNK and p38 MAPKs along with decreased PI3K/AKT signaling and NF- κ B activation should have profound anti-inflammatory effects. We propose that targeted delivery of these miRNAs into the lungs should result in significant anti-inflammatory effects. In this context, in our recent studies we examined the effect of transfection of human ASM cells with miR-140-3p and miR-708 on candidate inflammation-associated gene expression and validated the expression of some of these genes using qRT-PCR and release of some of the chemokines by ELISA (Dileepan, et al., 2016). Among the most significant biologic functions for the differentially expressed gene set following miRNA transfection in cells exposed to TNF- α were decreased inflammatory response, cytokine expression and signaling. In miR-708 transfected cells, qRT-PCR revealed inhibition of expression of the following chemokine genes: *CCL11*, *CXCL10*, *CCL2* and *CXCL8*, with inhibition of CCL11 release. In cells transfected with miR-140-3p on the other hand, there was inhibition of expression of *CCL11*, *CXCL12*, *CXCL10*, *CCL5* and *CXCL8*, with inhibition of CXCL12 release. Furthermore, miR-708 transfection also resulted in inhibition of expression of genes known to contribute to the pathogenesis of asthma such as *RARRES2*, *CD44* and *ADAM33*. These results showed that there are genes targeted by both miRNAs, while exerting distinct effects on inflammation-associated gene expression in human ASM cells (Dileepan, et al., 2016). Many of the chemokines whose expression is modulated by the miRNAs are involved in the recruitment of inflammatory cells into the airways such as eosinophils, basophils, mast cells and T lymphocytes in allergic airway disease. Furthermore, signaling through PI3K/AKT is also involved in ASM cell proliferation in asthma (Burgess, et al., 2008). Down-regulation of *PTEN* and *DUSP-1*, and p38 and JNK

MAP kinases as well as the transcription factor NF- κ B is involved in mediating inflammation. Several reports have demonstrated that airway smooth muscle has both contractile and immunomodulatory functions (Alrashdan, et al., 2012; Ammit, et al., 2000; Ammit, et al., 2002; Damera & Panettieri, 2011; Dekkers, et al., 2009; Ghaffar, et al., 1999; Hershenson, et al., 2008; John, et al., 2009; Knox, et al., 2001; Ozier, et al., 2011; Panettieri, et al., 1995; Tliba & Panettieri, 2009; K. Zhang, et al., 2007). During persistent airway inflammation, chemokines and cytokines elaborated by inflammatory cells and ASM cells can lead to cell proliferation and airway remodeling. miRNAs such as miR-140-3p and miR-708 play a critical role in the regulation of multiple of features of asthma pathogenesis. Most importantly, miR-140-3p and miR-708 regulate CD38 expression and cADPR production. Therefore, we propose that targeting these two miRNA networks may offer a novel therapeutic mechanism to regulate airway inflammation, ASM proliferation and airway remodeling via target proteins such as CD38 in diseases such as asthma.

Several recent reports provide evidence for altered expression of miRNAs in the induction of allergic airway inflammation. In a mouse model of allergic airway inflammation induced by house dust mite allergen, there was evidence for induction of expression of miR-126 with airway hyperresponsiveness to inhaled methacholine (Mattes, et al., 2009). In mice exposed to an antagomir specific for miR-126 followed by allergen sensitization and challenge, there was decreased release of IL-5 and IL-13 into the lungs, with decreased mucus hypersecretion, recruitment of eosinophils and attenuated methacholine responsiveness. In a chronic allergen challenge model, the authors noticed that anti-miR-126 had no significant effects on eosinophil recruitment into the lungs as well as the inflammatory response in the airway wall. In this chronic model of allergen challenge, miR-126 expression was elevated in the airway wall for a short time period and returned to baseline values, although the asthmatic phenotype of the mice was preserved beyond this period. These results suggest other mechanisms, such as multiple miRNAs may be involved in the switch from a normal to an asthmatic phenotype. In a study involving miR-155 knockout mice, there was indication for reduced Th2 cell numbers and chemokine expression in the lungs (Malmhall, et al., 2014). T cells deficient in miR-155 also produced significantly reduced quantities of IL-4. miR-155 knockout mice also exhibited reduced eosinophil numbers and mucus secretion compared with wild-type mice. miR-155 expression is higher in airway smooth muscle cells exposed to inflammatory cytokines than in cells from non-asthmatic subjects, with elevated COX-2 expression and PGE₂ secretion. Exposure of cells to anti-miR-155 resulted in decreased PGE₂ secretion. These results suggest that miR-155 may have a role in hyporesponsiveness to (β_2 -adrenoceptor agonists and targeting this miRNA may have a beneficial effect in restoring responsiveness to this class of bronchodilators. Other studies have shown specific effects of miR-21 in priming the lungs toward a Th2 and IL-13 skewed phenotype (Lu, et al., 2009). Our recent studies on miR-140-3p and miR-708 reveal differential expression in asthmatic vs. non-asthmatic ASM and significant down-regulation of expression following exposure to TNF- α (Dileepan, et al., 2014; Jude, et al., 2012). We believe that up-regulation of expression of these miRNAs should result in significant attenuation of airway hyperresponsiveness, a hallmark feature of asthma. This could stem from the direct inhibitory effect of miR-140-3p and miR-708 on CD38 expression, cADPR-mediated calcium regulation, and airway responsiveness. However, the challenge in this

approach is to deliver miRNAs into the lungs in sufficient quantities and over a long period of time to be effective in reversing the asthmatic response to allergen exposure.

7. Design and characterization of CD38 inhibitors

CD38 is an attractive drug target due to its involvement in airway inflammation and airway hyperresponsiveness and many other physiological processes (Figure 1). Several laboratories have developed small molecule CD38 inhibitors (Becherer, et al., 2015; Blacher, et al., 2015; Dong, et al., 2011; Escande, et al., 2013; Haffner, et al., 2015; Hara-Yokoyama, et al., 1996; Jiang, et al., 2009; Kellenberger, et al., 2011; Kwong, et al., 2012; Moreau, et al., 2013; Sauve & Schramm, 2002; Wall, et al., 1998; S. Wang, et al., 2014; Wu, et al., 2013; Zhou, et al., 2012). Currently over 200 compounds that inhibit CD38 have been reported (S. Zhang, et al., 2015). Chemically, these compounds are quite diverse and include NAD analogs, heterocyclic compounds and flavonoids (Table 2). The availability of the crystal structure of CD38 liganded with various substrates or products has aided in the rational design of CD38 inhibitors and in the identification of the key amino acid residues necessary for inhibitor interaction (Graeff, et al., 2006; Q. Liu, et al., 2005, 2006). The small molecule inhibitors can be categorized into two groups, covalent and non-covalent inhibitors (also known as irreversible and reversible inhibitors, respectively (Z. Liu, et al., 2013). The covalent group of inhibitors (also known as mechanism based inhibitors) take advantage of the catalytic mechanism of CD38 to form a covalent bond with the active site glutamic acid at position 226 of human CD38 (Sauve & Schramm, 2002). This group of inhibitors are NAD analogs with the 2'-position of the nicotinamide ribose modified with a fluoro-modified arabinose or with 2'-deoxyribose. These modifications can form the covalent reaction intermediate with CD38 but are hydrolyzed at a very slow rate, effectively inhibiting the enzyme activities of CD38 (Sauve & Schramm, 2002). The non-covalent inhibitors bind to the active site of CD38 in a reversible fashion. The covalent inhibitors in general are more potent than the non-covalent inhibitors described thus far. However, considering issues with stability and membrane permeability of the covalent inhibitors, it is thought that the non-covalent inhibitors will be better for future development as therapeutics and research tools (Z. Liu, et al., 2013; S. Zhang, et al., 2015).

Despite the growing number of small molecule CD38 inhibitors that have been reported, most of these compounds have low potency (IC_{50} of 10 μ M or greater) and there has been very little data on the selectivity and biological activity of these drugs. This section will focus on CD38 inhibitors whose activity has been examined in biological systems.

Dong et al. synthesized a series of membrane permeant N-substituted nicotinamide derivatives and found several to have moderate ability to inhibit CD38 (Dong, et al., 2011). Two of these compounds were tested for their ability to relax agonist induced muscle contraction in rat or guinea pig models. Compound 4 was able to relax rat aortic rings previously contracted by phenylephrine (Dong, et al., 2011). A structurally similar compound that did not inhibit CD38 was ineffective in this system. Compounds 4 and 7 were able to relax guinea pig tracheal strips previously contracted with acetylcholine (Dong, et al., 2011). In both these systems, the potency of the compounds was higher in the in vivo systems than in the in vitro CD38 inhibition assays. This is most likely due to the membrane

permeability of the drugs, which allow them to concentrate within the cells (Dong, et al., 2011). Deng et al. tested compound 4 from this study in an ozone-induced airway hyperresponsiveness mouse model and found that it was not effective (Deng, et al., 2013).

Two studies employing high throughput screening of large chemical libraries resulted in the identification of lead compounds that led to the synthesis of potent CD38 inhibitors with nanomolar potencies (Becherer, et al., 2015; Haffner, et al., 2015). Chemically these compounds were thiazoloquin(az)olin(on)es and 4-amino-8-quinoline carboxamides, respectively (Becherer, et al., 2015; Haffner, et al., 2015). Both studies chose compounds that effectively inhibited CD38 and also had promising pharmacokinetic data in mice (compound 78c, $IC_{50} = 7.3$ nM from Haffner et al. (Haffner, et al., 2015) and compound 1ah, $IC_{50} = 115$ nM and compound 1ai, $IC_{50} = 46$ nM from Becherer et al. (Becherer, et al., 2015)) to test for their ability to raise tissue NAD levels in a diet induced obesity mouse model, as an indication of CD38 inhibition. Compound 78c was able to increase NAD levels by 536% in liver after two hours (Haffner, et al., 2015). Compounds 1ai and 1ah were also effective in elevating NAD levels in liver (1483% and 515% after two hours by 1ai and 1ah, respectively (Becherer, et al., 2015). The compounds from these studies should be useful in studying their potential as therapeutics in a number of disease models, including asthma.

The usefulness of small molecule CD38 inhibitors has been tested in two other systems. Vaisitti et al. showed that kuromanin, a flavonoid CD38 inhibitor (Kellenberger, et al., 2011), effectively blocks the chemotaxis, adhesion and in vivo homing of chronic lymphocytic leukemia (CLL) cells (Vaisitti, et al., 2015). Blacher et al. recently identified the natural anthranoid, 4,5-dihydroxyanthraquinone-2-carboxylic acid (rhein) and its water soluble potassium salt (K-rhein) potent CD38 inhibitors ($IC_{50}s = 1.2$ and 0.8 μ M, respectively) (Blacher, et al., 2015). A glioma progression mouse model was used to show that K-rhein administration significantly inhibited glioma progression (Blacher, et al., 2015). This effect was observed in WT but not CD38 knockout mice, suggesting that the effect of K-rhein was due to inhibition of CD38 activity (Blacher, et al., 2015). These two studies provide further evidence that drugs that inhibit CD38 will be useful therapeutically.

A humanized monoclonal antibody against CD38, daratumumab, is FDA-approved for treatment of multiple myeloma (de Weers, et al., 2011; Laubach, et al., 2014). Multiple myeloma plasma cells express high levels of CD38. Daratumumab induces antibody dependent cell cytotoxicity and complement dependent cytotoxicity (de Weers, et al., 2011). The cells which mediate these responses to daratumumab are unknown.

It may also be possible to inhibit CD38 action by blocking its downstream signaling events. CD38 generates two signaling molecules, cADPR and NAADP, both of which are involved in calcium signaling. 8-bromo-cADPR is a cell permeant, competitive antagonist of cADPR action (Sethi, et al., 1997; Walseth & Lee, 1993). It has been used primarily in *in vitro* studies to dissect cADPR signaling pathways. Recently, Peng et al. demonstrated that the CD38/cADPR pathway was activated in sepsis using a cecal ligation and puncture rat model (Peng, Ai, et al., 2016). They also demonstrated that administration of 8-bromo-cADPR protected organs (heart, liver, kidneys) from sepsis-induced damage (Peng, Ai, et al., 2016; Peng, Zou, et al., 2016). These studies demonstrate that blocking cADPR action and/or

CD38 activity may be useful in treating sepsis. NAADP signaling has been shown to be involved in muscarinic receptor-induced contraction of guinea pig trachea (Aley, et al., 2013), NED-19, a NAADP antagonist (Naylor, et al., 2009) blocked carbachol-induced contraction of guinea pig trachea. To date, there are no studies that describe the use of any of the CD38 inhibitors and/or antibodies in animal models of allergic airway disease. CD38 and the signaling pathways it generates (cADPR and NAADP) play a role in the pathophysiology of airway diseases and are therefore attractive drug targets. Drugs that target the enzyme activities and/or the actions (cADPR and NAADP antagonists) should prove useful in the mitigation of airway hyperresponsiveness in diseases such as asthma.

8. Summary and Future Directions

CD38, a bifunctional membrane protein, is expressed on immune cells as well as on airway mesenchymal cells such as ASM cells. Experimental data thus far suggest multiple roles of CD38-cADPR pathway in the pathogenesis of asthma including in the regulation of allergen-induced airway inflammation and airway hyperresponsiveness. Additional studies are needed to determine if CD38-cADPR plays a role in the regulation of features of airway remodeling such as ASM hyperplasia. Expression and enzyme activities of CD38 and genetic variations in the *cd38* gene in healthy and asthmatic human subjects need to be established. Our studies on CD38-cADPR are restricted to immune cells and ASM cells. Functional role of CD38 in other airway cell types such as fibroblasts, mucus producing cells and epithelial cells needs to be investigated. The fact that these cells contribute significantly to asthma pathogenesis underscores the importance of studying CD38 in other airway cells. Multiple small molecule inhibitors described above have been developed for inhibiting CD38 function. These inhibitors can be exploited as novel therapeutics in asthma considering the multiple roles of CD38 in the asthma pathogenesis. In addition, the roles of microRNAs, specifically miR-140-3p and miR-708-5p, in the development and/or reversal of airway inflammation, airway hyperresponsiveness and airway remodeling following allergen sensitization and challenge is another area of future investigation.

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CD38 in asthma

Figure 1. CD38-cADPR in asthma pathogenesis

Allergen-induced inflammation, remodeling, and hyperresponsiveness are the major components of asthma. Resident airway cells such as ASM cells and migrated immune cells contribute to asthma pathogenesis. CD38 is expressed on immune cells and smooth muscle cells. CD38 on immune cells functions as cell surface marker and contributes to inflammatory response in asthma. ASM is the principal contractile component of the airways and CD38 via production of cADPR, a calcium elevating second messenger, contributes to smooth muscle contractility and airway hyperresponsiveness. Regulating CD38 expression or its enzyme activity or cADPR effect in effector cells may effectively mitigate multiple features of asthma.

Figure 2. Model depicting CD38/cADPR-mediated calcium release and functional effect in ASM
 Stimulation of ASM cells with Gq-coupled GPCR agonists results in the production of inositol 1,4,5 triphosphate (IP3) which in turn binds to its receptors on sarcoplasmic reticulum (SR). cADPR produced by the catalytic action of ADP-ribosyl cyclase (part of membrane bound protein CD38) acts as a calcium releasing second messenger presumably via ryanodine receptors (RyR) on the SR membrane. Extracellularly produced cADPR is believed to enter cell via membrane channel formed by connexin-43. Accessory proteins such as FKBP12.6 and calmodulin (CaM) are known to be involved in the cADPR-mediated calcium release. Recent studies have shown that cADPR sensitizes store-operated calcium entry via TRP channels on the plasma membrane to open and ADPR actually gates the channels. cADPR-mediated calcium release is involved in the regulation of global as well as local/compartmentalized (e.g. oscillations) calcium homeostasis in ASM cells. In vivo studies using CD38 null mice have confirmed the functional role of CD38/cADPR-mediated calcium release in the regulation of bronchomotor tone. Calcium sensitization via RhoA/ROCK contributes to ASM contraction. However, the role of cADPR in mediating calcium sensitization mechanism is not established (dotted lines). CD38 converts NADP into NAADP which is known to release calcium from intracellular stores such as lysosomes (shown on the right side of the figure).

Table 1

Regulation of CD38 expression in smooth muscle.

Cytokines	
IL-1 β	Up
UpTNF- α	Up
IFN- γ	Up
IL-13	Up
Hormones	
Glucocorticoids	Down
Estrogen	Up
Progesterone	Down
MicroRNAs	
miR-140-3p	Down
miR-708-5p	Down
miR-499-5p	Unknown
miR-155	Unknown
Transcription Factors	
NF- κ B	Up
AP-1	Up

Table 2

Drugs targeting CD38 or CD38 regulated signaling pathways.

Classification	Examples	Comments
CD38 inhibitors	Thiazoloquin(az)olin(on)es 4-amino-8-quinoline carboxamides Kuromanin (Flavinoid) K-rhein (Anthranoid)	IC50=7nM against human CD38 (Becherer et al.) IC50=76nM against human CD38 (Haffner et al.) Inhibits CLL chemotaxis, adhesion and in vivo homing (Kellenberger et al.) Inhibition of glioma progress in a mouse model (Blacher et al.)
Monoclonal antibody	Daratumumab	FDA approved for multiple myeloma. Stimulates complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity (de Weers et al., and Laubach et al.)
cADPR antagonist	8-bromo-cADPR	Attenuation of sepsis-induced organ damage in rats (Peng et al.)
NAADP antagonist	NED-19	Inhibition of muscarinic receptor-induced contractions of guinea pig tracheal contraction (Naylor et al.)