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CLCA Proteins in the Airways:
New Insights into their Expression Patterns and Role in Innate Immunity in Pneumonia

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Contents

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List of Abbreviations

AEC	**A**lveolar **E**pithelial **C**ells
AMC	**A**irway **M**ucus **C**ells
AWEC	**A**ir**w**ay **E**pithelial **C**ells
BALF	**B**ronchoalveolar **L**avage **F**luid
B cells	**B** lymphocytes
BEC	**B**ronchial **E**pithelial **C**ells
cAMP	**c**yclic **A**denosine **M**ono**p**hosphate
CC10	**C**lub (formerly Clara) **C**ell Protein **10**
CF	**C**ystic **F**ibrosis
CFTR	**C**ystic **F**ibrosis **T**ransmembrane conductance **R**egulator
CLCA	**C**h**l**oride channel regulator, **Ca**lcium-activated
CLCR	**C**h**l**oride **C**hannel **R**egulator
COPD	**C**hronic **O**bstructive **P**ulmonary **D**isease
CXCL	**CXC**-Motif **L**igand
CCL	Chemokine (**C-C** motif) **L**igand
ELISA	**E**nzyme **L**inked **I**mmunosorbent **A**ssay
et al.	**et al**ii (latin for "and others")
FACS	**F**luorescence **A**ctivated **C**ell **S**orting
FELASA	**F**ederation of **E**uropean **L**aboratory **A**nimal **S**cience **A**ssociations
GM-CSF	**G**ranulocyte **M**acrophage **C**olony-**S**timulating **F**actor
hCLCA	**h**uman **C**h**l**oride channel regulator, **Ca**lcium-activated
IL	**I**nter**l**eukin
IL-R	**I**nter**l**eukin **R**eceptor
KC	**K**eratinocyte-derived **C**hemokine, synonymously used for CXCL-1
LPS	**L**ipo**p**oly**s**accharide
LTA	**L**ipo**t**eichoic **A**cid
MAPK	**M**itogen-**a**ctivated **P**rotein **K**inase

LIST OF ABBREVIATIONS

mCLCA	**m**urine **Ch**loride channel regulator, **Ca**lcium-activated
mClca3^{-/-}	*mClca3*-knockout
MCP	**M**onocyte **C**hemotactic **P**rotein
MIP	**M**acrophage **I**nflammatory **P**rotein
mRNA	**M**essenger **R**ibo**n**ucleic **A**cid
Muc2	Mucin 2 gene
Muc5ac	Mucin 5ac gene
Muc5b	Mucin 5b gene
n.f.p.	**n**o **f**unctional **p**rotein
n.i.	**n**ot **i**nvestigated
NK cells	**N**atural **K**iller **cells**
n.s.	**n**ot **s**tated
PAS	**P**eriodic **A**cid-**S**chiff
PAMP	**P**athogen **A**ssociated **M**olecular **P**attern
PBS	**P**hosphate **B**uffered **S**aline
pCLCA	**p**orcine **Ch**loride channel regulator, **Ca**lcium-activated
PRR	**P**attern **R**ecognition **R**eceptor
RANTES	**R**egulated Upon **A**ctivation, **N**ormal **T** cell **E**xpressed, and **S**ecreted
RT-qPCR	**R**everse **T**ranscriptase-**q**uantitative **P**olymerase **C**hain **R**eaction
S. aureus	*Staphylococcus **aureus***
SMC	**S**mooth **M**uscle **C**ells
SMG	**S**ubmucosal **G**land
S. pneumoniae	*Streptococcus **pneumoniae***
T cells	**T** lymphocytes
Th cells	**T h**elper cell type lymphocytes
TLR	**T**oll **L**ike **R**eceptor
TNF	**T**umor **N**ecrosis **F**actor
WT	**W**ild-**T**ype

X

1 Introduction

During the past 15 years, the CLCA (chloride channel regulator, calcium-activated) proteins have been the main focus of our research group. To date, the tissue and cellular expression patterns and the protein structures of many members of this family have been characterized in different species in detail. Certain CLCA proteins have been identified as clinically relevant molecules in important human diseases of the respiratory tract and their respective animal models. However, their exact physiological and pathophysiological roles are widely still elusive.

The human hCLCA1 and its murine ortholog mCLCA3 have directly been linked to inflammatory airway diseases with mucus overproduction including cystic fibrosis (CF), asthma and chronic obstructive pulmonary disease (COPD; Brouillard et al. 2005; Hegab et al. 2004; Kamada et al. 2004). Both proteins have been implicated in the regulation of mucus cell metaplasia and mucus production (Alevy et al. 2012; Patel et al. 2006). In contrast to humans, it was demonstrated that the murine CLCA homolog, mCLCA5, is also able to induce mucus cell metaplasia (Patel et al. 2006). Thus, it has consequently been speculated that mCLCA3 and mCLCA5 may have redundant or mutually compensatory functions in the respiratory tract (Alevy et al. 2012; Mundhenk et al. 2012; Patel et al. 2009; Patel et al. 2006) which was not observed for their human orthologs hCLCA1 and hCLCA2, respectively (Alevy et al. 2012). However, to date the mCLCA5 protein expression pattern and the expressing cell type in the respiratory tract of mice are unknown.

In addition to its functional role in the induction of mucus cell metaplasia, mCLCA3 has recently been implicated to regulate tissue inflammation in innate immune responses (Long et al. 2006; Zhang and He 2010). However, the underlying pathomechanisms and pathophysiological function of mCLCA3 leading to this observation are still elusive.

1.1 <u>The CLCA Protein Family</u>

1.1.1 Background

The first two members of the CLCA protein family were isolated simultaneously in the early 1990ies by two independent research groups and were suspected to act either as modulators of calcium activated chloride conductance (Ran and Benos 1992, 1991) or as cellular adhesion molecules (Zhu et al. 1991). Since these initial descriptions, they have grown to a complex family whose initially suggested functions have dramatically been revised and

broadened. To date, up to eight CLCA family members in at least 30 species have been described, including four human, eight murine and five porcine members with broad tissue and cell type specific expression patterns (for review, see Patel et al. 2009; Plog et al. 2012a; Plog et al. 2009; Plog et al. 2012b). They were named after the species in which they are present (e.g., h = human, m = murine, p = porcine and so forth) and numbered according to the chronological order of their discoveries. The mouse is the prime organism in translational CLCA research, however, the pig is becoming a more suitable animal model especially for respiratory diseases like CF (Klymiuk et al. 2012; Meyerholz et al. 2010a; Meyerholz et al. 2010b; Ostedgaard et al. 2011; Plog et al. 2012a; Plog et al. 2009; Plog et al. 2012b; Rogers et al. 2008a; Rogers et al. 2008b; Stoltz et al. 2010). The orthologous CLCA proteins of different species are clustered based on sequence homologies (Song et al. 2012; Winpenny et al. 2009). The human, murine and porcine CLCA ortholog clusters are listed in Table 1.

Table 1. Clusters of Human, Murine and Porcine CLCA Orthologous Genes

	Cluster 1	**Cluster 2**	**Cluster 3**	**Cluster 4**
Human	*hCLCA1*	*hCLCA2*	*hCLCA3*	*hCLCA4*
Mouse	*mClca3*	*mClca5*	*mClca1, 2, 4*	*mClca6, 7, 8*
Pig	*pClca1*	*pClca2*	*pClca3*	*pClca4a, 4b*

Sources: Patel et al. 2009; Plog et al. 2012a; Plog et al. 2009; Plog et al. 2012b; CLCA = chloride channel regulator, calcium-activated

A common feature of all biochemically investigated CLCA proteins to date is their posttranslational cleavage of a ~ 140 kDa precursor protein into a large amino-terminal product of ~ 90 kDa and a small carboxy-terminal product of ~ 40 kDa (Gruber et al. 1998; Gruber and Pauli 1999; Mundhenk et al. 2006). Interestingly, the CLCA protein family can be classified biochemically into two subgroups according to the post-cleavage processing of these subunits. In one subgroup, including the proteins of cluster 1 (hCLCA1, mCLCA3 and pCLCA1) both subunits are fully secreted as heterodimers (Gibson et al. 2005; Mundhenk et al. 2006; Plog et al. 2009). In the second group, including CLCA proteins of cluster 2 (hCLCA2, mCLCA5 and pCLCA2) only the amino-terminal subunit is shed by the cell, whereas the carboxy-terminal subunit is anchored to the plasma membrane via a transmembrane domain (Braun et al. 2010a; Elble et al. 2006; Plog et al. 2012b).

It has initially been hypothesized that CLCA proteins act as functional chloride channels in cell membranes and were thus originally named "**ch**loride channels, **c**alcium-**a**ctivated"

(Dodge et al. 1997; Gruber et al. 1998). However, several independent studies have since then revealed that these proteins are no integral membrane proteins and thus cannot form ion-channels on their own (Elble et al. 2006; Gibson et al. 2005; Mundhenk et al. 2006). Instead, it has been hypothesized and shown that the secreted CLCA proteins modulate chloride conductance via a putative interaction with to date unknown chloride channels. Gibson and co-workers were the first to attempt renaming this protein family as "chloride channel regulators (CLCR)" (Gibson et al. 2005), but final designations will have to be established in the future following conclusive elucidation of CLCA protein function (Patel et al. 2009).

1.1.2 CLCA Proteins in Respiratory Diseases

The first link between respiratory diseases with mucus overproduction such as CF, asthma and COPD (Brouillard et al. 2005; Hauber et al. 2010; Hegab et al. 2004; Kamada et al. 2004) and CLCA proteins have been recognized based on the overexpression of certain *Clca* gene products in affected airways which was primarily regulated by T helper (Th)2 cytokine signals (Interleukin (IL)-4, IL-9 and IL-13; Hauber et al. 2010; Zhou et al. 2001). In particular, the function of the human hCLCA1 and the murine homologs mCLCA3 and mCLCA5 regarding mucus production and induction of mucus cell metaplasia, a common feature of all of the diseases, has previously been focused in CLCA research (Long et al. 2006; Mundhenk et al. 2012; Nakanishi et al. 2001; Patel et al. 2006; Robichaud et al. 2005; Thai et al. 2005; Zhang and He 2010).

CF is the most common lethal genetic disorder affecting the caucasian population with an incidence of 1 in 2,500 births (Dodge et al. 1997). It is an autosomal recessive disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene leading to defective functions of its gene product, a cyclic adenosine monophosphate (cAMP)-activated chloride channel. In the respiratory tract, these defects result in dramatic mucus accumulation with airway plugging and impaired mucociliary clearance predisposing to bacterial colonization (Brouillard et al. 2005; McAuley and Elborn 2000; Vankeerberghen et al. 2002). Severe and recurrent respiratory infections caused by pathogenic bacteria, such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* (*S. aureus*) are the most common cause of death in CF patients (Barnes 2008; Locksley 2010; Parameswaran et al. 1998).

Asthma is a chronic airway disease characterized by reversible airway obstruction, airway hyperresponsiveness, bronchial inflammation and mucus overproduction (Daser et al. 2001; McFadden and Gilbert 1992). This condition is caused by a combination of genetic and environmental factors (Barnes and Marsh 1998; Steinke et al. 2003; Tattersfield et al. 2002). It is highly prevalent with 330 million people affected worldwide and the incidence and severity of

asthma has steadily increased (Barnes 2008). This disease has become an expanding burden on public health services in both industrialized and developing countries (Locksley 2010; Pearce et al. 2007).

COPD is a major and increasing global health problem and the fourth common cause of death in the developed countries. It will account for over 6 million deaths per year by 2020 and is predicted to take a leap from the sixth- to the third-leading cause of death worldwide (Mannino and Buist 2007; Yao and Rahman 2011). COPD is characterized by persistent air-flow limitation, associated with mucus overproduction and an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases (Anthonisen 1988; Rouze et al. 2014; Vestbo et al. 2013).

A common trait of these diseases is mucus cell metaplasia with extensive mucus plugging, leading to airway obstruction, dyspnea and secondary bacterial infections.

1.1.3   CLCA Proteins in Mucus Cell Metaplasia

Experimental overexpression of the human hCLCA1 or its murine ortholog mCLCA3 in a mu-coepidermoid cell line (NCI-H292) caused a significant increase in mucin production and was the first observation on the role of CLCA proteins in mucus cell metaplasia (Nakanishi et al. 2001; Zhou et al. 2002). Similar observations have been made in various murine *in vivo* models. Specifically, experimental overexpression of mCLCA3 in ovalbumin-sensitized mice initiated mucus cell metaplasia (Nakanishi et al. 2001), whereas administration of a full-length *mClca3* antisense oligonucleotide inhibited the development of this condition. Moreover, it has also been demonstrated that a vector gene transfer of *mClca3* in mouse airways was sufficient to produce mucus cell metaplasia (Patel et al. 2006). More recently, it has been hypothesized that these CLCA proteins act as extracellular signaling molecules transforming airway mucus precursor cells to mature mucus cells, thereby regulating and driving mucus cell metaplasia (Patel et al. 2009). Specifically, hCLCA1, which is selectively expressed in mucus cells of the respiratory tract (Gruber et al. 1998), is known to regulate mucus cell metaplasia by inducing mucus gene transcription via a downstream mitogen-acti-vated protein kinase (MAPK)-13 signaling pathway (Alevy et al. 2012). While mCLCA3 has also been sufficient to induce mucus cell metaplasia in the respiratory tract, *mClca3* knockout mice (*mClca3$^{-/-}$*) did not show a corresponding phenotype (Patel et al. 2006). It has been hypothesized that other murine CLCA members which are also expressed in airway tissue may compensate for the lack of mCLCA3 and thereby led to mucus cell metaplasia (Patel et al. 2006). In particular, mCLCA5 has become the most prominent candidate for compensat-ing for the loss of mCLCA3 since a gene transfer with a *mClca5* encoding vector also in-duced mucus production (Patel et al. 2006). It has been speculated that mCLCA3 and

mCLCA5 may have redundant functions in the respiratory tract (Patel et al. 2006, Patel et al. 2009). Although mCLCA5 mRNA has been detected in the respiratory tract under naive conditions (Braun et al. 2010a), the mCLCA5 protein was only discovered after Th2-induced airway inflammation (Mundhenk et al. 2012). Hence, the expression pattern of mCLCA5 protein in the naive murine respiratory tract is still elusive; in particular the expressing cell type is unknown. Furthermore, in contrast to the murine CLCA homologs, mCLCA3 and mCLCA5, their human orthologs hCLCA1 and hCLCA2 did not compensate for each other during mucus cell metaplasia. While hCLCA2 was also expressed in the lung on mRNA level, no up-regulation under the condition of mucus cell metaplasia could be observed *in vitro* (Alevy et al. 2012). It has been argued that, due to these differences between murine and human orthologs, the value of mouse models for studying mucus cell metaplasia in translational medicine may be questionable (Patel et al. 2009; Patel et al. 2006).

1.1.4   CLCA Proteins in Pneumonia and Innate Immunity

Previous studies primarily focused on the role of hCLCA1 and its murine ortholog mCLCA3 on the induction of mucus cell metaplasia in complex inflammatory airway diseases (Long et al. 2006; Mundhenk et al. 2012; Nakanishi et al. 2001; Patel et al. 2006; Robichaud et al. 2005; Thai et al. 2005; Zhang and He 2010). In addition to this pathomechanism, CLCA proteins have recently been implicated in the regulation of tissue inflammation in the innate immune response (Long et al. 2006; Zhang and He 2010). Specifically, the group of Long showed that *mClca3⁻/⁻* mice challenged with ovalbumin or lipopolysaccaride (LPS) had increased numbers of neutrophils in bronchoalveolar lavage fluid (BALF) and that LPS-treated mice additionally exhibited enhanced CXC-motif ligand (CXCL)-1 protein levels (Long et al. 2006). Zhang et al. supposed that the overexpression of mCLCA3 in ovalbumin-challenged asthmatic mice may modulate the recruitment of inflammatory cells by regulating the function of chemokines derived from airway epithelial cells (Zhang and He 2010). Indeed, a recent study has demonstrated that hCLCA1 may act as an innate immune signaling molecule which activates airway macrophages and thereby enhances pro-inflammatory cytokine release, including CXCL-8, IL-6, IL-1ß, tumor necrosis factor (TNF)-α (Ching et al. 2013). Moreover, asthmatic mice treated with anti-mCLCA3-antibodies showed remarkable reduction of airway inflammation (Song et al. 2013). These findings have opened a new research field to investigate the function of CLCA proteins as modulators of the innate immune response. So far, only models of chronic and allergic airway inflammation (Long et al. 2006; Mundhenk et al. 2012; Patel et al. 2006) and acute inflammation due to LPS (Long et al. 2006) have been characterized in *mClca3⁻/⁻* mice. However, an acute bacterial infection with intact microorganisms instead of only partial virulence factors such as LPS would appear more suitable to investigate the role of mCLCA3 in modulating innate immune responses.

1.1.5 Expression of CLCA Family Members in Murine, Human and Porcine Airways

In the respiratory tract, mRNA expression was detected for all four human CLCA (hCLCA1 to 4), most murine (mCLCA1 to 5) and porcine (pCLCA1, pCLCA2, pCLCA4) members. However, the tissue and cellular expression patterns, particularly at the protein level, still need to be fully determined for most of them (Patel et al. 2009). The protein expressions of the orthologous proteins from CLCA cluster 1 (hCLCA1, mCLCA3 and pCLCA1) are well established, as they are selectively expressed in airway mucus cells (Gibson et al. 2005; Leverkoehne and Gruber 2002; Mundhenk et al. 2006; Plog et al. 2009). Furthermore, the orthologous human hCLCA4 and porcine pCLCA4a proteins have been found in the respiratory tract, specifically on the apical membranes of all airway epithelial cells (Agnel et al. 1999; Mall et al. 2003; Plog et al. 2012a). In contrast, several members such as the orthologs hCLCA2, mCLCA5 and pCLCA2 from CLCA cluster 2 were only detected on mRNA level in lung extracts so far (Braun et al. 2010a; Connon et al. 2005; Connon et al. 2004; Gruber et al. 1999). Up to now, neither their expressing cell type nor their protein have been established in the airways. Current knowledge on expressions of human, murine and porcine CLCA members in the airways on mRNA and protein level are summarized in Table 2.

Table 2: Expression of Human, Murine and Porcine CLCA Members in Airways

Gene	mRNA	Protein	Cell type	References
hCLCA1	+	+	AMC	(Hauber et al. 2004; Hoshino et al. 2002; Toda et al. 2002)
hCLCA2	+	-	unknown	(Agnel et al. 1999; Connon et al. 2004; Gruber et al. 1999)
hCLCA3*	+	n.f.p.	-	(Agnel et al. 1999; Gruber and Pauli 1999; Pauli et al. 2000)
hCLCA4	+	+	AWEC	(Agnel et al. 1999; Mall et al. 2003)
mClca1	+	-	unknown	(Gandhi et al. 1998; Gruber et al. 1998; Roussa et al. 2010)
mClca2	+	-	unknown	(Gandhi et al. 1998; Gruber et al. 1998; Leverkoehne et al. 2002)
mClca3	+	+	AMC	(Komiya et al. 1999; Leverkoehne and Gruber 2002; Zhou et al. 2001)
mClca4	+	+	SMC	(Braun et al. 2010b; Elble et al. 2002)
mClca5	+	-	unknown	(Beckley et al. 2004; Braun et al. 2010a; Evans et al. 2004a)
mClca6	-	-	-	(Bothe et al. 2008; Evans et al. 2004b)
mClca7	-	-	-	(Al-Jumaily et al. 2007)
mClca8	-	-	-	(Al-Jumaily et al. 2007)
pClca1	+	+	AMC, SMG	(Gaspar et al. 2000; Plog et al. 2009)
pClca2	+	-	unknown	(Plog et al. 2012b)
pClca3*	n.i.	n.f.p.	-	-
pClca4a	+	+	AWEC	(Plog et al. 2012a)
pClca4b	n.i.	n.i.	-	-

+ = expressed; - = not yet detected; AMC = airway mucus cells; AWEC = airway epithelial cells; SMC = smooth muscle cells; SMG = submucosal glands; n.i. = not investigated; *likely represents pseudogens; n.f.p. = no functional protein suspected, because of pseudogene status; CLCA = chloride channel regulator, calcium-activated

INTRODUCTION

In the next paragraphs, current knowledge on the expression patterns of the murine CLCA proteins mCLCA3 and mCLCA5 and their human and porcine orthologs as known to date will be described in detail.

1.1.5.1 Murine mCLCA3 and its Human and Porcine Orthologs, hCLCA1 and pCLCA1

The murine mCLCA3 and its human ortholog hCLCA1 are, to date, the best investigated CLCA family members in the respiratory tract. mCLCA3, as the third murine homolog, was first identified in goblet cells throughout the intestinal tract using in situ hybridization (Komiya et al. 1999). It was additionally found in the murine trachea and the uterus but without identification of the respective cell type (Komiya et al. 1999). Subsequent studies established the orthologous proteins of mCLCA3 and hCLCA1 being fully secreted and selectively expressed in mucus cells in various tissues, including the respiratory tract, the intestine and the uterus (Gibson et al. 2005; Leverkoehne and Gruber 2002; Mundhenk et al. 2006). Additionally, the porcine ortholog pCLCA1 is also expressed in mucin-producing cells of various organs, including the intestine and the airways, virtually identical to its murine and human orthologs (Plog et al. 2009). Therefore, it has been assumed that pCLCA1 possibly shares similar functions as shown for mCLCA3 and hCLCA1 in the respiratory tract (Plog et al. 2009).

hCLCA1 and mCLCA3 were found to be up-regulated in patients with chronic airway disease such as CF, asthma and COPD (Brouillard et al. 2005; Hegab et al. 2004; Kamada et al. 2004) or in experimentally induced challenge models, respectively (Long et al. 2006; Mundhenk et al. 2012; Patel et al. 2006).

1.1.5.2 Murine mCLCA5 and its Human and Porcine Orthologs, hCLCA2 and pCLCA2

The murine mCLCA5 has attracted notice by regulating airway mucus cell metaplasia in the absence of mCLCA3. It has therefore been speculated that mCLCA3 and mCLCA5 may have redundant functions with one compensating for the other. mCLCA5 mRNA was predominantly found in the eye and spleen (Evans et al. 2004b) as well as in the mammary gland (Beckley et al. 2004) and in tissues with squamous epithelium (Braun et al. 2010a). However, mCLCA5 has been detected in various tissues on the mRNA level, including the respiratory tract (Braun et al. 2010a). On the protein level, it was subsequently shown to be expressed only extrapulmonary in virtually all stratified squamous epithelia throughout the body, including the skin, the nonglandular part of the stomach, the oesophagus, the oral cavity and others (Braun et al. 2010a). Specifically, the protein was detected in late differentiated keratinocytes with a proposed function in growth arrest and maturation of squamous epithelial cells (Braun et al. 2010a). Similarly to mCLCA5, its human ortholog hCLCA2 was detected in the respiratory tract on mRNA level (Agnel et al. 1999; Gruber et al. 1999), whereas the protein of hCLCA2 appears to be expressed in basal epithelial cells of stratified

epithelia with a proposed role in stratification and basal cell-basement membrane adhesion (Carter et al. 1990; Connon et al. 2005; Connon et al. 2004). Furthermore, the porcine ortholog, pCLCA2, is also expressed in airways on mRNA level (Plog et al. 2012b) and, similarly to mCLCA5, its protein has so far only been found in mature keratinocytes of the epidermis and the inner root sheet of hair follicles (Plog et al. 2012b). After experimental induction of mucus cell metaplasia, mCLCA5 has been found to be up-regulated in respiratory epithelium *in vitro*, however, this could not be repeated for its human ortholog hCLCA2 (Alevy et al. 2012).

1.2 Species-Specific Anatomic and Histologic Differences between Murine, Human and Porcine Airways

Based on controversial findings concerning the expression of mCLCA5 and hCLCA2 in terms of mucus cell induction in the lung, possible species-specific differences in expression and function of CLCA proteins in the lung were discussed (Patel et al. 2009; Patel et al. 2006). In fact, several species-specific differences occur between murine, human and porcine lungs in both gross anatomy and histology, among other reasons likely due to the need for compensation for size differences and for a higher basal metabolic rate in mice in contrast to humans and pigs. Interestingly, the relative size of the airway lumen of mice is larger than those in humans and pigs and is proposed to decrease the airway resistance which would occur due to their rapid respiratory rate of 250 – 350 breaths per minute (Suarez 2012). Selected species-specific differences of airways are summarized in Table 3.

1.2.1 Comparative Anatomy

The murine lung comprises of the right lung which is divided into four lobes (cranial, middle, caudal and accessory) and the left lung with only one lobe. In contrast, the right lung of humans is divided into three lobes (upper, middle and lower) and the left lung is divided into two lobes (upper and lower; Fox 2007; Suarez 2012). The porcine lung is divided into the right lung with four lung lobes (cranial, middle, caudal and accessory) and the left lung with two lobes (cranial and caudal; König and Liebich 2005). In mice and pigs, the branching of the conducting airways occurs as an asymmetrical, monopodial branching pattern in which the larger parent airway segments gradually transists to a smaller segment. In humans, a dichotomous branching pattern occurs, in which the larger parent segments divides symmetrically at an approximately 45° angle into two equal but smaller daughter segments (Suarez 2012).

In mice the trachea contains 15 - 18 C-shaped rings with a rapid transition from complete cartilaginous rings to less organized cartilage plates. Above the heart, the trachea terminates at the carina, bifurcates and gives rise to the extrapulmonal right and left main bronchi which

divides into the intrapulmonal bronchi as they enter the lung. In the lung the intrapulmonal bronchi divides into segmental bronchi to supply the anatomic structures of the right and left lung, respectively, and further terminates as bronchioles. Due to the lack of cartilage in the intrapulmonal bronchi the clear differentiation between bronchi and bronchioles is impossible. In mice the terminal bronchioles often divide directly into alveolar ducts, while respiratory bronchioles are mostly lacking (Fox 2007; Suarez 2012).

In humans, the trachea has 15 - 20 incomplete U-shaped cartilaginous rings and after bifurcation in the upper thorax the main bronchus divides on the right side into three lobar bronchi and on the left side into two lobar bronchi which supply the pulmonary lobes, respectively. The lobar bronchi further branch into segmental bronchi which contain cartilage, next into primary bronchioles lacking cartilage and lastly to the terminal bronchioles which, in turn, divide into multiple respiratory bronchioles (Plopper and Hyde 2008). The respiratory bronchioles are the smallest anatomic unit of the lung. Furthermore, each respiratory bronchiole gives rise to 2 – 11 alveolar ducts which terminates in approximately six alveolar sacs in humans (Plopper and Hyde 2008; Suarez 2012).

The trachea in pigs contains 29 – 36 circularly arranged cartilaginous rings. In contrast to mice and humans, pigs have a *bronchus trachealis* which enters the cranial right lung lobe out of the trachea before bifurcating into two main bronchi. The cartilage surrounding airways are more widespread in distribution than in humans, extending further down into the tracheobronchial tree (Haworth and Hislop 1981; Rogers et al. 2008a). The primary organization of the bronchial structures is similar to that of mice and as in mice respiratory bronchioles are often lacking (Fox 2007; König and Liebich 2005; Plopper and Hyde 2008; Suarez 2012).

1.2.2 Comparative Histology

1.2.2.1 *Tracheal Epithelium*

The inner surface of the mouse trachea is lined by a pseudo-stratified columnar epithelium composed of six different cell types. Club (formerly known as Clara) cells are the predominant cell type and the primary secretory cell in mouse airways, followed by ciliated columnar epithelial cells (Suarez 2012). Furthermore, fewer basal cells, brush cells and neuroendocrine cells occur in descending numbers. Goblet cells are rare and, when present, located at the carina (Suarez 2012). In mice, submucosal glands (SMG) are restricted to the larynx and the proximal trachea with minor variations between different inbred strains (Lynch and Engelhardt 2014; Rawlins and Hogan 2005; Rock et al. 2009).

In humans, the trachea is lined by a pseudo-stratified and columnar epithelium predominantly consisting of ciliated cells (49 %), followed by basal cells (33 %) and mucous goblet cells (9

%). Similarly to mice, brush cells and neuroendocrine cells are present but in marginal numbers (Plopper and Hyde 2008). In contrast to mice, SMG are numerous, tightly packed and occur along the entire cartilaginous airways (Liu and Engelhardt 2008; Rock et al. 2010). Furthermore, contrary to mice, in human airways club cells are absent from the trachea and bronchi (Suarez 2012).

1.2.2.2 Intrapulmonary Airway Epithelium

In mice, the intrapulmonary proximal airway epithelium mainly consists of club cells (59 %) and ciliated cells (28 - 36 %; Fox 2007; Suarez 2012). The transition from bronchi to bronchioles is not easily delineated due to the lack of the usually used reference points cartilage, goblet cells and submucosal glands. The distal bronchial epithelium changes both in height and overall cell composition. The simple columnar epithelium with little pseudo-stratification turns into a simple cuboidal epithelium. While club cells dominate in the terminal bronchiole (60 - 80 %), ciliated cells (20 - 40 %) decrease (Fox 2007; Suarez 2012). Mice only have very few or no respiratory bronchioles, so terminal bronchioles often directly transit into alveolar ducts which terminate in alveolar sacs, the last segment of the respiratory zone. The alveolar wall is predominantly composed of a thin epithelial layer of alveolar epithelial cells (AEC) type I, interspersed with cuboidal cells known as AEC type II (Fox 2007; Suarez 2012).

In humans, ciliated cells (37 %) and basal cells (32 %) dominate in the intrapulmonary proximal airways with much less secretory goblet cells (10 %) and serous cells (3 %; Plopper and Hyde 2008; Rawlins and Hogan 2006; Rock et al. 2009; Suarez 2012; Wong et al. 2009). While the bronchi undergo successive branching, the epithelium diminishes in height, turns into a simply columnar one with little pseudo-stratification and the number of goblet cells decreases. The SMG become sparse and vanish in the distal segmental bronchi. Similarly, the cartilage rings turn into discontinuous plates and disappear with decreasing in size (Plopper and Hyde 2008; Suarez 2012). In the bronchioles, goblet cells disappear and serous cells strongly increase in number. In contrast to mice, humans have respiratory bronchioles in the first segment of the respiratory zone. The histological structure resembles that of the terminal bronchioles in mice. Respiratory bronchioles have occasional saccular outpouchings called alveoli which line the alveolar duct and terminate in the alveolar sacs with an identical structure and cell composition as described for mice (Plopper and Hyde 2008; Suarez 2012).

In pigs, the cellular lineages and compositions of the trachea and bronchi are comparable with those of human lungs (Jones et al. 1975; Mills et al. 1986; Winkler and Cheville 1984). Furthermore, similar to human lungs, there is an abrupt decrease in SMG in the smaller parts

of the segmental bronchi (Ballard and Inglis 2004). As shown for mice, pigs also have only few or no respiratory bronchioles and the terminal bronchioles transit into alveolar ducts which terminate in alveolar sacs. The cell composition of the alveolar wall is similar to the murine and human ones (König and Liebich 2005; Liebich 2010).

Table 3: Comparison of Selected Structures of Murine, Human and Porcine Airways

	Selected Structures	Mouse	Human	Pig
Anatomy	SMG	Proximal trachea	Along the cartilaginous airways	Along the cartilaginous airways
	Tracheal rings	15 - 18, C-shaped	15 - 20, U-shaped	29 - 36, circular
	Right lung lobes	Cranial, middle, caudal, accessory	Upper, middle, lower	Cranial, middle, caudal, accessory
	Left lung lobes	Only one	Upper and lower	Cranial and caudal
	Airway branching pattern	Monopodial	Dichotomous	Monopodial
	Bronchi / bronchioles	Not differentiable	Differentiable	Differentiable
	Respiratory bronchioles	Mostly absent	Present	Mostly absent
Composition of Airway Epithelial Cells	**Tracheal epithelium**	Pseudo-stratified, columnar	Pseudo-stratified, columnar	Pseudo-stratified, columnar
	Club cells*	49	n.s.	Similar to human
	Ciliated cells*	11 - 14	49	
	Mucus goblet cells*	< 1	9	
	Serous cells*	< 1	< 1	
	Basal cells*	10	33	
	Other*	1	n.s.	
	Proximal intrapulmonary epithelium	Simple columnar	Pseudo-stratified, columnar	Pseudo-stratified, columnar
	Club cells*	59 - 61	n.s.	Similar to human
	Ciliated cells*	28 - 36	40 - 50	
	Mucus goblet cells*	< 1	10	
	Serous cells*	< 1	3	
	Basal cells*	< 1	32	
	Other*	2 - 14	18	
	Terminal bronchioles	Simple cuboidal	Simple columnar	Simple columnar
	Club cells*	60 - 80	n.s.	Similar to human
	Ciliated cells*	20 - 40	52	
	Mucus goblet cells*	0	n.s.	
	Serous cells*	0	35	
	Basal cells*	< 1	< 1	
	Other*	0	13	

Sources: Fox 2007; Mills et al. 1986; Plopper and Hyde 2008; Suarez 2012; Winkler and Cheville 1984; n.s. = not stated; *approximate cell number in percent; SMG = submucosal glands

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The similarities between humans and pigs and fundamental differences between humans and mice regarding anatomy and histology of the respiratory tract supports the notion that the pig may become the preferred animal model in studying respiratory diseases, such as CF (Klymiuk et al. 2012; Meyerholz et al. 2010a; Meyerholz et al. 2010b; Ostedgaard et al. 2011; Plog et al. 2009; Rogers et al. 2008a; Rogers et al. 2008b).

1.3   Innate Immunity of the Lung

CLCA proteins have been implicated in the regulation of tissue inflammation in the innate immune response. In particular, it was demonstrated that hCLCA1 may act as an extracellular innate immune signaling molecule which activates airway macrophages and thereby enhances pro-inflammatory cytokine release (Ching et al. 2013). In asthmatic mice treated with anti-mCLCA3-antibodies a remarkable reduction of airway inflammation was observed (Song et al. 2013). However, the underlying pathomechanism and pathophysiological function of mCLCA3 are unknown. The innate immune system of the lung represents a complex network of diverse components as "actors" and various secreted molecules as "mediators" which are described in the following paragraphs in detail.

1.3.1   Innate Immune Response: Actors

The lung as the largest epithelial interface between the environment and the host is in permanent contact with toxins, infectious agents or allergens. The respiratory epithelium has an important function in innate immunity and host defense performing this in a complex fashion, including ciliary beat activity, mucus production but also production of chemokines, cytokines, antimicrobial peptides and proteins, proteinase inhibitors and surfactant proteins (Bals and Hiemstra 2004). Furthermore phagocytic cells, such as macrophages and neutrophils as well as natural killer cells, contribute equally to the first line of defense. These nonspecific immune mechanisms are focused on the detection of highly conserved antigen patterns which are common for several agents. These pathogen associated molecular patterns (PAMP), e.g., LPS, peptidoglycane, lipoteichoic acid (LTA) or other lipoproteins are detected by pattern recognition receptors (PRR). This in turn results in the activation of an intracellular downstream cascade which induces innate immune response genes, such as genes encoding for cytokines. PRR are widely expressed by immune cells, including macrophages and dendritic cells but also by airway epithelial cells and fibroblasts (Delclaux and Azoulay 2003; Medzhitov 2007; Mizgerd 2008). Besides these cellular components of the innate immune system, the complement system which consists of different serum proteins is also considered a functional part of the innate immune system regulating bacterial lysis, opsonisation of cells and even regulation of inflammation (Bals and Hiemstra 2004; Barthlott and Holländer 2006; Delclaux and Azoulay 2003). Following identification by resident macro-

phages, pathogens are phagocytized, degraded by reactive oxygen species, antimicrobial peptides or lysozymes. Epithelial cells, such as club cells, ciliated cells or mucus cells can be directly activated by pathogens themselves or indirectly by cytokine release from activated macrophages with a subsequent release of antimicrobial substances or inflammatory mediators. Similar to macrophages, neutrophils are recruited from the marginal capillary pool and contribute equally to the elimination of pathogens and to the cytokine and chemokine release which is necessary for further recruitment of immune cells, including monocytes and neutrophils from the blood into the lung.

1.3.2   Innate Immune Response: Mediators

Cytokines, as small molecular weight proteins, influence a plethora of biological processes, including cellular activation, recruitment, cell death, and repair. They play a critical role in the regulation of the innate immune response (Sagel and Accurso 2002). They are key modulators of inflammation, participating in acute and chronic inflammation via a complex and sometimes seemingly contradictory network of interactions (Turner et al. 2014). Several cytokines share similar functions to ensure redundancy in case of deficiency or loss of single players. The interaction of all cytokines is required for coordination of the innate immune response (Barthlott and Holländer 2006). Key pro-inflammatory cytokines in the innate immune response include IL-1ß, IL-6 and TNF-α. IL-1ß is released from macrophages/ monocytes and is actively involved in inflammatory cell recruitment and the amplification of the pro-inflammatory cytokine response (Dinarello 2011). It was originally identified as a pyrogen and its expression is mainly induced in response to microbial molecules (Dinarello 2009). IL-6 is expressed by an array of cells, including mononuclear phagocytes, T lymphocytes (T cells) and B lymphocytes (B cells) and induces the differentiation of B cells into plasma cells. Moreover, it is important in the secretion of acute phase proteins in the liver and is involved in hematopoesis (Kishimoto 2010). TNF-α is a potent inflammatory mediator that is central to the inflammatory action of the innate immune system, including induction of cytokine production, activation or expression of adhesion molecules or growth stimulation. It is primarily secreted from activated macrophages, but also monocytes, T-cells, natural killer (NK) cells and fibroblasts are able to produce TNF-α (Locksley et al. 2001; Tracey et al. 2008; Turner et al. 2014). Additionally to these key players, IL-17 is crucial for maintaining control of host defense against extracellular pathogens. Besides subsets of T-cells, including Th17 cells and γδT cells as the primary source of IL-17, alveolar macrophages are also competent of secreting IL-17 (Jin and Dong 2013; Song et al. 2008). This cytokine is involved in the regulation of both granulopoesis and the recruitment of neutrophils to inflamed tissues. IL-17 acti-

vates various cell types, including macrophages inducing chemokine release, such as CXCL-8 and its murine ortholog CXCL-1 (Korn et al. 2009; Laan et al. 1999).

Chemokines are chemotactic cytokines which orchestrate the recruitment and activation of inflammatory cells, mostly neutrophils to the sites of infection or injury (Charo and Ransohoff 2006; Turner et al. 2014). Although chemotaxis is the cardinal feature of chemokines, their physiological role is more complex, with many having additional functions, such as initiation of adaptive immune response and immune surveillance (Moser and Willimann 2004; Moser et al. 2004; Turner et al. 2014). One of the most widely studied chemokines is CXCL-8, an essential inflammatory mediator, and its murine ortholog CXCL-1, also termed keratinocyte-derived chemokine (KC). They are expressed by alveolar macrophages and epithelial cells in the lung and recruit neutrophils to sites of inflammation (Aujla et al. 2007; Becker et al. 1994; Hammond et al. 1995; Huang et al. 1992; Luster 1998).

Cytokines and chemokines determined in this study are summarized in Table 4.

Table 4: Select Cytokines and Chemokines in the Innate Immune Response

	Name	Main Source	Target Cell	Major Function	Type
Cytokines	IL-1ß	Macrophages, monocytes	Macrophages, B cells, NK cells	Pyrogenic, inflammatory cell recruitment, enhancement of cytokine response	Pro-inflammatory
	IL-6	Macrophages, fibroblasts	Activated B cells, plasma cells	Differentiation into plasma cells	
	IL-10	T cells, macrophages/ monocytes	B cells, macrophages	Inhibits cytokine production and mononuclear cell function	Anti-inflammatory
	IL-12p40	T cells, macrophages	NK cells	Activates NK cells	Pro-inflammatory
	IL-13	Th2 cells	B cells	Maturation and differentiation of B cells, inhibits cytokine release by macrophages	Pro- and anti-inflammatory
	IL-17	T cells, alveolar macrophages	Macrophages, neutrophils	Induction of cytokine release, granulopoesis, recruitment of neutrophils	Pro-inflammatory
	TNF-α	Macrophages, monocytes, NK cells	Macrophages	Phagocyte cell activation, induction of cytokine release	
Growth factor	GM-CSF	T cells, macrophages, fibroblasts,	Stem cells	Proliferation, differentiation and activation of neutrophils and macrophages	
Chemokines	MCP-1 (CCL2)	T cells, monocytes, fibroblasts	Monocytes, lymphocytes, granulocytes	Chemotaxis, recruitment of monocytes and lymphocytes	
	MIP-2	T cells, monocytes, fibroblasts	Monocytes, lymphocytes, granulocytes	Chemotaxis, recruitment of monocytes and lymphocytes	
	RANTES (CCL5)	Epithelial cells, T cells	Monocytes, neutrophils, T cells	Chemotaxis, recruitment of blood monocytes, memory T helper cells	
	CXCL-1 (KC)	Alveolar macrophages, epithelial cells	Neutrophils	Chemotaxis, recruitment of neutrophils	

Sources: Barthlott and Holländer 2006; Becker et al. 1994; Dinarello 2011, 2009; Hammond et al. 1995; Jin and Dong 2013; Korn et al. 2009; Laan et al. 1999; Locksley et al. 2001; Moser and Willimann 2004; Song et al. 2008; Tracey et al. 2008; Turner et al. 2014

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IL = interleukin; TNF = tumor necrosis factor; GM-CSF = granulocyte macrophage colony-stimulating factor; MCP = monocyte chemotactic protein; CCL = chemokine (C-C motif) ligand; MIP = macrophage inflammatory protein; RANTES = regulated upon activation, normal T cell expressed, and secreted; CXCL = CXC-motif ligand; KC = keratinocyte-derived chemokine; B cells = B lymphocytes, T cells = T lymphocytes; NK cells = natural killer cells; Th cells = T helper cell type lymphocytes;

1.4   Aims and Hypotheses of this Study

Members of the CLCA protein family have been linked to inflammatory airway diseases with increased mucus production (Brouillard et al. 2005; Hauber et al. 2010; Hegab et al. 2004; Kamada et al. 2004). In the respiratory tract, it has been hypothesized that select CLCA proteins may act as extracellular signaling molecules, transforming airway mucus precursor cells to mature mucus cells (Patel et al. 2009). In mice, it has been speculated that two CLCA members, mCLCA3 and mCLCA5, may have redundant and compensatory functions in the induction of airway mucus cell metaplasia (Patel et al. 2006). Furthermore, it has been repeatedly shown that mCLCA3 is expressed by mucus cells (Leverkoehne and Gruber 2002; Mundhenk et al. 2006). In contrast, neither the expressing cell type nor the protein expression pattern of mCLCA5 have been reported so far, although its mRNA has been detected in the respiratory tract of naive mice (Braun et al. 2010a). hCLCA2, the human ortholog to mCLCA5, is also expressed in the lung on mRNA level, however, in contrast to mice, no up-regulation under conditions of mucus cell metaplasia could be observed *in vitro* (Alevy et al. 2012), pointing towards possible species-specific differences in the expression of mCLCA5 and its human ortholog. **Thus, we hypothesized that the mCLCA5 protein is expressed in highly restricted areas of the naive murine lung and that this expression pattern is species-specific.** Consequently, as **first aim**, the protein expression pattern of mCLCA5 on entire lung sections of naive mice was systematically characterized. Therefore, the protein was localized by immunohistochemistry and confocal laser scanning immunofluorescence microscopy and immunohistochemical double staining for specific cell markers identifying other airway cells. Moreover, lung mRNA expression levels of selected genes of interest, including *mClca3*, *mClca5*, *Muc5ac* and *Muc5b*, were determined. Cells expressing mCLCA5, mCLCA3 and club (formerly Clara) cell-protein CC10 as well as periodic acid-schiff (PAS)-positive mucus cells from phosphate buffered saline (PBS)-treated or *S. aureus*-infected mice were quantified and compared to naive controls. Furthermore, the course of mCLCA5 protein expression in two other lung infection models using *Streptococcus pneumoniae* (*S. pneumoniae*) and influenza virus was investigated. To determine possible species-specific differences, the expression pattern of mCLCA5 was compared with those of

its human and porcine orthologs, hCLCA2 and pCLCA2, respectively. The porcine ortholog was included in this study since the pig is becoming an increasingly important animal model in translational medicine (Klymiuk et al. 2012; Meyerholz et al. 2010a; Meyerholz et al. 2010b; Ostedgaard et al. 2011; Plog et al. 2009; Rogers et al. 2008b; Stoltz et al. 2010).

In addition to the well-established role of CLCA proteins in mucus cell metaplasia and mucin production, mCLCA3 has been implicated in the regulation of tissue inflammation in the innate immune response (Long et al. 2006; Zhang and He 2010). To date, only models of chronic and allergic airway inflammation (Long et al. 2006; Mundhenk et al. 2012; Patel et al. 2006) and acute inflammation induced by LPS (Long et al. 2006) have been characterized in *mClca3*$^{-/-}$ mice. However, acute bacterial infection appears more suitable to test for a role of mCLCA3 in modulating innate immune responses. **Therefore, we hypothesized that lack of mCLCA3 has an impact on the innate immune response in acute *S. aureus* infection of the mouse lung.** Consequently, as **second aim**, investigations adopted infection of mice with *S. aureus* which is one of the most prevalent pathogens of community- and hospital-acquired infections in humans accounting for a significant health and economic burden (Gillet et al. 2002; Gillet et al. 2001; Kapetanovic et al. 2010). Besides septicemia, skin and soft tissue infections, *S. aureus* causes lower respiratory tract infections in humans, especially in infants and young children with CF (Ulrich et al. 1998; Wolter et al. 2013; Wong et al. 2013). To elucidate a modulatory role of the mCLCA3 protein in the innate immune response, we applied a loss-of-function study by using *mClca3*$^{-/-}$ mice which were available and characterized by our research group (Mundhenk et al. 2012). For this purpose, a non-bacteremic *S. aureus* pneumonia mouse model had to be established first and phenotyped. Subsequently, *mClca3*$^{-/-}$ mice and wild-type (WT) littermates were infected with an adequate infectious dose of *S. aureus* and the course of pneumonia was analyzed in comparison to uninfected, PBS-treated, mice regarding clinical signs, bacterial clearance, leukocyte immigration and cytokine response in the bronchoalveolar compartment, pulmonary vascular permeability, histopathology including morphometry, mucus cell quantification and respiratory tract mRNA expression levels of selected genes of interest, including *mClca1* to *7*, *Muc5ac*, *Muc5b*, *Muc2*, *Cxcl-1*, *Cxcl-2* and *Il-17*.

In conclusion, this study aimed at establishing whether the respiratory expression pattern of the mCLCA5 protein would enable its proposed function as an inducer of airway mucus cell metaplasia and compensatory candidate for mCLCA3 and, second, if mCLCA3 is an integral player of innate immunity in the lung, in addition to its known function as mucus cell inducer.

2   Own Research Publications in Scientific Journals

2.1   Murine CLCA5 is Uniquely Expressed in Distinct Niches of Airway Epithelial Cells

Authors: Dietert K, Mundhenk L, Erickson NA, Reppe K, Hocke AC, Kummer W, Witzenrath M, Gruber AD

Year: 2014

Journal: Histochemistry and Cell Biology

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Declaration of own portion of work in this research publication:

Contributions by K. Dietert: Design, preparation, completion and evaluation of all experiments including experimental animal procedures (preparation of lung tissues from mice) and processing of tissue samples from humans and pigs, lung expression pattern analyses on the mRNA and protein levels including quantitative RT-PCR, histochemistry, immunohistochemistry and quantification of cells, immunofluorescence for co-localization analyses by confocal laser scanning microscopy and double-stainings by immunohistochemistry. Independent subsequent preparation of the entire manuscript.

Contributions of other authors: All co-authors participated considerably to the study design, evaluation of experimental results and review of the manuscript. Andreas C. Hocke performed the spectral imaging and confocal laser scanning microscopy experiments.

Declaration on ethics:

All animal procedures were approved by the Ethics Committee of the Charité – Universitätsmedizin Berlin and local governmental authorities (Landesamt für Gesundheit und Soziales Berlin, approval ID naive mice: T 0104/06, PBS-treated and *Staphylococcus aureus*-infected mice: G 0358/11, *Streptococcus pneumoniae*- and influenza virus-infected mice: G 0044/11, G 0057/13) and were conducted in strict accordance with the Federation of European Laboratory Animal Science Associations (FELASA) guidelines and recommendations for the care and use of laboratory animals (Guillen 2012).

http://www.felasa.eu/recommendations/guidelines/felasa-guidelines-and-recommendations/

Human tissue samples were taken from body donors who devoted their corpses for teaching and research purposes by written declaration. The use of these samples was approved by the Ethics Committee of the Department of Medicine, Justus-Liebig University, Giessen, Germany (approval ID: 129/14), in strict accordance with the Declaration of Helsinki.

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ORIGINAL PAPER

Murine CLCA5 is uniquely expressed in distinct niches of airway epithelial cells

Kristina Dietert · Lars Mundhenk · Nancy A. Erickson · Katrin Reppe · Andreas C. Hocke · Wolfgang Kummer · Martin Witzenrath · Achim D. Gruber

Abstract The murine mCLCA5 protein is a member of the chloride channel regulators, calcium-activated (CLCA) family and is suspected to play a role in airway mucus cell differentiation. Although mCLCA5 mRNA was previously found in total lung extracts, the expressing cells and functions in the naive murine respiratory tract are unknown. Therefore, mCLCA5 protein expression was identified by immunohistochemistry and confocal laser scanning microscopy using entire lung sections of naive mice. Moreover, we determined mRNA levels of functionally related genes (mClca3, mClca5, Muc5ac and Muc5b) and quantified mCLCA5-, mCLCA3- and CC10-positive cells and periodic acid-Schiff-positive mucus cells in naive, PBS-treated or *Staphylococcus aureus*-infected mice. We also investigated mCLCA5 protein expression in *Streptococcus pneumoniae* and influenza virus lung infection models. Finally, we determined species-specific differences in the expression patterns of the murine mCLCA5 and its human and porcine orthologs, hCLCA2 and pCLCA2. The mCLCA5 protein is uniquely expressed in highly select bronchial epithelial cells and submucosal glands in naive mice, consistent with anatomical locations of progenitor cell niches. Under conditions of challenge (PBS, *S. aureus*, *S. pneumoniae*, influenza virus), mRNA and protein expression strongly declined with protein recovery only in models retaining intact epithelial cells. In contrast to mice, human and porcine bronchial epithelial cells do not express their respective mCLCA5 orthologs and submucosal glands had fewer expressing cells, indicative of fundamental differences in mice versus humans and pigs.

Keywords Airway epithelial cell · Murine lung · mCLCA3 · Mucus cell metaplasia · Translational medicine

Introduction

mCLCA5 is a murine member of the chloride channel regulators, calcium-activated (CLCA) protein family which has been linked to inflammatory airway diseases with increased mucus production such as asthma, cystic fibrosis and chronic obstructive pulmonary disease (Brouillard et al. 2005; Hegab et al. 2004; Kamada et al. 2004). It has also been hypothesized that CLCA proteins act as extracellular signaling molecules, transforming airway mucus precursor cells to mature mucus cells (Patel et al. 2009) or, as growing evidence suggests, modulating the innate immune response (Dietert et al. 2014; Long et al. 2006; Zhang and He 2010), pointing toward a pleiotropic function of these proteins (Patel et al. 2009).

The human CLCA1 (hCLCA1), expressed in mucus cells of the respiratory tract (Gibson et al. 2005), is known to regulate mucus cell metaplasia by inducing mucus gene transcription via a downstream mitogen-activated protein

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kinase (MAPK)-13 signaling pathway (Alevy et al. 2012). In the mouse lung, overexpression of mCLCA3, the murine ortholog of hCLCA1, also induces mucus cell metaplasia; however, mClca3 knockout mice do not show a corresponding phenotype (Patel et al. 2006). Loss of mCLCA3 in these mice has been discussed to be compensated by increased mCLCA5 expression in experimentally induced mucus cell metaplasia in vitro and in vivo (Alevy et al. 2012; Mundhenk et al. 2012; Patel et al. 2006). It has consequently been speculated that the two proteins may have a redundant function in the respiratory tract, which is also supported by the observation that gene transfer with a vector-encoding mClca5 also induces airway mucus production (Patel et al. 2006).

Interestingly, mCLCA5 mRNA has been detected in various tissues, including the respiratory tract of naive mice in which neither its expressing cell type nor its protein has been observed so far (Braun et al. 2010). Instead, mCLCA5 protein was only discovered after Th2-induced airway inflammation (Mundhenk et al. 2012). In unchallenged mice, the protein has only been detected outside the respiratory tract, specifically in late differentiated keratinocytes of all stratified squamous epithelial granular layers throughout the body with a proposed function in growth arrest and maturation of squamous epithelial cells (Braun et al. 2010). In contrast to mCLCA5, the human orthologous hCLCA2 protein appears to be expressed in basal epithelial cells of stratified epithelia, with a proposed role in stratification and basal cell-basement membrane adhesion (Carter et al. 1990; Connon et al. 2004, 2005). However, it has never been detected in airway epithelial cells and, in contrast to the murine mCLCA5, is not overexpressed following induction of mucus cell metaplasia (Alevy et al. 2012). It has been argued that due to these differences between murine and human CLCA orthologs, the value of mouse models for mucus cell metaplasia in translational medicine is questionable. Functional studies on mucus cell differentiation are still lacking, but it has been shown that the expression pattern of pCLCA1, the porcine ortholog to hCLCA1 and to mCLCA3, is virtually identical with that of hCLCA1, supporting the pig as the favored translational model (Plog et al. 2009).

Porcine pCLCA2, the ortholog of hCLCA2 and mCLCA5, is also expressed in airways on mRNA level, but the expressing cell type is still unknown (Plog et al. 2012b). Similarly to mCLCA5, its protein has, so far, only been found in mature keratinocytes of the epidermis and in the inner root sheath of hair follicles (Plog et al. 2012b).

We hypothesized that mCLCA5 protein is expressed in highly select areas of the naive murine lung, since it has only been found on the mRNA level in total lung extracts so far. We further speculated that its expression pattern may be different from that of human and porcine orthologs since

the expressional behaviors differ between the murine and the human orthologs following induction of mucus cell metaplasia (Alevy et al. 2012).

Consequently, we systematically characterized the protein expression pattern of mCLCA5 on entire mouse lung sections by immunohistochemistry and localized the protein by confocal laser scanning immunofluorescence microscopy and immunohistochemical double staining for specific cell markers. Since growing evidence additionally suggests a modulating role of CLCA proteins in innate immune response (Dietert et al. 2014; Long et al. 2006; Zhang and He 2010), we determined lung mRNA expression levels of selected genes of interest, including mClca3, mClca5, Muc5ac and Muc5b and quantified cells expressing mCLCA5, mCLCA3 and club (formerly Clara) cell protein CC10 as well as periodic acid-Schiff (PAS)-positive mucus cells from PBS-treated or *Staphylococcus aureus* (*S. aureus*)-infected mice in comparison with naive controls. We further investigated the course of mCLCA5 protein expression in two other lung infection models, *Streptococcus pneumoniae* (*S. pneumoniae*) and influenza virus. To determine possible species-specific differences, we compared the expression pattern of murine mCLCA5 with those of its human and porcine orthologs, hCLCA2 and pCLCA2.

Materials and methods

Naive mice and tissue processing

Naive female C57BL/6J wild-type mice, aged 8–9 weeks and weighing 18–20 g, were housed in individually ventilated cages under SPF conditions with a room temperature of 22 ± 2 °C and a relative humidity of 45–65 %. A 12-h light/dark cycle was maintained, and the animals had unlimited access to standard pelleted food and tap water. For experimental procedures, mice were anesthetized each by intraperitoneal injection of premixed ketamine (3.2 mg) and xylazine (1.5 mg) and sacrificed by exsanguination via the caudal *Vena cava*.

For lung tissue processing, whole lungs with tracheas were carefully removed, immersion fixed in 4 % formalin, pH 7.0, for up to 48 h, and subsequently embedded in paraffin. Multiple sections were cut from serial levels of the lung to ensure that the trachea and the complete bronchial stem including its branching points were available for systematic investigation.

Lung tissue of mouse models, pigs and humans

Similarly processed, formalin-fixed and paraffin-embedded (FFPE) lung tissues or snap-frozen lungs from PBS-treated,

S. aureus-, *S. pneumoniae*- or influenza virus-infected mice from previous studies (Dames et al. 2014; Dietert et al. 2014; Reppe et al. 2009) were used. Samples from human tracheas and lungs, obtained from body donors corpses at the Institute of Anatomy and Cell Biology, Justus-Liebig University, Giessen, Germany, as well as from healthy porcine tracheas and lungs, taken from the routine necropsy pool of the Department of Veterinary Pathology, Freie Universität Berlin, Germany, were also fixed in 4 % buffered formalin and embedded in paraffin.

RNA isolation and quantitative RT-PCR

Total RNA was isolated from snap-frozen, murine lungs using the Nucleo Spin RNA/Protein isolation Kit (Macherey–Nagel, Düren, Germany), quality checked and quantified using the NanoDrop ND-100 Spectrophotometer (Peqlab, Wilmington, USA). Transcript expression levels of murine Clca3, Clca5, Muc5ac and Muc5b, normalized to the reference genes elongation factor 1α (Ef-1α), β-2 microglobulin (B2m) and glyceraldehyde-3-phosphate dehydrogenase (Gapdh), were determined as described (Dietert et al. 2014). RT-qPCR and data analyses were conducted using the CFX96 Touch Real-Time PCR Detection System and CFX Manager software 1.6 (BioRad). Relative quantification and comparison of groups were performed by the $\Delta\Delta$Ct method using naive animals as controls.

Histochemistry, immunohistochemistry and quantification of cells

For visualization of mucus cells, sections were processed as described and the PAS reaction was conducted (Leverkoehne and Gruber 2002). Immunohistochemical analyses were performed as described (Braun et al. 2010; Leverkoehne and Gruber 2002). Briefly, entire murine FFPE lungs with tracheas or FFPE tissue samples from humans and pigs were cut at 2 μm thickness and mounted on adhesive glass slides. After dewaxing in xylene and rehydration in decreasing ethanol concentrations, antigen retrieval was performed with 0.1 % protease pretreatment for 10 min at 37 °C (AppliChem, Darmstadt) for the detection of mCLCA5, mCLCA3 or club cell protein 10 and with microwave heating (600 W) in 10-mM citric acid (750 ml, pH 6.0) for 12 min for the detection of cytokeratin 5. For single stainings, slides of murine lungs were incubated with immunopurified rabbit antibodies for mCLCA5 (α-mCLCA5-C1-ap, 1:300) (Braun et al. 2010), mCLCA3 (α-m3-C-1p, 1:600) (Bothe et al. 2011) or cytokeratin 5 (1:1,000; ab24647, Abcam) or with the immunopurified goat antibody for club cell 10 protein (CC10, 1:1,500; sc-9772, Santa Cruz Biotechnology). To exclude cross-reactivity of the mCLCA5 antibody with other murine

CLCA members, immunohistochemical stainings of the intestine, expressing mCLCA3, mCLCA4, mCLCA6 and mCLCA7 (Patel et al. 2009), and the pancreas, expressing mCLCA1/2 (Roussa et al. 2010), were performed and yielded negative results. Furthermore, specificity of the mCLCA5 antibody was previously verified by immunoblot analysis (Braun et al. 2010). Slides of porcine lungs were incubated with the immunopurified rabbit antibody for pCLCA2 (p2-C-1a, 1:300) (Plog et al. 2012b). The human lung sections were incubated at 4 °C over night with the pCLCA2 antibody which yielded an identical, specific, cellular staining pattern, pointing toward cross-reactivity with the human hCLCA2. Incubation with an immunopurified, irrelevant rabbit or goat antibody at similar dilutions served as negative control for all immunohistochemical stainings on murine, porcine and human tissue samples. The slides were incubated with biotinylated, secondary goat anti-rabbit IgG (1:200, BA 1000, Vector, Burlingame, CA) or rabbit anti-goat IgG (1:200, BA 5000, Vector, Burlingame, CA) antibodies and HRP- or AP-coupled streptavidin. Diaminobenzidine (DAB) or triamino-tritolyl-methanechloride (Neufuchsin) was used as substrates for color development, respectively. The slides were counterstained with hematoxylin or PAS reaction where indicated, dehydrated through graded ethanol, cleared in xylene and coverslipped.

Immunohistochemical double staining of mCLCA5 and mCLCA3 was performed using the H_2O-elution method in accordance with the instructions of zytomed systems (Zytomed 2009). This method is suitable for double staining using primary antibodies from the same species. Therefore, slides were prepared as described above and incubated with the mCLCA5 antibody (1:300) at 4 °C over night. After incubation with the biotinylated, secondary goat anti-rabbit IgG antibody (1:200), DAB was used for color development. Due to using two secondary antibodies against the same species, unspecific binding was excluded by washing the slides in heated, deionized water (750 ml microwaved at 600 W for 10 min) to eliminate remaining unbound primary antibodies with a consecutive rinse in water at 4 °C for 5 min. Following incubation with the purified mCLCA3 antibody (1:600) at 4 °C over night and with the secondary, goat anti-rabbit IgG alkaline phosphatase-conjugated antibody (1:500, AP-1000, Vector, Burlingame, CA), triaminotritolyl-methanechloride (Neufuchsin) was used as substrate for color development. Slides that were incubated with an irrelevant immunopurified rabbit antibody served as negative controls. To ensure specific binding of the secondary either HRP- or AP-conjugated antibody with the mCLCA5- or mCLCA3-specific primary antibody, respectively, slides were incubated with only one primary but with both secondary antibodies. Finally, slides were counterstained with hematoxylin, dehydrated, cleared and coverslipped. PAS-, mCLCA5-, mCLCA3- and CC10-positive cells were
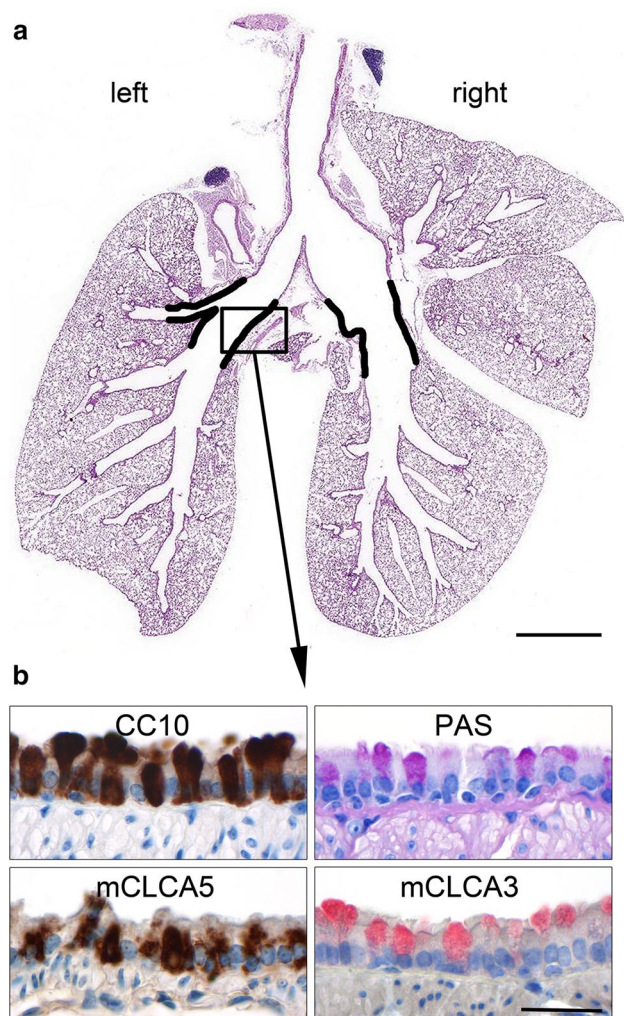
Fig. 1 mCLCA5 is expressed in lungs of naive mice in a highly selective pattern. **a** Whole lungs of naive mice were embedded in paraffin, and multiple sections of defined layers were prepared to ensure that the entire bronchial stem and its branching points are available for systematic investigation. mCLCA5 protein is expressed in bronchial epithelial cells at the transition from the extrapulmonary main bronchi to the intrapulmonary bronchi (*black lines*) only. HE staining. **b** This region was characterized by CC10-positive club cells, PAS-positive mucus cells as well as mCLCA5- and mCLCA3-expressing cells. *Bar* (**a**) 2 mm, *bar* (**b**) 20 μm

counted per millimeter of basement membrane at four anatomically defined regions of the bronchial epithelium at the extra- to intrapulmonary junction (Fig. 1, black lines) of naive, PBS-treated or *S. aureus*-infected mice using digital image software (AnalySIS docu 5.0., SIS).

Immunofluorescence and spectral confocal laser scanning microscopy

For immunofluorescence co-localization analyses, slides were incubated with the purified, primary mCLCA5

antibody (1:50) over night at 4 °C as described above and with Alexa Fluor 488-conjugated, secondary donkey anti-rabbit IgG antibody (1:2,000, Invitrogen) for 1 h at room temperature. Slides were then incubated with the purified, primary CC10 antibody (1:50) at 4 °C over night, incubated with Alexa Fluor 594-conjugated, secondary donkey anti-goat IgG antibody (1:2,000, Invitrogen) for 1 h at room temperature and mounted with Roti-Mount FluorCare DAPI (4,6-diaminidino-2-phenylindole) (Carl Roth, Karlsruhe, Germany). Adequate negative controls, including incubation of slides with only one primary but both secondary antibodies, were conducted. Slides were analyzed by spectral confocal microscopy with a LSM 780 microscope (objective 40×, Plan-Neofluar/oil, NA 1.3; Zeiss, Jena, Germany).

Data analysis

Data are expressed as mean $\pm$ SEM. Statistical analyses were performed using the Mann–Whitney test. $p < 0.05$ was considered significant.

Results

mCLCA5 is expressed in select bronchial epithelial cells at the transition from the extrapulmonary main bronchi to the intrapulmonary bronchi

For systematic expression analyses of mCLCA5 in the naive murine respiratory tract, trachea and entire lungs including bronchial stem and its branching points were investigated (Fig. 1a). The mCLCA5 protein was exclusively localized in bronchial epithelial cells of a defined region of approximately 2 mm in length (black lines) at the extra- to intrapulmonary junction as well as in epithelial cells of the SMGs which are, in mice, restricted to the larynx and the proximal trachea. For quantification of the different cell types of the bronchial epithelium in this specific region, the numbers of mCLCA5-, mCLCA3-, CC10-positive cells as well as PAS-positive mucus cells per millimeter of basement membrane were determined (Fig. 1b). In this specific location, 70.9 ± 2.7 % of the bronchial epithelial cells were positive for the club cell marker CC10, followed by 51.5 ± 1.7 % of mCLCA5-positive cells, 23.2 ± 3.2 % of PAS-positive cells and 20.5 ± 2.9 % of mCLCA3-positive cells (mean $\pm$ SEM, $n = 4$). Cytokeratin 5 expressing basal cells was localized only in the tracheal epithelium as well as in the cartilaginous bronchial epithelium and was clearly absent from the regions that possess mCLCA5-expressing cells (data not shown).
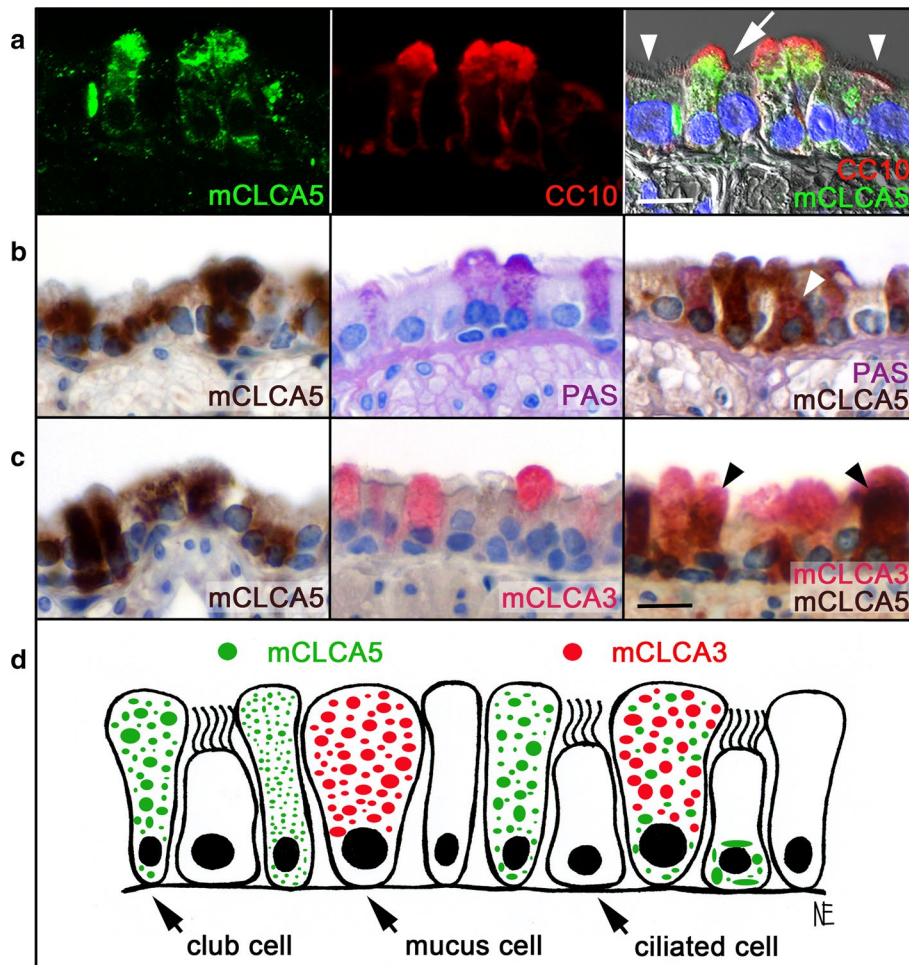
Fig. 2 mCLCA5 is predominantly located in club cells, to lesser extent in mucus cells and ciliated cells. **a** For co-localization studies of mCLCA5 and club cell protein CC10, immunofluorescence and spectral confocal laser scanning microscopy was performed. (mCLCA5: *left*, *green*; CC10: *center*, *red*; merged image: *right*). *Blue* DAPI (4,6-diaminidino-2-phenylindole) staining of the DNA in the nuclei. **b**, **c** Double staining of mCLCA5 either with PAS reaction, identifying mucus cells, or with mCLCA3 by immunohistochemistry was conducted. mCLCA5 is primarily located in club cells (**a** *arrow*), followed by fewer ciliated cells (**a** *arrowhead*) and mucus cells (**b** *arrowhead*). In mucus cells, mCLCA5 was occasionally co-localized with mCLCA3 (**c** *arrowhead*). **d** Club cells and mucus cells showed an either fine or coarse, diffuse, granular, cytoplasmatic, subcellular labeling pattern of mCLCA5, in contrast to ciliated cells, which displayed a clumpy and perinuclear labeling pattern. However, only a limited number of the investigated cells were positive for mCLCA5 protein expression. *Bar* (**a**) 5 μm, *bar* (**b**, **c**) 10 μm

mCLCA5 is predominantly located in club cells and, to a lesser extent, in ciliated cells and mucus cells of the bronchial epithelium, with a cell type-specific, subcellular expression pattern

For identification of mCLCA5-expressing cells, immunofluorescence and spectral confocal laser scanning microscopy was performed. mCLCA5 was predominantly expressed in CC10-positive club cells (Fig. 2a, arrow) with a diffuse, either finely or coarsely granular, cytoplasmic pattern. However, only a limited number of club cells were positive for mCLCA5 protein expression. The protein was also detected in a few ciliated cells (Fig. 2a, arrowhead), displaying a clumpy and perinuclear expression signal.

Further co-localization studies revealed mCLCA5 expression in few mucus cells (Fig. 2b, arrowhead), occasionally co-localized with the mucus cell marker mCLCA3 (Fig. 2c, arrowhead, d).

mCLCA5 mRNA and protein strongly decrease after various challenges

mRNA levels of Muc5ac, Muc5b, mClca3 and mClca5 were quantified in lungs from naive, PBS-treated and *S. aureus*-infected mice by RTq-PCR. After 24 h, expression of both the mucin genes Muc5ac and Muc5b as well as of mClca3 was not altered compared to naive controls, independently of the type of challenge (Fig. 3a,
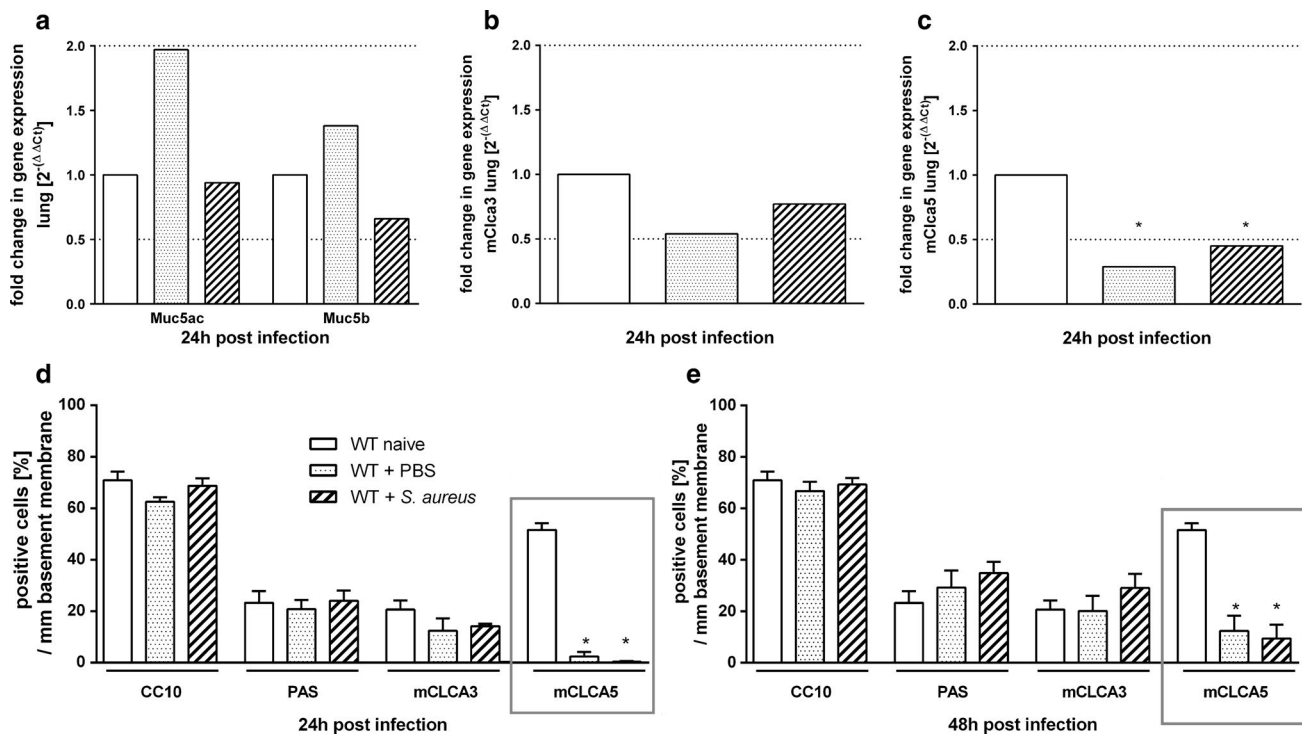
Fig. 3 mCLCA5 mRNA and protein are strongly decreased in challenged lungs. **a–c** 24 h after mice were treated with PBS or infected with *S. aureus*, lung mRNA expression levels of Muc5ac, Muc5b, mClca3 and mClca5 were determined by RT-qPCR in comparison with naive mice. Only mClca5 mRNA was significantly decreased in both challenge models (**c**). *Dotted lines* indicate fold changes of 0.5 and 2, respectively, as limits for valid statement of lowered and elevated parameters. Values are given as mean $\pm$ SEM ($n = 8$ each group). *Ct* cycle threshold. $*p < 0.05$ versus the naive control group. **d**, **e** Numbers of CC10-, PAS-, mCLCA3- and mCLCA5-positive cells per mm basement membrane were quantified by immunohistochemistry or PAS reaction. mCLCA5-positive cells were significantly reduced in both challenge models at indicated time points, without any further changes in number or composition of the bronchial epithelial cells. Values are given as mean $\pm$ SEM ($n = 4$ each group). $*p < 0.05$ versus the naive control group

b). Only mClca5 was significantly decreased on mRNA level after PBS treatment and *S. aureus* infection (Fig. 3c). Quantification of CC10-, PAS- and mCLCA3-positive cells per mm basement membrane revealed no differences between PBS-treated or *S. aureus*-infected mice compared to naive controls at all time points investigated (Fig. 3d, e). In contrast, mCLCA5-positive cells were significantly reduced 24 h after PBS treatment and *S. aureus* infection compared to naive mice (Figs. 3d, 4a, b). Despite this significant decrease which was still present after 48 h, the epithelium showed a slight tendency toward increasing numbers of mCLCA5-positive cells (Figs. 3e, 4b) which were significantly elevated ($*p < 0.05$) in *S. aureus*-infected mice. Additionally, after infection of mice with *S. pneumoniae* (Fig. 4c) or influenza virus, which both caused significant cell damage and loss in this area (Fig. 4d), a gradual reduction of mCLCA5-positive cells was observed over time without returning, possibly due to the initiated epithelial damage by these two pathogens.

Human and porcine mCLCA5 orthologs are expressed in submucosal glands but not in bronchial epithelial cells

In order to determine possible species-specific differences as seen for other CLCA gene family members, the respiratory expression patterns of the mCLCA5 orthologs, hCLCA2 and pCLCA2, were immunohistochemically examined in human or porcine lungs, respectively. In mice, SMGs are only present in the upper part of the trachea (Fig. 5a, blue lines), whereas in the human and porcine respiratory tracts, these glands line the entire cartilaginous airways down to their branching into segmental bronchi (Fig. 5b, c, blue lines). The epithelial cells of these species-specifically distributed submucosal glands were positive for the respective CLCA orthologs in mice, humans and pigs in which the murine mCLCA5 signal was much stronger than in those of the respective orthologs (Fig. 5d–f, left picture). In contrast to the murine mCLCA5, neither its human nor its porcine ortholog was expressed in bronchial epithelial cells or other cell types throughout the entire lungs (Fig. 5d–f, right picture).

Fig. 4 mCLCA5 protein expression disappeared in various challenge models. Lungs from naive ($n = 4$) and PBS-treated ($n = 4$) mice as well as from mice infected with *S. aureus* ($n = 4$), *S. pneumoniae* ($n = 2$) and influenza virus ($n = 2$) were examined at the extrapulmonary to intrapulmonary junction to characterize the presence and the course of mCLCA5 protein expression in this specific location at various time points. **a**, **b** Comparison of naive lungs to lungs from PBS-treated or *S. aureus*-infected mice revealed a significant reduction in mCLCA5 protein expression 24 h after infection, with a slight tendency toward a return after 48 h. **c**, **d** After infection with *S. pneumoniae* and influenza virus, the immunosignal of mCLCA5 disappeared over time. *Bar* 20 μm
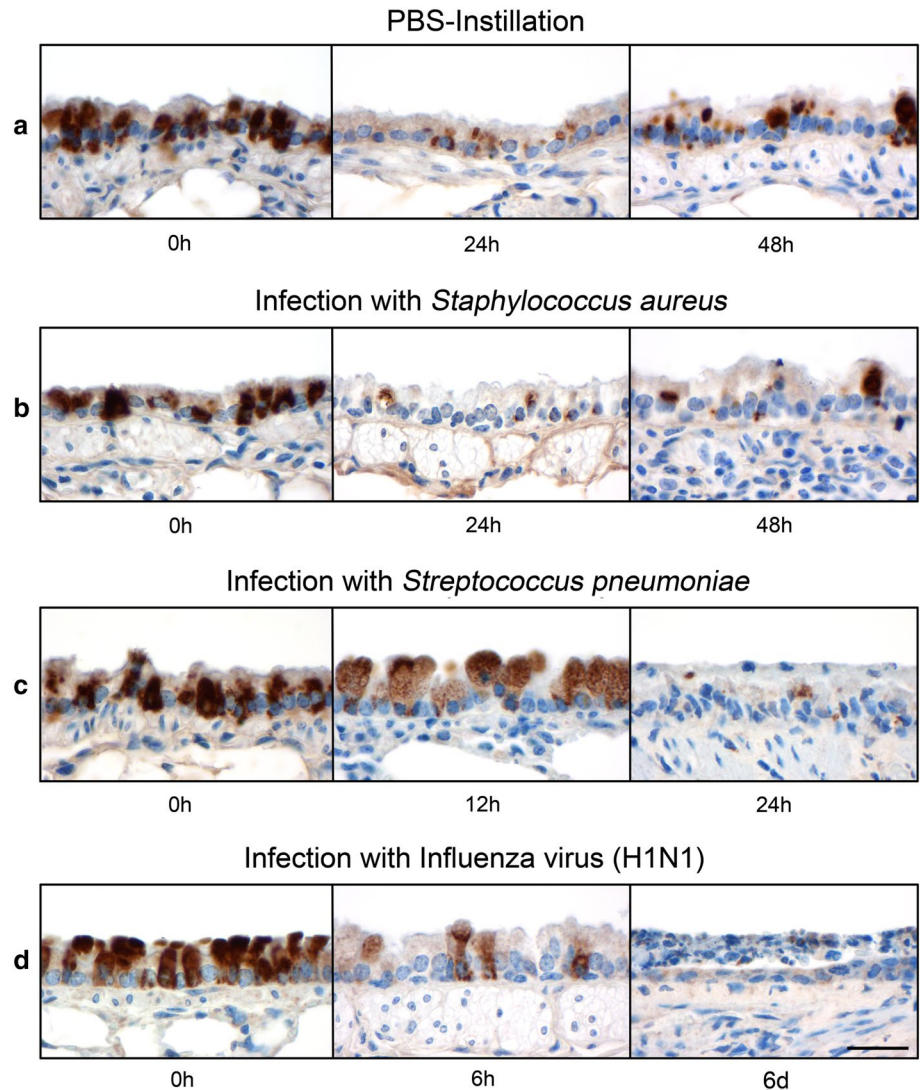
Discussion

In the current study, we identified a unique mCLCA5 expression pattern in mouse airways which is restricted to two specific locations. On the one hand, mCLCA5 is expressed in the epithelial cells of the SMGs and, on the other hand, in the bronchial epithelium, specifically at the transition of the extrapulmonary main bronchi into the intrapulmonary bronchi. Interestingly, both regions are anatomically described as progenitor cell niches which have been characterized by several studies in detail (Liu and Engelhardt 2008; Rawlins and Hogan 2006; Roomans 2010; Warburton et al. 2008).

Club cells were the predominant cell type in the bronchial epithelia intensely expressing mCLCA5, whereas mucus cells and ciliated cells showed a reduced or absent expression of the mCLCA5 protein. Based on this apparently unique and highly specific distribution pattern of the mCLCA5 protein in airway epithelial cells, we can virtually exclude mCLCA5 protein expression in other known select, functionally distinctive airway epithelial cells with a characteristic tissue distribution. In particular, basal cells in the tracheal and cartilaginous bronchial epithelium as well as club cells located more distally from this location, and alveolar epithelial cells type II clearly do not express mCLCA5. In particular, a specific subset of club cells, the variant club cell type, which is primarily located in the non-cartilaginous bronchi and bronchioles (Liu and Engelhardt 2008; Rawlins and Hogan 2006; Roomans 2010; Warburton et al. 2008), is a known progenitor cell for non-ciliated club cells, ciliated cells and mucus cells (Pardo-Saganta et al. 2013; Rawlins and Hogan 2006; Rawlins et al. 2009; Reader et al. 2003; Reynolds and Malkinson 2010; Roomans 2010; Wong et al. 2009). A previous study identified mCLCA5 as being sufficient to induce mucus production and responsible for mucus cell metaplasia (Patel et al. 2009). Thus, the predominant expression of mCLCA5 in club cells as putative progenitors of mucus

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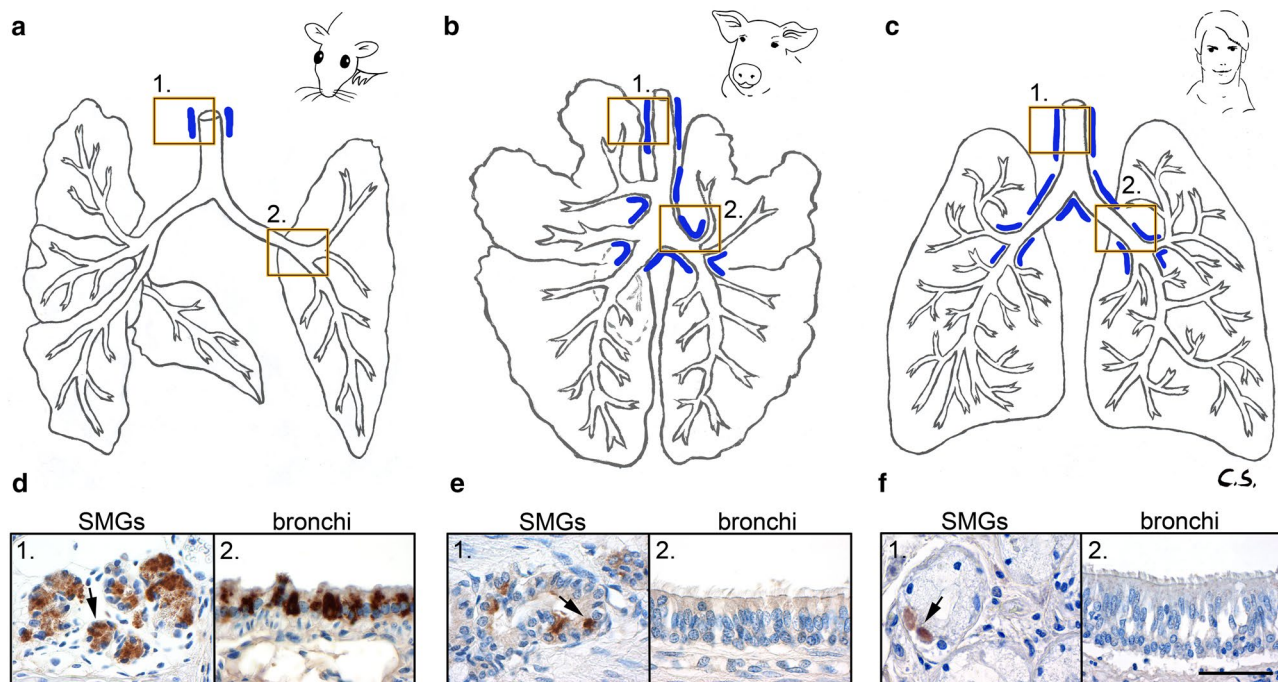
Fig. 5 Species-specific differences in expression patterns of mCLCA5 and its human and porcine orthologs. Murine ($n = 4$), human ($n = 2$) and porcine ($n = 3$) lung tissues were investigated by immunohistochemistry. **a–c** A species-specific distribution pattern in the submucosal glands (*blue lines*) was observed, and all species investigated had mCLCA5-, hCLCA2- or pCLCA2-positive cells, respectively, in the epithelial cells of these SMGs (*d1*, *e1*, *f1*, *left picture*). However, only the mouse had mCLCA5-positive cells in this specific location within the bronchial epithelium (*d2*, *e2*, *f2*, *right picture*). *Bar* 40 μm

cells and its presence in known anatomical locations of progenitor cell niches would be in line with its proposed function in mucus cell differentiation. It is conceivable that mCLCA5 expression by precursor mucus cells drives their differentiation into mature mucus cells, as it has already been hypothesized for the closely related proteins hCLCA1 and mCLCA3 (Alevy et al. 2012; Patel et al. 2006, 2009). These findings were confirmed by the fact that a role in epithelial differentiation of other lineages has already been shown for several CLCA members (Alevy et al. 2012; Patel et al. 2006, 2009; Walia et al. 2012; Yu et al. 2013) and is also consistent with the suspected role of mCLCA5 in growth arrest and maturation processes of squamous epithelial cells of the skin (Beckley et al. 2004; Braun et al. 2010). The identified expressing cell types showed a distinct intracellular mCLCA5 protein distribution pattern. In club cells and in the few mucus cells, mCLCA5 displayed a diffuse, finely granular, cytoplasmatic pattern as well as an evenly distributed but coarse and clumpy pattern throughout the entire cytoplasm. In ciliated bronchial cells, a large, clumpy, perinuclear pattern dominated, suggesting that the subcellular protein distribution may depend on the differentiation status of the respective cell type or on a cell type-specific arrangement of organelles. Moreover, it is well conceivable that the mCLCA5 protein in mucus cells and ciliated cells is lost with progressing differentiation from airway precursor to mature cells.

Similar to mCLCA5, the murine mCLCA3 is a known inducer of mucus cell metaplasia (Patel et al. 2006) and it has been suspected that mCLCA5 and mCLCA3 may have redundant functions, with one compensating for the loss of the other (Patel et al. 2006, 2009). In our study, mCLCA5 and mCLCA3 had only partially overlapping expression patterns in mucus cells and mCLCA3 was neither expressed in club cells nor in ciliated cells (Leverkoehne and Gruber 2002). This may be suggestive of different functional relevance of these two related proteins in their respective cellular microenvironments.

Expression of mCLCA5 was further characterized in lung tissues that were available from previous challenge models (Dames et al. 2014; Dietert et al. 2014; Reppe et al. 2009). Under challenged conditions and independently of the type of challenge (PBS or *S. aureus*), mCLCA5 strongly decreased both on mRNA and protein levels at 24 h after challenge. Interestingly, after 48 h, the mCLCA5 protein signal reappears, possibly due to the epithelium remaining intact in these two models. While mCLCA5 mRNA expression and number of mCLCA5-positive cells decreased, the overall number and cell type composition of club cells and mucus cells, the major expressing cell types of mCLCA5,

remained constant, pointing toward a selective transcriptional regulation of mCLCA5 with subsequent loss of the protein. Secretory processes by club cells and mucus cells which have been observed under challenged conditions (Davis and Dickey 2008; Evans et al. 2004; Pack et al. 1980; Reader et al. 2003; Reynolds and Malkinson 2010) may have contributed to the reduction of mCLCA5 on the protein level.

Under conditions of challenge, the downregulation of specific proteins involved in cellular differentiation is a known phenomenon of cells reacting to a specific type of challenge (Das et al. 2011; Zheng et al. 2013) which is consistent with our findings and the proposed role of mCLCA5 in cellular differentiation.

A similar effect on the mCLCA5 protein level was seen after infections with *S. pneumoniae* or influenza virus where a gradual reduction of mCLCA5-positive cells was observed over time without reappearance, possibly due to the initiated epithelial cell damage and death inflicted by these two pathogens. However, we cannot exclude that other, more specific factors may have contributed to the loss of mCLCA5 expression under the challenges used.

In a recent study comparing IL-13-challenged mice with PBS-treated controls, mCLCA5 protein was found in airway mucus cells, interpreted as a de novo expression (Mundhenk et al. 2012). However, mCLCA5 expression level and pattern in naive mice were not assessed in that study which would explain the fact that no differential upregulation of mCLCA5 mRNA was seen under challenged conditions (Mundhenk et al. 2012).

The murine lung, including the two niches that selectively express mCLCA5, differs from the lungs of other species in several anatomical and functional aspects. Murine SMGs are restricted to the larynx and the proximal trachea, whereas in humans and pigs, SMGs occur along the entire cartilaginous airways (Liu and Engelhardt 2008; Lynch and Engelhardt 2014; Rawlins and Hogan 2005; Rock et al. 2010; Suarez et al. 2012). The murine proximal airway epithelium predominantly consists of club cells, the principal secretory cell type (Liu et al. 2006; Rawlins and Hogan 2006; Reynolds and Malkinson 2010; Rock and Hogan 2011), followed by ciliated and fewer mucus cells (Pack et al. 1980; Wong et al. 2009). In contrast, ciliated and basal cells dominate in the human lung with much less secretory goblet cells (Rawlins and Hogan 2006; Rock et al. 2010; Wong et al. 2009). It is important to note that bronchial club cells, the major mCLCA5-expressing cell type in the mouse, do not exist in humans (Suarez et al. 2012). Furthermore, basal cells can only be found in the murine trachea and proximal cartilaginous airways (Rawlins and Hogan 2006; Rock et al. 2009), whereas in humans, they extend down to small bronchi (Fox 2007; Suarez et al. 2012; Wetzels et al. 1992). Based on these species-specific differences in airway anatomy and the suspected redundant

functions of murine CLCA homologs (Patel et al. 2009), mice may not be the most suitable model for studying CLCA gene products in mucus cell metaplasia.

We therefore tested whether other species also express mCLCA5 orthologs in these specific niches of the respiratory tract. Specifically, we examined the protein expression patterns of hCLCA2 and pCLCA2, the direct orthologs to the murine mCLCA5, in human and porcine lungs. Interestingly, only very few human and some porcine SMG cells but no bronchial epithelial cells were found to express hCLCA2 or pCLCA2, respectively. It is tempting to speculate that this unique niche of mCLCA5-expressing cells in murine bronchial epithelium compensates for the lack of SMGs in the lower segments of murine airways.

The lack of hCLCA2 and pCLCA2 expression in the bronchial epithelium may point toward a species-specific function and is in line with the observation that hCLCA2 is not upregulated under mucus cell metaplasia, in contrast to its murine ortholog mCLCA5 (Alevy et al. 2012). Controversially, the similarity of mCLCA5 ortholog expression pattern between humans and pigs supports the notion that CLCA genes may be more closely related to the human than to the murine species as one would expect from the degree of sequence similarities (Plog et al. 2009, 2012a, b). The pig may thus become the preferred model in studying mucus cell metaplasia.

In summary, our results yielded several surprising observations on the distribution of mCLCA5 in the mouse lung and its human and porcine orthologs, hCLCA2 and pCLCA2. First, naive mice express mCLCA5 in very distinct niches of their bronchial epithelium and in epithelial cells of the SMGs. Second, under conditions of challenge, including instillation of PBS and infection with *S. aureus*, *S. pneumoniae* or influenza virus, mCLCA5 mRNA and protein expression strongly declined with protein reappearance only after challenges without epithelial cell damage. Third, the mCLCA5 orthologs, hCLCA2 and pCLCA2, are not expressed by bronchial epithelial cells in human and porcine lungs, respectively. Here, the orthologous proteins are present in SMG epithelial cells only, which, however, decorate the entire bronchial branchings. We speculate that the lack of these glands in most segments of the murine bronchial tree is compensated by additional mCLCA5 expression in a highly select area of the murine bronchial epithelium. Together with the results of previous studies on mCLCA5 and other CLCA homologs, our results raise several questions as to the role of these proteins in the maturation and differentiation of mucus cells. An approach including an ovalbumin challenge as the preferred model for studying mucus cell differentiation and mucus cell metaplasia in mice (Long et al. 2006; Nakanishi et al. 2001; Robichaud et al. 2005; Zhang and He 2010) may become of special interest in this issue.

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2.2   mCLCA3 Modulates IL-17 and CXCL-1 Induction and Leukocyte Recruitment in Murine *Staphylococcus aureus* Pneumonia

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Declaration of own portion of work in this research publication:

Contributions by K. Dietert: Design, preparation, completion and evaluation of all experiments including experimental animal procedures (infection and preparation of mice), determination of bacterial loads in lungs, liver and blood; cell counting procedures, manually and with fluorescent-activated cell sorter (FACS) of BALF and blood, cytospin preparation of BALF, Enzyme linked immunosorbent assay (ELISA) analyzes of BALF and plasma, quantitative RT-qPCR analyzes of lungs and tracheas, immunoblot analyzes of lungs, tracheas and BALF, histological examination and immunohistochemistry, lung morphometry and volume estimation. Subsequent creation of the entire manuscript.

Contributions of other authors: All co-authors participate considerably to the study design, evaluation of experimental results, and review of the manuscript. Technical assistance and supervising for infection, preparation and evaluation of experimental procedures and results was performed by Katrin Reppe relating to the analyses as mentioned above.

Declaration on ethics:

All animal procedures were approved by the Ethics Committee of the Charité - Universität Berlin and local governmental authorities (Landesamt für Gesundheit und Soziales Berlin, approval ID: G 0358/11). The animal studies were conducted in strict accordance with the FELASA guidelines and recommendations for the care and use of laboratory animals (Guillen 2012).

http://www.felasa.eu/recommendations/guidelines/felasa-guidelines-and-recommendations/

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mCLCA3 Modulates IL-17 and CXCL-1 Induction and Leukocyte Recruitment in Murine *Staphylococcus aureus* Pneumonia

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Abstract

The human hCLCA1 and its murine ortholog mCLCA3 (calcium-activated chloride channel regulators) are exclusively expressed in mucus cells and linked to inflammatory airway diseases with increased mucus production, such as asthma, cystic fibrosis and chronic obstructive pulmonary disease. Both proteins have a known impact on the mucus cell metaplasia trait in these diseases. However, growing evidence points towards an additional role in innate immune responses. In the current study, we analyzed *Staphylococcus aureus* pneumonia, an established model to study pulmonary innate immunity, in mCLCA3-deficient and wild-type mice, focusing on the cellular and cytokine-driven innate inflammatory response. We compared clinical signs, bacterial clearance, leukocyte immigration and cytokine responses in the bronchoalveolar compartment, as well as pulmonary vascular permeability, histopathology, mucus cell number and mRNA expression levels of selected genes (mClca1 to 7, Muc5ac, Muc5b, Muc2, Cxcl-1, Cxcl-2, Il-17). Deficiency of mCLCA3 resulted in decreased neutrophilic infiltration into the bronchoalveolar space during bacterial infection. Only the cytokines IL-17 and the murine CXCL-8 homolog CXCL-1 were decreased on mRNA and protein levels during bacterial infection in mCLCA3-deficient mice compared to wild-type controls. However, no differences in clinical outcome, histopathology or mucus cell metaplasia were observed. We did not find evidence for regulation of any other CLCA homolog that would putatively compensate for the lack of mCLCA3. In conclusion, mCLCA3 appears to modulate leukocyte response via IL-17 and murine CXCL-8 homologs in acute *Staphylococcus aureus* pneumonia which is well in line with the proposed function of hCLCA1 as a signaling molecule acting on alveolar macrophages.

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All data are included within the manuscript or supporting Information file.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

The human hCLCA1 and its murine ortholog mCLCA3 are members of the CLCA (calcium-activated chloride channel regulator) family with a well established role in inflammatory airway diseases with increased mucus production such as asthma, cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) [1–4]. The link between CLCA proteins and inflammatory airway diseases has been recognized based on overexpression of Clca gene products in affected airways which is regulated by Th2 cytokine signals (IL-4, IL-9, and IL-13) [4,5]. The secreted proteins hCLCA1 and mCLCA3, which are selectively expressed in mucus cells of airways and other tissues [6–8] have been directly linked to the trait of mucus cell metaplasia in inflammatory airway diseases [9]. Specifically, it was previously demonstrated that hCLCA1 acts as an extracellular signaling protein, inducing mucus gene transcription via a downstream mitogen-activated protein kinase (MAPK)-13 signaling pathway and hereby regulating mucus cell metaplasia [10]. Hence, hCLCA1 and its ortholog mCLCA3 have been proposed as biomarkers of inflammatory airway diseases [11] and as targets for therapeutic intervention in mucus overproduction [11,12].

However, in addition to the modulation of mucus production and the strong link to mucus cell metaplasia, CLCA-proteins have been implicated in the regulation of tissue inflammation in the innate immune response [13,14]. Indeed, recent studies have demonstrated that hCLCA1 may act as an innate immune signaling molecule which activates airway macrophages and thereby enhances pro-inflammatory cytokine release (IL-8, IL-6, IL-1β, TNF-α) [15]. Moreover, asthmatic mice treated with anti-mCLCA3-antibodies showed remarkable reduction of airway inflammation [16].

So far, only models of chronic and allergic airway inflammation [9,13,17] and acute inflammation due to LPS [13] have been characterized in mCLCA3-deficient mice. However, acute bacterial infection appears more suitable to test for a role of mCLCA3 in modulating innate immune responses.

Consequently, this study adopted infection of mCLCA3-deficient mice with *Staphylococcus aureus* (*S. aureus*) which is one of the most prevalent pathogens of community- and hospital-

acquired infections in humans accounting for a significant health and economic burden [18–20]. Besides septicemia, skin and soft tissue infections, *S. aureus* causes lower respiratory tract infections in humans, especially in infants and young children with CF [21–23].

Here, we hypothesized that mCLCA3 has an impact on the innate immune response in acute *S. aureus* infection of the lung. mCLCA3-deficient mice (mClca3$^{-/-}$) and wild-type (WT) littermates were infected with *S. aureus* and the course of pneumonia was analyzed in comparison with uninfected mice regarding clinical signs, bacterial clearance, leukocyte immigration and cytokine response in bronchoalveolar lavage fluid (BALF), pulmonary vascular permeability, histopathology including morphometry, mucus cell quantification and respiratory tract mRNA expression levels of selected genes of interest, including mClca1 to 7, Muc5ac, Muc5b, Muc2, Cxcl-1, Cxcl-2 and Il-17. We show that mCLCA3 modulates the cellular leukocyte recruitment via IL-17 and CXCL-1 in bacterial pneumonia and thus appears to have an impact on the early innate immune response following *S. aureus* lung infection.

Materials and Methods

Ethics Statement

All animal procedures were approved by the ethics committee of the Charité - Universität Berlin and local governmental authorities (Landesamt für Gesundheit und Soziales Berlin, approval ID: G 0358/11). The animal studies were conducted in strict accordance with the FELASA guidelines and recommendations for the care and use of laboratory animals.

Mice

Female mCLCA3-deficient mice (mClca3$^{-/-}$) and C57BL/6J WT littermates (aged 8–9 weeks and weighing 18–20 g) were randomly assigned to groups of four and housed in individually ventilated cages under SPF conditions with a room temperature of $22\pm2°C$ and a relative humidity of 45%–65%. A 12 hour light/12 hour dark cycle was maintained and the animals had unlimited access to standard pelleted food and tap water. mClca3$^{-/-}$ mice were generated on a C57BL/6J background by replacing parts of exons 7–11 with a neomycin cassette [9]. For all experimental procedures, excluding the infection procedure, mice were anes-

Table 1. Lung Scoring Parameters.

Parameter	Scale/score
Lung inflammation:	
Bronchitis	0–4
Peribronchial inflammation	0–4
Interstitial inflammation	0–4
Intraalveolar inflammation	0–4
Alveolar necrosis	0–4
Alveolar edema	0–4
Perivascular edema	0–4
Perivascular inflammation	0–4
Infiltration by neutrophils	0–4
Infiltration by macrophages	0–4
Lung area affected	0–100%

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thetized by intraperitoneal injection of premixed ketamine (3.2 mg) and xylazine (1.5 mg) and sacrificed by exsanguination via the caudal *Vena cava*.

Bacterial Growth and Infection

Staphylococcus aureus Newman (*S. aureus*) from –80°C glycerol stocks was plated on Columbia agar with 5% sheep blood and incubated overnight at 37°C with 5% CO_2. Following incubation, single colonies were picked and incubated in 25 ml Caso-bouillon (Carl Roth, Karlsruhe, Germany) for 2 to 3 hours at 220 rpm until midlog phase ($A_{600} = 0.5$). After centrifugation at $800\times g$, the pellet was resuspended in sterile PBS to an adjusted infectious dose (5×10^7 cfu *S. aureus*). For infection, mice were anesthetized by intraperitoneal injection of premixed ketamine (1.6 mg) and xylazine (0.5 mg) and transnasally inoculated [24] with 5×10^7 cfu *Staphylococcus aureus* Newman in 20 µl sterile PBS. Controls received 20 µl of sterile PBS. Body weight and rectal temperature (BAT-12 Microprobe, Physitemp, Clifton, NJ, USA) were recorded every 12 hours.

Bacterial Burden in Lungs, Liver and Blood

Mice were intraperitoneally anesthetized, heparinized, and blood was taken from the caudal *Vena cava*. Sterilely dissected lungs and livers were homogenized, serial dilutions of homogenates and blood were plated on blood agar and incubated (37°C, 5% CO_2) for 24 h for subsequent counting of colony forming units.

Quantification and Differentiation of Leukocytes in BALF and Blood

At indicated time points, mice were intraperitoneally anesthetized, tracheotomized, ventilated and intracardially heparinized as described [24]. Blood was taken from the caudal *Vena cava* and lungs were perfused with 0.9% NaCl via the pulmonary artery. Bronchoalveolar lavage was performed twice with 800 µl ice-cold PBS. After spinning, supernatant was snap frozen for cytokine analyses. Total leukocytes were counted manually on Neubauer Chamber and differentiated by fluorescent-activated cell sorter (FACS) analysis (FACS Calibur, BD Biosciences, Heidelberg Germany) using forward versus side scatter characteristics and the specific antibodies CD45 PerCP (clone 30-F11, BD Biosciences), GR-1 PE (clone RB6-8C5, BD Biosciences) and F4-80 APC (clone BM8, Invitrogen, Karlsruhe, Germany) as described [25]. Total blood leukocytes were counted and differentiated by FACS analysis using BD TruCOUNT Tubes, forward versus side scatter characteristics and specific antibody staining with CD45 PerCP and GR-1 PE [25]. Cytospins from BALF were obtained by centrifugation of 100 µl BALF cell suspension at $20\times g$ for 10 minutes (Cytospin 3, Shandon Ltd, Runcorn, UK) and subsequently stained with May-Grünwald Giemsa.
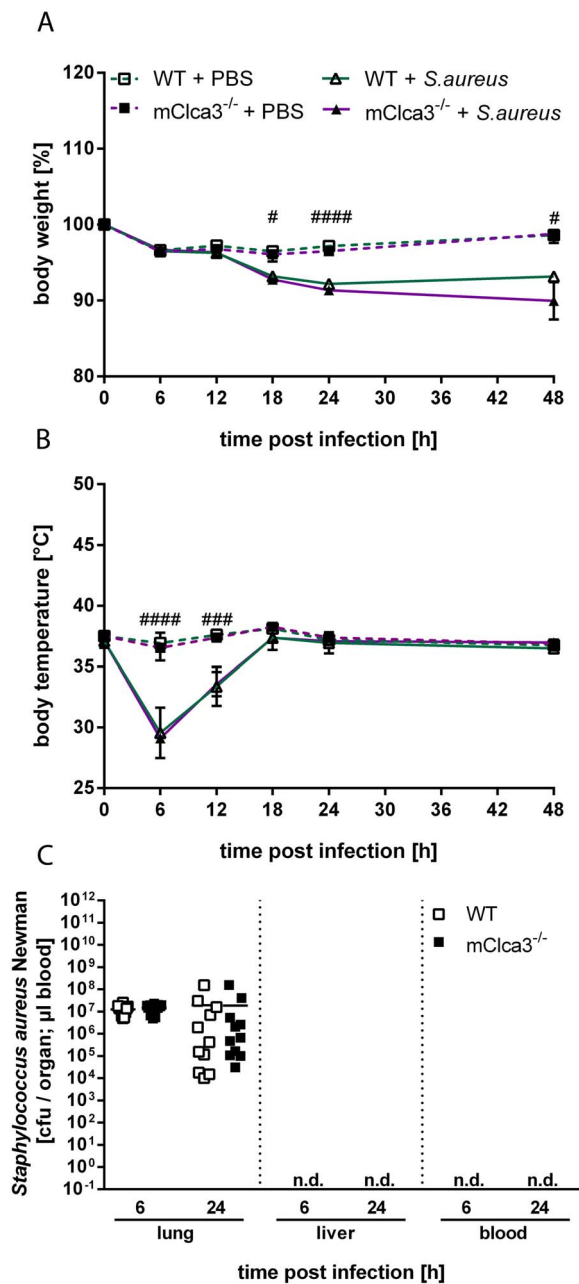
Pulmonary Vascular Permeability and Protein Quantification

Mouse albumin (MA) was quantified in BALF and plasma by ELISA (Biomol, Hamburg, Germany) according to the manufacturer's instructions. Pulmonary vascular permeability was assessed by calculating the MA BALF/plasma ratio. Total protein of BALF was measured via bicinchoninacid-assay (BCA) according to the manufacturer's instructions using Pierce BCA Protein Assay (Thermo Scientific, Rockford, USA).

36

A

B

C

Figure 1. mCLCA3-deficiency had no impact on clinical outcome of pneumonia or bacterial loads in lungs. mClca3^{−/−} and WT mice were transnasally infected with 5×10^7 *Staphylococcus aureus* Newman or received PBS (controls) and body weights (A) and temperatures (B) were measured every 6 hours for 24 hours and once after 48 hours. Values are given as mean ± SEM (n = 8 each group). [#]p<0.05, [##]p<0.01, [###]p<0.001, [####]p<0.0001 versus the PBS control group. (C) Lung, liver and blood bacterial loads were determined 6 hours and 24 hours after infection. Values are given as individual data and mean (n = 11 each group). n.d. = not detected.
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Quantification of Cytokines in BALF

Cytokines (IL-1β, IL-6, IL10, IL12p40, IL-13, MCP-1, GM-CSF, RANTES, CXCL-1 and TNF-α) were measured in the supernatant of BALF using a cytokine protein multiplex assay (Bioplex, Bio-Rad, Hercules, CA) and IL-17 was quantified by

ELISA (ab100702, Abcam, Cambridge, UK) according to the manufacturer's instructions.

RNA Isolation and Quantitative RT-PCR

Total RNA was isolated from lungs and trachea using Nucleo Spin RNA/Protein isolation Kit (Macherey Nagel, Düren, Germany). Total isolated RNA was quality checked and quantified using the NanoDrop ND-100 Spectrophotometer (Peqlab, Wilmington, USA). Transcript expression levels of murine Clca1 to 7, Muc5ac, Muc5b, Muc2, Il-17, Cxcl-1 and Cxcl-2, normalized to the reference genes elongationfactor 1α (Ef-1α), β-2 microglobulin (B2m) and glyceraldehyde-3-phosphate dehydrogenase (Gapdh), were determined. For exact transversion, 100 ng of RNA from lungs and trachea were reverse transcribed twice, each time in 20 μl reaction volume using Iscript (BioRad) according to manufacturer's instructions. Primers and probes for mClca1, mClca2, mClca3, mClca5 mClca6, mClca7, Ef1-α [26], Muc5ac, Muc5b [17], Muc2 [27], Il-17, Gapdh [28] and B2m [29] were used as described. Primers and probes for mClca4, Cxcl-1 and Cxcl-2 were designed using Primer3 software (WWW primertool, Whitehead Institute of Biomedical Research). All primer pairs encompassed an intron to avoid amplification of genomic DNA. In silico analysis of primer sequences and probes using NCBI nucleotide BLAST searches revealed 100% identity only with the expected DNA sequence. Agarose gel electrophoresis and sequencing of all PCR-products confirmed an amplification of a single product of expected size and sequence. For mClca4, no cross-reactions were detected when linearized, fully cloned cDNA samples of mClca1 and mClca2 were used as templates. Gapdh, Ef1-α and B2m were used as internal reference genes. Primer and probe sequences as well as amplicon sizes and annealing temperatures are listed in Table S1. An optimized reaction-mix in a total volume of 15 μl containing Maxima Probe qPCR Master Mix (Thermo Scientific, Oxford, USA), forward and reverse primers (each 0.3 μM), probe (0.2 μM), nuclease free water to adjusted volume as well as 1 μl of template cDNA was applied and cycling conditions according to manufacturer's instructions were used. RT-qPCR protocols were established using 10 fold serial dilutions ranging from 10^2 to 10^6 copies of purified (Nucleo Spin Gel and PCR Clean-up, Macherey-Nagel) PCR-derived fragments of each mRNA. RT-qPCR and data analyses were conducted using the CFX96 Touch Real-Time PCR Detection System and CFX Manager software 1.6 (BioRad). Relative quantification and comparison of groups were performed by the ΔΔCt method using uninfected wild-type animals as controls.

Western Blot Analysis

BALF proteins were separated by denaturating SDS (10%) PAGE followed by immunoblotting with mCLCA3 specific antibodies as described [6].

Histology and Immunohistochemistry

In a separate set of experiments, mice were intraperitoneally anesthetized, heparinized and blood was taken from the caudal *Vena cava*. After careful removal, lungs were immersion-fixed in 4% formalin (pH 7.0) and processed as described [6]. Briefly, sections were stained with hematoxylin and eosin and periodic acid-Schiff (PAS) reaction was performed. Three evenly distributed sections from each lung were scored for various parameters [30–32] (Table 1), and PAS-positive cells per millimeter of basement membrane were counted. *S. aureus* was detected by immunohistochemistry as described [33].
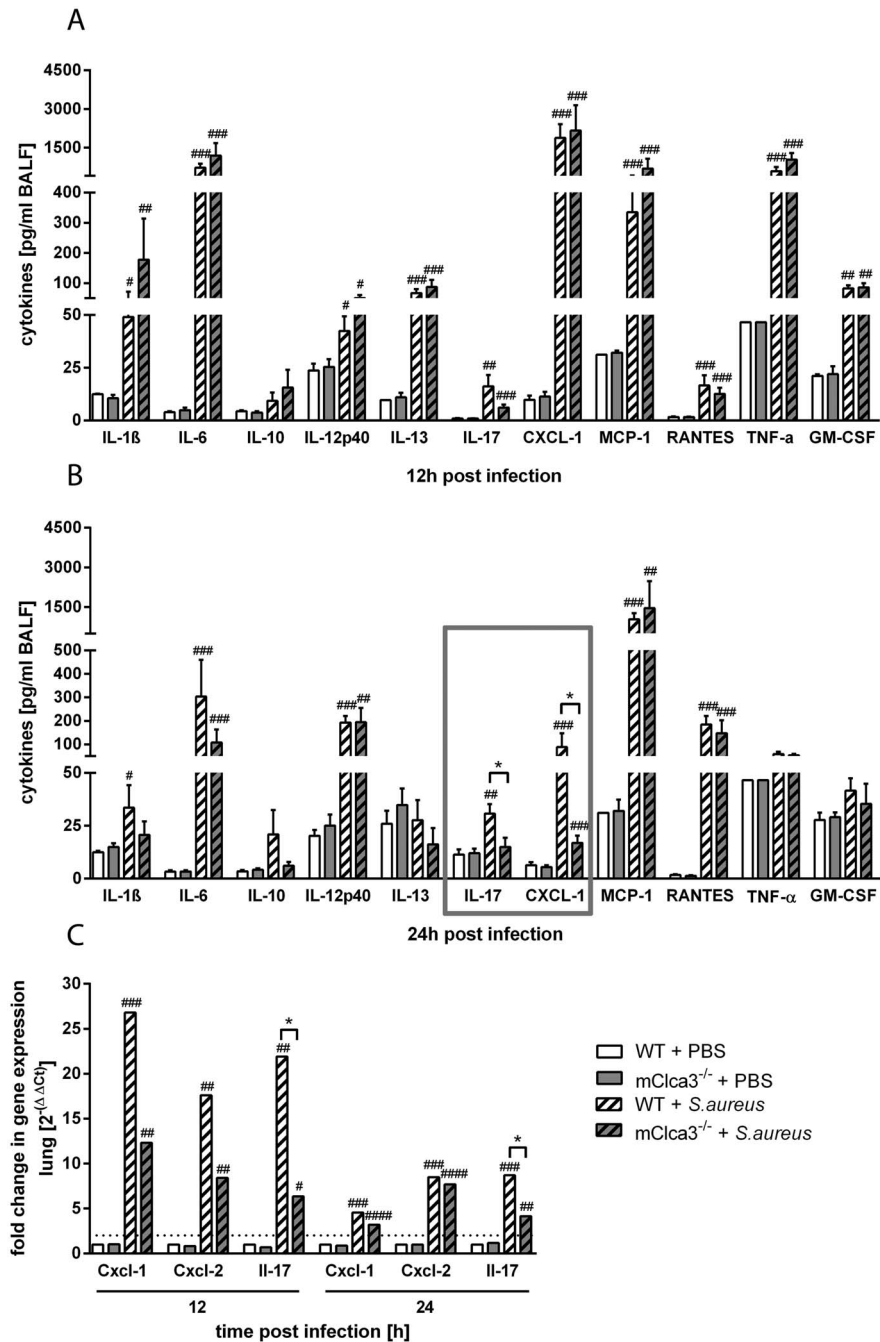
Figure 2. Chemokine CXCL-1 and cytokine IL-17 were significantly decreased in BALF of infected mCLCA3-deficient mice. (A, B) Protein levels of cytokines were measured at 12 hours and 24 hours post infection by multiplex assay technique (IL-1β, IL-6, IL-10, IL-12p40, IL-13, CXCL-1, MCP-1, RANTES, TNF-α, GM-CSF) or by ELISA (IL-17) in BALF. (C) Expression levels of Cxcl-1, Cxcl-2 and Il-17 were quantified by RTq-PCR in the lungs. Dotted line indicates a fold change of 2 as limit for valid statement of elevated parameters. Values are given as mean ± SEM (n = 6 to 8 each group). #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$, ####$p < 0.0001$ versus PBS control group. *$p < 0.05$ as indicated.
doi:10.1371/journal.pone.0102606.g002

Lung Morphometry for Volume Estimation by the Cavalieri Principle

Total volume of left lungs from infected mice and corresponding lesions were assessed by Cavalieri principle [34,35]. Left lungs of *S. aureus* infected mice (24 hours post infection, n = 4) were cut into 140 sections of equal thickness (10 μm). Each seventh section was stained with hematoxylin and eosin for subsequent determination of total cut face areas (A_{CF}) as well as total areas of lung lesions (A_L) using digital image analysis software (AnalySIS docu 5.0., SIS). An estimated volume of the left lungs and of left lung lesions was calculated by multiplying the slab thickness ($T = 10 \ \mu m \times 7$) with the sum of the cut face areas (A_{CF}) or lesion areas (A_L) of each particular section ($V = T \times \sum A_{CF}$; $V = T \times \sum A_L$) [34–36]. Finally, the percentages of lesions were calculated.

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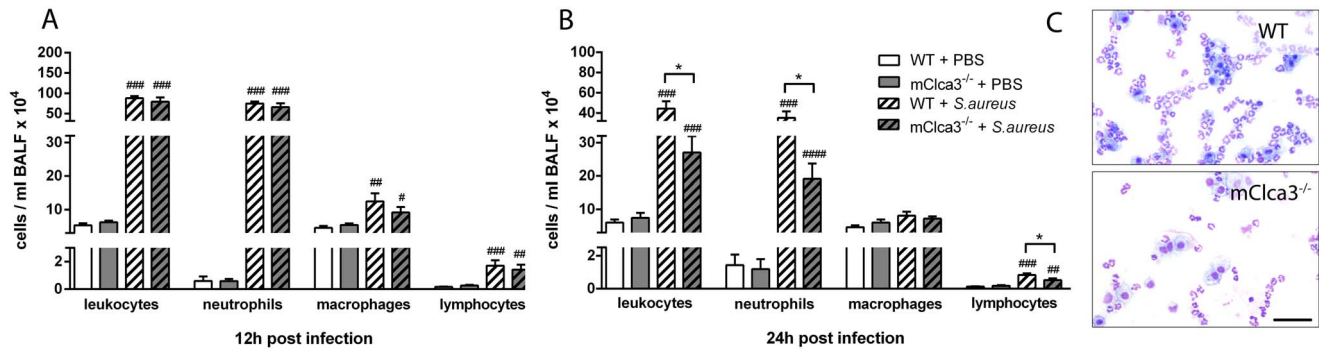
Figure 3. Pulmonary neutrophils and lymphocytes were significantly decreased in mCLCA3-deficient mice 24 hours after infection. (A, B) At 12 hours and 24 hours after infection, leukocytes in bronchoalveolar lavage fluid (BALF) were manually counted and subpopulations were analyzed by fluorescence-activated cell sorter (FACS). Values are given as mean ± SEM (n = 8 each group). #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 versus PBS controls. *p<0.05 as indicated. (C) Cytospins from BALF 24 hours post infection were obtained by centrifugation and BALF cell suspension was subsequently stained with May-Grünwald Giemsa. Leukocyte numbers were reduced in BALF from mClca3−/− mice (n = 8 each group). *Bar*, 50 µm.
doi:10.1371/journal.pone.0102606.g003

Data Analysis

Data are expressed as mean ± SEM. Statistical analyses were performed using the Mann-Whitney test. P<0.05 was considered significant.

Results

mCLCA3 deficiency had no impact on clinical outcome of *Staphylococcus aureus* pneumonia

After infection of mCLCA3-deficient and wild-type mice with *S. aureus* Newman, both genotypes equally developed acute pneumonia clinically hallmarked by significant loss of body weight (Figure 1A) and temperature (Figure 1B) compared to PBS controls. Infected animals showed similarly constant bacterial loads after 6 and 24 hours which were restricted to the lungs (Figure 1C). Neither bacteremia nor bacterial spread into the liver were present at any time investigated confirming a non-bacteremic *S. aureus* pneumonia.

mCLCA3 modulated a selective pulmonary inflammatory cytokine release in acute staphylococcal pneumonia

Inflammatory cytokines were quantified on protein level in BALF (IL-1β, IL-6, IL-10, IL-12p40, IL-13, IL-17, CXCL-1, MCP-1, RANTES, TNF-α, GM-CSF) or on mRNA level in lung homogenates (Cxcl-1, Cxcl-2, Il-17) of *S. aureus* infected mice and PBS controls. In infected animals, all cytokine protein levels analyzed, except for IL-10, were increased 12 hours after infection as compared to uninfected mice, independently of genotype (Figure 2A). In contrast, after 24 hours, infected mCLCA3-deficient mice showed a significant decrease of the cytokines CXCL-1 and IL-17 compared to infected wild-type mice. Furthermore, levels of IL-1β, IL-6, IL-13, CXCL-1, TNF-α and GM-CSF slightly declined, whereas levels of IL-12p40, MCP-1 and RANTES increased over time in infected mice (Figure 2B). RTq-PCR analysis of lung homogenates after 12 hours showed a 2.17 fold decrease of Cxcl-1-mRNA and a significant 3.4 fold decrease of Il-17-mRNA in infected mCLCA3-deficient mice compared to infected wild-type mice. Additionally, Cxcl-2-mRNA was decreased by a factor of 2.04 in infected mCLCA3-deficient
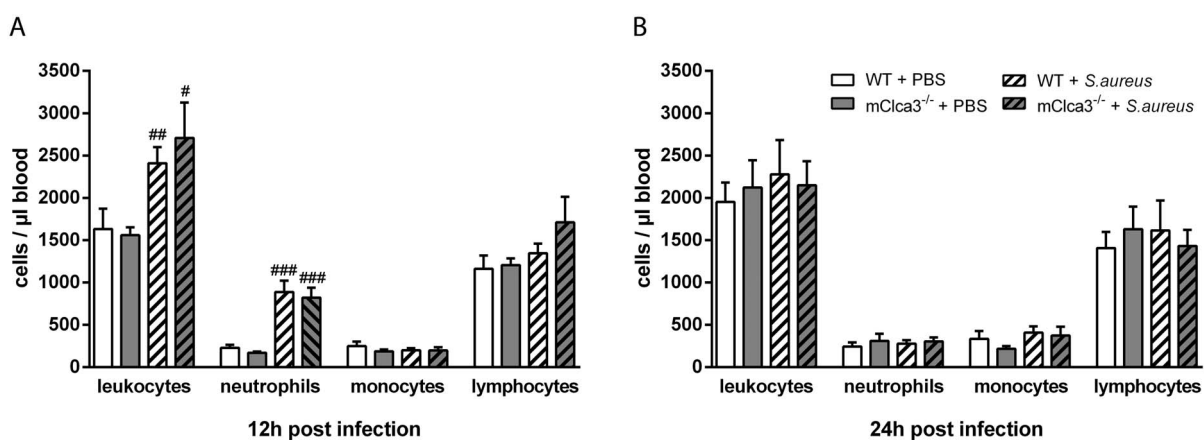
Figure 4. Early infection with *S. aureus* led to a comparable increase in systemic leukocyte response. (A, B) At indicated time points after infection, blood leukocyte numbers and subpopulations were determined by fluorescence-activated cell sorter (FACS). Values are given as mean ± SEM (n = 8 each group). #p<0.05, ##p<0.01, ###p<0.001 versus the PBS-treated control group.
doi:10.1371/journal.pone.0102606.g004
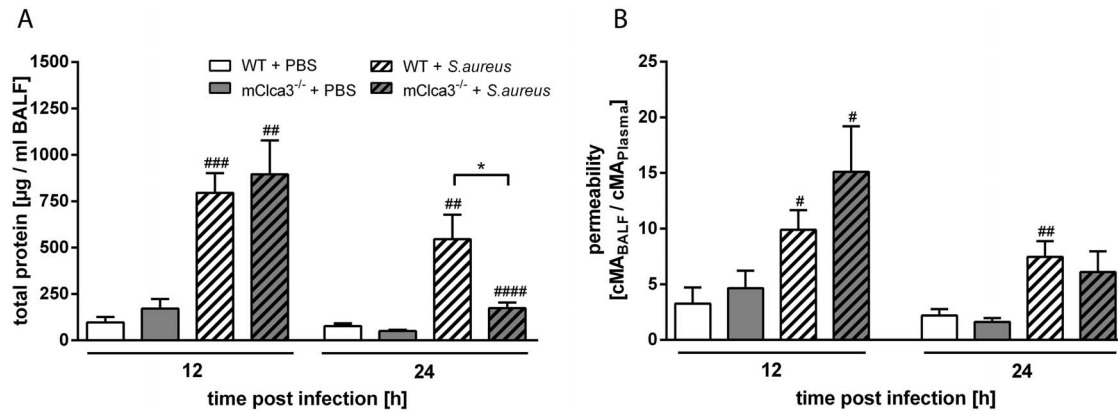
Figure 5. mCLCA3-deficiency led to reduced protein quantities in BALF during infection without altered pulmonary vascular permeability. (A) Total protein of BALF was examined at indicated time points by BCA-assay. (B) Mouse albumin (MA) of transnasally infected or uninfected mice was measured by ELISA in BALF and plasma. Pulmonary vascular permeability was calculated from the MA BALF/plasma ratio. Values are given as mean ± SEM (n = 8 each group). #p<0.05, ##p<0.01 versus the PBS control group. *p<0.05 as indicated.
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mice compared to infected wild-type mice. After 24 hours, Il-17 mRNA levels remained significantly decreased in infected mCLCA3-deficient mice compared to infected wild-type mice whereas the mRNA expression of Cxcl-1 and Cxcl-2 of the infected groups showed similar levels. Moreover, at indicated time points, mRNA levels of all parameters investigated were significantly elevated compared to PBS controls (Figure 2C).

Leukocyte recruitment into *Staphylococcus aureus*-infected lungs was dependent on mCLCA3

At indicated time points, leukocyte numbers and subsets were examined in BALF by fluorescent-activated cell sorter (FACS) analysis. Total leukocyte numbers increased significantly within 12 hours in infected animals independently of genotype compared to PBS controls (Figure 3A). Predominantly neutrophils, as well as macrophages and lymphocytes to a lesser extent, contributed to the observed leukocyte influx into the bronchoalveolar space without any genotype difference in numbers. In contrast, 24 hours after *S. aureus* infection, mCLCA3-deficient mice showed significantly decreased numbers of total leukocytes, neutrophils and lymphocytes compared to infected wild-type mice while numbers of macrophages remained equal. In uninfected

mCLCA3-deficient mice, leukocyte numbers were comparable to those of uninfected wild-type mice (Figure 3B). Cytospin analysis from BALF cell suspensions of infected mice confirmed the results (Figure 3C). However, only 12 hours after *S. aureus* infection systemic leukocyte response in the blood was altered. Numbers of leukocytes and specifically neutrophils were significantly increased in infected mice compared to PBS controls, however, independently of genotype (Figure 4).

Infected mCLCA3-deficient mice had reduced protein amounts in BALF without genotype specific changes in pulmonary vascular permeability

Total protein amount in BALF was determined with the BCA-method. Infected mice had increased quantities of total protein 12 hours after infection with *S. aureus* compared to PBS controls without genotype differences. In contrast, after 24 hours, infected mCLCA3-deficient mice showed a significant 3-fold reduction of BALF proteins compared to infected wild-type mice while PBS controls did not show genotype-specific differences (Figure 5A). As an indicator for the loss of vascular integrity, mouse albumin (MA) concentrations were quantified in BALF and plasma. Pulmonary
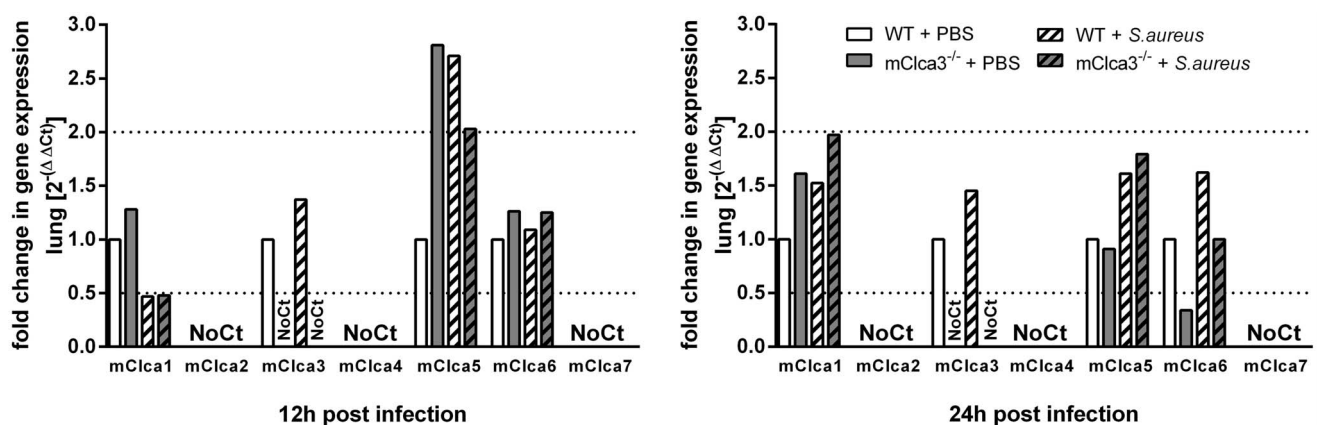
Figure 6. mCLCA3-deficiency in *S. aureus* infection was not compensated by regulation of other CLCA members. Lung expression mRNA levels of mClca1 to 7 were quantified by RTq-PCR. Dotted lines indicate a fold change of 0.5 and 2, respectively, as limits for valid statement of lowered and elevated parameters. Values are given as mean ± SEM (n = 8 each group). Ct, cycle treshold.
doi:10.1371/journal.pone.0102606.g006

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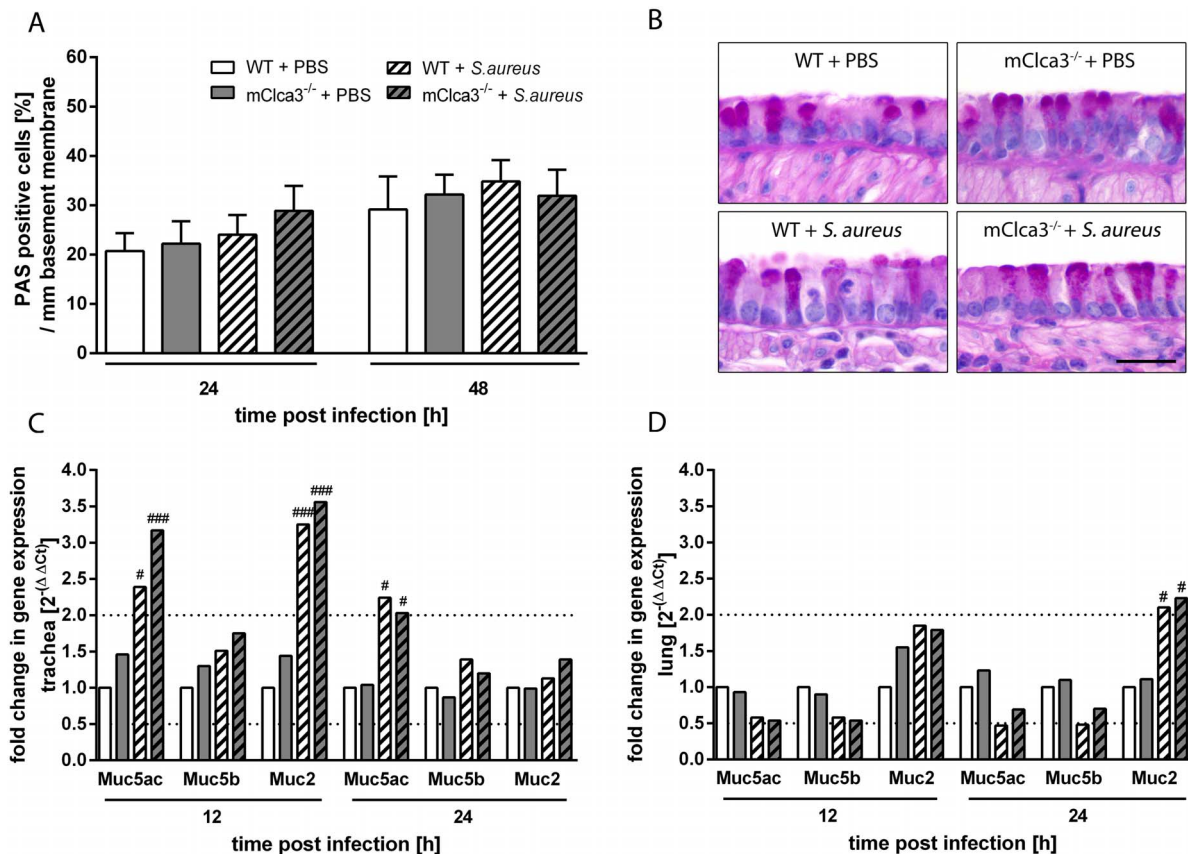
Figure 7. mCLCA3-deficiency had no influence on mucus cell number and mucin expression in infected mice. (A) Mucus cells were quantified by calculating the percentage of periodic acid Schiff (PAS)-positive cells per mm basement membrane. (B) No differences in number or distribution of mucus cells were observed in *S. aureus* infected or uninfected mice. (C, D) Expression levels of Muc5ac, Muc5b and Muc2 were quantified at indicated time points in trachea and lung by RTq-PCR. Dotted lines indicate fold change of 0.5 and 2, respectively, as limit for valid statement of lowered and elevated parameters. Values are given as mean $\pm$ SEM (n = 4 each group (A, B), n = 8 each group (C, D)). [#]p<0.05, [##]p<0.01, [###]p<0.001, versus the PBS control group. *Bar* (B), 20 μm.
doi:10.1371/journal.pone.0102606.g007

vascular permeability, assessed by calculating the MA BALF/plasma ratio, was significantly increased in *S. aureus* infected mice compared to PBS controls independently of genotype (Figure 5B).

Staphylococcus aureus infection did not alter mCLCA3 protein and mRNA expression or induce differential regulation of other CLCA family members in mCLCA3-deficient mice

Immunoblotting of BALF using mCLCA3-specific antibodies as well as RTq-PCR analysis of mClca3-mRNA in lungs (Figure 6) failed to reveal differences on protein or mRNA levels in infected wild-type mice compared to PBS controls at all time points investigated. Furthermore, no mCLCA3 mRNA (Figure 6) or protein was detected in mCLCA3-deficient mice confirming the knockout status. Additionally, mRNA levels of mClca1 to 7 were measured in lungs of *S. aureus* infected mice and PBS controls by RTq-PCR. mClca1, mClca3, mClca5, and mClca6 were expressed in the lungs, whereas mClca2, mClca4 and mClca7 were not detected. After 12 hours, exclusively mClca5 was increased during infection independently of genotype, therefore no significant changes in expression levels of putative compensatory Clca homologs were observed in the lung (Figure 6).

Mucus cell number and mucin expression were independent of mCLCA3 during *Staphylococcus aureus* infection

Quantification of PAS-positive mucus cells per mm basement membrane revealed no differences between infected or uninfected mice independently of genotype at investigated time points (Figure 7A, 7B). Tracheal mRNA levels of Muc5ac and Muc2 were significantly increased 12 hours after infection in mCLCA3-deficient mice and wild-type mice compared to PBS controls, whereas 24 hours after infection only Muc5ac was slightly elevated in the trachea of infected animals (Figure 7C). In contrast, only Muc2 was significantly up-regulated on mRNA level in the lungs of *S. aureus* infected mice, whereas gene expression levels of the other mucin genes tested were not differentially regulated in lung tissue (Figure 7D).

mCLCA3-deficiency had no effect on histological and morphometric parameters in acute staphylococcal pneumonia

Course of pneumonia, lung damage and lesion distribution were further analyzed by pathological investigation. Macroscopical examination revealed first lesions in infected mice independently of genotype as early as 6 hours after infection with progressive exacerbation over time. The upper third of the left lung was

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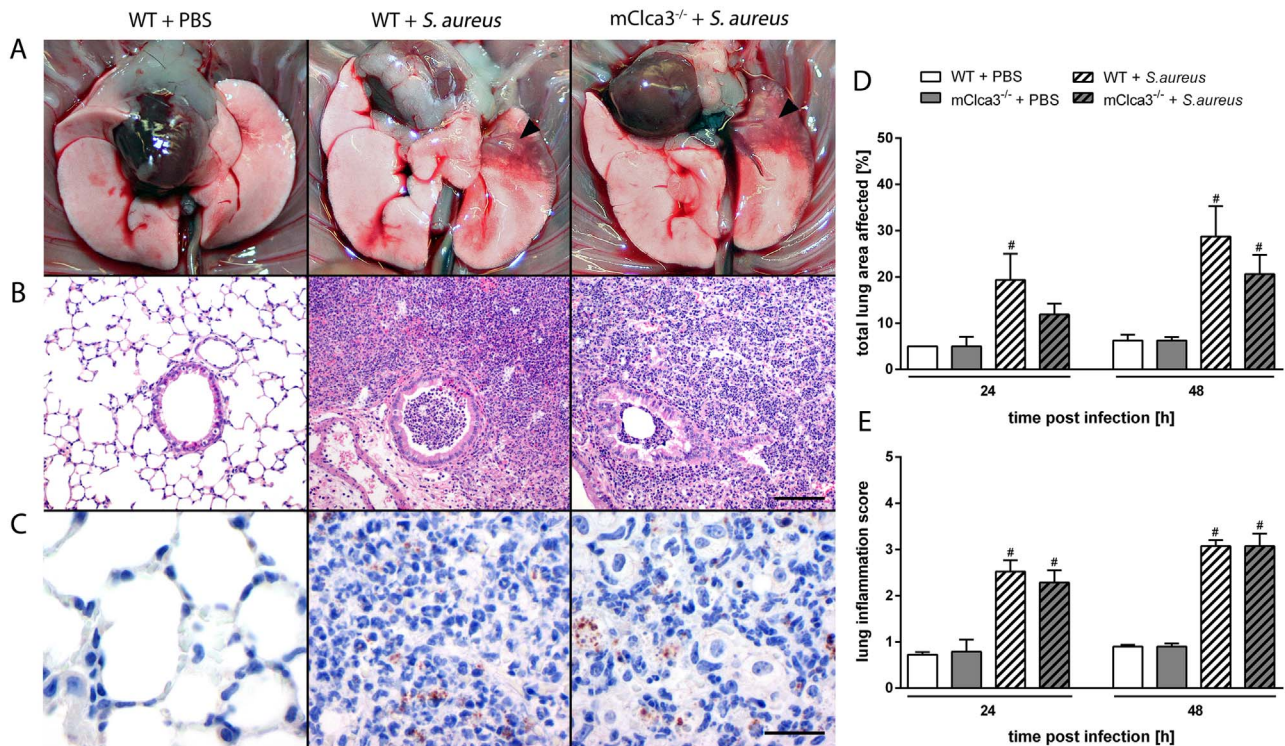
Figure 8. No genotype differences in lung inflammation, lesion distribution or bacterial loads were observed during infection. Mice were transnasally infected with 5×10^7 *Staphylococcus aureus* Newman and killed at indicated time points. (A) Macroscopic examination revealed deeply red consolidated areas in the infected lungs (arrowhead) in contrast to PBS controls which behaved virtually identical. (B) Subsequently, lungs were fixed, embedded in paraffin and stained with hematoxylin and eosin for histopathological analyses. (C) Evidence of bacteria was assessed by immunohistochemistry with anti-*Staphylococcus aureus* antibody. Uninfected animals (left panel) served as negative controls. *Brown*, 3,3′-diaminobenzidine; *blue*, hematoxylin counterstain. (D) The total lung area affected by inflammation and (E) a lung inflammation score were determined. Values are given as mean ± SEM (n = 4 each group). $^{\#}p < 0.05$ versus the PBS control group. *Bar* (B), 100 μm. *Bar* (C), 20 μm.
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predominantly affected. The altered lung tissue was colored deeply red and, in few cases, visible accumulations of suppurative exsudate were present within the pneumonic areas (Figure 8A). Histologically, infected lungs from both genotypes showed a moderate to severe, acute, multifocal, necro-suppurative bronchopneumonia with prominent perivascular edema, multifocal hem-

orrhage and massive accumulation of neutrophils and macrophages in the consolidated areas (Figure 8B). *S. aureus* was detected by immunohistochemistry using a specific anti-*S. aureus* antibody exclusively in infected animals mostly within macrophages and neutrophils at each investigated time point (Figure 8C). For semiquantification of histologic lesions, total affected lung
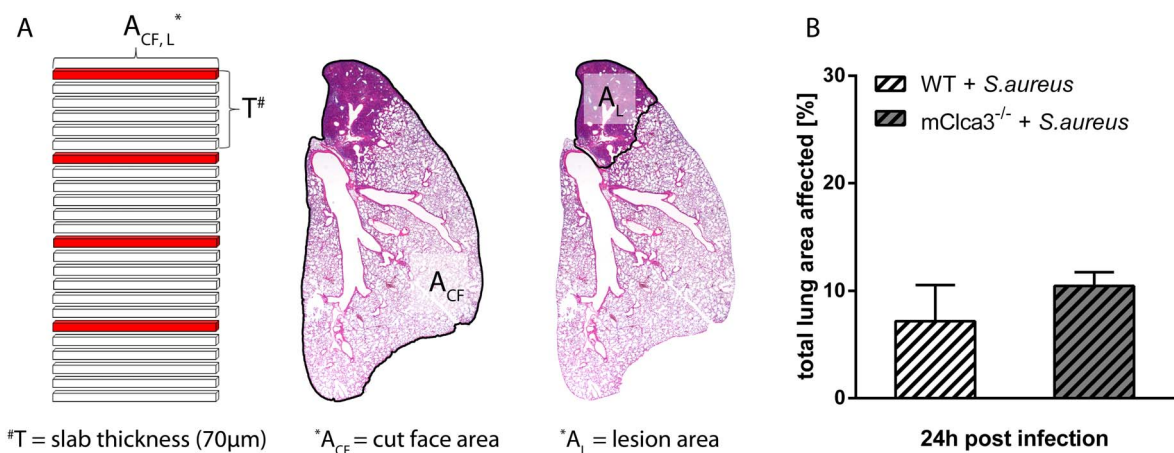
Figure 9. mCLCA3 had no impact on severity or expansion of lung inflammation. (A) 24 hours after infection, left lungs of *Staphylococcus aureus* infected mice were used for quantification of lung lesions by Cavalieri principle. (B) Percentages of estimated lung lesion volumes and total lung volumes of the left lungs were calculated. Values are given as mean ± SEM (n = 4 each group).
doi:10.1371/journal.pone.0102606.g009

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areas were determined (Figure 8D). Additionally, for evaluation of severity, several parameters were defined (Table 1) and a lung inflammation score was assessed (Figure 8E). No differences between genotypes, neither in severity nor in expansion of lung lesions were observed at indicated time points in *S. aureus* infected mice. Only mild infiltration by macrophages close to the hilum of the lungs was observed in uninfected controls independently of genotype, likely due to the application of PBS. To examine the lung as a 3-dimensional structure and to warrant complete measurement of lung lesions, volume was estimated by Cavalieri principle in *S. aureus* infected lungs. Whole left lungs were cut into consecutive sections of equal thickness and each seventh slide was analyzed at indicated time points (Figure 9A). No genotype differences, neither in volume of examined lungs nor in percentages of lesions were observed (Figure 9B).

Discussion

Several previous studies have analyzed the role of hCLCA1 and its murine ortholog mCLCA3 which were overexpressed in complex inflammatory airway diseases, primarily focusing on mucus cell regulation and induction of mucus cell metaplasia [9,13,14,17,37–39]. More recently, it has been reported that both proteins may have an impact on airway inflammation and regulation of innate immune responses [13–16].

In the present study, we describe an inflammatory phenotype of mCLCA3-deficient mice in an acute bacterial lung infection model with *Staphylococcus aureus* Newman. mCLCA3-deficiency was associated with decreased protein-levels of CXCL-1 and IL-17 in the BALF compared to wild-type mice 24 hours post infection. CXCL-1 is a murine homolog to human CXCL-8 and a potent chemoattractant for neutrophils [40,41] and IL-17 mediates proinflammatory responses primarily by inducing the expression of other cytokines and chemokines, including CXCL-8 [42]. In contrast, all other cytokines tested (IL-1β, IL-6, IL-10, IL-12p40, IL-13, MCP-1, RANTES, TNF-α and GM-CSF) were significantly increased after infection, independently of mCLCA3-deficiency, pointing towards selective modulation of a specific subset of cytokines in mCLCA3-deficient mice after bacterial challenge. Consistent with our protein data, markedly decreased mRNA-levels of Cxcl-1 and Il-17 were observed as early as 12 hours post infection. Similarly to Cxcl-1, Cxcl-2, another murine CXCL-8 homolog [43], showed a similar transcriptional regulation in challenged mCLCA3-deficient mice. The reduced induction of these cytokines on the mRNA and protein levels points towards aberrant transcriptional regulation in mCLCA3-deficient mice after *S. aureus* challenge. As a consequence of the low CXCL-1 levels, neutrophilic recruitment to the site of infection was decreased after 24 hours. However, as an alternative explanation for reduced leukocyte numbers in bronchoalveolar spaces, capillary-alveolar transmigration may also have been reduced. Although we failed to observe differences in plasma protein extravasation, this additional effect cannot be fully excluded. Besides neutrophils, lymphocytes were also significantly reduced in the BALF of mCLCA3 deficient mice 24 hours after infection. The cytokines CXCL-1 and IL-17 found dysregulated in this study are neither known to be chemoattractants for lymphocytes nor inducers of lymphocyte proliferation. It is more likely that a yet unidentified factor influencing lymphocyte recruitment and/or proliferation may also be modulated after *S. aureus* challenge in the context of mCLCA3 deficiency.

We hypothesized that attenuation of coordinated leukocyte recruitment in mCLCA3-deficient mice was accompanied by reduced lung barrier destruction. However, the albumin BALF/

plasma ratio, quantified for determination of pulmonary endo-epithelial barrier failure, was not reduced in mCLCA3-deficient mice. It thus appears likely that reduction of proteins from viable and degenerate leukocytes due to decreased bronchoalveolar leukocyte influx was the main contributor to the diminished total protein content in BALF of mCLCA3-deficient mice.

The predominant cell types in the lung expressing the two CXCL-8 homologs CXCL-1 and CXCL-2 during bacterial infections are alveolar macrophages and pulmonary epithelial cells [41,44]. Although subsets of T-lymphocytes are the primary source of IL-17, it was previously demonstrated that alveolar macrophages are also competent of secreting IL-17 [45,46]. The altered transcriptional regulation of the CXCL-8-homologs and IL-17 identified in this study is in line with the recently hypothesized additional role of CLCA-proteins as signaling molecules in inflammation [10,15]. Specifically, these studies demonstrated that hCLCA1, the human ortholog of mCLCA3 with virtually identical structure and cellular expression pattern, acts as a signaling molecule that induces mucus gene transcription via a downstream MAPK-13 signaling pathway [10]. Furthermore, hCLCA1 activates alveolar macrophages resulting in pro-inflammatory cytokine induction, including CXCL-8 [15]. It thus appears reasonable to assume that mCLCA3 may have a similar effect on mouse alveolar macrophages after bacterial challenge with *S. aureus* via murine CXCL-8 homologs and IL-17. However, we cannot exclude that the reduced murine CXCL-8 homolog expression in mCLCA3-deficient mice is a downstream event due to the aberrant induction of IL-17, which is a known inducer of CXCL-8 [42,47] instead of being directly regulated by mCLCA3.

A link between IL-17 and mCLCA3 has recently been suggested. In that study, neutralization of IL-17 in a murine viral lung infection model decreased mRNA-levels of mClca3 and also decreased Cxcl-1-mRNA levels with reduced infiltration of neutrophils into the bronchoalveolar space [48]. Further studies should elucidate the relation between IL-17 and mCLCA3, especially the effect of mCLCA3 on IL-17-expressing cell types.

In contrast to our study, a previous study revealed that mCLCA3-deficient mice challenged with ovalbumin or LPS had increased numbers of neutrophils and that LPS-treated mice additionally showed enhanced CXCL-1 protein levels without alterations in CXCL-2 and IL-17 protein levels [13]. This seemingly contradictory result may be due to the different inducers of inflammation (LPS vs. bacterial infection) and/or different mouse strains used (129SvEvBrd vs. C57BL/6J). Moreover, LPS is an endotoxin from Gram-negative bacteria whereas in this study we used a Gram-positive bacterial species which could also account for the seemingly contradictory results.

The acute *S. aureus* pneumonia in mice is an appropriate model for studying innate immune responses [18,49–51]. However, based on the well established role of CLCA-proteins in Th2 cytokine driven inflammatory airway diseases with increased mucus production and mucus cell metaplasia [9,13,14,17,37–39], we also analyzed the effect of mCLCA3 on mucus cells and mucin gene expression in this acute *S. aureus* pneumonia. No differences in the extent of mucus cell metaplasia or mucin gene induction were observed after infection with *S. aureus* between mCLCA3-deficient and wild-type mice. The increase of Muc5ac mRNA levels without significant changes in Muc5b gene expression in infected mice compared to PBS controls are consistent with previous reports [9,10,17]. In addition, Muc2 mRNA levels were found to be increased in *Staphylococcus aureus* infected animals compared to PBS controls independent of genotype corresponding to previously reported results of lipoteichoic acid (LTA) from gram

positive bacteria strongly inducing Muc2 expression [52]. The induction of mucus production under bacterial challenges is thus a known innate immune response irrespective of the mCLCA3 status.

Interestingly, mCLCA3 independent mucin regulation in airway diseases shows a discrepancy between mice and humans [10]. It was previously hypothesized that upregulation of other members of the murine CLCA family may possibly compensate for the lack of mCLCA3 [9,10,17]. In addition to mCLCA3, the murine CLCA5, -6, and -7 are potential candidates to drive mucus cell metaplasia in mice [11]. Therefore, we analyzed the lungs regarding a possible compensatory differential regulation of other murine CLCA homologs. mClca1, mClca3, mClca5, and mClca6 were expressed, whereas mClca2, mClca4 and mClca7 were not detected. Of these, only mClca5 was increased during infection, however, independently of genotype. Thus, no differentially regulated and putatively compensatory CLCA member with regard to mucus cell regulation was found, even under challenged conditions which is in line with previous reports [17,53].

In contrast to the observed reduction in neutrophil infiltration, our pathologic examination failed to reveal differences in lung inflammation or lung lesion expansion between genotypes despite using up-to-date morphometric methods [34]. Infected animals developed an acute, marked, suppurative and necrotizing bronchopneumonia with consolidation and destruction of inflamed areas. Due to this tissue destruction, an exact separation between lung parenchyma and alveolar spaces was impossible and histological quantification of cell types in the two separate compartments that could have confirmed the results of BALF analyses was impossible. Furthermore, no differences in clinical course (physical constitution, behavior, body temperature, changes in body weight) or lung bacterial loads were observed following infection, indicating that the effect of mCLCA3 on the molecular and subsequent cellular responses had no obvious impact on clinical and pathological outcome at the times investigated. Obviously, additional determinants other than leukocyte number decide on the overall severity of pneumonia and clinical outcome.

In conclusion, our data suggest that mCLCA3 modulates the cellular leukocyte recruitment via IL-17 and CXCL-1 in acute *S. aureus* pneumonia. Lack of mCLCA3 led to a selectively decreased induction of IL-17 and CXCL-8 homologs and decreased numbers of neutrophils and total protein in BALF in *S. aureus* infected mice. During bacterial infection, no differences were observed in mucin regulation and no other mCLCA family members were differentially regulated. Thus, mCLCA3 seems to have an impact on the early innate immune response via direct or indirect induction of select cytokines during *S. aureus* infection.

Further studies should characterize the specific cytokine pathways and the main target cells activated by mCLCA3 under physiological condition and infection. Prospectively, mCLCA3 may even become a potential therapeutic target for the modulation of inflammation in lung infections.

Supporting Information

Table S1 Quantitative Real Time RT-PCR: Sequences and Specifications.
(DOCX)

Acknowledgments

Author Contributions

Conceived and designed the experiments: KD KR MW LM ADG. Performed the experiments: KD KR. Analyzed the data: KD KR LM. Contributed reagents/materials/analysis tools: KD KR MW ADG. Wrote the paper: KD LM KR MW ADG.

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SUPPORTING INFORMATION

Table S1

Quantitative Real Time RT-PCR: Sequences and Specifications

Gene	Genbank Accession no.	Oligonucleotide	Sequences (5'-3')	Amplicon Size, bp	Annealing Temp., °C
mClca1	NM_009899	Primer (upstream)	GTGGACCAGCCTTTCTACATGTCTAG	114	60
		Primer (downstream)	TGTGACACAGTTGCCTCTCTCA		
		TaqMan Probe	FAM-ATCACTGGCACCAATGTGGTTCACAAC-BHQ		
mClca2	NM_030601	Primer (upstream)	GGACCGGCCTTTCTACATTTCTAG	109	60
		Primer (downstream)	CACAGGCAAGAAGGTGGTCCACGA		
		TaqMan Probe	FAM-CACACAGCTGCCTCTCTGACA-BHQ		
mClca3	NM_017474	Primer (upstream)	GAAATTCCCCAGCCCTGTAACAG	144	60
		Primer (downstream)	CAAGCATTCGCCAAGGAGCCTCGCC		
		TaqMan Probe	FAM-TGCGCACCTGCTCCGTTATC-BHQ		
mClca4	NM_139148	Primer (upstream)	ACTAACCTAATAAGGATCATCAATGA	81	60
		Primer (downstream)	CTCCTACCTAGCGATCAGCACAAAGC		
		TaqMan Probe	FAM-AGTTCCGCCATTTGGGTATT-BHQ		
mClca5	NM_178697	Primer (upstream)	ACGATGACCGGAAGCTGCTG	100	60
		Primer (downstream)	CCTGCCGACCGCCGTGTCCAC		
		TaqMan Probe	FAM-ACCACCTCAAAGCCTTTCTTAACC-BHQ		
mClca6	NM_207208	Primer (upstream)	CATAATAAAAAGTGCAATTACAG	91	60
		Primer (downstream)	CTTGGGAAGTAATCAGCACTTCTG		
		TaqMan Probe	FAM-AGGGTGACGTCTCCAT-BHQ		
mClca7	NM_001033199.3	Primer (upstream)	ATGGAGACACCACCGG	114	60
		Primer (downstream)	CTTCTTCTCCCTGCTGAGGATCAG		
		TaqMan Probe	FAM-TCTATTCAGACGGTCAGAAGAAC-BHQ		
Muc5ac	NM_010844.1	Primer (upstream)	GCGTGGAGAATGAAAAGTATGCT	120	60
		Primer (downstream)	CCCAGTGCCACGCCACCGTG		
		TaqMan Probe	FAM-CATACATGCAGTTCGAGAAGAAG-BHQ		
Muc5b	NM_028801	Primer (upstream)	GCACGTAAATGCGACTGTCT	154	60
		Primer (downstream)	TATCCAAGTACTCCATGGAGGCCC		
		TaqMan Probe	FAM-ATGGACCTTGCTCTCCTGAC-BHQ		
Muc2	NM_023566	Primer (upstream)	GAGGCAGTACAAGAACCGGA	104	60
		Primer (downstream)	TTCGGCTCGGTGTTCAGAG		
		TaqMan Probe	FAM-CCATTGAGTTTGGGAACATGC-BHQ		

Cxcl1	NM_008176.3	Primer (upstream) GATGCTAAAAGGTGTCCCCA	83	60
		Primer (downstream)AGACTGCTCTGATGGCACCT		
		TaqMan Probe FAM-GTCAGAAGCCAGCGTTCAC-BHQ		
Cxcl2	NM_009140.2	Primer (upstream) CTGAACAAAGGCAAGGCTAACT	73	60
		Primer (downstream)ACCTGGAAAGGAGGAGCCT		
		TaqMan Probe FAM-CTTTGGTTCTTCCGTTGAGG-BHQ		
Il-17A	NM_010552	Primer (upstream) GCTCCAGAAGGCCCTCAGA	142	60
		Primer (downstream)CTCTCCACCGCAATGAAGACCCTGA		
		TaqMan Probe FAM-AGCTTTCCCTCCGCATTGA-BHQ		
Ef1a	NM_010106.2	Primer (upstream) AAAAACGACCCACCAATGG	67	60
		Primer (downstream)GGCCTGGATGGTTCAGGATA		
		TaqMan Probe FAM-AGCAGCTGGCTTCACTGCTCAGGTG-BHQ		
B2m	NM_009735.3	Primer (upstream) ATTCACCCCCACTGAGACTGA	86	60
		Primer (downstream)CTCGATCCCAGTAGACGGTC		
		TaqMan Probe FAM-TGCAGAGTTAAGCATGCCAGTATGGCCG-BHQ		
Gapdh	NM_008084.2	Primer (upstream) TCACCACCATGGAGAAGG	169	60
		Primer (downstream)GCTAAGCAGTTGGTGGTGCA		
		TaqMan Probe FAM-ATGCCCCCATGTTTGTGATGGGTGT-BHQ		

3 Concluding Discussion

For almost two decades, CLCA proteins have been in the focus of several research groups worldwide. During this time, a paradigm shift has emerged regarding their protein structures and functions. Although these proteins have been linked to important human and animal respiratory diseases with secretory dysfunctions from early on, their roles in normal tissues and their specific contributions to certain pathomechanisms in these diseases are still elusive.

Initially, it was suggested that CLCA proteins are integral membrane proteins anchored to the cell membrane with several transmembrane domains, likely representing chloride channels on their own (Cunningham et al. 1995; Gruber et al. 1998). However, in recent years, several studies have revealed that the CLCA protein family can be classified into two groups according to their protein structure and processing after the cleavage of a precursor protein into two subunits which is a common event of the CLCA family members. For some proteins, including hCLCA1 and mCLCA3, it was shown that both subunits are fully secreted as heterodimers (Gibson et al. 2005; Mundhenk et al. 2006), whereas other members such as hCLCA2 and mCLCA5 are anchored to the cell membrane via one carboxy-terminal transmembrane domain and only the amino-terminal cleavage product is shed by the cell (Braun et al. 2010a; Elble et al. 2006). These biochemical studies clearly showed that CLCA proteins are no integral membrane proteins with multiple transmembrane domains and so cannot form ion-channels on their own (Elble et al. 2006; Gibson et al. 2005; Mundhenk et al. 2006). Instead, it was shown that the secreted hCLCA1 protein may act as an extracellular signaling molecule modulating chloride conductance by interacting with to date unidentified chloride channels in an as yet unknown fashion (Gibson et al. 2005).

Over the years, more and more family members in different species have been identified and various putative functions have been proposed, including adhesion molecule function (Abdel-Ghany et al. 2003), bicarbonate channel activity (Thevenod et al. 2003), modulation of other channel proteins (Greenwood et al. 2002; Loewen et al. 2002), inhibition of tumor growth by promoting apoptosis (Elbe and Pauli 2001), and protease activity (Pawlowski et al. 2006). However, the mechanisms of how these functions may be conveyed were also still elusive.

Recently, a role of human and murine CLCA members in differentiation and modulation of mucus cells in the respiratory tract have become the main focus of CLCA research (Long et al. 2006; Mundhenk et al. 2012; Nakanishi et al. 2001; Patel et al. 2009, Patel et al. 2006; Robichaud et al. 2005; Thai et al. 2005; Zhang and He 2010; Zhou et al. 2002). It has re-

peatedly been shown that the human hCLCA1 acts as an extracellular signaling molecule inducing mucus gene transcription and thereby regulating and driving mucus cell metaplasia (Alevy et al. 2012). This was of tremendous importance for the currently far improved understanding of the pathophysiological role of at least some CLCA proteins in inflammatory airway diseases with secretory dysfunctions.

Mouse models have been the most commonly used animal models in CLCA research. Specifically, many similar observations, as observed for hCLCA1 regarding airway mucus regulation, have been made for its murine ortholog, mCLCA3, in mouse models of airway diseases (Long et al. 2006, Mundhenk et al. 2012; Nakanishi et al. 2001; Patel et al. 2006; Zhang and He 2010). However, species-specific differences among members of the CLCA family are among of the major challenges and barriers in translational CLCA research until today. Unique genetic duplications and intraspecies evolution of CLCA homologs have occurred in each species studied to date and several significant species-specific differences have been observed in genomic structures, tissue expression patterns and probably functions of direct CLCA orthologs. For example, the human family consists of only four members whereas there are eight members in mice and five members in pigs known to date (Patel et al. 2009; Plog et al. 2009, Plog et al. 2012a; Plog et al. 2012b). Among the consequences of this complexity is a still preliminary and somewhat confusing nomenclature and seemingly inconsistent numbering of direct CLCA orthologs between different species. For example, the first discovered human CLCA family member, hCLCA1, is the direct ortholog to the murine gob-5 which has been renamed to mCLCA3 because it was the third CLCA member to be discovered in the mouse. For a systematic explanation of the current nomenclature, see Table 1 on page 2 in the introduction to this thesis.

As a second consequence of the apparent evolutionary complexity and diversity of CLCA molecules, the translation of experimental results from one species to another should be practiced with caution which also became important for this study. While loss-of-function studies with *mClca3$^{-/-}$* mice had failed to show a corresponding phenotype regarding mucus cell metaplasia, a possible compensatory or overlapping function of other murine CLCA members had been suspected for the species mouse (Patel et al. 2006). It had been previously shown that mCLCA5, a homolog of mCLCA3 in the mouse, was also capable of inducing mucus production and mucus cell metaplasia in the respiratory tract in contrast to its human ortholog hCLCA2. Thus, this protein became a potential candidate for possibly compensating for the loss of mCLCA3 in the respective knockout-mice (Patel et al. 2006). Therefore, the murine homologs mCLCA3 and mCLCA5 appeared among the most interesting members in murine models of respiratory diseases regarding mucus overproduction.

CONCLUDING DISCUSSION

CLCA homologs had a cell-type specific expression pattern in each species studied so far. It thus seems that these proteins occupy different structural and functional niches in particular organs. For example, the intestinal expression patterns of the murine CLCA members are well known. mCLCA1 and mCLCA2 proteins are only expressed in basal crypt epithelial cells (Gruber et al. 1998; Leverkoehne et al. 2006) while mCLCA3 is exclusively expressed in goblet cells (Leverkoehne and Gruber 2002; Romio et al. 1999). Moreover, mCLCA6 can only be found on the apical surface of non-goblet cell enterocytes (Bothe et al. 2008) and mCLCA4 was proposed to be expressed by smooth muscle cells (Elble et al. 2002), possibly pointing towards a cell-type specific function. In contrast, the knowledge on the expression pattern of murine CLCA proteins in the respiratory tract was largely incomplete prior to this study. Although it has been well established that mCLCA3 is exclusively expressed in airway mucus cells (AMC; Leverkoehne and Gruber 2002, Romio et al. 1999), the expressing cell type of mCLCA5 or other CLCA homologs in this organ system was unknown.

In addition to its proposed role in mucin gene regulation, it was recently demonstrated that hCLCA1 also induces pro-inflammatory cytokine release by activating alveolar macrophages *in vitro* (Ching et al. 2013). This has now turned out to be a further important aspect for understanding the complex role of CLCA proteins in inflammatory airway diseases. Similar observations have been made for its murine ortholog mCLCA3 in various mouse models. Here, several studies have implicated that mCLCA3 possibly regulates tissue inflammation in the innate immune response (Long et al. 2006, Zhang and He 2010). However, the underlying pathomechanisms for hCLCA1 and mCLCA3 in innate immunity remained widely elusive.

Thus, the first aim of this study was to identify the cellular expression pattern and intracellular distribution of the mCLCA5 protein in the naive murine respiratory tract. Although this CLCA member has experimentally been identified as an inducer of mucus cell metaplasia and mucus production in previous studies under conditions of diseases (Patel et al. 2006). However, the expressing cell type in murine airways under unchallenged conditions has been unknown to date. In addition, we also investigated the mCLCA5 distribution pattern and expression level under conditions of inflammatory challenges since recent studies have suggested a possible immune-modulatory function for certain CLCA proteins (Ching et al. 2013; Long et al. 2006; Zhang and He 2010). Furthermore, the expression patterns of the mCLCA5-orthologous proteins in humans and pigs, hCLCA2 and pCLCA2, respectively, were established and compared with that of mCLCA5 as species-specific differences in terms of mucus cell metaplasia have been observed between these species (Alevy et al. 2012).

As a second aim, a possibly modulatory role of the mCLCA3 protein in the innate immune response in acute *S. aureus* infection of the lung was characterized in order to establish its

alleged role in innate immunity, as suggested in previous studies (Ching et al. 2013; Long et al. 2006; Zhang and He 2010).

3.1 Expression of Murine CLCA5 in the Respiratory Tract and its Putative Role in Mucus Cell Differentiation

mCLCA5 and its murine homolog mCLCA3 have been speculated to play a role in mucus cell differentiation due to an observed overexpression in Th2-mediated conditions of mucus cell metaplasia and mucus overproduction (Hauber et al. 2010; Patel et al. 2009; Zhou et al. 2001). Therefore, these two mouse CLCA members as well as the human ortholog of mCLCA3, hCLCA1, have been linked to various inflammatory airway diseases with secretory dysfunctions including CF, asthma and COPD (Brouillard et al. 2005; Hegab et al. 2004; Kamada et al. 2004).

Based on the observation that mCLCA5 gene expression is increased in experimentally induced mucus cell metaplasia in *mClca3*$^{-/-}$ mice, a putative compensatory role of these homologous proteins has been suspected (Mundhenk et al. 2012; Patel et al. 2006). It has thus consequently been speculated that the two proteins may have somewhat redundant functions in mucus cell differentiation in the respiratory tract of mice (Patel et al. 2006). While only mCLCA5 mRNA has been detected in total extracts from mouse airways under naive conditions (Braun et al. 2010a), neither its expressing cell type nor its protein have been localized so far.

Since growing evidence had suggested an additional modulating role of select CLCA proteins in the innate immune response (Ching et al. 2013; Long et al. 2006; Zhang and He 2010), the expression of mCLCA5 was assessed after Th2-induced airway inflammation (Mundhenk et al. 2012). However, the role and expression pattern of mCLCA5 in acute inflammatory airway diseases which mirror an innate immune response, were elusive prior to this study.

In contrast to the murine CLCA homologs mCLCA3 and mCLCA5, their human counterparts hCLCA1 and hCLCA2 failed to show a similarly redundant or compensatory behavior in previous studies. Specifically, while hCLCA2, the human ortholog to mCLCA5, is also expressed in the lung on mRNA level, no up-regulation under mucus cell metaplasia conditions was observed (Alevy et al. 2012). It thus appeared conceivable that the protein expressions of mCLCA5 and its human and porcine orthologs, hCLCA2 and pCLCA2, respectively, may also be different. Consequently, this notion was also addressed in this study.

CONCLUDING DISCUSSION

3.1.1 Characterization of the Expression Pattern and Cellular Localization of mCLCA5 in Murine Airways

mCLCA5 mRNA has been detected in various tissues, including the respiratory tract of naive mice in which neither its expressing cell type nor its protein have been observed so far (Beckley et al. 2004; Braun et al. 2010a; Evans et al. 2004b). Nevertheless, experimental data clearly showed that the protein is capable of inducing airway mucus cell metaplasia (Patel et al. 2006). Consequently, the mCLCA5 protein expression pattern was immunohisto-chemically characterized in tracheas and entire lungs from the naive murine respiratory tract. Therefore, multiple sections were cut from serial levels of the lung to ensure that the trachea and the complete bronchial stem including its branching points were available for a complete analysis. A strikingly unique expression pattern of mCLCA5 protein was identified, restricted to two very specific locations in the airways: first, in a distinct niche of bronchial epithelial cells, approximately 2 mm in length, at the transition of the extrapulmonary main bronchi into intrapulmonary bronchi and second, in the epithelial cells of the SMG which were only present in the larynx and the proximal tracheal segment in mice.

In bronchial epithelial cells at the extra- to intrapulmonary junction the mCLCA5 protein was predominantly expressed in CC10-positive club cells as identified by immunofluorescence and spectral imaging and confocal laser scanning microscopy. Furthermore, both ciliated and mucus cells were positive for the mCLCA5 expression as determined by immunohisto-chemistry. However, only a limited number of all investigated cells showed mCLCA5 protein expression in this specific region. Basal cells in the tracheal and cartilaginous bronchial epithelium as well as club cells located more distally from this location, and AEC type II clearly lacked mCLCA5. In mucus cells, the mCLCA5 protein was occasionally co-localized with the mucus cell marker and murine homolog mCLCA3 as visualized by double immunostaining in this study. Interestingly, the subcellular protein expression pattern differed between the in-vestigated cell types. While club cells and mucus cells possess a diffuse, either finely or coarsely granular cytoplasmatic pattern, ciliated cells displayed a more clumpy and peri-nuclear expression signal of mCLCA5 protein. This difference may point towards their presence in different intracellular compartments, possibly more restricted to the endoplasmic reticulum or Golgi in ciliated cells versus more apical or systematically secretory granules-associated location in club cells. This differentiation, however, needs to be addressed in the future using either high resolution immuno-co-localization techniques with organelle-specific markers or immune-electron microscopy.

Anatomically, both mCLCA5 expressing regions are strongly suggestive of progenitor cell niches which have been characterized in detail in several previous studies (Liu and

Engelhardt 2008; Rawlins and Hogan 2006; Roomans 2010; Warburton et al. 2008). In particular, a specific subset of club cells located in the non-cartilaginous bronchi, the variant club cell type, is a well established progenitor cell for non-ciliated club cells, ciliated cells and mucus cells (Pardo-Saganta et al. 2013; Rawlins and Hogan 2006; Rawlins et al. 2009; Reader et al. 2003; Reynolds and Malkinson 2010; Roomans 2010; Wong et al. 2009). Interestingly, a previous study identified mCLCA5 as sufficient for the induction of mucus production and responsible for mucus cell metaplasia (Patel et al. 2006). Thus, the predominant expression of mCLCA5 in club cells as putative progenitor cells and its presence in known anatomical locations of progenitor cell niches are in line with its proposed function in mucus cell differentiation. It is well conceivable that mCLCA5 expression by progenitor cells drives their differentiation into mucus cells, as already hypothesized for the closely related proteins hCLCA1 and mCLCA3 (Alevy et al. 2012; Patel et al. 2009; Patel et al. 2006). These findings were consistent with previous observations on CLCA members playing a role in epithelial differentiation of other lineages (Alevy et al. 2012; Patel et al. 2009; Patel et al. 2006; Walia et al. 2012; Yu et al. 2013). This is also consistent with the suspected role of mCLCA5 in growth arrest and maturation processes of squamous epithelial cells of the skin (Beckley et al. 2004; Braun et al. 2010a). Furthermore, the different subcellular expression patterns in the expressing cell types may be dependent on the differentiation status or arrangement of cell organelles in the respective cell type. Moreover, the mCLCA5 protein may even be lost in mucus and ciliated cells with progressing differentiation. Since mCLCA3 is, similarly to mCLCA5, a known inducer of mucus cell metaplasia (Patel et al. 2006), it has been speculated that both proteins may have redundant functions in the respiratory tract with one compensating for the loss of the other (Patel et al. 2009; Patel et al. 2006). However, this study clearly showed that mCLCA5 and mCLCA3 have only partially overlapping expression patterns in different sets of mucus cells which may somewhat limit and redefine the notion of mutually overlapping or even compensatory functions. Furthermore, the observation that mCLCA3 is neither expressed in club cells nor in ciliated cells (Leverkoehne and Gruber 2002) may point towards a different functional relevance of these two related proteins in their respective cellular microenvironments and argues against a complete redundancy of both proteins. On the one side, it appears reasonable to speculate that mCLCA5 may be highly relevant in mucus cell differentiation of club cells as putative progenitors for mucus cells. On the other side, under challenged conditions mCLCA3 could possibly act as extracellular signaling molecule on both the innate immune response and on mucus cell differentiation.

3.1.2 Expression of the mCLCA5 Protein in Challenged Lungs

Recent studies have indicated a possible role of CLCA proteins in the inflammatory response to various chronic and allergic challenges, including ovalbumin (Long et al. 2006; Mundhenk

et al. 2012; Patel et al. 2006) and to acute inflammation due to exposure to LPS as virulence factor of gram-negative bacteria (Long et al. 2006). Up to now, mCLCA5 expression has only been studied in Th2-mediated challenges, mimicking more chronic, lymphocyte driven in-flammation, where the mCLCA5 protein was found in airway mucus cells (Mundhenk et al. 2012).

Consequently, in this study the expression pattern of mCLCA5 was analyzed in various models of acute lung challenge, including PBS-treatment and infection with *S. aureus*, *S. pneumoniae* and influenza virus, and compared to naive controls. Furthermore, lung mRNA expression levels of functionally related genes, including *mClca3*, *mClca5*, *Muc5ac* and *Muc5b,* were determined by RT-qPCR. Cells expressing mCLCA5-, mCLCA3- and club cell-protein CC10 were quantified by immunohistochemistry as well as PAS-positive mucus cells from naive, PBS-treated or *S. aureus* infected mice. This study clearly showed that under challenged conditions and independently of the type of challenge the mRNA and protein ex-pression of mCLCA5 strongly declined at all time points investigated. Interestingly, mCLCA5 expression reappeared over time in PBS-treated and *S. aureus* infected mice, possibly due to regeneration of the epithelium which remained intact in these two models. After infection with *S. pneumoniae* or influenza virus, a gradual reduction of the mCLCA5 protein without reappearance was observed, possibly due to the initiated irreversible cell damage and death inflicted by these two pathogens. After challenge, the number of mCLCA5-positive cells de-creased while the overall number and cell type composition of club cells and mucus cells, as the major mCLCA5 expressing cell types, remained constant. This observation may point towards a selective transcriptional down-regulation of mCLCA5 with subsequent loss of the protein, independently of the different challenge types used. It is well conceivable that se-cretory processes of club cells and mucus cells, which are observed under challenged condi-tions (Davis and Dickey 2008; Evans et al. 2004a; Pack et al. 1980; Reader et al. 2003; Reynolds and Malkinson 2010), may have contributed to the reduction of mCLCA5 on the protein level. Furthermore, the down-regulation of proteins involved in cellular differentiation (Das et al. 2011; Zheng et al. 2013) is a well established phenomenon of cells reacting to a specific type of challenge which supports our findings and the proposed role of mCLCA5 in mucus cell differentiation.

3.1.3 Species-Specific Differences in Expression Patterns of mCLCA5 Versus its Human and Porcine Orthologs, hCLCA2 and pCLCA2

Since changes of mRNA expression levels differ between mCLCA5 and its human ortholog hCLCA2 following induction of mucus cell metaplasia (Alevy et al. 2012), the expression pattern of mCLCA5 was compared to those of its human and porcine orthologs, hCLCA2 and

pCLCA2, respectively, to determine putative species-specific differences. Healthy human and porcine airways were examined by immunohistochemistry, similar to the murine airways before. Of note, the murine lung, including the two niches that express mCLCA5, differs from the lungs of other species in several anatomical and functional aspects. For example, murine SMG are only present in the upper part of the trachea, whereas in humans and porcine respiratory tracts, they line the entire cartilaginous airways (Liu and Engelhardt 2008; Lynch and Engelhardt 2014; Rawlins and Hogan 2005; Rock et al. 2010; Suarez 2012). Here, the epithelial cells of the SMG were found to be positive for the respective orthologs in mice, humans and pigs in which the murine mCLCA5 signal was much stronger than in those of the respective orthologs. Moreover, the murine proximal bronchial epithelium predominantly consists of club cells as the principal secretory (Liu et al. 2006; Rawlins and Hogan 2006; Reynolds and Malkinson 2010; Rock and Hogan 2011) and major mCLCA5-expressing cell type which do not exist in humans (Suarez 2012). In contrast to the murine mCLCA5, neither its human nor its porcine ortholog was expressed in bronchial epithelial cells nor in other cell types throughout the entire lungs. One could thus speculate that mCLCA5-expression in a highly select segment of murine bronchial epithelial cells may in some aspects compensate for the lack of SMG in the lower segments of murine airways. The cellular expression patterns of mCLCA5, hCLCA2 and pCLCA2 in murine, human and porcine airways, respectively, as established in this study, are summarized in Table 5.

Table 5: Extended Expression Patterns of Human, Murine and Porcine CLCA Members in unchallenged Airways

Gene	mRNA[a]	Cell Types Immunohistochemically Identified to Express the Protein[b]
hCLCA2	+	SMG only
mClca5	+	SMG and highly select BEC
pClca2	+	SMG only

SMG = submucosal glands, BEC = bronchial epithelial cells (club-, ciliated- and mucus cells);
[a] = as identified in previous studies (Agnel et al. 1999; Beckley et al. 2004; Braun et al. 2010a; Connon et al. 2004; Evans et al. 2004b; Gruber et al. 1999; Plog et al. 2012b);
[b] = as identified in this study

These observations again clearly point towards species-specific differences in cellular expression patterns of CLCA proteins that parallel species-specific anatomic differences. This particular difference may also explain the previous observation that hCLCA2, in contrast to its murine ortholog mCLCA5, was not up-regulated under conditions of mucus cell metaplasia (Alevy et al. 2012). Furthermore, due to these species-specific differences in airway anatomy and expression of mCLCA5 orthologs, the mouse may not be a suitable model in translational medicine to study the roles of CLCA proteins in mucus cell differentiation and metaplasia, in particular for studying the suspected redundant functions of mCLCA3 and mCLCA5 (Patel et al. 2006). In contrast, the similarity of the expression patterns of the mCLCA5 orthologs in humans and pigs supports the notion that porcine CLCA genes may share more similarities with the human species (Plog et al. 2012a; Plog et al. 2009; Plog et al. 2012b). The pig may thus indeed become the preferred animal model for studying mucus cell differentiation and metaplasia (Klymiuk et al. 2012; Meyerholz et al. 2010a; Meyerholz et al. 2010b; Plog et al. 2009; Rogers et al. 2008a; Rogers et al. 2008b; Stoltz et al. 2010).

3.2   mCLCA3 in the Innate Immune Response

In addition to the modulation of mucus production and the impact on mucus cell metaplasia, select CLCA proteins have been implicated in the regulation of tissue inflammation in the innate immune response (Ching et al. 2013; Long et al. 2006; Zhang and He 2010). However, none of the previous studies have addressed the role of CLCA proteins in acute bacterial infection, a more appropriate model to test for modulating properties in innate immunity. Consequently, the course of acute bacterial pneumonia after infection of $mClca3^{-/-}$ mice and WT controls with *S. aureus* was investigated here. *S. aureus* which is one of the most prevalent pathogens of community- and hospital-acquired infections (Gillet et al. 2002; Gillet et al. 2001; Kapetanovic et al. 2010) causes lower respiratory tract infections in humans, especially in infants and young children with CF (Ulrich et al. 1998; Wolter et al. 2013; Wong et al. 2013). Specifically, the acute *S. aureus* pneumonia in mice has previously been proposed as appropriate model for studying innate immune responses in this species (Banerjee et al. 2010; Bragonzi 2010; Kapetanovic et al. 2010; Kohler et al. 2011).

After infection of mice with 5×10^7 *S. aureus* Newman, both $mClca3^{-/-}$ and WT genotypes equally developed acute pneumonia with significant loss of body weight and body temperature and with equally constant bacterial loads. In addition, the lesions and site of bacterial infection were restricted to the lungs, confirming a non-bacteremic *S. aureus* pneumonia.

However, only mCLCA3-deficiency was associated with significantly decreased protein levels of selected cytokines in the BALF compared to WT mice at 24 hours post infection. Specifically, CXCL-1, the murine homolog to human CXCL-8 and a potent chemoattractant for neu-

trophils (Huang et al. 1992; Luster 1998), and IL-17 which mediates pro-inflammatory responses by inducing other cytokines, including CXCL-8 (Korn et al. 2009), were clearly reduced. All other cytokines tested (IL-1ß, IL-6, IL-10, IL-12p40, IL-13, MCP-1, RANTES, TNF-α and GM-CSF) were significantly increased after infection compared to PBS-treated controls, independently of the genotype used. These results clearly pointed towards a selective modulation of CXCL-1 and IL-17 responses in the *mClca3*^{-/-} genotype after bacterial challenge. Moreover, markedly decreased mRNA levels of *Cxcl-1* and *Il-17* were observed as early as 12 hours post infection which was consistent with the protein data. Additionally, *Cxcl-2*, another murine CXCL-8 homolog (Bozic et al. 1994) showed a similar transcriptional regulation in infected *mClca3*^{-/-} mice only. The reduced induction of these cytokines on mRNA and protein levels at staggered time-points obviously indicates their aberrant transcriptional regulation in *mClca3*^{-/-} mice after *S. aureus* infection. As a coherent consequence of this reduced cytokine induction of CXCL-1, CXCL-2 and IL-17, a significant decrease of neutrophil recruitment to the site of infection was observed in *mClca3*^{-/-} only. This reduction in cellular trafficking into the infected airways most likely also account for the reduction in total protein amounts in the BALF.

It was recently demonstrated that hCLCA1, the human ortholog to mCLCA3, activates alveolar macrophages, resulting in pro-inflammatory cytokine induction, including CXCL-8 (Ching et al. 2013). Interestingly, the predominant cell types in the lung expressing the two murine CXCL-8 homologs, CXCL-1 and CXCL-2, during infection are alveolar macrophages and pulmonary epithelial cells (Becker et al. 1994; Huang et al. 1992). In addition to subsets of T cells as primary source of IL-17, alveolar macrophages are also competent in secreting IL-17 (Jin and Dong 2013; Song et al. 2008). It thus seems plausible to assume that mCLCA3 may have similar effects on alveolar macrophages after *S. aureus* infection as it was shown for its human ortholog hCLCA1 *in vitro* (Ching et al. 2013).

In addition to the decrease of neutrophils in the BALF of *mClca3*^{-/-} mice, the numbers of lymphocytes in the BALF were also significantly reduced. Both cytokines CXCL-1 and IL-17 which were found to be dysregulated after infection are neither known to be chemoattractants nor inducers of lymphocyte proliferation. This raises the question of whether an as yet unidentified factor or mechanism may influence lymphocyte recruitment after bacterial infection in *mClca3*^{-/-} mice. Alternatively, we cannot exclude that the reduced CXCL-8 homolog expression is a consequence of the aberrant induction of IL-17, a known inducer of CXCL-8, instead of being directly regulated by mCLCA3.

Given the well-established role of mCLCA3 in mucus production and mucus cell metaplasia (Alevy et al. 2012; Patel et al. 2006), the effect of mCLCA3 on mucus cells and mucin genes in acute *S. aureus* pneumonia was analyzed here by histochemistry and RT-qPCR. Solely

the mucin genes *Muc5ac* and *Muc2* were elevated independently of the genotype after infection compared to PBS-controls which is in line with the notion that induction of mucus production is a canonical innate immune response to infections (Lemjabbar and Basbaum 2002) and other challenges (Alevy et al. 2012; Mundhenk et al. 2012; Patel et al. 2006).

Due to the different expressional behaviors of CLCA proteins in humans and mice regarding mucus cell metaplasia (Alevy et al. 2012) and a previously hypothesized compensatory regulation of further members of the murine CLCA family in the absence of mCLCA3 (Patel et al. 2009; Patel et al. 2006), other murine homologs (*mClca1* to *mClca7*) in lungs were analyzed in this study. However, no single other murine CLCA member that potentially could have compensated for the lack of mCLCA3 was differentially regulated under challenged conditions, which is again consistent with previous reports (Braun et al. 2010a; Mundhenk et al. 2012).

In contrast to our observed reduction of neutrophils in BALF of infected *mClca3$^{-/-}$* mice, our pathologic examinations, including contemporary morphometric methods (Ochs 2006; Schneider and Ochs 2013) failed to reveal differences in lung inflammation or lesion expansion between genotypes. This may have been due to the consolidation and destruction of inflamed areas and therefore impeded lung structure differentiation. Alternatively, the histomorphometrical approaches in the destroyed tissues may have not been sensitive enough to pick up the differences. Similarly, no differences were observed in all clinical parameters recorded. Thus, obviously, additional determinants other than leukocyte numbers and aberrant regulation of selected cytokines decide on the overall severity and clinical outcome of *S. aureus*-induced pneumonia. Taken together, mCLCA3 seems to have an impact on the early innate immune response in mice via direct or indirect induction of CXCL-8 homologs and IL-17, however, without affecting the general clinical and pathological outcome at all time points investigated here.

3.3   New Insights from this Study in Context of CLCA Research

A diverse cell type specific expression pattern of different family members in one organ is a well established feature of the CLCA family. For example, in the murine intestinal tract, different CLCA family members are expressed in basal versus apical enterocytes, goblet cells and likely even in smooth muscle cells (Bothe et al. 2008), pointing towards cell-type specific functions for each of these related molecules. Nevertheless, what these functions are and whether they are identical or at variance among the different cell types is still poorly understood. This study revealed that in the respiratory tract, the mCLCA5 protein is predominantly expressed by club cells while mCLCA3 is exclusively expressed by mucus cells. Interestingly, mCLCA5 was also detected in select ciliated cells and mucus cells with a partially

overlapping expression with mCLCA3. Furthermore, all SMG epithelial cells also strongly expressed mCLCA5. Of note, the presence of a single CLCA homolog in different cell types of one organ and overlapping expression of two CLCA homologs in one cell type have not been observed for other CLCA members before. Even more challenging, the mCLCA5 protein was previously found in squamous epithelial cells throughout the entire body (Braun et al. 2010). This apparent discrepancy raises the question of whether it may serve one function in all different cell types or whether its functional pathways vary between the different cell types. Its predominant expression in club cells as putative progenitors of mucus cells and ciliated cells at defined regions may point toward a function in cellular differentiation with subsequent loss of the protein in mature cells. Similar thoughts have been raised for squamous epithelia of the skin (Braun et al. 2010) where the protein was suspected be involved in growth arrest and maturation of keratinocytes. The results of this study thus seem to support the hypothesis that mCLCA5 may function as cellular differentiation protein, both in the skin and in the respiratory tract. Alternatively, we still cannot exclude the possibility that mCLCA5 possesses different functions in the respective cell types which could then also be consistent with its alleged compensatory role for its homolog mCLCA3 in mucus cell regulation (Patel et al. 2006).

As it was the case for other CLCA family members before, we observed clear species-specific differences between the expression pattern of mCLCA5 in mice and its orthologs in human and porcine airways. Species-specific variations in cellular expression patterns appear to be another consensus hallmark of the CLCA family (Agnel et al. 2006; Alevy et al. 2012; Anton et al. 2005; Connon et al. 2004; Evans et al. 2004). The differences we observed here between mCLCA5 and hCLCA2 might even explain why the murine mCLCA5 in progenitor cells is capable of inducing mucus cell metaplasia in airways of mice, contrary to its ortholog hCLCA2 (Alevy et al. 2012) which is expressed in human SMG only. It thus appears noteworthy that such species-specific differences between direct CLCA orthologs must be considered in translational CLCA research. In particular, the value of mouse models for studying mucus cell metaplasia in translational medicine becomes more and more questionable.

Furthermore, we have shown that mCLCA3 is required for modulating pro-inflammatory cytokine induction *in vivo* as it was first proposed for its human ortholog hCLCA1 *in vitro* (Ching et al. 2013). Our results thus confirm the role of hCLCA1 and mCLCA3 in modulating the innate immune response, likely by acting as extracellular signaling molecules secreted by mucus producing epithelial cells. The target receptor and target cell type, however, remain to be identified in future studies. Taken together, our current knowledge supports dual functions of mCLCA3 and possibly hCLCA1, including modulation of mucus cell differentiation and an

integral role in leukocyte recruitment during the early innate immune response. This again supports the assumption that at least some CLCA family members possess pleiotropic functions (Patel et al. 2009).
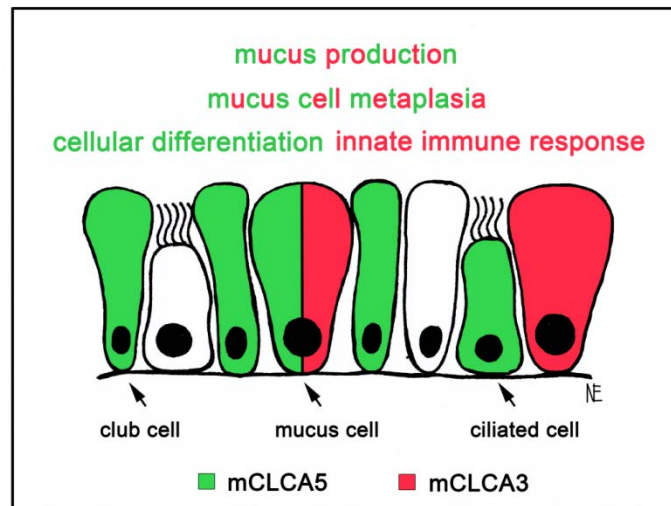

Figure 1: Expression patterns and putative functions of the mCLCA3 and mCLCA5 proteins in murine airways. mCLCA3 (in red) and mCLCA5 (in green) have both similarities and differences in expression and in putative functions in the murine airways. While mCLCA3 is exclusively expressed by mucus cells, mCLCA5 is predominantly expressed by club cells followed by fewer mucus cells and ciliated cells. Thus, both proteins showed only a partially overlapping expression pattern in some mucus cells of defined regions. Furthermore, the two proteins were implicated in the regulation of mucus production and mucus cell metaplasia, whereas only mCLCA5 was suspected to play a role in cellular differentiation as well as mCLCA3 in modulating the innate immune response.

3.4 __Conclusions__

In the first part of this study, it was established that naive mice express the mCLCA5 protein in highly select bronchial epithelial cells and in SMG, consistent with anatomical locations of progenitor cells. Interestingly, in the bronchial epithelium the mCLCA5 protein is predominantly expressed in club cells, followed by fewer mucus and ciliated cells in very restricted regions. In contrast, the mCLCA3 protein is only expressed by mucus cells throughout the entire respiratory tract and in these occasionally co-localized with the mCLCA5 protein. The predominant expression of mCLCA5 in club cells as putative progenitors of mucus cells and ciliated cells, points toward a possible role in cellular differentiation with subsequent loss of the protein in differentiated cells. This would explain the observation that most club cells but only few mucus and ciliated cells express mCLCA5. This notion would also be well in line

with the proposed and partially compensatory or redundant function of mCLCA5 and its homolog mCLCA3 in regulating experimentally induced mucus cell metaplasia.

Nevertheless, mCLCA3 and mCLCA5 only partially overlap in their expression patterns in mucus cells which argues against a complete functional redundancy of these two proteins *in vivo* as suggested before (Patel et al. 2006). While mCLCA3 and mCLCA5 are obviously capable of similar *in vitro* functions in mucus production and mucus cell metaplasia, they likely permit more independent functions *in vivo*.

Moreover, as revealed in the second part of this study, mCLCA3 seems to have an impact on the early immune response in bacterial infections via induction of selected cytokines and a subsequent cellular response. This result is consistent with the proposed function of the orthologous hCLCA1 protein acting as extracellular signaling molecule modulating innate immunity (Ching et al. 2013). In the bacterial infection model employed here, no differential expression or other evidence of the alleged compensatory action of other CLCA family members (Patel et al. 2009) was found, including mCLCA5.

Taken together, as first proposed by Bothe et al., 2008, our results support the notion that different CLCA family members within one species occupy specific cellular niches with only partially overlapping and possibly even compensatory or redundant functions. This is most likely the result of a long term and complex evolution of the CLCA gene family with species-specific gene duplication events and radiation into different cellular and functional niches that are specific for one several mammalian species but not others. Clearly, we are still at the beginning of our understanding of the complex physiological and pathological roles and mechanisms of CLCA proteins. In terms of mCLCA3 and mCLCA5 and their orthologs in other species including man, these exact functions and pathways involved remain to be established on the molecular level in future *in vitro* and *in vivo* studies. *In vivo* studies should clearly include a *mClca5$^{-/-}$* knockout mouse model as well as double *mClca3$^{-/-}$* and *mClca5$^{-/-}$* knockout mice with relevant challenges in each model and independent overexpression of mCLCA3 and mCLCA5.

3.5 <u>Outlook</u>

Further investigations should clarify the alleged role of mCLCA5 in the maturation and differentiation processes of progenitor club cells to mucus cells. For example, early time points of the mucus cell metaplasia development, i.e., using an ovalbumin-challenge, could be investigated in detail comparing WT and *mClca3$^{-/-}$* mice with particular focus on the unique and restricted expression regions of mCLCA5. Specifically, the protein expression of mCLCA5 during differentiation of club cells as putative precursor mucus cells, to mature mucus cells would be of special interest. Is the protein lost with progressive differentiation and

maturation? As known from earlier studies, *mClca3*^{-/-} mice did not show a pathologic pheno-type (Long et al. 2006; Robichaud et al. 2005), possibly because of the suggested compensation of the lack of the mCLCA3 protein by mCLCA5 (Patel et al. 2006). Consequently, the generation of a *mClca5*^{-/-} knockout mouse model should be considered in the future. However, if compensatory functions should indeed be independently of the respective protein, only a double *mClca3*^{-/-} and *mClca5*^{-/-} knockout mouse could address several questions concerning the alleged redundant or compensatory functions of these two homologs. Furthermore, lineage tracing of cells during development is one of the most powerful tools available for identifying stem/ progenitor cells and analyzing their functions (Fox et al. 2008; Kretzschmar and Watt 2012; Pardo-Saganta et al. 2013). Lineage tracing of progenitor club cells with special regard to mCLCA5 expression would be of tremendous interest in future studies. Moreover, to evaluate possible progenitor functions of mCLCA5 in club cells in mice, a naphthalene-challenge has been shown to be the preferred model (Hong et al. 2001; Mahvi et al. 1977; Reynolds et al. 2000; Stripp et al. 1995; Stripp and Reynolds 2008; Van Winkle et al. 1995). This approach may become of interest in addressing the following questions. Are there any changes in the expression pattern and cellular distribution of mCLCA5 during epithelial renewal? Is there any transcriptional or translational up- or down-regulation of mCLCA5 and its homolog mCLCA3 compared to untreated controls? It is noteworthy that naive mice have to be included as negative controls in all models as any instillation of substances to murine lungs, including PBS, obviously affect the expression of mCLCA5. Moreover, expressional characteristics of hCLCA2 and pCLCA2 in the SMG and bronchial epithelium in diseased human and porcine lungs, respectively, with mucus cell metaplasia and mucus overproduction as well as under infectious conditions would be of special interest. However, in humans, these analyses will be highly limited by the fact that very specific locations such as the main bronchi and trachea would have to be investigated which are only available post mortem. Moreover, naive controls are extremely difficult to obtain from these particular anatomic regions from healthy donors.

To further elucidate the role of mCLCA3 in innate immunity, further studies should primarily identify the main target cell type and receptor of mCLCA3. As it was shown for the human ortholog hCLCA1 (Ching et al. 2013), isolated murine alveolar macrophages should be stimulated with the mCLCA3 protein to determine a specific subsets of cytokines on the protein level using a multiplex assay and on the mRNA level by RT-qPCR. Does mCLCA3 activate alveolar macrophages and induce cytokine release of CXCL-1, CXCL-2 and IL-17 *in vitro*? Do alveolar macrophages of WT mice and *mClca3*^{-/-} mice behave identically? A similar approach could be employed to determine this for isolated subsets of lymphocytes, specifically Th-17 and δγT cells which are the primary source of IL-17. Moreover, we would like to

know if and how mCLCA3 modulates the innate immune response in other infection models, possibly using other gram-positive or gram-negative bacteria, or even other pathogens. As one possibility, the activation of a specific toll like receptor, in case of *S. aureus* toll like receptor (TLR)-2, may decide on the modulatory effect of mCLCA3-dependent cytokine release and cellular recruitment.

4   Summary

CLCA Proteins in the Airways: New Insights into their Expression Patterns and Role in Innate Immunity in Pneumonia

Kristina Dietert

For more than 15 years, the CLCA (chloride channel regulator, calcium-activated) protein family has been in the focus of several research groups worldwide due to their strong implication in several important animal and human diseases. However, the functions of these molecules in normal tissues and their exact roles in diseases are still incompletely understood. Some CLCA proteins possess a well established role in inflammatory airway diseases with mucus overproduction, such as asthma, cystic fibrosis and chronic obstructive pulmonary disease. In the respiratory tract, the human hCLCA1 and its mouse ortholog mCLCA3 are selectively expressed in mucus cells and have directly been linked to the trait of mucus cell metaplasia, a common feature of these diseases. In addition to mCLCA3, the murine mCLCA5 has also been associated with airway mucus cell metaplasia and a redundant or overlapping function of the two murine members was previously proposed. However, the cell types that express mCLCA5 in the airways were unknown.

Consequently, in this study the cellular expression pattern of mCLCA5 was determined under healthy and challenged conditions in murine lungs. Since differences in expression patterns between different species have previously been observed for other CLCA proteins, the expression patterns of the mCLCA5 orthologous proteins in humans and pigs, hCLCA2 and pCLCA2, respectively, were also established to allow for a better understanding of animal models for human diseases. In healthy mice, mCLCA5 was found to be uniquely expressed in highly select regions of bronchial epithelial cells and in submucosal glands (SMG), consistent with the canonical anatomical locations of progenitor cell niches. Since club cells were the predominantly mCLCA5 expressing cell type, followed by fewer mucus cells and ciliated cells, it appears unlikely that mCLCA5 has a fully redundant function with mCLCA3 which is expressed in mucus cells only. Under conditions of challenge including instillation of phosphate buffered saline (PBS), *Staphylococcus aureus* (*S. aureus*), *Streptococcus pneumoniae* (*S. pneumoniae*) or influenza virus, mCLCA5 mRNA and protein expression strongly declined. Protein reappearance was observed only in models retaining intact epithelial cells (PBS, *S. aureus*). The unique localization of mCLCA5 to murine airway epithelial progenitor cell niches and the observation that mCLCA5 but not mCLCA3 is present in club cells as putative progenitors for mucus cells suggest that mCLCA5 but not mCLCA3 is the prime

CLCA protein involved in mucus cell differentiation from precursor cells in mice. Of note, normal human and porcine bronchial epithelial cells did not express their respective mCLCA5 orthologs and SMG of both species had fewer expressing cells, indicative of fundamental differences in mice on the one side versus human and pigs on the other.

In addition to their modulation of mucus production, the human hCLCA1 and possibly other CLCA proteins have also been implicated *in vitro* in the regulation of tissue inflammation in the innate immune response. Consequently, early immune responses were characterized in this study *in vivo* using a mouse model that lacks expression of mCLCA3, the mouse ortholog to hCLCA1. A *S. aureus* pneumonia model was employed in *mClca3* knockout (*mClca3*$^{-/-}$) mice and wild-type (WT) littermates. Experimental readouts included clinical symptoms, bacterial clearance, leukocyte immigration and cytokine responses in the bronchoalveolar compartment, pulmonary vascular permeability and histopathological changes. Furthermore, effects on mucus cell number and mucin gene expression levels as well as possibly compensatory differential regulation of other murine CLCA homologs were determined. Deficiency of mCLCA3 resulted in decreased neutrophilic immune cell infiltration into the bronchoalveolar space after *S. aureus* infection when compared to WT controls. Only the cytokines IL-17 and the murine CXCL-8 homolog CXCL-1, also termed KC, were decreased on mRNA and protein levels in infected *mClca3*$^{-/-}$ mice compared to WT controls. However, no differences were observed in clinical outcome, histopathology or mucus cell metaplasia. No evidence was found for regulation of other CLCA homologs that would putatively compensate for the lack of mCLCA3. In summary, this *in vivo* study clearly revealed that mCLCA3 plays a significant role in the early innate immune response in a *S. aureus* pneumonia mouse model via induction of select cytokines with subsequent immune cell recruitment. These data confirm and extend the functional understanding of previous *in vitro* observations on its human ortholog hCLCA1.

Taken together, the results gained from this study substantially add to our knowledge on the expression patterns, functions in healthy and diseased airways as well as differences in expression between mice, humans and pigs for the two CLCA members most relevant for respiratory diseases. Still, other important challenges remain and several new questions were raised. Future studies should more closely define the putative role of mCLCA5 as modulator of progenitor cells in mucus cell differentiation and mucus cell metaplasia. Furthermore, the mechanisms, target cells and pathways of mCLCA3 modulating the inflammatory innate immune response will have to be established. Finally, we hope that this research on CLCA molecules and their roles in such devastating respiratory diseases will contribute to the development of more powerful therapeutic approaches in the future.

5   Zusammenfassung

CLCA Proteine in den Atemwegen: Neue Erkenntnisse über ihre Expressionsmuster und Rolle bei angeborener Immunität im Pneumoniemodell

Kristina Dietert

Seit mehr als 15 Jahren steht die CLCA (chloride channel regulators, calcium-activated)-Proteinfamilie aufgrund ihrer Bedeutung bei verschiedenen Erkrankungen von Mensch und Tier im Focus mehrerer Forschergruppen weltweit. Dennoch sind die Funktionen dieser Moleküle in gesundem Gewebe sowie deren genaue Rolle bei krankhaften Veränderungen bislang weitgehend unklar. Einzelne Proteine dieser Familie spielen insbesondere bei Atemwegserkrankungen mit sekretorischer Dysfunktion wie dem Asthma, der zystischen Fibrose oder der chronisch-obstruktiven Lungenerkrankung eine große Rolle. In der Lunge konnten hierbei vor allem das humane hCLCA1 und sein muriner Orthologer mCLCA3, welche ausschließlich in schleimproduzierenden Zellen exprimiert werden, mit der Entstehung von Mukuszellmetaplasien, als eine der Haupteigenschaften der genannten Erkrankungen in Verbindung gebracht werden. Neben mCLCA3 wurde jüngst ein weiterer CLCA-Vertreter der Maus, mCLCA5, ebenfalls mit der Entstehung einer Mukuszellmetaplasie in Verbindung gebracht und somit eine mögliche Kompensation und Redundanz beider Proteine vermutet. Jedoch waren die mCLCA5-exprimierenden Zelltypen bisher völlig unbekannt. Folglich wurde in dieser Studie das zelluläre Expressionsmuster von mCLCA5 in der gesunden und in der modellhaft erkrankten Mauslunge untersucht. Da unterschiedliche Expressionsmuster zwischen verschiedenen Spezies bereits für andere CLCA-Proteine beschrieben wurden, untersuchten wir auch die Expressionsmuster der mCLCA5-orthologen Proteine von Mensch und Schwein, hCLCA2 und pCLCA2, um eine bessere Beurteilung von Tiermodellen für humane Erkrankungen vornehmen zu können. Bei gesunden Mäusen konnte ein einzigartiges Expressionsmuster des mCLCA5-Proteins in hoch selektiven Bronchialepithelzellen sowie in den epithelialen Zellen der submukosalen Drüsen nachgewiesen werden. Diese anatomischen Lokalisationen entsprechen hierbei weitgehend dem Vorliegen von Progenitorzellnischen. Da mCLCA5 vorrangig in Club-Zellen und weniger in Mukus- und zilierten Zellen exprimiert wird und damit ein nur partiell überlappendes Expressionsmuster mit seinem Verwandten mCLCA3, der ausschließlich in Mukuszellen exprimiert wird, aufweist, erscheint ein vollwertiger Ersatz des einen Proteins durch das andere kaum möglich. Nach der Behandlung von Mäusen mit phosphatgepufferter Salzlösung (PBS) oder nach deren Infektion mit *Staphylococcus aureus* (*S. aureus*), *Streptococcus pneumoniae* (*S. pneumoniae*) oder In-

fluenzaviren konnte eine deutliche Verminderung der mRNA- und Proteinexpression von mCLCA5 beobachtet werden. Die Proteinexpression erholte sich jedoch nach einiger Zeit ausschließlich in solchen Modellen mit intakt gebliebenem Epithel (PBS und *S. aureus*). Die einzigartige Expression des mCLCA5-Proteins in ausgewählten Atemwegsepithelien der Maus in anatomischen Nischen von Progenitorzellen sowie die Tatsache, dass mCLCA5 und nicht mCLCA3 in Club-Zellen als mögliche Vorläufer für Mukuszellen exprimiert wird, spricht vielmehr für mCLCA5 als dasjenige CLCA-Molekül, welches an der Differenzierung von Mukuszellen beteiligt ist. Interessanterweise wiesen das normale Bronchialepithel von Mensch und Schwein keine Expression der mCLCA5-Orthologen hCLCA2 und pCLCA2 auf, und auch die submukosalen Drüsen zeigten eine weitaus geringere Expression im Vergleich zur Maus. Dieses teils deutlich abweichende Expressionsmuster weist auf nicht zu vernachlässigende Unterschiede zwischen den einzelnen Spezies hin.

Zusätzlich zu ihren vermuteten modulatorischen Funktionen in der Mukusproduktion wurden CLCA-Proteine, wie hCLCA1 und mögliche andere, bereits mit der Regulation von Entzündungen im Rahmen der angeborenen Immunität *in vitro* in Verbindung gebracht. Folglich wurde in dieser Studie eine frühe Immunantwort im *in vivo* Mausmodell charakterisiert. Dieses erfolgte in knockout-Mäusen, die das mCLCA3-Protein, also den Maus-Orthologen des humanen hCLCA1, nicht exprimieren. Hierbei wurde ein *S. aureus*-Pneumoniemodell in *mClca3*$^{-/-}$-Mäusen und Wildtyp (WT)-Kontrolltieren eingesetzt. Die Schwerpunkte der Untersuchungen lagen auf der zellulären und Zytokin-bedingten unspezifischen Immunantwort. Im Detail wurden der Verlauf der Lungenentzündung in Bezug auf klinische Symptome, die Beseitigung der bakteriellen Last, Leukozyteneinwanderung und Zytokinantwort im bronchoalveolären Raum, die vaskuläre Permeabilität der Lungengefäße sowie die histopathologischen Veränderungen der Lungen untersucht. Weiterhin wurden mögliche Effekte auf die Mukuszellzahl, die Muzingenexpression sowie auf die mögliche unterschiedliche Regulation der Expression anderer CLCA-Vertreter der Maus untersucht. Der Verlust des mCLCA3-Proteins in den *mClca3*$^{-/-}$-Tieren führte nach Infektion mit *S. aureus* im Vergleich zu WT-Kontrolltieren zu einer verminderten Einwanderung von neutrophilen Granulozyten in den bronchoalveolären Raum. Ausschließlich die Zytokine IL-17 sowie der murine CXCL-8-Homologe CXCL-1, auch KC genannt, waren deutlich in ihrer mRNA-Expression sowie in ihrem Proteingehalt in den infizierten *mClca3*$^{-/-}$-Mäusen, verglichen mit den WT-Kontrolltieren, erniedrigt. Es konnten keine Unterschiede im klinischen Verlauf, in der Histopathologie sowie in der Ausbildung einer Mukuszellmetaplasie festgestellt werden. Ferner fanden sich keine Hinweise für das Vorliegen einer Kompensation der mCLCA3-Defizienz durch andere CLCA-Homologe im Sinne einer Regulation ihrer Genexpressionen. Zusammengefasst weisen die *in vivo* Ergebnisse deutlich darauf hin, dass das mCLCA3-Protein einen erheblichen Einfluss auf die frühe, unspezifische Immunantwort durch die Induktion ausgewählter Zytokine mit

konsekutiver Immunzellrekrutierung nimmt. Diese Daten bestätigen und erweitern somit die funktionellen Aspekte, die bereits für den Orthologen hCLCA1 *in vitro* beobachtet worden waren.

Insgesamt konnten hier substanzielle Ergänzungen zu den früher bekannten Expressions-mustern und möglichen Funktionen von CLCA-Proteinen bei gesunden und erkrankten Atemwegen sowie deutliche Unterschiede zwischen Mäusen, Menschen und Schweinen für die zwei wichtigsten CLCA-Mitglieder in der Lunge gewonnen werden. Dennoch bleiben wichtige Herausforderungen bestehen und die neuen Erkenntnisse werfen auch viele neue Fragen auf. Zukünftige Untersuchungen sollten nun zum einen die Rolle von mCLCA5 als möglicher Modulator von Progenitorzellen insbesondere bei ihrer Differenzierung zu Mukus-zellen und die Entstehung einer Mukuszellmetaplasie beinhalten. Zum anderen sollten die Mechanismen und Zielzellen der immunmodulatorischen Funktionen von mCLCA3 im Rah-men der unspezifischen Immunität aufgedeckt werden. Schließlich besteht die Hoffnung, dass die Ergebnisse dieser Arbeit über zwei wichtige CLCA-Moleküle sowie deren Rolle bei verheerenden Atemwegserkrankungen von Tier und Mensch zu zukünftigen Entwicklungen effektiverer Therapieformen einen Beitrag leisten können.

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7   List of Publications

Oral presentations:

- **Dietert K**, Reppe K, Mundhenk L, Witzenrath M, Gruber AD: mCLCA3 beeinflusst die Induktion der Zytokine IL-17 und CXCL-1 sowie die leukozytäre Immunantwort bei der murinen Staphylokokken-Pneumonie. 57. Annual Conference of the German Veterinary Medical Society, Section Veterinary Pathology, Fulda, Germany (08.03.-09.03.2014) Tierärztliche Praxis / Ausgabe K, Kleintiere, Heimtiere; 42(2) - S. A16; ISSN: 1434-1239

Poster presentations:

- **Dietert K**, Bothe M, Mundhenk L, Lübke-Becker A, Gruber AD: Weisen CLCA-Proteine antibakterielle Eigenschaften auf? 6th Post graduate research symposium at the Freie Universität Berlin, Berlin, Germany (01.07.2011)

- **Dietert K**, Bothe M, Mundhenk L, Kaup M, Gruber AD: Affinitätschromatografische Aufreinigung aktiver, komplexer Proteine. 7th Post graduate research symposium at the Freie Universität Berlin, Berlin, Germany (13.07.2012)

- **Dietert K**, Reppe K, Witzenrath M, Gruber AD: *Staphylococcus aureus* Newman: Establishing a mouse model of acute pneumonia. 8th Post graduate research symposium at the Freie Universität Berlin, Berlin, Germany (15.07.2013)

Research publications in scientific journals:

- **Dietert K**, Mundhenk L, Erickson NA, Reppe K, Hocke AC, Kummer W, Witzenrath M, Gruber AD (2014) Murine CLCA5 is Uniquely Expressed in Distinct Niches of Airway Epithelial Cells. Histochemistry and Cell Biology:1-11 doi:10.1007/s00418-014-1279-x

- **Dietert K**, Reppe K, Mundhenk L, Witzenrath M, Gruber AD (2014) mCLCA3 Modulates IL-17 and CXCL-1 Induction and Leukocyte Recruitment in Murine Staphylococcus aureus Pneumonia. PLoS ONE 9(7): e102606. doi:10.1371/journal.pone.0102606

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Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, 2014   Kristina Dietert