Institute of Veterinary Pathology
Department of Veterinary Medicine
Freie Universität Berlin

The CLCA1 Protein in Innate Immunity – A Mucus Barrier Component or Signaling Molecule?

Thesis submitted for the fulfillment of a
Doctor of Philosophy (PhD) degree in Biomedical Sciences
at the
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submitted by
Nancy Ann Erickson
Veterinarian from Papenburg

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Für meine Eltern

Contents

List of Abbreviations

Agr2	**A**nterior **gr**adient protein 2 homolog
alvM	**alv**eolar **M**acrophages
ANOVA	**An**alysis **O**f **Va**riance
Aoah	**A**cyl**o**xy**a**cyl **h**ydrolase
AP	**A**lkaline **P**hosphatase
B2m	**B**eta-**2 m**icroglobulin
BAL	**B**roncho**a**lveolar **L**avage
BALF	**B**roncho**a**lveolar **L**avage **F**luid
bCLCA	**b**ovine **C**h**l**oride channel regulator, **C**alcium-**a**ctivated
BMDM	**B**one **M**arrow-**D**erived **M**acrophages
BPI	**B**acterial/**P**ermeability **I**ncreasing
BPIFA1	(**BPI**) **F**old-containing protein family **A1** protein (also termed SPLUNC1 in humans)
Bpifa1$^{-/-}$	***Bpifa1***-deficient
BSA	**B**ovine **S**erum **A**lbumin
CaCC	**C**alcium-**a**ctivated **C**hloride **C**urrent
cAMP	**c**yclic **A**denosine **M**ono**p**hosphate
CBA	**C**ytometric **B**ead **A**rray
CCh	**C**arba**ch**ol
CCL	Chemokine (**C**-**C** motif) **L**igand
CCR	Chemokine (**C**-**C** motif) **R**eceptor
CF	**C**ystic **F**ibrosis
CFTR	**C**ystic **F**ibrosis **T**ransmembrane **C**onductance **R**egulator
Cftr$^{-/-}$	*Cftr*-deficient
CHO	**C**hinese **H**amster **O**vary
CLCA	**C**h**l**oride channel regulator, **C**alcium-**a**ctivated (formerly known as Chloride channel, Calcium-activated)

Clca1$^{-/-}$	***Clca1***-deficient
CM	**C**onditioned **M**edium
COPD	**C**hronic **O**bstructive **P**ulmonary **D**isease
Ct	**C**ycle **t**hreshold
CXCL-1	Chemokine (**C**-**X**-**C** motif) **L**igand
DAB	**D**i**a**mino**b**enzidine
DAI	**D**isease **A**ctivity **I**ndex
DIOS	**D**istal **I**ntestinal **O**bstruction **S**yndrome
DOG1	**D**iscovered **O**n **G**astrointestinal Stromal Tumors Protein-**1**
DSS	**D**extran **S**odium **S**ulfate
DTT	**D**i**t**hio**t**hreitol (also termed Cleland´s reagent)
e.g.	**e**xample **g**iven
E-cad	**E**-**cad**herin (also termed Cadherin-1)
EDTA	**E**thylene**d**iamine**t**etraacetic **A**cid
Ef-1α	**E**longation **f**actor-**1a**lpha
Eif2s3y	**E**ukaryotic translation **i**nitiation **f**actor **2**, **s**ubunit **3**, structural gene **Y**-linked
ELISA	**E**nzyme **L**inked **I**mmunosorbent **A**ssay
Emr4	**E**GF-like **m**odule containing, mucin-like, hormone **r**eceptor-like sequence **4**
ENaC	**E**pithelial **Na**trium (Latin for “sodium”) **C**hannel
Eotaxin-2	Eosinophil chemotactic protein 2 (also termed CCL24)
et al.	***et al****ii* (Latin for “and others”)
FACS	**F**luorescence **A**ctivated **C**ell **S**orting
FASP	**F**ilter-**a**ided **S**ample **P**reparation
FBS	**F**etal **B**ovine **S**erum
FCGBP	**Fc**-**G**amma **B**inding **P**rotein
FCS	**F**etal **C**alf **S**erum

FELASA	**F**ederation of **L**aboratory **A**nimal **S**cience **A**ssociations
Fig.	**Fig**ure
FISH	**F**luorescence-***I**n **S**itu*-**H**ybridization
FnIII	**F**ibro**n**ectin type **III**
Gapdh	**G**lycer**a**ldehyde-3-**p**hosphate **d**e**h**ydrogenase
Glu	**Glu**tamic acid
GM-CSF	**G**ranulocyte **M**acrophage **C**olony-**S**timulating **F**actor
Gob-5	**Gob**let cell protein-**5**
GPI	**G**lycosyl**p**hosphatidyl**i**nositol
GuHCl	**G**uanidinium **H**ydro**c**h**l**oride
H2-M2	**H**istocompatibility **2**, **M** region locus **2**
hCLCA	**h**uman **C**h**l**oride channel regulator, **C**alcium-**a**ctivated
HEK	**H**uman **E**mbryonal **K**idney
HEXXH	Abbreviation for the amino acids: Histidine, Glutamic acid, 2 unspecified amino acids, Histidine
His	**His**tidine
HRP	**H**orse**r**adish **P**eroxidase
HUGO	**Hu**man **G**ene N**o**menclature Committee
i.e.	***id est*** (Latin for “that is to say”)
IF	**I**mmuno**f**luorescence
IFNγ	**I**nter**f**ero**n** **g**amma
Ig	**I**mmuno**g**lobulin
IL	**I**nter**l**eukin
KC	**K**eratinocyte **C**hemoattractant (also termed CXCL-1)
kDa	**k**ilo **D**alton
Klk1	**K**a**l**li**k**rein-**1**
LCM	**L**aser **C**apture **M**icrodissection
LFQ	**L**abel-**F**ree **Q**uantification
LPS	**L**ipo**p**oly**s**accharide

LTA	**L**ipo**t**eichoic **A**cid
Lu-ECAM-1	**L**ung **E**ndothelial **C**ell **A**dhesion **M**olecule-**1** (also termed bCLCA2)
Ly6c1	**Ly**mphocyte **a**ntigen **6** complex, locus **C1**
LysC	Endoproteinase cleaving proteins on the C-terminal side of lysine residues
M	**M**olar
m/z	mass-to-charge ratio
MALDI-TOF	**M**atrix **A**ssisted **L**aser **D**esorption **I**onization-**T**ime **o**f **F**light
MAPK	**M**itogen-**A**ctivated **P**rotein **K**inase
mCLCA	**m**urine **C**h**l**oride channel regulator, **C**alcium-**a**ctivated
MCP	**M**onocyte **C**hemoattractant **P**rotein
MGNC	**M**ouse **G**ene **N**omenclature **C**ommittee
MIDAS	**M**etal **I**on–**D**ependent **A**dhesion **S**ite
MIP-2α	**M**acrophage **I**nflammatory **P**rotein **2**-**a**lpha (also termed Cxcl2)
MLN	**M**esenteric **L**ymph **N**odes
mRNA	**m**essenger **R**ibo**n**ucleic **A**cid
Muc2	murine **Muc**in **2** protein (termed MUC2 in humans)
Muc2$^{-/-}$	*Muc2*-deficient
MW	**M**olecular **W**eight
MyD88	**My**eloid **D**ifferentiation primary response **88**
n.i.	**n**ot **i**nvestigated
NanoLC-MS/MS	**Nano**-**L**iquid-Chromatography **M**ass-**S**pectrometry
Nas1	sodium-sulfate-cotransporter gene
NEA	**N**on-**E**ssential **A**mino Acids
NF	**N**eu**f**uchsin (triaminotritolyl-methanechloride)
Nhe3	sodium-hydrogen exchanger 3 gene
nl/min	**n**ormal **l**iters per **min**ute
NM	curated accession number
ODF2L	**O**uter **D**ense **F**iber of Sperm Tails **2**-**L**ike

O-glycan	mucin-type O-linked oligosaccharides
OVA	**Ova**lbumin
P	**P**seudogene
P. aeruginosa	***P**seudomonas aeruginosa*
PBS	**P**hosphate-**B**uffered **S**aline
pcDNA	**pcDNA**3.1+ vector
PLUNC	**P**alate, **Lu**ng, and **N**asal Epithelium **C**lone Protein
PRIDE	**PR**oteomics **IDE**ntifications
Rag	**R**ecombination-**a**ctivating **g**ene
RANTES	**R**egulated on **A**ctivation, **N**ormal **T** Cell **E**xpressed and **S**ecreted (also termed CCL5)
RGD	**R**at **G**enome **D**atabase
RNA	**R**ibo**n**ucleic **A**cid
RT-qPCR	**R**everse **T**ranscriptase **q**uantitative **P**olymerase **C**hain **R**eaction
S. aureus	***S**taphylococcus aureus*
S. pneumoniae	***S**treptococcus pneumoniae*
SCFA	**S**hort-**C**hain **F**atty **A**cids
Scid	**S**evere **c**ombined **i**mmuno**d**eficiency
SDS-PAGE	**S**odium **D**odecyl **S**ulfate **P**olyacrylamide **G**el **E**lectrophoresis
SEM	**S**tandard **E**rror of the **M**ean
SH3GLB1	**SH3**-Domain **G**RB2-**L**ike Endophilin **B1**
SPLUNC1	**S**hort **P**alate, **Lu**ng, and **N**asal Epithelium **C**lone **1**
spp.	***sp**ecies **p**luralis* (Latin for “multiple species”)
STAT6	**S**ignal **T**ransducer and **A**ctivator of **T**ranscription **6**
Tff	**T**re**f**oil **f**actor
Th	**T h**elper
TLR	**T**oll-**L**ike **R**eceptor
TMEM16A	**T**rans**mem**brane protein **16A** (also termed Anoctamin1/DOG1)
TNBS	2,4,6-**T**ri**n**itro**b**enzene **S**ulfonic Acid

TNF-α	**T**umor **N**ecrosis **F**actor-**a**lpha
Tris	**Tris**(hydroxymethyl)aminomethane
UC	**U**lcerative **C**olitis
VLE DMEM	**V**ery **L**ow **E**ndotoxin **D**ulbecco´s **M**odified **E**agle´s **M**edium
vs.	***ver**s**us*** (Latin for “against”)
VWA	**V**on **W**illebrand factor type **A**
VWD	**V**on **W**illebrand factor type **D**
WT	**W**ild **T**ype
Xaa	Unspecified amino acid
Zg16	**Z**ymogen **g**ranule protein **16**
16S rRNA	**16S r**ibosomal **R**ibo**n**ucleic **A**cid

1 Introduction

The chloride channel regulator, calcium-activated (CLCA) proteins have been an intriguing subject to research for over 25 years. To date, up to eight highly conserved members have been discovered in at least 30 species and their tissue and cellular expression pattern is largely known. Certain members have been linked to inflammatory diseases. In particular, CLCA1, which is secreted by mucus-producing cells e.g. of the respiratory and intestinal tract, is differentially expressed in respiratory diseases characterized by mucus overproduction and is discussed as a potential therapeutic target, e.g. modulating disease severity in mucoviscidosis (cystic fibrosis, CF), asthma, or chronic obstructive pulmonary disease (COPD). Currently, the two most promising and substantiated hypothetic functions of CLCA1 include it being a structure-associated component of the mucus barrier or a modulator of cytokine expression. However, its specific physiologic and pathophysiologic functions are far from being resolved.

1.1 The CLCA Family – Background

The first two CLCA founding members were simultaneously discovered by two independent working groups in 1991 [1, 2]. Whilst analyzing the biochemical basis of chloride channel function, Ran and Benos isolated a CLCA member from bovine apical tracheal membranes, which mediated a calcium-dependent chloride conductance *in vitro* and named it bovine tracheal chloride channel [1, 3]. This induction of a calcium-activated chloride current (CaCC) was later also shown for further CLCA isoforms, including CLCA1, across different species in Human Embryonic Kidney (HEK) cell culture [4-7]. Hence, it was initially thought that these proteins function as a chloride channel *per se*. The second founding member was localized to bovine endothelial cells of distinct pulmonary blood vessels and promoted the selective attachment of lung-metastatic melanoma cells in a murine model [2]. Therefore, this member was initially named bovine lung endothelial cell adhesion molecule-1 (Lu-ECAM-1) [2].

The nucleotide and amino acid sequences of the two bovine founding members showed a high conformity of 92 % [8], by which a novel protein family was established. In the course of time, more members of this family were discovered in, to date, at least 30 different species with up to eight species-specific numbers of homologs [9, 10]. In the light of various putative functions, time point of discovery, and species-specific number, the nomenclature has grown increasingly confusing. In a first attempt toward nomenclature standardization, this protein family was originally termed “chloride channels, calcium activated” and the two founding members, bovine tracheal chloride channel and bovine Lu-ECAM-1 were renamed bCLCA1 and bCLCA2, respectively [11]. Accordingly, newly discovered CLCA members were named

after the species in which they are present, such as b = bovine, h = human, m = murine, p = porcine, and numbered according to their chronological discovery.

However, more recent *in silico* analyses did not predict the presence of several transmembrane domains [12] as expected for a channel protein. Even more so, CLCA proteins are entirely or partially secreted into the extracellular environment [12-18]. Hence, these proteins do not form anion channels *per se*, but may rather function as soluble signaling molecules [12, 13]. Therefore, the abbreviation of CLCA was changed to “chloride channel regulator, calcium-activated”. The nomenclature, however, was still confusing as direct orthologs, such as the human CLCA1 and the murine CLCA3, were numbered differently from another and, hence, were not readily associable. Due to this inconsistency, the Mouse Gene Nomenclature Committee (MGNC) renamed the *Clca* genes due to realignment with respect to the human nomenclature in accordance with the Human Gene Nomenclature Committee (HUGO) and the Rat Genome Database (RGD) (see Table 1). The new CLCA nomenclature was published by Erickson *et al.* [19], which is part of this cumulative thesis.

Table 1. Revised murine CLCA nomenclature

Human symbol	New mouse symbol	Previously used mouse symbols	Gene bank accession number
CLCA1	**Clca1**	**mClca3, gob-5**	**NM 017474**
CLCA2	**Clca2**	**mClca5**	**NM 178697**
CLCA3P	**Clca3a1**	**mClca1**	**NM 009899**
	Clca3a2	**mClca2**	**NM 030601**
	Clca3b	**mClca4**	**NM 139148**
CLCA4	**Clca4a**	**mClca6**	**NM 207208**
	Clca4b	**mClca7 (AI747448)**	**NM 0001033199**
	Clca4c	**mClca8 (Gm6289, EG622193, A730041H10Rik)**	**NM 001039222**

Source: MGNC, Erickson *et al.* [19]; P = pseudogene; NM = curated accession number; gob-5 = goblet cell protein-5

The *Clca* genes are located in a conserved and dense single locus between the two flanking genes, *outer dense fiber of sperm tails 2-like* (*ODF2L*) and *SH3-domain GRB2-like endophilin B1* (*SH3GLB1*) [9]. Nonetheless, the number of *CLCA* genes differs between species, e.g. eight in mice, four in humans, and five in pigs, which are grouped in four different clusters [20]. In humans, each cluster is comprised of only one *CLCA* member. Also, for clusters 1 and 2, only one member exists in every species investigated so far. In contrast, the number of *CLCA* members differs species-specifically in clusters 3 and 4, e.g. mice exhibit three members each whilst the pig has one and two, respectively [9, 10, 21].

In addition to the genomic organization, the CLCA proteins had been intensively analyzed *in silico* and *in vitro*. The most striking feature of all CLCA proteins is the posttranslational cleavage of the primary glycosylated translation product into a larger amino-terminal and a smaller carboxy-terminal subunit [9, 11]. The cleavage site of one bovine CLCA member, formerly known as Lu-ECAM-1, and the murine CLCA1, alias mCLCA3 or gob-5, had been experimentally determined via matrix assisted laser desorption ionization-time of flight (MALDI-TOF) and/or Edman-degradation [8, 22]. This site seems to be conserved in all CLCA proteins [9].

Depending on the homolog, the cleaved subunits are either fully secreted as a soluble heterodimer, e.g. the human and murine CLCA1 [9, 12, 13, 20], or only the amino-terminal subunit is secreted, whilst the carboxy-terminal remains associated with the plasma membrane. This anchoring can be conferred either by a transmembrane domain, as in human CLCA2 [14] or murine CLCA2, 4a, and b - previously termed mCLCA5 [17], mCLCA6 [15], and mCLCA7 [9], respectively - or via a glycosylphosphatidylinositol (GPI) anchor, as in human CLCA4 [9]. This single carboxy-terminal anchoring region, however, lacks the structural requirements of an ion channel [12].

A cysteine-rich domain was identified in the amino-terminal part of all CLCA proteins, which is uniquely conserved in this family [11]. This region contains a zinc-dependent metalloproteinase domain [23], which seems to be responsible for the posttranslational autocatalytic cleavage of the precursor protein as was experimentally shown for the murine CLCA1 [22]. Directly adjacent to this cysteine-rich domain, a von Willebrand factor type A (VWA) domain is located. VWA domains are generally known to mediate protein-protein interactions [24] via a metal ion–dependent adhesion site (MIDAS). The VWA domains of CLCA proteins contain an intact MIDAS, except for CLCA2 [25]. Instead, the latter has a β4-integrin binding motif [26-28], which, however, can additionally be found in other CLCA members. The carboxy-terminal CLCA domain includes a fibronectin type III (FnIII) domain, which is a protein-protein interaction module of the immunoglobulin (Ig) superfamily [9]. As their amino acid sequence also contains known protein-protein interaction motifs, it is

tempting to speculate that CLCA proteins could interact with other proteins, such as in terms of a signaling function [25].

The tissue expression pattern of nearly all human and murine CLCA members had been investigated at least on the messenger ribonucleic acid (mRNA) level using whole tissue samples (reviewed in [9]). The cellular expression of certain human and murine CLCA members is largely known. CLCA2, for example, is expressed in cells of stratified squamous epithelia [17, 29] and unique niches of the respiratory tract [30] with species-specific differences between humans and mice [30] whilst murine CLCA4a was located in the apical surface of non-mucus cell enterocytes [15], but the cellular expression pattern of its human ortholog is still unknown.

The murine CLCA1 protein is expressed in mucus-producing cells of the respiratory, gastrointestinal, and reproductive tract [31]. A virtually identical expression pattern had been reported for its human [6], equine [32], and porcine ortholog [20]. Other secretory tissues containing mucus cells, including gallbladder, kidney, pancreas, sublingual salivary glands, oviduct, mammary gland, and prostate are devoid of CLCA1 in the mouse [31]. Both CLCA1 cleavage products are secreted extracellularly [12, 13, 20, 32] and the protein had been found to be associated with the mucus layer lining the intestinal epithelium [31].

Its most abundant expression is located in the colon of humans and mice [6, 31, 33]. CLCA1 was, however, not detectable in specific subsets of intestinal mucin-producing cells, including mucus cells at crypt base. Interestingly, goblet cells are made up of two distinct populations, one migrating downwards toward the crypt base and undergoing apoptosis, the other migrating upwards to the lumen and sloughing off [34]. CLCA1 has been shown to be exclusively associated with the latter in the colon [31].

In the respiratory tract, human CLCA1 and its murine and porcine orthologs are also selectively expressed in mucus cells and secreted extracellularly [12, 13, 20, 31]. Specifically, human and murine CLCA1 were found in mucus cells of the trachea and major bronchi as well as, to a lesser degree, in submucosal glands of the upper trachea [31, 35-39], whilst its porcine homolog was also found in submucosal glands of the upper respiratory tract [20, 40].

Apart from this expression pattern under steady state conditions, CLCA1 was repeatedly found to be differentially expressed on the mRNA and protein level in significant human respiratory diseases such as in CF, asthma, or COPD [9, 35, 41-44] and their corresponding mouse models [9, 36, 38, 42]. Due to its possible relevance in these diseases, CLCA1 came into focus of biomedical research and *Clca1*-deficient (*Clca1*$^{-/-}$) mouse models based on different exon-specific knockouts, i.e. knockout of exon 7 [45], exons 7 and 8 [46], and exons

7 to 11 [25] were established. CLCA1 is the most intensively studied CLCA family member to date.

1.2 CLCA1 and its Significance in Disease

Increased expression of CLCA1 has consistently been associated with inflammatory conditions such as asthma, COPD, or CF [9, 12, 36-38, 47-49], in which its expression exceeded that of other mediators of inflammation by far [35, 44]. These diseases are predominantly characterized by mucus cell meta- and hyperplasia, mucus overproduction, and disturbed clearance.

Specifically, expression levels of human CLCA1 mRNA and protein were significantly increased in the airway epithelium of asthma patients [37, 38, 48, 49] and CLCA1 mRNA levels decreased after treatment with inhaled glucocorticoids [48]. Coinciding, high amounts of CLCA1 were also present in the bronchoalveolar lavage fluid (BALF) of asthma [12] and CF patients [36-38, 47-49]. In the latter, CLCA1 upregulation in the bronchial mucosa was also associated with mucus overproduction [36, 47]. Furthermore, human CLCA1 mRNA and protein, beside human CLCA2 and 4, was predominantly overexpressed in lung samples of COPD patients [9, 49]. Thus, expression of CLCA1 may serve as a useful biomarker of inflammatory airway disease [9].

Asthma arises from multiple genetic and environmental factors [50, 51] and has a high prevalence with about 330 million people affected worldwide and its incidence and its severity are continuously increasing [52]. This reversible airway obstruction is characterized by airway hyperresponsiveness, bronchial inflammation, and mucus overproduction [53, 54].

Similarly to asthma, COPD is a widespread disease. The World Health Organization predicts that COPD will become the third leading cause of death worldwide by 2030 with currently more than 64 million people affected [55]. Patients suffering from COPD experience airflow limitation, mucus overproduction, and enhanced chronic inflammatory response due to noxious particles or gases [56-58].

In contrast to asthma and COPD, CF is an autosomal recessive disorder and the most common lethal genetic disease [59] with a carrier rate of ~ 5 % in Caucasians [52]. Underlying causes are mutations in the *cystic fibrosis transmembrane conductance regulator* (*Cftr*) gene, rendering its product, a cyclic adenosine monophosphate (cAMP)-activated chloride channel, defective [60]. Consecutive clinical manifestations are primarily due to dehydration of liquid layers lining epithelial surfaces with their respective onset varying with the patient's age [61]. Meconium ileus may occur within two days after birth and an intestinal obstruction is seen in ~ 15 % of CF infants [60-63]. It may be followed by pancreatic

insufficiency, distal intestinal obstructive syndrome (DIOS), respiratory disease, liver disease, and infertility [61-63]. Respiratory disease is due to mucus plugging with impaired clearance and secondary bacterial infection leading to chronic airway inflammation occurring months to years after birth, which is the major cause of CF morbidity and mortality [41, 60, 63-67]. Disease severity seems to be modulated by several factors, including CaCCs [68-71]. This alternative chloride conductance may partially compensate for loss of CFTR function and is thought to regulate mucus hydration together with CFTR [68-72].

In addition to the significant overexpression of CLCA1, several genetic studies have associated the molecule with it possibly modulating the severity of these diseases. Genomewide linkage analyses of COPD patients, for example, showed that the degree of airflow obstruction may be linked to genetic changes in a region of human chromosome 1 containing the CLCA family locus [73]. In detail, haplotype analyses of single-nucleotide polymorphisms suggest that human *CLCA1* gene variations are linked to asthma and COPD susceptibility in the Japanese population [42, 43].

Studies on genetic variations in the *CLCA* gene locus showed that certain *CLCA* gene family members, e.g. CLCA1 and 4, seem to modulate the residual gastrointestinal chloride current and hence, the phenotype, in a certain subset of CF patients with mild disease [74]. Specific allelic variants of the human *CLCA1* gene had been identified in a subset of CF patients with aggravated intestinal disease [75], whilst variants of the CLCA1-homolog *CLCA4* determine the manifestation of the CF basic defect by modulating the capability to express residual chloride secretion in colonic tissue [76].

In order to genetically segregate the influence of CLCA proteins on the two main disease characteristics of asthma and COPD - mucus cell metaplasia and airway hyperreactivity - a mouse model of Sendai virus-induced bronchiolitis was established on differently susceptible genetic backgrounds [25]. This study linked CLCA1 exclusively to mucus cell metaplasia, but not to airway hyperreactivity.

A modulation of the diseases by CLCA1 had also been experimentally investigated in mouse models of asthma and CF. Murine CLCA1 plasmid transfected to the airways of healthy mice induced asthmatic airway inflammation independently of an allergen exposure [77]. CLCA1 was highly present in the BALF of an ovalbumin (OVA)-induced mouse model of bronchial asthma, and its overexpression in the airway epithelium was sufficient to cause an asthma phenotype including mucus cell metaplasia [78], which was ameliorated by intratracheal administration of CLCA1 antisense RNA [78] or via antibody-treatment [79]. Particularly, treatment of transgenic mice overexpressing the T helper (Th) 2 cytokine interleukin (IL) 9, which develop a human asthma-like phenotype, with neutralizing IL-9 antibody suppressed

CLCA1 mRNA expression, whereas IL-9 treatment of wild type (WT) mice enhanced its expression [35]. Also, a significant increase of equine CLCA1 mRNA and protein was present in horses with recurrent airway obstruction, which shares characteristic features with human asthma and COPD [18, 32].

In mouse models of cystic fibrosis, *Cftr*-deficient (*Cftr*$^{-/-}$) mice exhibit severe intestinal lesions - particularly mucus overproduction and accumulation - similarly to meconium ileus in CF patients [80-82]. A marked reduction of CLCA1 protein expression was identified in this model [41], and restoration of the CLCA1 protein led to an amelioration of the intestinal phenotype [83]. These findings suggest that a physiological level of CLCA activity is necessary for normal mucus cell function [9].

It is therefore established that CLCA1 is overexpressed in and modulates the severity of these complex diseases with mucus overproduction. However, its pathophysiological role and the pathways in which it interacts are unknown to date.

1.3 Functions Hypothesized for CLCA1

Originally, CLCA proteins were thought to act as chloride channels, since certain CLCA members evoke a chloride current upon calcium addition *in vitro* [1-7]. Anyhow, it was later shown that CLCA proteins lack multiple transmembrane domains necessary for genuine channel formation [12], and its physiological and pathophysiological function is far from being resolved. It has been hypothesized that CLCA1 - the member investigated in most detail so far – functions as a structure-associated component of the mucus barrier or as a modulator of cytokine expression. Other functions, such as acting as a zinc-dependent metalloproteinase, modulating CaCCs, or driving mucus cell metaplasia have also been discussed. Since the latter three hypothesized functions of CLCA1 may only indirectly influence the mucus structure, these are discussed separately from it perhaps acting as a structure-associated mucus barrier component *per se*.

1.3.1 CLCA1 as a Structure-associated Component of the Mucus Barrier

Numerous observations have led to the hypothesis that CLCA1 may play a significant role as a fundamental component of the mucus barrier, particularly in the intestinal tract [12, 13, 31, 33, 84]. However, one must hereby carefully differentiate between two putative distinct functions of CLCA1, firstly as structure-forming component itself (as described here) or secondly as a key player in mucus structure modulation (see also 1.3.2 to 1.3.4).

CLCA1 is secreted by mucus-producing cells throughout the body [31]. It is most highly expressed in the colon [31], in which both cleavage products are secreted into the mucus layer [12, 13, 33]. Here, CLCA1 represents the most abundant protein alongside the main structural component of the protective colonic mucus, the murine mucin 2 (Muc2, termed MUC2 in humans) protein [33]. Both proteins have a conspicuous overlapping expression profile (Fig. 1, representative images of distal colon) with similar abundancy along the gastro-intestinal axis [84]. Specifically, the duodenum and ileum contain larger amounts of both proteins in contrast to the stomach and jejunum. Anyhow, their most abundant expression is located in the colon with a distinct increase from proximal to distal [33, 84]. Consequently, it was hypothesized that CLCA1 may function as a structural component of the intestinal mucus barrier [33, 84] and hence, may also have an impact on its protective function of the intestinal epithelium.

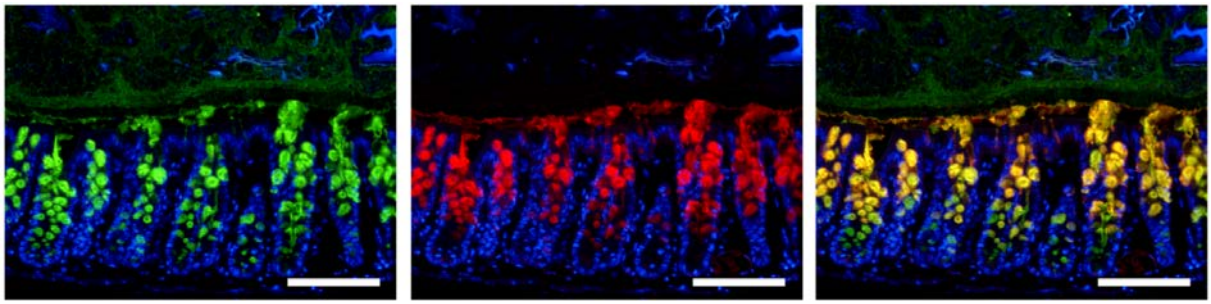

Fig. 1 Expression patterns of intra- and extracellular Muc2 (green) and CLCA1 (red) in WT mice in distal colon with co-expression being confirmed by co-staining (yellow, merge). Bars = 20 µm. Fig. from Erickson *et al.* [19]

Besides CLCA1, the Fc-gamma binding protein (FCGBP) is another major protein secreted by mucus cells. Interestingly, FCGBP shows a structural parallel to CLCA1 in terms of the presence of von Willebrand domains. FCGBP is not an immunoglobulin-binding protein [85]. Instead, it can covalently bind and cross-link mucus proteins via its autocatalytically cleaved von Willebrand D domains [86], which are also present in MUC2 [87] and to which binding of FCGBP has been confirmed [86]. Besides these similarities and putative interactions between CLCA1 or Muc2 and FCGBP, the MIDAS and FnIII domain would also enable the interaction with other proteins [23, 24].

Apart from these structural prerequisites, further findings also point toward the proposed role of CLCA1 as an extracellular structure-modulating protein, i.e. in terms of an enzymatic function as zinc-dependent metallohydrolase (further referred to in paragraph 1.3.2), in terms of liquid homeostasis modulation as a CaCC-regulator (further referred to in paragraph 1.3.3) or driving mucus cell metaplasia (further referred to in paragraph 1.3.4).

However, to date, no experimental analysis of the functional role of CLCA1 in mucus barrier structure, formation, and integrity has been performed and which has been addressed in this work for the first time.

1.3.2 CLCA1 as a Zinc-dependent Metalloproteinase

A further possible function of CLCA1 came into play when a HEXXH zinc-binding amino acid motif was discovered *in silico* in CLCA proteins [23]. This HEXXH motif, consisting of the amino acids His-Glu-Xaa-Xaa-His [88], is suggestive of a metalloprotease [23], which typically confers zinc-dependent cleavage [89]. Consecutive MALDI-TOF and Edman-degradation analysis of murine CLCA1 protein fragments identified a single cleavage site in CLCA1 between amino acids 695 and 696 in the carboxy-terminal region [22]. Interestingly, this consensus cleavage motif, which is contained in all CLCA proteins [9], is hypothesized to be recognized by the zincin metalloprotease domain located within the amino-terminus of the CLCA protein itself, pointing toward a self-cleavage mechanism (see 1.1). The zinc-dependency of the self-cleavage event was further proven by cleavage abrogation using cation-chelating metalloproteinase inhibitors [22].

In order to support this self-cleavage hypothesis, an E157Q mutation, which is a common molecular manipulation to study the enzymatic function of a HEXXH motif, was introduced into this zinc-binding amino acid motif of human CLCA1 and its murine and porcine orthologs and was capable of abrogating their cleavage [22, 23]. This furthermore suggests a universal and evolutionarily conserved event due to its presence in many species, whilst post-translational glycosylation, cellular transport or secretion were not impaired [22]. After co-transfection with WT murine CLCA1 in HEK293 cells, however, the CLCA1E157Q mutant was cleaved despite its own cleavage-deficiency, pointing toward cross-proteolytic capability [22], as also shown for other proteases [90].

These findings support the notion of CLCA1 acting as a zinc-dependent metallohydrolase, which is known to alter mucus properties [83, 91]. Since CLCA1 is stored in mucin granules and secreted together with the mucus, it may directly alter mucus properties enzymatically, i.e. its viscosity or rheology or the protein processing [83]. It is, however, unknown to date if CLCA1 has other substrates besides itself. It remains to be investigated if extracellular mucin glycoproteins or other components of the mucus layer are enzymatically processed by CLCA1 [22].

1.3.3 CLCA1 as a Modulator of Calcium-activated Chloride Currents (CaCCs)

The observations that CLCA1 is a fully secreted protein devoid of transmembrane domains disproved the initial hypothesis of it being a chloride channel *per se* [12, 13]. However, the protein seems to regulate other chloride channels, which would explain the occurrence of a chloride conductance after transfection. Porcine CLCA1 was shown to enhance cAMP-activated chloride conductance in cell culture [92, 93] with subsequent electrophysiological studies pointing toward an indirect activation of the chloride secretory pathway [94]. Human CLCA1 also enhanced the conductance of endogenous CaCCs by lowering the energy barriers for ion translocation through the pore [95]. This non-CFTR-mediated, alternative CaCC is of biomedical importance as it is thought to regulate hydration of the airway mucus layer together with CFTR [72]. It has therefore been discussed as an important modulator of disease, at least partially compensating for defective CFTR function, and as a therapeutic target in CF patients and murine CF models [68-71]. In detail, the carboxy-terminal subunit of CLCA1 was found to be released upon cleavage, unmasking the amino-terminal fragment, which then independently gates calcium-activated chloride channel-mediated chloride transport [96]. Hence, the self-cleavage process is required for calcium-activated chloride channel activation and mediated solely through the amino-terminus. This mechanism has been verified by mutations inhibiting self-cleavage and calcium-activated chloride channel activation simultaneously [96].

Transmembrane protein 16A (TMEM16A, also known as Anoctamin1/ Discovered On Gastrointestinal Stromal Tumors Protein-1 (DOG1)) was recently discovered as the first genuine calcium-activated chloride channel in mammals by three independent groups [97-99] and further identified as a molecular candidate for CLCA1-mediated CaCC. In particular, CLCA1 modulated TMEM16A to activate CaCCs in a paracrine and self-cleavage–dependent fashion [100] by directly engaging with the channel at the cell surface via its VWA domain, which was necessary and sufficient for this interaction [101]. Hence, CLCA1 may be a potential therapeutic target due to its selective VWA-mediated activation of TMEM16A.

Similar to CLCA1, TMEM16A expression was also found to be significantly increased by IL-13 and IL-4 in *in vitro* models of chronic inflammatory airway disease [97, 102], and its overexpression was also linked to mucus cell metaplasia and airway hyperreactivity [103, 104]. In addition, TMEM16A-specific inhibitors decrease mucus secretion and airway hyperreactivity *in vitro* [103], which may implicate that these CLCA1-related findings may be downstream effects of TMEM16A. As a possible explanation, CLCA1 may increase the TMEM16A protein level at the cell surface without increasing protein expression, either by dimerization of monomeric TMEM16A or by stabilizing dimeric TMEM16A, thereby preventing its removal and, consequently, increasing calcium-dependent chloride currents,

which may represent a novel mechanism of channel regulation by a secreted protein [100]. Hence, TMEM16A may be the first downstream target of CLCA proteins to be found [100].

In the mouse, however, tracheal instillation of IL-13 resulted in overexpression of CLCA1, but failed to induce CaCC activity [105]. Moreover, the CaCC was unchanged in the airways of *Clca1*$^{-/-}$ mice after IL-13 stimulus, which induced goblet cell hyperplasia as well as mucin gene expression to similar levels in both *Clca1*$^{-/-}$ and WT mice. Similarly, under naive conditions, the lack of murine CLCA1 did not reveal any bioelectric differences in basal currents, sodium absorption, calcium-activated chloride secretion, nor did it entail any histologic differences in respiratory or other tissues. It therefore seems that, in contrast to human airways, CLCA1 may not play a role in CaCC-mediated chloride secretion in the mouse [105]. This observation of CLCA1 not contributing to chloride secretion may also be translated to the intestinal tract, in which restoration of decreased CLCA1 protein levels in a mouse model of CF has been shown to ameliorate intestinal mucus plugging, which is a common trait of human and murine CF [41, 83], which, however, may not be due to CLCA1 rectifying the CF electrophysiology defect in the intestine or by it activating CaCCs, but due to CLCA1-induced mucus property alteration [83].

Nonetheless, compensation for lack of CLCA1 by other murine CLCA members in the respiratory tract of *Clca1*$^{-/-}$ mice cannot be ruled out. Although no differential expression of other genes compensating for the lack of CLCA1 in *Clca1*$^{-/-}$ respiratory tracts had been found [105], it cannot be excluded that other CLCA members may compensate at physiologic levels.

In summary, CLCA1, which was originally thought to be a calcium-activated chloride channel [6], is now recognized as a chloride channel modulator, in particular of CaCC, by which, for example, it could consecutively regulate mucus volume, composition, and viscosity. However, species-specific discrepancies, which complicate the translation of animal models to human disease [10], demand a detailed functional investigation for every species.

1.3.4 CLCA1 as a Driver in Mucus Cell Metaplasia

Experiments in cell models revealed first evidences that CLCA may play a key role in the induction of mucin expression. Experimental overexpression of human or murine CLCA1 in the mucoepidermoid cell line NCI-H292 as well as in OVA- [78] or IL-9-challenged mice [35] led to a significant increase in mucin production and expression of MUC family members or mucus cell metaplasia [35, 78], respectively. This CLCA1 overexpression aggravated the asthma phenotype, characterized by both airway hyperreactivity and mucus overproduction, which was abrogated by administration of a full-length CLCA1 antisense oligonucleotide [78,

79]. In order to segregate these two phenotypical traits, the CLCA1 gene was transferred to mouse airway epithelium *in vivo* by an Adenoviral-associated virus vector and was sufficient to induce mucus cell metaplasia, but not airway hyperreactivity [25].

Recently, first insights into underlying pathways of mucin gene induction were revealed. It could be shown that the secreted human CLCA1 protein stimulated an increase in mucus production, particularly of MUC5AC, by initiating a mitogen-activated protein kinase (MAPK) signaling pathway. *In vitro*, CLCA protein expression is mainly driven by Th2 cytokines, particularly by IL-13 [35, 106-108]. It has been hypothesized that a primary environmental stimulus (e.g., an allergen or virus) triggers IL-13 production in human airway epithelial cells, which then initiates a signal transducer and activator of transcription 6 (STAT6)-CLCA1-MAPK13-signaling pathway, leading to MUC5AC expression [102]. Anyhow, the receptor mediating the CLCA1 signaling is unknown to date. Concluding, CLCA1 may act as a key regulator of mucus cell metaplasia in inflammatory airway disease by transforming airway mucus precursor cells to mature mucus cells and thereby regulating and driving mucus cell metaplasia [9].

Mouse models, however, failed to mirror the human IL-13-dependency of CLCA1-mediated airway mucus production [102]. More importantly, *Clca1*$^{-/-}$ mice showed the same degree of mucus cell metaplasia as WT mice [25], even after intratracheal IL-13 induction [105]. This species-specific difference may be due to compensatory effects by other murine CLCA members - particularly of CLCA 2 and 4a - which are also expressed in airway tissue [9, 25, 102] and which were shown to be sufficient to produce mucus cell metaplasia [25].

Concluding, CLCA1 was shown to be associated with mucus production and mucus cell metaplasia, again showing species-specific discrepancies specifically concerning the IL-13-dependency of human CLCA1-mediated airway mucus production, which could not be proven in the mouse.

1.3.5 CLCA1 as a Modulator of Cytokine Expression

Recent *in vivo* studies using *Clca1*$^{-/-}$ mice showed a differential cytokine expression after certain respiratory stimuli. Lipopolysaccharide (LPS)-challenged *Clca1*$^{-/-}$ mice exhibited significantly greater levels of murine chemokine (C-X-C motif) ligand CXCL-1 (alias KC, keratinocyte chemoattractant), a potent neutrophil chemoattractant, which significantly increased neutrophilic inflammation in BAL, but less perivascular inflammation in lung tissue than WT mice [45]. An identical cellular reaction was also observed in an OVA-model of *Clca1*$^{-/-}$ mice [45]. LPS-treated WT mice additionally showed enhanced CLCA1 protein levels [45]. Coinciding, OVA-challenge of WT mice revealed a strong upregulation of CLCA1 mRNA

and protein expression in lung tissue in tight correlation with upregulated chemokine (C-C motif) ligand (CCL) 17 and RANTES (regulated on activation, normal T cell expressed and secreted; also known as CCL5) expression. Consecutively, inflammatory cell infiltrates, especially of lymphocytes and eosinophils, were significantly increased in the corresponding BALF [109]. These findings suggest that CLCA1 may play a role in cytokine regulation of the innate immune response.

The induction of *Staphylococcus* (*S.*) *aureus* pneumonia in *Clca1*$^{-/-}$ mice also led to differential Cxcl-1 expression [110]. However, in contrast to OVA- and LPS-challenge, this challenge resulted in decreased Cxcl-1 and Cxcl-2 (alias MIP-2α, macrophage inflammatory protein 2-alpha) responses with decreased neutrophil recruitment and reduced expression of the pro-inflammatory cytokine Il-17 [110]. These seemingly contradictory results may be due to CLCA1-mediated cytokine modulation being dependent on the stimulus used - acute bacterial infection with intact, Gram-positive bacteria *versus* (*vs.*) partial virulence factors of Gram-negative bacteria such as LPS – or the different mouse strains used - C57BL/6J *vs.* 129SvEvBrd [110].

Preliminary *in vitro* studies showed that the human CLCA1 induced an upregulation of IL-8, the human ortholog to the murine CXCL-1 and CXCL-2, as well as of IL-6, IL-1β, and tumor necrosis factor (TNF)-α in primary porcine alveolar macrophages and in a human monocyte cell line U-937, which had been artificially differentiated into airway macrophage-like cells [111].

In conclusion, CLCA1 seems to act on macrophages as a signaling molecule to induce a pro-inflammatory cytokine response, which may be responsible for the cytokine-based phenotype observed in *in vivo* studies.

1.4 The Intestinal Mucus Barrier

CLCA1 has been intensively studied in the respiratory tract [9, 25, 37, 45, 78, 102, 105, 110]. Nonetheless, it is expressed in much higher amounts in the colonic mucus of humans and mice compared to the airway mucus [6, 31, 33]. Since mucus composition and structure are tissue-specific, the intestinal mucus barrier will be described in more detail as the model to investigate the function of CLCA1 in this study.

The epithelium of the gastrointestinal tract is made up predominantly of only a single layer of epithelial cells with an extracellular mucus layer protecting it against the environment. This intestinal mucus barrier lining these epithelial surfaces is an essential component and first

line of defense of innate immunity and has become of increasing interest in recent research, particularly of mucus-based diseases [112, 113].

The maintenance of its proper structure and function is essential in sustaining homeostasis and health and there is strong evidence of mucus layer disruption contributing to the pathogenesis of ulcerative colitis (UC) [114]. Furthermore, experimentally altered mucus properties consistently induce colitis in mice due to intestinal mucus barrier disintegration [114-116].

The healthy intestinal mucus layer is mainly comprised of mucins, which are large, highly glycosylated proteins [117]. Glycans protect the central mucin domains - the protein core - from proteolytic degradation and bind high amounts of water, rendering them gel-like [112] and thereby creating a dynamic and motile diffusion barrier. Mucins can be subdivided into two main groups, the transmembrane and the gel-forming mucins. Transmembrane mucins such as Muc1, -3, and -4 are produced by intestinal epithelial cells, form the glycocalyx on microvilli of enterocytes [118-120], and may be involved in sensing and regulating the local milieu at the enterocyte surface. Gel-forming mucins such as Muc2 originate from goblet cells and form large polymers for protection and lubrication [86, 121-124].

Muc2 is the main structural intestinal mucus component. It is a highly glycosylated, multimerizing protein, which creates net-like complexes with other secreted proteins and forms two distinct mucus layers exclusively in the colon. The inner, stratified, and adherent mucus layer [33, 86, 125, 126] acts as a protective gel, providing an effective and, during steady-state conditions, impermeable barrier against the commensal colonic bacteria [33, 112].

Proteolytic cleavage within the Muc2 network converts the inner layer at about 50 µm distance from the murine colonic epithelium into a fully soluble outer layer four times its volume, in which bacteria reside and which is held together by cysteine disulphide bonds [33]. This cleavage is controlled by the host itself and not by gut bacteria, as germ-free mice may also exhibit an outer mucus layer [33]. Interestingly, the small and large intestinal mucus structures fundamentally differ from each other, despite both being composed of Muc2, which, hence, behaves differently in the small and large intestine [33, 119, 127]. The small intestine exhibits only a single, non-adherent mucus layer, which is porous for nutrient absorption and, hence, resembles the colonic outer mucus layer [33, 119, 127]. Therefore, the protective mechanisms must also diverge. The large intestine´s distal propulsion with a high turnover rate of the inner mucus layer of once per hour is the main protective mechanism [128]. In contrast, the small intestine is kept sterile by the continuous mucus flow and, even more importantly, by high concentrations of antibacterial peptides or proteins such

as IgA and defensins [129-132], which are, though to a lesser degree, also present in the colon. CLCA1 was found to be the second most abundant protein in both colonic mucus layers showing the same trend of Muc2 expression [33, 84].

The colonic mucus layer represents a selective microbial habitat, which only specific bacteria bind to via bacteria-specific lectins and glycosidases [133, 134]. Besides their main digestive function, the intestinal microbiota proteolytically degrade mucins to produce short-chain fatty acids (SCFA), which are an essential nutritional component for the underlying epithelium [135]. They also have major effects on mucus thickness and composition, with certain types of bacteria possessing mucus-stimulating properties [136] and providing colonization resistance, immune system education, and tolerance. Germ-free mice, which are devoid of any microbiota, are known to have fewer goblet cells, a thinner mucus layer, and also a higher percentage of neutral mucins in the colon [137]. Furthermore, mice with different genetic backgrounds or originating from different animal facilities differ in their susceptibility to experimental colitis induction via dextran sodium sulfate (DSS) [138-140], most likely due to variation in the bacterial flora [123]. Interestingly, stimulation with bacterial products such as LPS already suffices to establish normal mucus properties in germ-free mice [141], the exact mechanism, however, being unclear to date. Notably, *Muc2*-deficient (*Muc2*$^{-/-}$) mice experience bacterial overgrowth and develop spontaneous colitis, which is ameliorated by antibiotics [142-144], pointing towards a reciprocal interaction between the mucus layer and the gut microbiota [145]. It remains unclear if the onset of inflammatory disease depends on selectively altered and thus more colitogenic microbiota, on mislocalization of the same microbiota or merely on increased bacterial load [145]. In conclusion, the intestinal microbiota is a sculpting component of the mucus and is a modifier of intestinal disease. Its analysis should therefore be a crucial part in investigating potentially mucus-based phenotypes, as was performed in the present study.

Proper mucus layer structure and function are impaired in numerous diseases. Due to a defective colonic mucus layer, bacteria or bacterial antigens come into contact with the epithelium, thereby triggering an inflammatory response [146], which is the main mechanism behind UC. *Muc2*$^{-/-}$ mice exhibit a complete loss of the mucus barrier [114, 147], hence, leading to inflammation and long-term to colon cancer [33, 114, 147, 148].

CF, another mucus-related disease, is characterized by meconium ileus in newborns or DIOS in adults, in which ileocaecal mucus is adherent to the epithelium and possesses an increased bacterial load [149-151]. For correct mucin unfolding and gel formation, bicarbonate is necessary to precipitate calcium ions [126]. Bicarbonate secretion is, however, impaired in CF-patients due to the defective CFTR protein, which normally mediates chloride and bicarbonate secretion. Hence, in a CF mouse model, this phenotype can be normalized

by exposure to bicarbonate buffer [152]. Apart from the dysfunctional CFTR channel, it has also been speculated that Muc2 remains anchored to the goblet cell after secretion and needs to be cleaved by a specific protease in order to be released into the mucus [153], but detailed data on the identity and function of this protease in health and disease is lacking.

MUC2 biosynthesis is relatively slow under steady state conditions with mucus cell refill-time being more than four to five hours [154]. Under inflammatory conditions, such as in patients with active UC or UC in remission, the mucus layer is thinner and penetrable with fewer filled goblet cells, perhaps due to an increased turnover rate generating mucus of poorer quality and limiting MUC2 mucin availability [112, 155]. The bacteria can penetrate the inner mucus layer and trigger inflammation, which is also demonstrated in various mouse models of spontaneous colitis displaying characteristics of UC, as mentioned earlier.

In studying the function of the mucus barrier, various chemically induced murine models of intestinal inflammation have been established for specific types of colitis and with strain-dependent susceptibility.

DSS colitis is the most broadly employed rodent model. In mice, it is generally induced orally via drinking water as 3 to 5 % DSS solution with colitis development after four to seven days [156]. The inflammation is limited to the colon, since water absorption leads to high DSS concentrations, especially in the distal colon [123]. As severe combined immunodeficiency (Scid)- or recombination-activating gene (Rag)-deficient mice that lack T- and B-cells also develop severe intestinal inflammation after DSS treatment, the adaptive immune system does not seem to play a role in the acute phase of this colitis [157]. Hence, this model is particularly useful to study the contribution of innate immune mechanisms in colitis such as the mucus barrier [156], as performed in this study.

In contrast to other chemically induced colitis models, such as 2,4,6-trinitrobenzene sulfonic acid (TNBS) or Oxazolone, which are given with ethanol to break the mucus barrier and additionally haptenize autologous proteins [156], DSS is the sole model with an impact on the mucus barrier itself and without a primary effect on the epithelium or on its mucus secretion [123]. Hence, this model is pivotal for investigating an impact of CLCA1-deficiency on the mucus barrier and the consecutive inflammatory response.

Until recently, the exact mechanism of DSS-induced colitis was unclear. Germ-free or *Muc2*$^{-/-}$ mice which have a thinner or no functional mucus layer, respectively, show massive bleeding after DSS administration long before inflammation [144, 158, 159]. This may be due to direct DSS contact with the epithelium or higher DSS concentrations or both, and possibly triggered by the toxic effect of the calcium-chelating DSS to epithelial cells of the basal crypts [123, 139].

Johansson *et al*., however, demonstrated a rapid effect of DSS decreasing mucus thickness and increasing bacteria permeability of its inner layer already at 15 minutes post-DSS-exposure [123]. The DSS-induced loss of stratified lamellar organization enables bacteria to penetrate the inner mucus layer as early as four hours and come into epithelial contact at 12 hours, whilst the inner layer vanishes at 24 hours and is completely dissolved at 120 hours. At this latter stage, first signs of inflammation become histologically evident. Of note, the murine diurnal drinking rhythm has a tremendous impact on the mucus penetrability, which alternates biphasically due to fluctuating relative amounts of DSS in the mucus [123]. It is assumed that DSS alters the biophysical mucus structure, possibly due to it being highly sulfated and therefore readily soluble in the also highly sulfated MUC2 network. This was demonstrated *in vivo* by mice lacking the sodium-sulfate-cotransporter gene *Nas1*, which show less sulfation of their mucins, and hence, are more susceptible to colitis [159]. Contrarily, dextran alone, which, in contrast to DSS, is devoid of sulfate groups, does not affect mucus organization [123]. Also, DSS is more acidic than dextran and may have a pore-forming effect, which has also been observed for hydrochloric stomach acid experimentally applied to colonic mucus [160], both allowing for bacteria to come into contact with the epithelium and to elicit a fulminant inflammation.

The molecular mass of DSS also plays an important role in the induction of colitis. DSS with a molecular mass of 500 kDa does not cause any inflammation, perhaps since it is too large to diffuse into the mucus network [123]. Surprisingly, in contrast to the 40 to 50 kDa variants used for colitis induction, variants of even smaller molecular weights, for example of 5 kDa, induce a less severe colitis [161]. Due to the multiple variables of DSS susceptibility, such as species-specific susceptibility, genetic background, intestinal microflora composition, as well as molecular weight, sulfation degree, and hence, specific stock of DSS employed [123, 138, 139, 156, 159, 161], it is crucial to perform a DSS concentration pilot study in order to assess the DSS colitis susceptibility of the animals under the conditions used.

In conclusion, the mucus layer is an essential protective barrier and exhibits a unique architecture in the colon. The DSS colitis model is a widely used model to study the function of this barrier and the impact on inflammatory disease.

1.5 Aims and Hypotheses

CLCA1 has been an intriguing subject of research over the past two decades. It has been clearly associated with mucus-based diseases such as asthma, COPD, and CF whilst numerous possible roles have been postulated. However, its exact function remains elusive to date. **The aim of this study is to more specifically elucidate the function of CLCA1**, focusing on the two most promising functions of CLCA1 - as a structural mucus component or as signaling protein modulating cytokine expression.

CLCA1 is most highly expressed and secreted by mucus cells in the colon. Due to its striking similarities in abundance and expression profile compared to the main structural protein of the intestinal mucus barrier, Muc2 [33, 84], **it can be hypothesized that CLCA1 functions as a structural protein**, i.e. influences the formation, structure, and integrity of the mucus barrier.

The mucus barrier is easily modifiable in the intestine with well-established methods. Murine DSS colitis, the most commonly used *in vivo* model of mucus barrier disruption, is restricted to the colonic mucosa [138, 139, 162, 163], in which CLCA1 is most highly expressed [31, 33]. Furthermore, the acute DSS colitis model is most favorable in studying probable effects of the lack of CLCA1 in the colonic mucus at relevant early points of time during mucus layer disruption [123], as well as during full colitis. Using this standard model of DSS colitis also allows for comparability with numerous other published studies.

Hence, *Clca1*$^{-/-}$ and WT mice were comparatively investigated in terms of key clinical parameters – weight loss, fecal consistency, and presence of occult or macroscopic blood in the feces – over the entire course of the experiment (see 2.1 and 2.2). At necropsy at three highly relevant points in time, which were chosen in order to investigate early changes during mucus barrier disruption after 24 and 48 hours as well as during full colitis after seven days of DSS treatment followed by two consecutive days of normal drinking water, additional in-depth read-outs were applied. Expression profiles of select mucin, *Clca* and cytokine genes were measured at all three predetermined times (see 2.1 and 2.2). The intestinal mucus barrier was analyzed comparatively in terms of mucus penetrability, mucus layering, and goblet cell filling by fluorescence-*in situ*-hybridization (FISH) at early points in time (see 2.1), whilst the barrier integrity was determined by analysis of bacterial translocation into sentinel organs such as blood, mesenteric lymph nodes, liver, and spleen during fulminant colitis (see 2.1). Additionally, colon weight-length-ratio and histopathology concerning immune cell infiltration, erosion or ulceration, and regeneration were also analyzed during full colitis (see 2.2).

The mucus barrier is furthermore dependent on the microflora, i.e. a change in intestinal microbiota composition gives rise to an altered chemical mucus composition and goblet cell function [164]. Animals from different facilities show striking differences in their gut microflora composition, and, hence, different susceptibility to DSS colitis [138, 139]. During colitis conditions, the microflora undergoes significant changes, shifting towards pro-inflammatory and less diverse intestinal bacteria [165, 166]. Therefore, fecal microbiota composition was investigated in this study with a focus on main gut bacterial groups (see 2.1), which are known to be altered during colitis development [165, 166].

In challenged mouse models of the respiratory tract, certain cytokines were differentially expressed between *Clca1*$^{-/-}$ and WT mice. After infection with *S. aureus*, *Clca1*-deficiency resulted in decreased *Cxcl-1* and *Cxcl-2* responses with decreased neutrophil recruitment and reduced expression of the pro-inflammatory cytokine *Il-17* in the lung [110]. Contrarily, intranasal LPS challenge in *Clca1*$^{-/-}$ mice induced CXCL-1 upregulation and consecutively increased neutrophil recruitment [45]. Hence, CLCA1 may act as signaling molecule, modulating early inflammatory responses, depending on the stimuli used and with a specific effect on *Cxcl-1* and *Il-17*. The exact mechanisms of CLCA1-mediated cytokine regulation are unclear to date. Nevertheless, a first notion was gained by investigating potential effects of human CLCA1 on macrophages, which elicited a pro-inflammatory cytokine response [111]. Whether this cellular response is also mediated by CLCA1 in the mouse remains to be elucidated. However, this is of prime importance when employing animal models in translational research as it could be shown that other hypothesized functions of human CLCA1 - such as CaCC modulation - are not reproducible in the mouse [105]. So far, only circumstantial evidence has pointed towards a role of CLCA1 in early airway inflammation of the mouse as described above. Clearly, the exact mechanisms of how murine CLCA1 may execute such effects remain to be established, with careful consideration of possible differences between humans and mice.

Hence, **it can also be hypothesized that murine CLCA1** acts on macrophages and thereby modulates inflammatory responses. This correlation was investigated in this study concerning direct effects of murine CLCA1 on cytokine expression levels *ex vivo* in alveolar and bone-marrow derived macrophages in a solely murine model (see 2.3).

Macrophages were stimulated with CLCA1-conditioned medium and certain cytokines were quantified on the mRNA level via reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) and, on the protein level, via cytometric bead array and multiplex assay. A microarray analyses identified further genes differentially expressed by CLCA1-stimulated macrophages. One of these genes was investigated in detail using biomaterial of an *in vivo* pneumonia study, which had been conducted on CLCA1$^{-/-}$ mice previously [110].

2 Research Publications in Journals with Peer-Review

2.1 The Goblet Cell Protein Clca1 (Alias mClca3 or Gob-5) is Not Required for Intestinal Mucus Synthesis, Structure and Barrier Function in Naive or DSS-Challenged Mice

Authors: Erickson NA, Nystrom EEL, Mundhenk L, Arike L, Glauben R, Heimesaat MM, Fischer A, Bereswill S, Birchenough GMH, Gruber AD, Johansson MEV

Year: 2015

Journal: PLoS ONE

Bibliographic Source: *Erickson NA, Nystrom EEL, Mundhenk L, Arike L, Glauben R, Heimesaat MM, Fischer A, Bereswill S, Birchenough GMH, Gruber AD, Johansson MEV. The Goblet Cell Protein Clca1 (Alias mClca3 or Gob-5) Is Not Required for Intestinal Mucus Synthesis, Structure and Barrier Function in Naive or DSS-Challenged Mice. PloS One (2015);10(7):e0131991*

Declaration of own portion of work in this research publication:

Contributions by N. A. Erickson: Independent design, preparation, completion, and analysis of all experimental procedures involving the DSS-challenge model in comparison to naive mice. Assessment of clinical parameters (fecal blood and stool consistency) during the entire course of the experiment, preparation and processing of colon tissue samples, assessment of mucus penetrability, mucus layering, and goblet cell filling by FISH, analysis of bacterial translocation into sentinel organs such as blood, mesenteric lymph nodes, liver, and spleen, analysis of fecal microbiota composition via RT-PCR quantification of 16S rRNA gene sequences, RNA isolation of proximal and distal colon and RT-qPCR mucin gene expression analyses, statistical analysis, and interpretation. Subsequent preparation of the manuscript.

Contributions of other authors: Shared first authorship with E. E. L. Nyström who independently designed, prepared, completed, and analyzed naive mouse intestinal mucus in terms of immunofluorescence, proteomics of mucus samples, mucus thickness

measurements and responsiveness to induced secretion, mucus penetrability assay and statistical analysis and interpretation. Subsequent preparation of the manuscript.

All co-authors participated considerably to the study design, evaluation of experimental results, and the creation and review of the manuscript.

Declaration on ethics: All animal studies involving solely naive mice were approved by the Swedish Laboratory Animal Ethical Committee in Gothenburg, Sweden (number: 280–2012) and conducted at the Department of Medical Biochemistry, University of Gothenburg, Sweden, in full compliance with Swedish animal welfare legislation. Animal studies involving DSS treatment were approved by the local governmental authorities (State Office of Health and Social Affairs Berlin, approval IDs: T 0394/12; G 0170/12) and conducted at the Research Institutes for Experimental Medicine, Charité - Universitätsmedizin Berlin, Campus Benjamin Franklin, Germany, in strict accordance with the FELASA guidelines and recommendations for the care and use of laboratory animals [167].

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

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The Goblet Cell Protein Clca1 (Alias mClca3 or Gob-5) Is Not Required for Intestinal Mucus Synthesis, Structure and Barrier Function in Naive or DSS-Challenged Mice

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Abstract

The secreted, goblet cell-derived protein Clca1 (chloride channel regulator, calcium-activated-1) has been linked to diseases with mucus overproduction, including asthma and cystic fibrosis. In the intestine Clca1 is found in the mucus with an abundance and expression pattern similar to Muc2, the major structural mucus component. We hypothesized that Clca1 is required for the synthesis, structure or barrier function of intestinal mucus and therefore compared wild type and *Clca1*-deficient mice under naive and at various time points of DSS (dextran sodium sulfate)-challenged conditions. The mucus phenotype in *Clca1*-deficient compared to wild type mice was systematically characterized by assessment of the mucus protein composition using proteomics, immunofluorescence and expression analysis of selected mucin genes on mRNA level. Mucus barrier integrity was assessed *in-vivo* by analysis of bacterial penetration into the mucus and translocation into sentinel organs combined analysis of the fecal microbiota and *ex-vivo* by assessment of mucus penetrability using beads. All of these assays revealed no relevant differences between wild type and *Clca1*-deficient mice under steady state or DSS-challenged conditions in mouse colon. Clca1 is not required for mucus synthesis, structure and barrier function in the murine colon.

Introduction

The goblet-cell derived protein Clca1 (chloride channel regulator, calcium-activated-1, previously termed mClca3 or gob-5, see revised nomenclature S1 Table) has repeatedly been linked

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to several human respiratory conditions such as asthma, cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) as well as to corresponding mouse models [1–4]. Common features related to disease severity are goblet cell hyperplasia, mucus overproduction and disturbed clearance, together with Clca1 protein and mRNA overexpression which has led to the conclusion that Clca1 is involved in mucin gene expression and/or altering mucus properties [4]. Experimental overexpression of murine Clca1 aggravates the asthma phenotype in ovalbumin- or interleukin (IL)-9-challenged mouse models [5,6]. Conversely, neutralization via antisense- or antibody-treatment of Clca1 ameliorates disease severities [5,7].

Indeed, it was recently shown that the human CLCA1 induces airway goblet cell metaplasia via an extracellular MAPK (mitogen-activated protein kinase)-signaling pathway that drives mucus gene expression [8]. Therefore, CLCA1 may be key to the pathogenesis of conditions with mucus overproduction, possibly suggesting a role in novel therapeutic concepts [4]. However, the function of CLCA1 is still far from being understood.

CLCA1 is abundantly secreted by goblet cells of the intestinal tract together with the MUC2 mucin [9–13]. MUC2 is a highly glycosylated, multimerizing protein and the main structural mucus component, creating net-like sheets that stack upon each other after secretion, thus forming a sieving mucus structure that is normally impenetrable to bacteria [11,14]. It thereby acts as a protective gel, providing an effective barrier against the commensal colonic bacteria [11,15]. In patients with ulcerative colitis, there is strong evidence for mucus layer disruption contributing to the disease pathogenesis [16]. Also, experimentally altered mucus properties consistently induce colitis in mice due to breakdown of the intestinal mucus barrier [16–18].

Several other proteins have been identified in the mucus which are thought to also contribute to mucus structure formation [11,13]. One of these is Clca1, a major constituent of the extracellular intestinal mucus, with a similar expression pattern to Muc2 along the intestinal axis [13]. During its biosynthesis, the primary Clca1 translation product is autocatalytically cleaved within its carboxy-terminal region into two secreted cleavage products by a self-contained zinc-dependent metallohydrolase [19–22]. Such metallohydrolases are thought to critically alter mucus properties [23]. Additionally, a conserved von Willebrand factor type A (vWA) domain with a metal-dependent adhesion site is contained in the amino-terminal cleavage product of CLCA1 which implies that it may interact with other proteins [20,24,25], supporting the notion of a structural role in mucus formation. Furthermore, restoration of decreased Clca1 protein levels in a mouse model of CF has been shown to ameliorate intestinal mucus plugging which is a common trait of human and murine CF [23,26]. It has consequently been hypothesized that CLCA1 may play a role in mucus formation [5,10,21,24,27].

In this study we characterized the intestinal mucus phenotype and microbiota composition of wild type (WT) versus *Clca1*-deficient (*Clca1*$^{-/-}$) mice under naive conditions, in a set of *ex vivo* experiments and under conditions of DSS (dextran sodium sulfate) challenge. DSS destabilizes biophysical mucus layer properties but is thought not to affect mucin biosynthesis [28]. It is commonly used to experimentally induce colitis via its rapid deleterious effects on mucus integrity which causes mucus barrier disruption and allows bacterial penetration and subsequent epithelial barrier disruption [28,29]. The consequent inflammatory response results in clinically manifest colitis after a treatment course of several days that in many ways resemble and can be used as a model for Ulcerative colitis [29]. Here, mucus barrier integrity was also determined by quantification of bacterial translocation into sentinel organs. Furthermore, the intestinal microbiota composition was analyzed in terms of main gut bacterial groups since these are known to be sensitively altered during development of experimental colitis [30,31].

Much to our surprise, we failed to detect any differences in all of our experimental readout parameters between *Clca1*$^{-/-}$ and wild type mice, regardless of whether unchallenged or DSS-challenged tissues were employed.

Materials and Methods

Ethics statement, mice and DSS treatment

All animal studies involving naive mice were conducted at the Department of Medical Biochemistry, University of Gothenburg, Sweden, in full compliance with Swedish animal welfare legislation and approved by the Swedish Laboratory Animal Ethical Committee in Gothenburg, Sweden (number: 280–2012). Animal studies also involving DSS treatment were conducted at the Research Institutes for Experimental Medicine, Charité - Universitätsmedizin Berlin, Campus Benjamin Franklin, Germany, in strict accordance with the FELASA guidelines and recommendations for the care and use of laboratory animals [32] and approved by the local governmental authorities (State Office of Health and Social Affairs Berlin, approval IDs: T 0394/12; G 0170/12).

Naive *Clca1*$^{-/-}$ mice, generated on a C57BL/6J background [33] and control mice of the same genetic background were allowed an acclimatization period of at least 10 days and housed under standardized SPF conditions. Animals of both genders between 6 and 12 weeks of age were used, except for DSS experiments involving only weight- and age-matched female mice. For experimental procedures, mice were given 2.5% DSS (MW 36,000–50,000, 17.1% sulfur substitution, no detectable free sulfate, pH 7.1, MP Biomedicals, LLC., Illkirch, France) in their drinking water for 24 (24 h-group) or 48 (48 h-group) hours to allow for the investigation of initial mucus barrier disruptions. In addition, DSS administration for 7 days with 2 consecutive days of tap water only (7 d-group) allowed the monitoring of effects with a completely disrupted barrier and assessment of clinical parameters in terms of fecal blood content [34], using the hemoCARE Guaiac testing method (CARE diagnostic, Voerde, Germany) and stool consistency [35]. All mice were anesthetized with isoflurane prior to euthanasia by cervical dislocation.

Immunofluorescence

Intestinal tissues were fixed in Methanol-Carnoy and immunostained as described before [36]. Sections were immunostained with a rabbit anti-MUC2-C3 antibody (1:500) [11] and co-stained with a mouse anti-human CLCA1 antibody (Abcam, Cambridge, UK, ab129283, 1:1,000). Goat anti-rabbit IgG conjugated to Alexa Fluor 488 (Molecular Probes, Thermo Fisher, Waltham, MA USA, 1:1,000), or goat anti-mouse IgG conjugated to Alexa Fluor 546 (Molecular Probes, Thermo Fisher, 1:1,000) were used as secondary antibodies, respectively. WT and *Clca1*$^{-/-}$ intestinal sections were stained with a rabbit anti-mouse CLCA1 antibody (amino-terminal reactive; Abcam ab46512, 1:1,000) or a mouse anti-human CLCA1 antibody (carboxy-terminal reactive; Abcam ab129283, 1:2,000) combined with an appropriate Alexa Fluor 546-conjugated secondary antibody to verify the antibody specificity. Other primary antibodies used were: goat anti-Agr2 (Santa Cruz Biotechnology, Dallas, USA, sc-54561, 1:100), rabbit anti-Fcgbp (Sigma-Aldrich, HPA003517, 1:100), rabbit anti-Klk1 (Boster Immunoleader, Pleasanton, CA, PA1709, 1:1,000) or rabbit anti-Zg16 (made in house, PH8292B-11, 1:600) [13] followed by the appropriate Alexa Fluor 546-conjugated secondary antibodies. DNA was stained for 5 min using 1 μg/ml Hoechst 34580 (Molecular Probes, Thermo Fisher). Micrographs were obtained using a Nikon eclipse E1000 fluorescence microscope (Nikon, Tokyo, Japan) with the NIS elements software (Nikon) or a LSM700 Axio Examiner Z.1 confocal microscope (Zeiss, Göttingen, Germany) with the ZEN 2010 software (Zeiss).

Proteomics

Sample collection. Mucus samples were collected from flushed colonic tissue of WT and *Clca1*$^{-/-}$ mice mounted in a horizontal perfusion chamber as described for mucus thickness measurements below but without removal of the muscle layer. 10 μm polystyrene beads

(FluoSpheres, Molecular Probes, Thermo Fisher) were used to visualize the mucus layer. Mucus was gently scraped off the epithelium into the apical buffer and was collected using a micropipette [37]. 2x cOmplete EDTA (ethylenediaminetetraacetic acid)-free protease inhibitor (Roche, Mannheim, Germany) was added to the samples.

Sample preparation. Mucus samples were incubated overnight at 37°C in reduction buffer (6 M GuHCl, 0.1 M Tris/ HCl pH 8.5 (Merck), 5 mM EDTA, 0.1 M DTT (Merck)) followed by a FASP (Filter-aided Sample Prep) digestion protocol [38] using 6 M guanidinium hydrochloride (GuHCl) instead of urea. Briefly, proteins were alkylated on 30 kDa cut-off filters and subsequently digested for 4 h with LysC (Wako, Richmond, VA, USA) followed by an overnight trypsin (Promega, Fitchburg, WI, USA) digestion. Peptides released from the filter after centrifugation were cleaned with StageTip C18 columns as previously described [13,39].

Nano-Liquid-Chromatography Mass-spectrometry (NanoLC–MS/MS). NanoLC–MS/MS was performed on an EASY-nLC system (Thermo Scientific, Odense, Denmark), connected to a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific) through a nanoelectrospray ion source. Peptides were separated with reverse-phase column (150 × 0.075 mm inner diameter, C18-AQ 3 μm) by 95-minute gradient from 5 to 30% B A (A: 0.1% formic acid, B: 0.1% formic acid/80% acetonitrile 200 nl/min). Full mass spectra were acquired from 350–1,600 m/z with resolution of 70,000 (m/z 200). Up to twelve most intense peaks (charge state $\geq$ 2) were fragmented and tandem mass spectrum was acquired with a resolution of 35,000 and dynamic exclusion 30 s.

Data analysis. Data was analyzed with the MaxQuant program (version 1.4.1.2 [40]), as previously described [13].

Label-free quantification was performed by loading LFQ-intensity data from the MaxQuant output to the Perseus software (version 1.4.1.3). The data was filtered based on the presence of a protein group in at least four out of five samples in at least one group. Each group (WT and *Clca1*$^{-/-}$mice) was averaged using the median with the median values being presented in a scatterplot.

The mass spectrometry proteomics data were deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository [41] with the dataset identifier PXD001804.

Mucus thickness measurements and responsiveness to induced secretion

Procedure, material and equipment were employed as previously described [37]. Briefly, the distal colon was dissected and thoroughly flushed with ice-cold, oxygenated Krebs' solution. It was then opened up and the longitudinal muscle layer removed before being mounted in a 2.5 mm horizontal perfusion chamber with oxygenated basolateral Krebs' glucose solution and apical Krebs' mannitol solution. Activated charcoal particles (Fluka) were added to the apical surface to visualize the mucus layer. The mucus thickness was measured every 15 min for 1 h after an initial measurement at time 0 h (t0). To investigate the responsiveness of the tissue to induce mucus secretion, carbachol (CCh, 1mM, Sigma Aldrich) was added to the basolateral buffer after 30 min in one of the two specimen-containing chambers.

Mucus penetrability assay

The mucus penetrability assay was performed on distal colon tissue as previously described [16,37]. Briefly, the colonic explants were mounted in the perfusion chamber with basolateral Kreb's glucose solution containing 1μg/ml Calcein violet tissue stain (CellTrace Calcein Violet, Molecular Probes, Thermo Fisher) and a suspension of far red fluorescent beads (FluoSpheres

Carboxylate-Modified Microspheres, 1.0 μm, red fluorescent (580/605), 2% solids, Molecular Probes, Thermo Fisher) and Kreb's mannitol solution was added to the apical side and allowed to sediment for 5 min before it was refilled with Kreb's mannitol solution. The distribution of the beads in the mucus was investigated by acquiring confocal images in XY stacks (320 x 320 μm) 30min after tissue mounting. These were obtained using a LSM700 Axio Examiner Z.1 confocal microscope with Plan-Apochromat x 20/1.0 DIC water objective (Zeiss) and the ZEN 2010 software (Zeiss). Average bead distance from the tissue surface was calculated by combining bead fluorescence intensity data for each z-plane above the tissue surface.

Fluorescence-*in situ*-hybridization

This was performed as previously described [36]. In brief, de-waxed and washed Methanol-Carnoy fixed tissues were hybridized with the general bacterial probe, EUB338-I (250 ng, 5′ –GCT GCC TCC CGT AGG AGT–3′ , Eurofins genomics, Ebersberg, Germany), conjugated to Alexa Fluor 555 (Molecular Probes, Thermo Fisher) [11,36]. For mucus visualization slides were incubated with a rabbit anti-MUC2-C3 antibody (1:500) followed by incubation with goat anti-rabbit IgG conjugated to Alexa Fluor 488 (1:1000, Molecular Probes, Thermo Fisher). Nuclei were stained with Hoechst 34580 (Molecular Probes, Thermo Fisher) at 1 μg/ml in PBS. Slides were dried and cover-slipped with ProLong Antifade (Molecular Probes, Thermo Fisher). Micrographs were obtained using an Eclipse E1000 (Nikon) fluorescence microscope with the NIS element software (Nikon).

Mucus penetration, mucus layering and goblet cell filling score

Bacterial penetration of the inner mucus layer was scored using the fluorescence-*in situ*-hybridization (FISH)-, anti-Muc2- and DNA-stained sections as described previously (S2 Table) [28]. The mucus layering score, focusing solely on the layering and structure of the inner, stratified mucus layer and the goblet cell filling score [16] were applied (S3 Table and S4 Table, respectively). Sections were scored independently by two blinded individuals, except for the naive controls which were scored by one blinded individual using the Eclipse E1000 and Eclipse90i (Nikon) fluorescence microscope as well as the Axiovert 200M microscope (Zeiss) with the EXFO X-Cite 120 Fluorescence Illumination System (Olympus, Hamburg, Germany). Micrographs were obtained using the LSM700 Axio Examiner Z.1 confocal microscope (Zeiss) with the ZEN 2010 software (Zeiss).

Bacterial translocation

Blood derived via sterile cardiac puncture and biopsies of spleen, liver and mesenteric lymph-nodes (MLN) draining the large intestine were taken from WT and *Clca1*$^{-/-}$ controls under naive conditions as well as after DSS challenge (7 d-group; n = 10 per group) and cultivated in thioglycolate enrichment broths (Thioglycolat-Bouillon, Oxoid, Wesel, Germany) as described [42]. Bacterial growth was monitored daily by turbidity assessment. Aliquots from turbid broths were cultivated on solid media under aerobic, microaerophilic and obligate anaerobic conditions and the bacterial species were identified microbiologically and biochemically as described earlier [43].

Fecal microbiota composition—Real-time PCR and quantification of 16S rRNA gene sequences

Real-time PCR-based quantification of main gut bacterial groups—*Enterobacteriaceae*, Enterococci, lactic acid bacteria, bifidobacteria, *Bacteroides/ Prevotella spp*., *Clostridium leptum*

group, *Clostridium coccoides* group, mouse intestinal bacteroidetes and total eubacterial load—was performed.

DNA was extracted from colonic fecal samples as described previously (Heimesaat 2006) with minor modifications. Here, the sediment was incubated with 50 µl of lysozyme (20 mg/ml) and proteinase K (20 mg/ml; Sigma-Aldrich) for 30 min at 37°C and resuspended with 0.5 ml of lysis buffer (0.01 M Tris pH 9.0, 0.02 M EDTA, 0.15 M NaCl, 0.5% SDS).

Total DNA was quantified via Quant-iT PicoGreen dsDNA reagent (Invitrogen, Paisley, UK) as described [44] and adjusted to 10 ng DNA/µl. The abundance of specific intestinal bacterial groups was measured by quantitative real-time PCR as described [42,44–46]. Targets and 16S rRNA gene group-specific primers are listed in S5 Table. Data are expressed as median in a scatterplot. A minimum cut-off value of 10^0 gene copy numbers/ng DNA and a minimum log-change of 1 were set as limits of statistically significant relevance with $p < 0.05$.

RNA isolation and quantitative reverse transcriptase-qPCR

Sections of the distal colon were opened longitudinally, flushed briefly in ice-cold PBS before snap freezing in liquid nitrogen for subsequent storage at -80°C. Total RNA isolation using the Nucleo Spin RNA isolation Kit (Macherey Nagel, Düren, Germany), primer and probe design, quantitative Reverse Transcriptase-PCR (RT-qPCR) protocols, RT-qPCR and data analysis was performed as previously described [47].

Transcript expression levels of murine *Muc1*, *-2*, *-3*, *-4*, *-5ac*, *-5b* and *Muc6* were determined and normalized to the internal reference genes glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), elongationfactor 1α (*Ef-1α*) and ß-2 microglobulin (*B2m*) as previously described [47]. Primers and probes for *Ef-1α* [48], *Gapdh* [49], *B2m* [50], *Muc4* [51] and *Muc5ac* [52] were used as described. Primers and probes for *Muc1*, *-2*, *-3*, *-5b* and *-6* were designed using Primer3 software (WWW primertool, Whitehead Institute of Biomedical Research). Primer and probe sequences are listed in S6 Table.

The ΔΔCt method based on data obtained from naive WT control animals as reference allowed for relative expression level quantification and group comparison. Significance levels (p-values) were determined by Mann-Whitney-U test. Data are expressed as fold changes. Fold changes of 0.5 and 2 are considered as limits for valid statement of lowered and elevated parameters, respectively.

Statistics

Data are expressed as mean ± SEM, except for the proteomics log2 intensity, fecal microbiota composition and gene expression level data which are expressed as indicated. Pearson's correlation coefficient and the fraction of shared variance, R squared, were calculated for mucus proteomics data. Statistical analysis of mucus thickness and growth was performed with 2-way ANOVA followed by Tukey's HSD test for multiple comparisons. Impenetrable mucus thickness was compared by unpaired two-tailed t-test with 95% confidence interval. Other statistical analyses were performed using the Mann-Whitney-U test. $P < 0.05$ was considered significant. Statistical analysis and graphical illustrations were performed using GraphPad PRISM 6 (GraphPad Software Inc., La Jolla, USA).

Results

Expression of the major intestinal mucin Muc2 and mucus structure are not altered in *Clca1*$^{-/-}$ mice

Previous studies have identified the highest levels of murine Clca1 expression as being located in the intestinal tract where it is restricted to mucin producing goblet cells in the upper half of

the colonic crypts and to villus goblet cells of the small intestine [10,13]. In order to investigate if Clca1 deficiency affected the Muc2 expression pattern or mucus structure in the large intestine, immunofluorescence (IF) using an anti-MUC2-C3 antibody was performed on sections from proximal, mid and distal colon. The anti-MUC2-C3 antibody failed to reveal any difference in staining patterns between the genotypes, neither in the crypt goblet cells nor in the secreted mucus (Fig 1A and 1B, green). Moreover, the stratified structures of the inner mucus were very similar (Fig 1B and 1i). These findings indicate normal Muc2 protein expression and mucus structure in *Clca1*$^{-/-}$ mice. Staining with an anti-Clca1 antibody revealed, in contrast to previous data [13], a low expression of Clca1 in the proximal colon and higher expression in the distal colon (Fig 1A, red). We therefore focused the following experiments on the distal colon. The merged picture of Muc2 and Clca1 in WT confirmed co-localization of Clca1 to Muc2 producing goblet cells and presence of Clca1 in the secreted mucus (Fig 1A merge).

The specificity of the anti-CLCA1 antibody specificity was verified by staining *Clca1*$^{-/-}$ distal colon sections as negative controls and comparing the staining pattern between antibodies directed against either the amino- or the carboxy terminus of Clca1 in WT sections (Fig 1C).

Lack of Clca1 has no effect on the abundance of other proteins in the intestinal mucus

To study possible further effects of Clca1-deficiency on Muc2 and other important components of the mucus, such as Agr2, Fcgbp, Klk1 and Zg16, a proteomics study was performed. The relative abundances of proteins were compared in the mucus layer, displayed in a scatter plot showing a tight and significant correlation between WT and *Clca1*$^{-/-}$ mucus proteins (Pearson´s $r = 0.931$, $r^2 = 0.868$, $p < 0.0001$, based on 809 valid pairs; Fig 2A). No significant differences in mucus protein expression were observed between the genotypes, except for the complete absence of the Clca1 protein in *Clca1*$^{-/-}$ mice. The major mucus components Agr2, Fcgbp, Klk1 and Zg16 were also investigated by IF in distal colon sections with no differences found in their expression patterns or signal intensity between *Clca1*$^{-/-}$ and wild type mice (Fig 2B).

Of the eight murine Clca family members, except for Clca1 in WT mice, only Clca4a and Clca4b (formerly known as mClca6 and mClca7, respectively, see revised nomenclature S1 Table) were identified in the mucus; however, they were only detected in a few samples with very few unique peptides and without any difference between the genotypes. The mass spectrometry proteomics data are presented in S7 Table and have been deposited to the ProteomeXchange Consortium with the dataset identifier PXD001804.

Mucus thickness, growth rate and impenetrability do not differ between WT and *Clca1*$^{-/-}$ mice

The mucus phenotype of WT and *Clca1*$^{-/-}$ mice was characterized in a set of *ex vivo* experiments examining the distal colonic mucus in which Clca1 is most abundantly detected.

Mucus was measured to investigate the initial thickness of the inner, firmly attached mucus as well as the secreted and expanding mucus over time both under normal and stimulated conditions. The outer, non-adherent mucus layer was flushed away prior to removal of the longitudinal muscle layer, providing an initial measurement representing the inner, adherent mucus thickness. Neither the thickness of the adherent mucus layer (65.3 ± 5.1 μm and 68.6 ± 5.8 μm for WT and *Clca1*$^{-/-}$ mice, respectively; Fig 3A) nor the spontaneous mucus growth in the explant chamber system during 1 h (total thickness of 202.0 ± 15.8 μm and 180.7 ± 19.0 μm at t = 60 min with an average growth rate of 2.40 ± 0.21 μm/min and 1.89 ± 0.21 μm/min for WT and *Clca1*$^{-/-}$ mice, respectively; Fig 3A and 3B) differed between the two genotypes. Increased mucus secretion was evident in both WT and *Clca1*$^{-/-}$ tissue after addition of CCh, a cholinergic

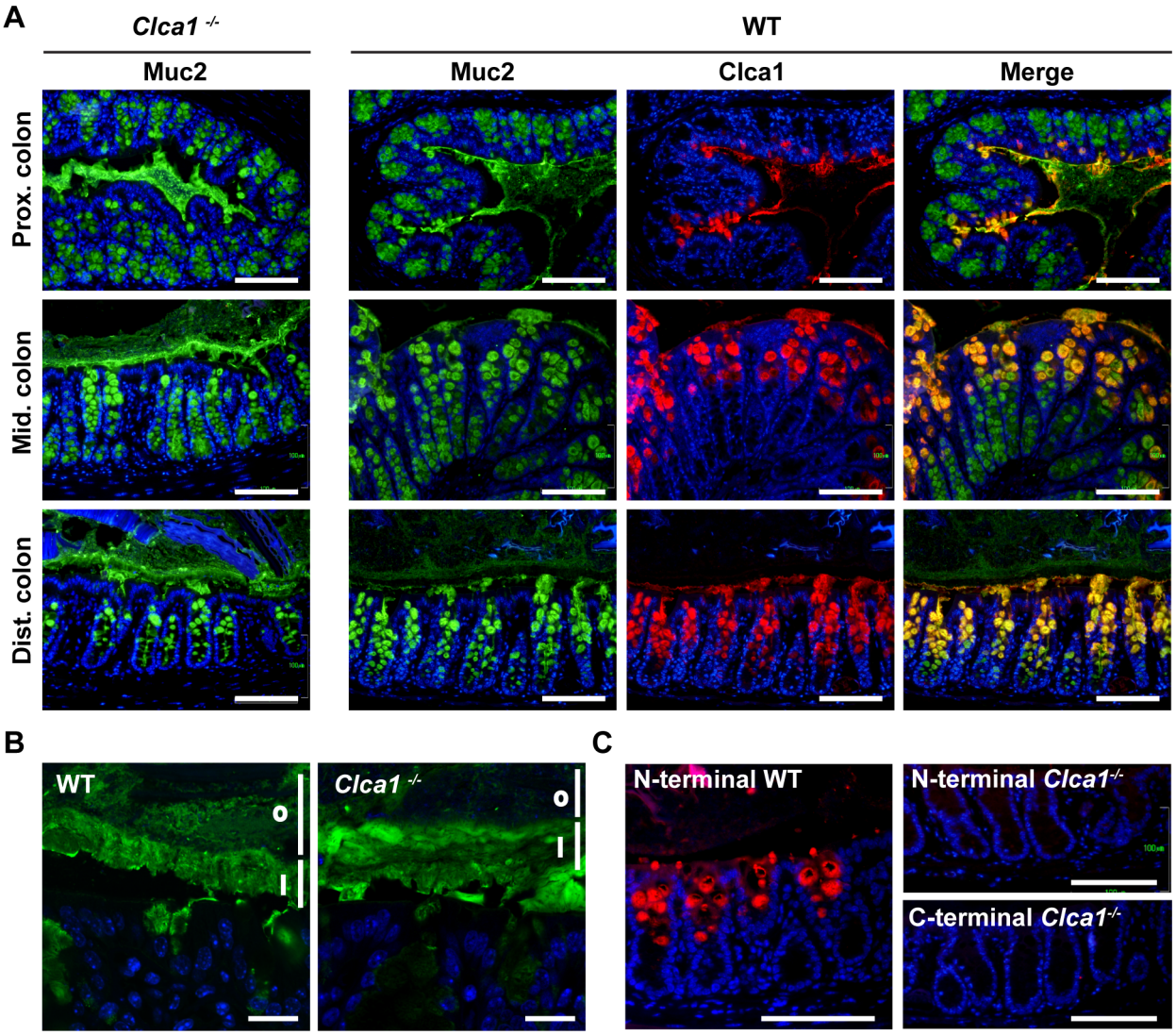

Fig 1. No difference of Muc2 expression pattern was observed by immunofluorescence in WT and *Clca1*$^{-/-}$ mice. (A) The expression patterns of Muc2 (green) were investigated in the large intestine in *Clca1*$^{-/-}$ mice and WT mice as control. No difference in Muc2 expression pattern could be detected between WT and *Clca1*$^{-/-}$ mice in any location. Co-expression of Clca1 and Muc2 were confirmed by co-staining (yellow, merge with Clca1 carboxy-terminus stained red). The highest expression of CLCA1 was found in distal colon of WT mice and was restricted to goblet cells of the upper parts of the crypts. (B) Higher magnification revealed a stratified inner mucus layer by Muc2 staining (green) in both WT and *Clca1*$^{-/-}$ distal colon sections. i = inner mucus layer, o = outer mucus layer. (C) Primary antibodies directed against either the amino- or carboxy-terminus of Clca1 (red) were used to confirm the absence of murine Clca1 expression in the distal colon of *Clca1*$^{-/-}$ mice. All sections were stained with Hoechst 34580 DNA stain (blue), n = 4 per group. Scale bars in A and C 100 μm, in B 20 μm.

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agonist known to induce intestinal epithelial ion and mucus secretion, and a significantly thicker mucus layer compared to unstimulated tissue was measured after a total of 60 min in the chamber (261.6 ± 20.0 μm vs. 202.0 ± 15.8 μm in WT, $p < 0.01$ and 258.1 ± 15.0 μm vs. 180.7 ± 19.0 μm in *Clca1*$^{-/-}$ mice, $p < 0.01$; Fig 3A). No difference in total mucus thickness before and after CCh stimulation was noted between WT and *Clca1*$^{-/-}$ mice (261.6 ± 20.0 μm vs. 258.1 ± 15.0 μm, respectively). The mucus growth rate showed an immediate peak response to CCh at 30–45 min in tissue explants of both genotypes (Fig 3B). The difference observed could indicate a small shift in peak response time that might be from variation in administration in the basolateral perfusate. In addition, a drop in the transepithelial potential difference,

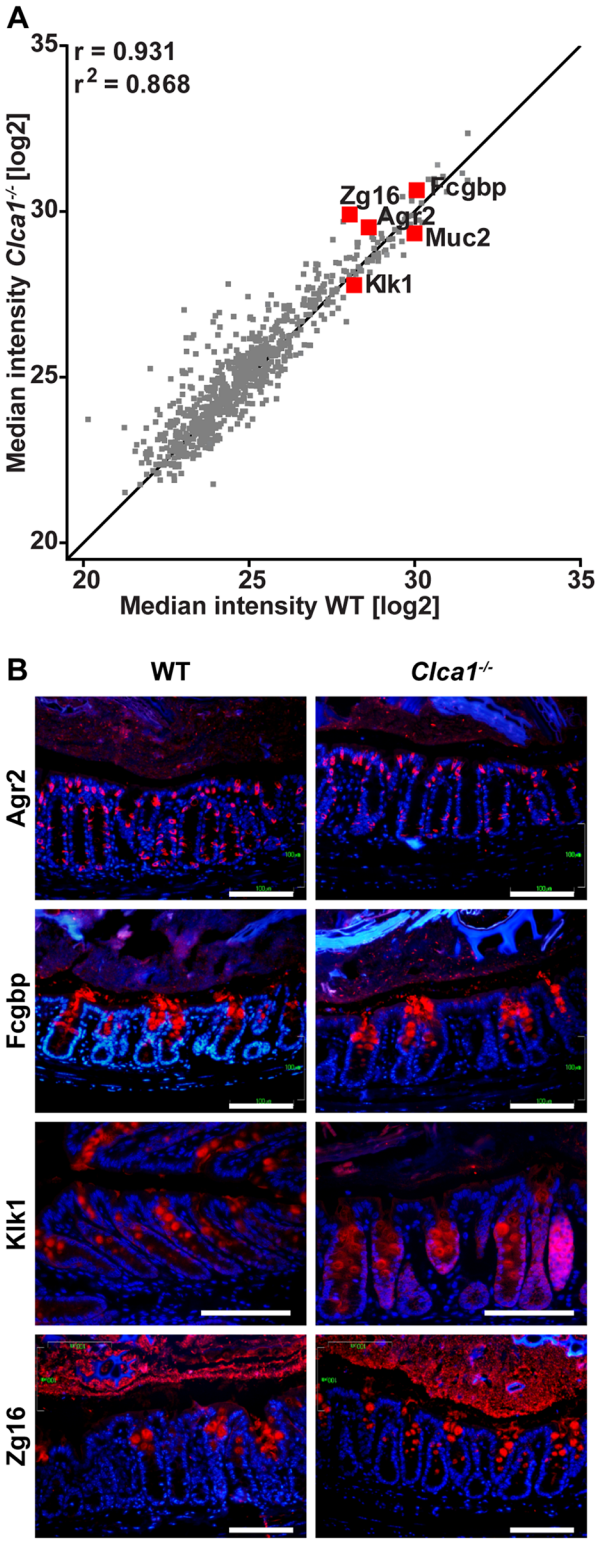

Fig 2. Major mucus components—Agr2, Fcgbp, Klk1, Muc2 and Zg16 –are unaltered in *Clca1*$^{-/-}$ colonic mucus compared to WT controls. (A) Scatter plot depicting correlation in the median label-free intensity of all mucus proteins between WT and *Clca1*$^{-/-}$ mice by proteome analysis using mass spectrometry with the correlation coefficient (Pearson's r) and r^2 shown. Proteins marked with red represent known major mucus components (Agr2, Fcgbp, Klk1, Muc2 and Zg16). Line = x + 1; n = 5 per group. (B) IF of Agr2, Fcgbp, Klk1 or

Zg16 (red) in WT and *Clca1*$^{-/-}$ distal colon sections, confirmed that there is no difference in protein expression in any of the major mucus proteins in *Clca1*$^{-/-}$ mice compared to WT, n = 2–3 per group. Blue = Hoechst 34580 DNA-stain, scale bars 100 μm.

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indicating the presence of an ion transport, was noted concurrently with the increase in mucus growth rate both in WT and *Clca1*$^{-/-}$ mice.

To test whether Clca1 has any structural relevance for mucus barrier formation, a penetrability and distribution assay was performed in which bacteria-sized beads were applied on top of the mucus and their distribution in the mucus was investigated by confocal microscopy. Both in WT and in *Clca1*$^{-/-}$ mice, a clear separation between the epithelium and the beads sedimenting on top of the mucus was observed (Fig 3C). Impenetrable mucus thickness was the same for WT and *Clca1*$^{-/-}$ mice (115.0 ± 10.2 μm and 128.6 ± 8.1 μm in WT and *Clca1*$^{-/-}$ mice, respectively; Fig 3D). The exclusion of bacteria from the inner mucus layer was confirmed for both genotypes with FISH using a general bacterial probe and Muc2 counterstaining (Fig 3E).

The mucus barriers of WT and *Clca1*$^{-/-}$ mice are identically affected under DSS challenge

Mucus penetration and layering of the inner, stratified mucus layer and goblet cell filling were assessed via FISH and IF microscopy after 24 and 48 h of DSS application. Overall, the mucus penetrability increased significantly during DSS treatment from 1.1 ± 0.6 and 1.0 ± 0.5 (naive conditions) to 2.8 ± 0.4 and 2.9 ± 0.4 (24 h DSS) and to 3.4 ± 0.3 and 3.6 ± 0.3 (48 h DSS) for WT and *Clca1*$^{-/-}$ mice, respectively ($p < 0.01$ for all groups except WT naive vs. 24 h DSS $p < 0.05$; Fig 4A). This clearly reflects the expected loss of mucus barrier integrity within the first 48 h of DSS administration [28]; however, without any significant difference between the two genotypes.

The mucus layering score increased under the influence of DSS without any observable difference between the genotypes from 0.9 ± 0.4 and 0.8 ± 0.3 (naive conditions) to 1.9 ± 0.3 and 1.8 ± 0.3 (24 h DSS) and to 2.1 ± 0.2 and 1.8 ± 0.3 (48 h DSS) for WT and *Clca1*$^{-/-}$ mice, respectively ($p < 0.05$; Fig 4B), due to loss of mucus structure which correlates well with the penetration score.

The goblet cell filling score remained similar from 1.3 ± 0.3 and 1.0 ± 0.2 (naive conditions) to 1.0 ± 0.2 and 1.6 ± 0.2 (24 h DSS) and to 1.1 ± 0.1 and 0.6 ± 0.2 (48 h DSS) for WT and *Clca1*$^{-/-}$ mice, respectively. A significant increase in goblet cell filling was observed only at the single time point of 48 h for *Clca1*$^{-/-}$ compared to WT mice ($p < 0.05$; Fig 4C).

Loss of Clca1 has no effect on bacterial translocation in naive and DSS-challenged mice

In addition to characterizing bacterial mucus penetration via FISH, we also determined the relative translocation frequencies of live bacteria into selected sentinel organs such as blood, mesenteric lymph nodes, liver and spleen. Overall, there was neither a difference between the genotypes nor between naive and colitic mice of the same genotype (data not shown).

Fecal microbiota composition, blood content and stool consistency are not altered during acute colitis in WT and *Clca1*$^{-/-}$ mice

Since a change in intestinal microbiota composition gives rise to an altered chemical mucus composition and goblet cell function [53], we quantified selected species of the fecal microbiota

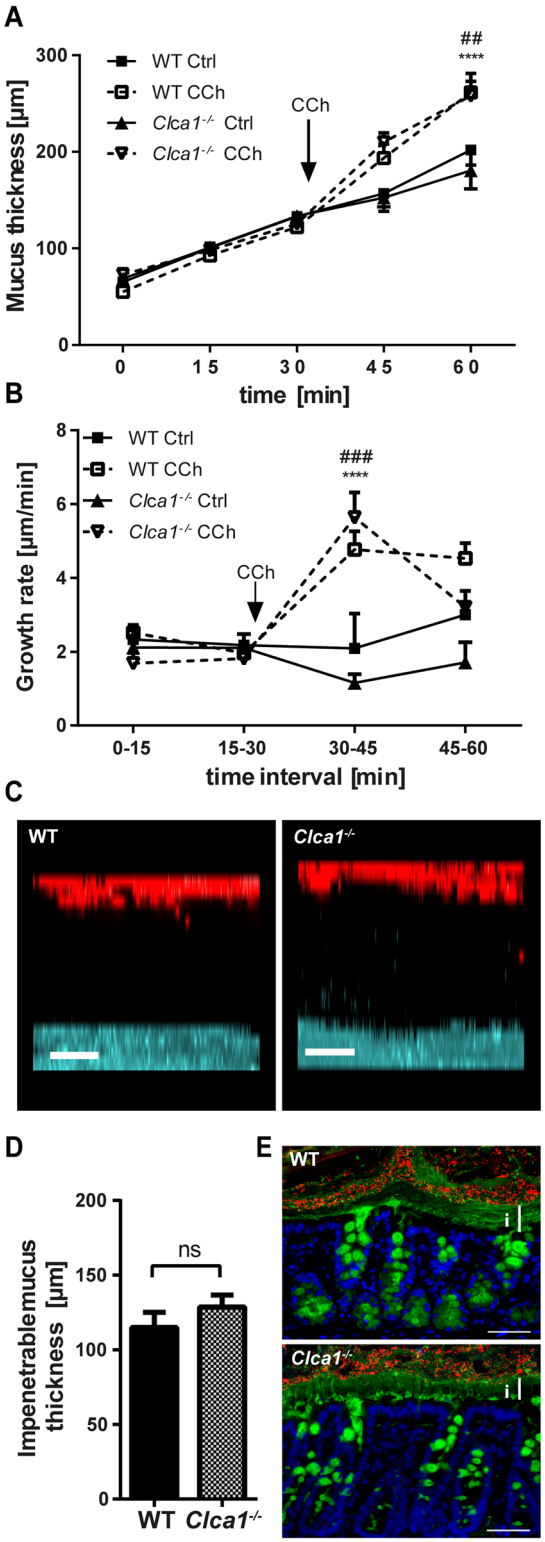

Fig 3. *Clca1*$^{-/-}$ has no effect on mucus growth, responsiveness and penetrability in *Clca1*$^{-/-}$ mice. (A) After flushing distal colon explants, mucus thickness and growth were similar in *Clca1*$^{-/-}$ mice (dotted lines) compared to WT (filled lines). After CCh stimulation, an increase in mucus thickness compared to unstimulated explants was observed both in WT and in *Clca1*$^{-/-}$ mice. Ctrl = control. (B) The growth rate was constant during unstimulated conditions whereas a significant growth rate increase in response to CCh was

evident in both groups shortly after its addition. No significant difference was observed between the groups. Ctrl = control. (C) *Ex vivo* mucus penetrability assessment using bacteria-sized beads (1 μm, red) and confocal microscopy was performed. Representative z-stack projections from WT and *Clca1*$^{-/-}$ mucus 30 minutes after tissue mounting both showed a clear separation between the tissue (blue) and the beads (red). Scale bars 50 μm. (D) The impenetrable mucus thickness, measured as the distance between the tissue and the sedimented beads in the confocal z-stacks did not differ between WT and *Clca1*$^{-/-}$ mice. ns = non-significant. (E) FISH with a general bacterial 16S probe (EUB338, red), counterstained for Muc2 (anti-MUC2-C3, green) and DNA (Hoechst 34580, blue) in sections from distal colon confirmed the impenetrability of the inner mucus layer both in WT and *Clca1*$^{-/-}$ mice with a clear separation of the tissue and bacteria. i = inner mucus layer. Scale bars 100 μm. n = 5 per group. Data are presented as mean ± SEM. $^{\#\#}$ p < 0.01, $^{\#\#\#}$ p < 0.001 for WT Ctrl vs. WT CCh; ****p < 0.0001 for *Clca1*$^{-/-}$ Ctrl vs. *Clca1*$^{-/-}$ CCh.

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in colonic fecal samples of mice before and after treatment with DSS (7 d-group) by real-time PCR, with a comparison between WT and *Clca1*$^{-/-}$ mice (Fig 5A).

In the naive state, mice of either genotype had a very similar microbiota composition. Following colitis induction, increases in the colonic *Clostridium leptum* group and in the *Clostridium coccoides* group were statistically significant. Bifidobacteria were only detectable above the cut-off value of 10^0 gene copy numbers/ng DNA during colitis with no statistically significant difference between the genotypes.

Fecal blood content (Fig 5B) and stool consistency scores (Fig 5C) revealed an overall increase over the course of DSS administration, but without any statistically significant differences between genotypes, except for a higher fecal blood content score of WT vs. *Clca1*$^{-/-}$ mice at day 4.

Clca1 deficiency has no effect on the mRNA expression of mucin genes under naive and DSS challenged conditions

After treatment with DSS for 24 or 48 h as well as for 7 d (n = 9–16 for each group), mRNA expression levels of selected mucin genes (*Muc1*, *2*, *3*, *4*, *5ac*, *5b* and *6*) were compared by RT-qPCR in distal colon samples to those of respective controls (Fig 6A–6D). *Muc1* gene expression (Fig 6A) did not show any statistically significant difference between the genotypes or between naive and DSS-challenged conditions.

Only in the 7 d-group was a statistically significant difference seen compared to water controls with *Muc2* and *Muc3* (Fig 6B and 6C, respectively) both down-regulated in WT ($p < 0.05$ and < 0.01, respectively) as well as in *Clca1*$^{-/-}$ mice ($p < 0.05$ and < 0.0001, respectively). Furthermore, a slightly lower expression of *Muc2* and slightly higher expression of *Muc3* was noted in *Clca1*$^{-/-}$ mice compared to WT mice under naive conditions which levelled out in the course of DSS challenge.

No significant differential expression was observed for *Muc4* (Fig 6D). Overall, *Muc5ac*, *-5b* and *-6* failed to show any relevant expression in the distal colon of WT and *Clca1*$^{-/-}$ mice under naive and DSS challenged conditions.

Discussion

CLCA1 is expressed and secreted by goblet cells in all investigated species so far including humans [9], mice [10], horses [54], and pigs [55] and is a major constituent of the intestinal mucus [10,11,13]. In CF knockout mice Clca1 expression is reduced [26] while experimental overexpression in CF ameliorates the murine intestinal mucus phenotype, characterized by mucus inspissation and intestinal obstruction [23]. A similar correlation has been found in humans where a certain allelic variant of CLCA1 is significantly overrepresented in CF patients with aggravated intestinal disease [56]. These observations have given rise to the hypothesis that Clca1 play a role in intestinal goblet cell function, mucus properties or modulation of

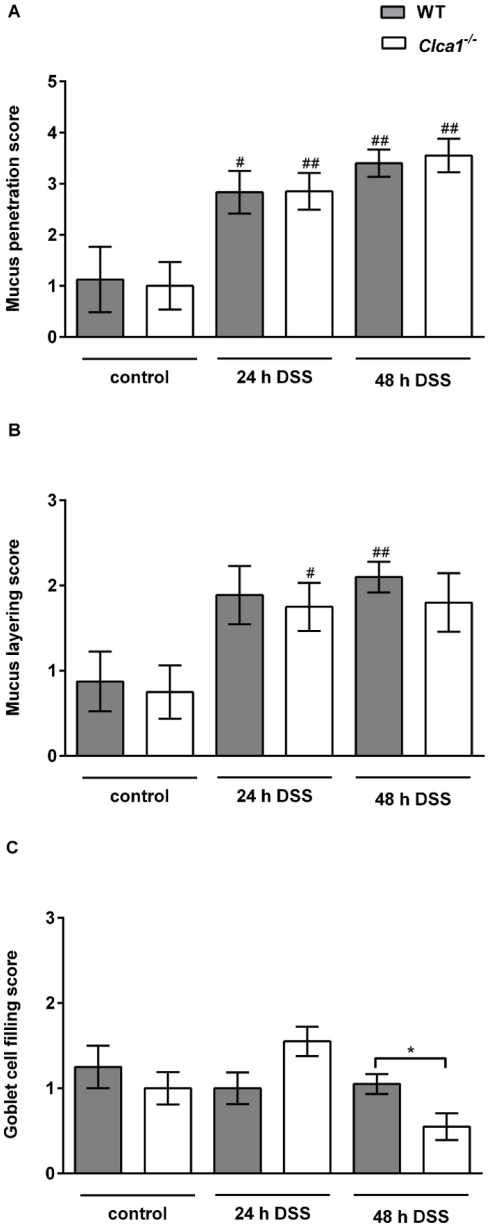

Fig 4. The mucus barrier of WT and *Clca1*$^{-/-}$ mice are identically affected during DSS challenge. Mucus penetration, mucus layering and goblet cell filling in the colon of mice after 24 and 48 h DSS treatment were assessed using IF microscopy. (A) Mucus penetration increased identically under DSS treatment, however, without any significant difference between the genotypes. (B) The mucus layering score also increased under DSS influence without any observable difference between the genotypes. (C) The goblet cell filling score did not show any statistically significant differences, neither between genotypes nor between naive vs. DSS-treated animals, except for WT vs. *Clca1*$^{-/-}$ mice after 48 hours. Mean values (n = 8 to 10). The scoring system is depicted in S3 Table and S4 Table. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ versus the naive control group. $^{*}p < 0.05$ as indicated. Scale bars 50 μm.

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secretion [23]. Consequently, we also speculated that it may have an impact on the protective function of the intestinal mucus barrier.

This study therefore focused on the characterization of the intestinal mucus phenotype in WT controls versus *Clca1*$^{-/-}$ mice. The expression of Clca1, which colocalizes with Muc2

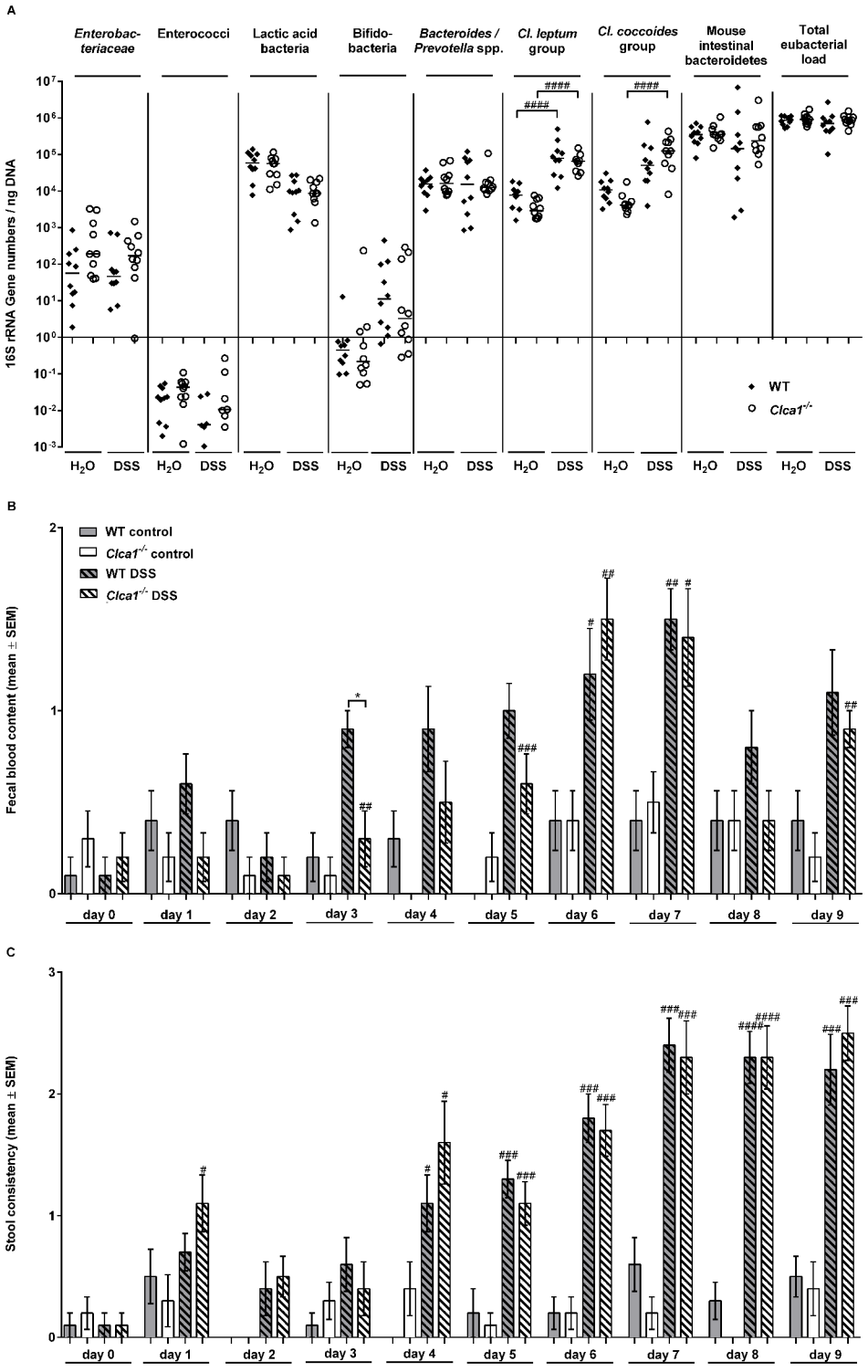

Fig 5. The fecal microbiota composition, blood content and stool consistency are altered during DSS-colitis without differences between *Clca1*$^{-/-}$ and wild type mice. (A) Real-time-PCR analyses, amplifying bacterial 16S rRNA variable regions and 16S rRNA gene numbers/ng DNA derived from colon samples, were employed in order to analyze colonic microbiota composition regarding main intestinal bacterial groups—*Enterobacteriaceae*, enterococci, lactic acid bacteria, bifidobacteria, *Bacteroides/Prevotella spp.*, *Clostridium leptum* group, *Clostridium coccoides* group, mouse intestinal bacteroidetes, and total eubacterial load—whilst comparing WT and *Clca1*$^{-/-}$ fecal samples collected before and after DSS treatment (7 d-group). In the

naive state, mice of either genotype had a comparable fecal microbiota composition. Following colitis induction, increases in the colonic *Clostridium leptum* and *Clostridium coccoides* group as well as in bifidobacteria were evident, yet without any statistically significant difference between the genotypes. A minimum cut-off value of 10^0 gene copy numbers/ng DNA and a minimum log-change of 1 were set as limits of statistically significant relevance. Medians (n = 9 to 10) and significance levels (p values) determined by the Mann-Whitney-U test are indicated. $^{\#\#\#\#}p < 0.0001$ vs. before DSS application. (B) Fecal blood content and (C) stool consistency scores revealed an overall increase in the course of DSS administration without any statistically significant differences between genotypes, except for a higher fecal blood content score in WT vs. *Clca1*$^{-/-}$ mice at day 4. Mean values (n = 9 to 10) ± SEM and significance levels (p values) determined by the Mann-Whitney-U test are indicated. No boxplot = scores of all animals in this group were zero. $^{\#}p < 0.05$, $^{\#\#}p < 0.1$, $^{\#\#\#}p < 0.01$ and $^{\#\#\#\#}p < 0.001$ versus the naive control group. *p < 0.05 as indicated.

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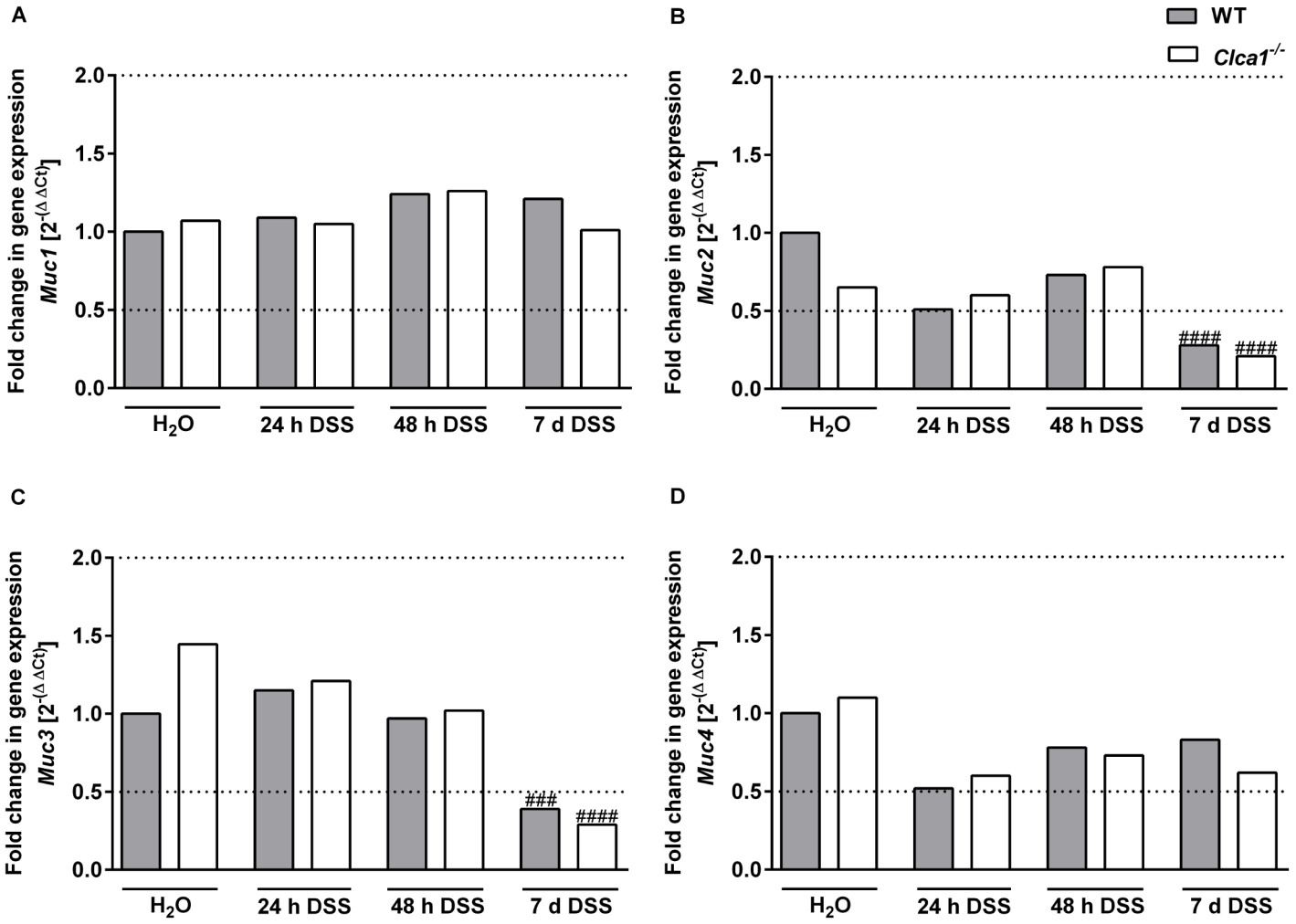

Fig 6. Clca1-deficiency has no effect on the mRNA expression of mucin genes in the intestinal tract under naive and DSS challenged conditions. After treatment of mice with DSS for 24 or 48 h as well as for 7 d with respective controls (n = 9–16 per group), mRNA expression levels of mucin genes were determined by quantitative RT-PCR. (A) *Muc1* gene expression did not show any statistically significant difference, neither between the genotypes nor during challenge. For (B) *Muc2* and (C) *Muc3*, a statistically significant down-regulation in WT and *Clca1*$^{-/-}$ was seen in the 7 d-group compared to water controls. (D) *Muc4* also did not show any significant differential expression, neither between nor amongst the genotypes. Dotted lines indicate a fold change of 0.5 and 2, respectively, as limits for valid statement of lowered and elevated parameters. Ct, cycle threshold. $^{\#\#\#}p < 0.001$ and $^{\#\#\#\#}p < 0.0001$ vs. the naive control group.

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increases along the intestinal axis and thus parallels the increasing impenetrability of the mucus [57]. This also supported our hypothesis that CLCA1 may have a function in the intestinal mucus barrier.

Much to our surprise, the data presented here failed to reveal any involvement in normal mucus structure and function in the model and under the conditions used. No difference in mucus thickness, adherence to the epithelium or transformation from inner to outer mucus was observed between the genotypes, indicating normal mucus secretion and processing in the absence of Clca1. Additionally, an equal responsiveness to the secretagogue carbachol and quality of the mucus preventing bacterial penetration towards the epithelium was observed in *Clca1*$^{-/-}$ and WT mice. The relative protein abundance and expression pattern of the main structural mucus component Muc2 and of other mucus proteins, such as Agr2, Klk1, Fcgbp, Zg16 were unaltered in the Clca1-deficient tissues. The proteomics data also failed to reveal significant changes in mucus composition of *Clca1*$^{-/-}$ mice except for the lack of Clca1.

Our data analysis panel is well in line with and complements previous, solely histological investigations which failed to reveal any intestinal phenotype of various unchallenged *Clca1*$^{-/-}$ models [52,58]. In addition to unchallenged conditions, we also tested for effects of lack of Clca1 in comparison to WT mice after DSS-challenge. The highly sulphated dextran of DSS has a dramatic and rapid destructive impact on the mucus structure and is commonly used as an inducer of colitis in mice [28,59]. Overall, the statistically significant bacterial penetrability increase between the respective naive and DSS-treated groups of the same genotype confirmed the expected loss of mucus barrier integrity within the first 48 h after initial DSS administration [28], but without any significant difference between the genotypes. The overall loss of layering structure within the first 48 h is also in line with previous observations [28]. The only statistically significant difference observed was the higher filling status of *Clca1*$^{-/-}$ goblet cells after 48 h of DSS administration. This could either reflect a decreased mucus release in *Clca1*$^{-/-}$ mice which is contradicted by the mucus structure analysis or may result from an increased synthesis which is contradicted by the gene expression data, both of which show no difference between the genotypes. We therefore feel that the statistical significance of the goblet cell filling score may be biologically debatable. However, we cannot exclude that Clca1 plays a role in exocytosis of mucin granules.

Furthermore, we quantified the induction of selected mucin genes in the distal colon. Mucins are subdivided into the goblet cell secreted gel-forming mucins such as Muc2, Muc5ac, Muc5b and Muc6 and the transmembrane mucins, expressed by epithelial cells, such as Muc1, Muc3 and Muc4 [60–62]. Of these Muc1 to 4 are expressed in the large intestine [63]. No significant alterations in intestinal mucin gene expression were observed between the genotypes in unchallenged conditions although a slightly lower expression of the secreted mucin *Muc2* and slightly higher expression of the membrane-bound *Muc3* were noted in *Clca1*$^{-/-}$ mice compared to WT mice. This difference disappeared during the course of DSS application. In the 7 d-group, *Muc2* and *-3* were, in a very similar fashion, significantly down-regulated in WT and *Clca1*$^{-/-}$ mice compared to water controls which is in line with decreased Muc2 mucin synthesis observed under pathological conditions [64]. It should also be mentioned that RNA levels often poorly correlate with protein levels in the mucus since goblet cells are capable of storing large amounts of mucus. We also failed to detect any changes in Muc2 expression along the intestinal tract by IF or in the relative abundance of Muc2 in the secreted mucus as observed by our proteomics data.

Muc5ac and 5b are the main respiratory mucins. Muc5ac is also expressed in the stomach and is transported through the intesinal tract [13] while MUC5B is expressed at low levels in human colon [65,66] but is not normally observed in the murine gastrointestinal tract [67,68]. MUC6 expression is normally restricted to the stomach and duodenum [69]. Muc5ac

expression was of particular interest due to its observed induction by CLCA1 overexpression in airway cell culture systems and in the respiratory tract [5,33]. In Crohn´s disease, MUC5AC expression is known to be induced in the intestine together with MUC5B and MUC6 [70]. Muc5ac is also expressed in the intestine upon clearance of helminth infections [71]. Besides Muc5ac, Muc6 was also of interest as it exhibited a transient *de novo*-expression in *Muc2*-deficient mice, possibly due to a compensatory protective mechanism within the colonic epithelium [72] although this has never been verified at protein level. In our study, *Muc5ac* and *-5b* as well as *Muc6* failed to show any significant expression in the distal colon, neither in the *Clca1*$^{-/-}$ model nor during pathological conditions regardless of the genotype used. The lack of *Muc5ac*, *-5b* and *-6* on RNA-level is in line with previous reports that failed to detect these mucins at the protein level in unchallenged WT mice [13]. With this data we failed to observe any impact of *Clca1*-deficiency on mucin gene expression in the distal colon. Similar results had been obtained for the murine respiratory tract under naive and challenged conditions [47,52,58]. In contrast, it had been shown that human CLCA1 activates an Il-13-dependent MAPK-mediated airway mucin expression. These differences in mucin gene expression between mice and humans are perhaps due to compensatory effects of certain murine CLCA members [8]. It has previously been argued that, additionally to Clca1, the murine Clca2, -4a and -4b may also induce airway gene expression [8]. Of note, Clca4a and -4b have previously been found to be upregulated at the mRNA level in *Clca1*$^{-/-}$ mice [52]. Here, however, only minute traces of Clca4a and -4b were identified in the secreted mucus without any difference between the genotypes. Clca4a is known to be expressed by enterocytes and its amino-terminal part is released into the mucus [21]. Clca4b is also expressed in the intestine [52,73]. However, its expressing cell type is unknown to date. The Clca2 protein which is expressed in keratinocytes of stratified epithelia and in distinct niches of the respiratory tract [48,74] has not been detected in the intestinal tract to date which is consistent with our findings [48]. The absence of any differential Clca expression pattern in the mucus on the protein level suggests that the lack of a phenotype may not be due to a phenotype rescue by other Clca homologs.

Intestinal microbiota composition was investigated with a focus on main gut bacterial groups which are known to be altered during colitis development [30,44]. After induction of acute DSS colitis, an increase in the colonic clostridia and bifidobacteria was observed which is consistent with previous findings, yet without significant differences between the genotypes [30]. The expected increased scores of fecal blood content and stool consistency, monitored as clinical parameters of colitis occurred independently of the genotype [34,35].

Taken together our data point towards a function of CLCA1 other than in mucus structure, function and barrier integrity. As an alternative function a role for CLCA1 as a signaling molecule regulating cytokine expression has become the focus of recent research. In the respiratory tract, *Clca1*$^{-/-}$ mice develop decreased levels of Cxcl-1, a potent neutrophil chemoattractant [75,76], and of Il-17, a proinflammatory cytokine [77] at the mRNA and protein levels with a consequent decrease in neutrophil response during *Staphylococcus aureus* pneumonia [47]. This observation is in line with reduced Clca1 and CXCL-1 in an IL-17 neutralization study [78]. Consistent with this notion is that the human CLCA1 presumably induces a proinflammatory macrophage response [79]. It has therefore been speculated that CLCA1 may play a role in the modulation of an innate immunity response or cytokine regulation. As a second possible function it has been suggested that CLCA1 may play a role in the differentiation and proliferation of intestinal epithelial cells and colorectal cancer cells [80,81]. Finally, it was recently discovered that secreted CLCA1 increases the surface expression of TMEM16A (Anoctamin1/DOG1), the first genuine calcium-activated chloride channel, in a paracrine fashion and thereby increases the calcium-dependent chloride current *in vitro* [82]. Future experiments will have to clarify whether and how CLCA1 actually fulfills and possibly even

combines several of these suggested functions and how they may relate to the proposed roles of CLCA1 in maladies with secretory dysfunctions. A role for CLCA1 as a signaling molecule affecting the integrity of the epithelial barrier could also be a new area of interest. In conclusion, our results show that Clca1 is not required for intestinal mucus synthesis, structure and barrier function in naive or DSS-challenged mice.

Supporting Information

S1 Table. Revised CLCA nomenclature.
(PDF)

S2 Table. Scoring system for bacterial penetration of the inner mucus layer.
(PDF)

S3 Table. Scoring examples for the inner mucus layer.
(PDF)

S4 Table. Scoring system for the goblet cell filling.
(PDF)

S5 Table. 16S rRNA gene group-specific primers for quantitative Real Time-PCR.
(PDF)

S6 Table. Primers and probes for quantitative Real Time-PCR.
(PDF)

S7 Table. Mucus proteome in wild type and *Clca1*$^{-/-}$ distal colon.
(PDF)

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Author Contributions

Conceived and designed the experiments: NAE EELN LM RG MMH AF SB ADG MEVJ. Performed the experiments: NAE EELN LM LA RG MMH AF GMHB. Analyzed the data: NAE EELN LM LA MMH AF GMHB MEVJ. Contributed reagents/materials/analysis tools: RG AF MMH SB ADG MEVJ. Wrote the paper: NAE EELN LM LA MMH AF SB GMHB ADG MEVJ.

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S1 Table. Revised CLCA nomenclature

Human symbol	New mouse symbol[1]	Previously used mouse symbols	Gene bank accession number
CLCA1	**Clca1**	**mClca3, gob-5**	**NM 017474**
CLCA2	**Clca2**	**mClca5**	**NM 178697**
CLCA3P	**Clca3a1**	**mClca1**	**NM 009899**
	Clca3a2	**mClca2**	**NM 030601**
	Clca3b	**mClca4**	**NM 139148**
CLCA4	**Clca4a**	**mClca6**	**NM 207208**
	Clca4b	**mClca7 (AI747448)**	**NM 0001033199**
	Clca4c	**Clca8 (Gm6289, EG622193, A730041H10Rik)**	**NM 001039222**

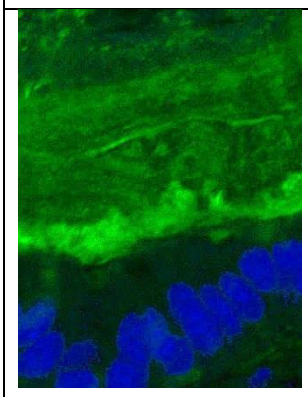
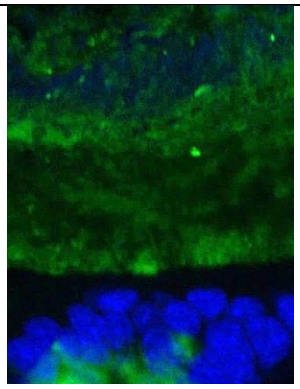
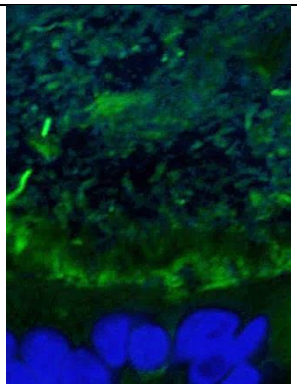
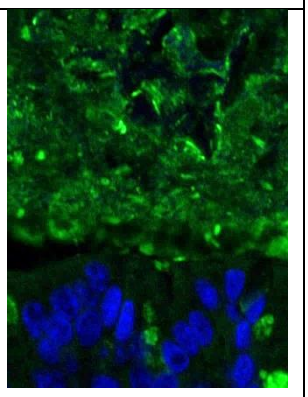
[1]Renamed by the Mouse Gene Nomenclature Committee (MGNC) due to a realignment of the *Clca* genes with respect to human and rat nomenclature in accordance with HUGO (Human Gene Nomenclature Committee) und RGD (Rat Genome Database).

S2 Table. Scoring system for bacterial penetration of the inner mucus layer

Bacterial penetration score[1]	
0	**The inner mucus layer separates bacteria from epithelium**
1	**Few bacteria in the inner mucus layer**
2	**More bacteria entering the mucus but are not in contact with the epithelium**
3	**Some few bacteria are in contact with the epithelium**
4	**A lot of bacteria are in contact with epithelium**
5	**All epithelial surfaces have contact with bacteria**

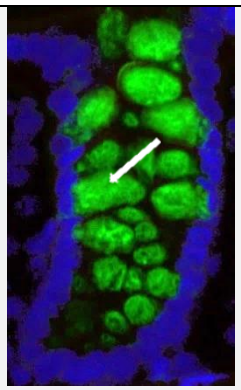
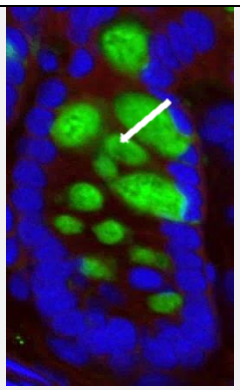
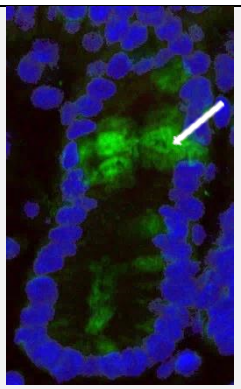
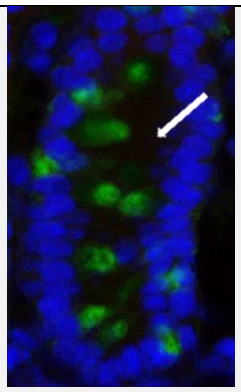
[1] According to Johansson 2010 and 2014 [1;2]

S3 Table. Scoring examples for the inner mucus layer

Mucus layering score			
Score 0	**Score 1**	**Score 2**	**Score 3**
			
Clearly visible layering with stratified structure of the inner mucus layer	Observable layering in the inner mucus layer but with less overall structure	Remains of layering in the inner mucus are visible	No structure at all in the mucus can be observed

Green: Muc2; blue: nuclei

S4 Table. Scoring system for the goblet cell filling

Goblet cell filling score[1]			
Score 0	**Score 1**	**Score 2**	**Score 3**
			
Highly filled and densely packed goblet cells	Moderately filled and loosely packed goblet cells	Minimally filled goblet cells	Depleted goblet cells

[1] Modified from Johansson 2014 [2]

Green: Muc2; blue: nuclei; arrows: representative goblet cell filling status.

S5 Table. 16S rRNA gene group-specific primers for quantitative Real Time-PCR[1]

Main bacterial groups (target)	Bacterial reference strain	Forward primer (5´ - 3´)	Reverse primer (5´ - 3´)	References
Enterobacteriaceae (γ-Proteobacteria/ *Enterobacteriaceae*)	*Escherichia coli* ATCC 25922	AAACTCAAATGAATTGACGG	CTTTTGCAACCCACTCC	Kühbacher 2006 [3]
Enterococci	*Enterococcus gallinarium*	ATCAGAGGGGGATAACACTT	ACTCTCATCCTTGTTCTTCTC	Matsuda 2009 [4]
Lactic acid bacteria (*Lactobacillus* group[2])	*Lactobacillus acidophilus* DSM 20079	AGCAGTAGGGAATCTTCCA	CACCGCTACACATGGAG	Heilig 2002 [5] Walter 2001 [6]
Bifidobacteria (*Bifidobacterium* genus)	*Bifidobacterium* sp. (murine origin)	CTCCTGGAAACGGGTGG	GGTGTTCTTCCCGATATCTACA	Matsuki 2002 [7]
Bacteroides/ Prevotella spp. (*Bacteroides* group[3])	*Bacteroides ovatus* DSMZ 1896	GAAGGTCCCCCACATTG	CAATCGGAGTTCTTCGTG	Bartosch 2004 [8]
Clostridium leptum group (*Clostridium leptum* subgroup[4])	*Clostridium leptum* DSMZ 753	TTACTGGGTGTAAAGGG	TAGAGTGCTCTTGCGTA	Van Dyke 2002 [9]
Clostridium coccoides group (*Clostridium coccoides*[5])	*Clostridium coccoides* DSMZ 935	AAATGACGGTACCTGACTAA	CTTTGAGTTTCATTCTTGCGAA	Matsuki 2002 [7]
Mouse intestinal bacteroidetes (Mouse Intestinal Bacteroides)	MIB plasmid 16-1	CCAGCAGCCGCGGTAATA	CGCATTCCGCATACTTCTC	Barman 2008 [10]
Total eubacterial load (Domain Bacteria, targets 16S V3 region)	*Escherichia coli* ATCC 25922	CGGYCCAGACTCCTACGGG	TTACCGCGGCTGCTGGCAC	Lee 1996 [11]

[1] from Rausch 2013 [12]

[2] including *Leuconostoc*, *Pediococcus*, *Aerococcus* and *Weissella* but not *Enterococcus* or *Streptococcus*

[3] including *Prevotella* and *Porphyromonas*

[4] including *Faecalibacterium* (Fusobacterium) *prausnutzii Clostridium* 16S rRNA cluster IV

[5] *Eubacterium rectale* subgroup (*Clostridium* 16S rRNA cluster XIVa/b)

S6 Table. Primers and probes for quantitative Real Time-PCR

Gene	Forward primer (5´ - 3´)	Reverse primer (5´- 3´)	Probe	References
Gapdh	TCACCACCATGGAGAAGGC	GCTAAGCAGTTGGTGGTGCA	ATGCCCCCATGTTTGTGATGGGTGT	Giulietti 2001 [13]
Ef-1α	CAAAAACGACCCACCAATGG	GGCCTGGATGGTTCAGGATA	AGCAGCTGGCTTCACGCTCAGGTG	Braun 2010 [14]
B2m	ATTCACCCCCACTGAGACTGA	CTCGATCCCAGTAGACGGTC	TGCAGAGTTAAGCATGACAGTATGGCCG	Norris 2000 [15]
Muc1	TATGGGCAGCTGGACATCTT	ATTACCTGCCGAAACCTCCT	ATGAGTGAATACCCTACCTACCACA	*
Muc2	CTCCATTGAGTTTGGGAACAT	TTCGGCTCGGTGTTCAGAG	CTGAGGCAGTACAAGAACCG	*
Muc3	GGAACTGGTGGAGAGCGTAG	CGGTCCTGTAGCTTCTCACTG	TCCGTGGAAGTGAGTGTGAC	*
Muc4	GTCCACTTCTTCCCCATCTC	GTAGCCTTTGTAGCCATCACAT	CCAGGACCAGATGGCTCTGAACCT	Tetaert 2007 [16]
Muc5ac	GCGTGGAGAATGAAAAGTATGCT	CCCAGTGCCACGCCACCGTG	CATACATGCAGTTCGAGAAGAAG	*
Muc5b	GCACGTAAATGCGACTGTCT	TATCCAAGTACTCCATGGAGGCCC	ATGGACCTTGCTCTCCTGAC	Mundhenk 2012 [17]
Muc6	CACCAACACAGAAGGCTGTT	CTGTGTACCCTGTGCACCAC	CTGTGTACCCTGTGCACCAC	*

*Primers and probes designed by authors.

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2.2 Role of Goblet Cell Protein CLCA1 in Murine DSS Colitis

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Contributions of other authors: All co-authors participated considerably to the study design, evaluation of experimental results, and the creation of the manuscript.

Declaration on ethics: All animal procedures were approved by the local governmental authorities (State Office of Health and Social Affairs Berlin, approval IDs: T 0394/12; G 0170/12) and conducted at the ‘‘Research Institutes for Experimental Medicine”, Charité - Universitätsmedizin Berlin, Germany, in strict accordance with the FELASA guidelines [167].

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Journal of Inflammation

SHORT REPORT Open Access

Role of goblet cell protein CLCA1 in murine DSS colitis

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Abstract

Background: The secreted goblet cell protein CLCA1 (chloride channel regulator, calcium-activated-1) is, in addition to its established role in epithelial chloride conductance regulation, thought to act as a multifunctional signaling protein, including cellular differentiation pathways and induction of mucus production. Specifically, CLCA1 has recently been shown to modulate early immune responses by regulation of cytokines. Here, we analyze the role of CLCA1, which is highly expressed and secreted by colon goblet cells, in the course of murine dextran sodium sulfate-induced colitis.

Findings: We compared *Clca1*-deficient and wild type mice under unchallenged and DSS-challenged conditions at various time points, including weight loss, colon weight-length-ratio and histological characterization of inflammation and regeneration. Expression levels of relevant cytokines, trefoil factor 3 and E-cadherin were assessed via quantitative PCR and cytometric bead arrays. Lack of CLCA1 was associated with a more than two-fold increased expression of *Cxcl-1*- and *Il-17*-mRNA during DSS colitis. However, no differences were found between *Clca1*-deficient and wild type mice under unchallenged or DSS-challenged conditions in terms of clinical findings, disease progression, colitis outcome, epithelial defects or regeneration.

Conclusions: CLCA1 is involved in the modulation of cytokine responses in the colon, albeit differently than what had been observed in the lungs. Obviously, the pathways involved depend on the type of challenge, time point or tissue environment.

Keywords: *Clca1*$^{-/-}$, CXCL-1, Keratinocyte chemottractant, CXCL-2, MIP-2α, Inflammation, Signaling molecule, IL-17, E-cadherin, TFF3

Introduction

The goblet cell-derived protein CLCA1 (chloride channel regulator, calcium-activated 1) is thought to act as a multifunctional signaling protein via as yet unidentified molecular pathways. Originally, CLCA1 had been thought to modulate epithelial cell chloride conductance. Subsequent work has shown, however, that it may also induce airway mucus production through an interleukin (IL)-13-mediated cascade [1] and promote spontaneous differentiation while reducing proliferation of Caco-2 cells [2]. In a similar context, CLCA1 expression was proposed as a prognostic factor in colorectal cancer [2, 3].

Recently, CLCA1 was shown to modulate pulmonary cytokine expression in early immune responses, specifically the pro-inflammatory response of human airway macrophages in vitro [4], whereas more complex results have been obtained from mouse models [5, 6]. In *Clca1*-deficient (*Clca1*$^{-/-}$) mice, experimental *Staphylococcus (S.) aureus* pneumonia was associated with decreased responses of chemokine (C-X-C motif) ligand (CXCL)-1, a potent neutrophil chemoattractant, with consequently decreased neutrophil recruitment [5]. Furthermore, lack of Clca1 expression yielded reduced responses of the pro-inflammatory cytokine IL-17. In contrast, following intranasal ovalbumin or lipopolysaccharide (LPS) challenge, Clca1-deficiency resulted in increased neutrophil recruitment preceded by CXCL-1 upregulation in the LPS model [6]. Thus, the role of CLCA1 in cytokine

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modulation seems to be complex and dependent on the stimulus used.

Here, we investigated the proposed function of CLCA1 in modulating the early immune response in the colon, the tissue in which CLCA1 is most highly expressed in man [7] and mice [8]. We chose the dextran sodium sulfate (DSS) challenge model which is commonly used to study early immune reactions in mouse intestine [9]. In a previous DSS colitis study, we failed to observe any effects of Clca1-deficiency on mucus barrier integrity and mucin gene expression [10] which likely would have affected secondary immune responses. Additionally, recent studies have indicated that CLCA1 does not play a role in calcium-activated chloride secretion in the respiratory tract nor does restoration of reduced Clca1 expression rectify the cystic fibrosis electrophysiology defect in the intestine [11, 12]. In light of the absence of such possibly interfering effects, we postulated that possible differences in inflammatory parameters during DSS colitis in the *Clca1*$^{-/-}$ model would be due to primary CLCA1 effects on the immune response. We thus compared *Clca1*$^{-/-}$ and wild type (WT) mice under unchallenged and DSS-challenged conditions in terms of key clinical and histopathological parameters as well as expression profiles of select cytokines. Furthermore, we determined expression levels of goblet cell-derived trefoil factor (*Tff*)-3, a key regulator in mucosal repair and protection [13], and E-cadherin (E-cad) which is down-regulated after *Clca1*-knock-down in vitro [3].

Materials and methods

Ethics statement, mice and DSS treatment

Clca1$^{-/-}$ and WT mice were given 2.5 % DSS for 24 (24 h-group), 48 h (48 h-group) or for 7 days with 2 consecutive days of water (7 d-group) as described [10]. For ethics statement, selection and treatment see Additional file 1.

Weight loss, colon weight-length-ratio and sampling

In the 7 d-group, weight loss was determined in the course of DSS administration and colon weight-length-ratio at necropsy. Colons of all groups were equally sectioned from proximal to distal for histopathology which were immediately fixed in 4 % buffered formalin. For Reverse Transcriptase-quantitative PCR (RT-qPCR) and organ culture, sections were opened longitudinally, flushed with ice-cold Dulbecco's phosphate buffered saline (biowest, Nuaille, France) and either snap frozen in liquid nitrogen and stored at − 80 °C or immediately processed, respectively.

Histopathology

4 μm thick formalin-fixed, paraffin-embedded, hematoxylin and eosin-stained sections of proximal and distal colon were evaluated separately by veterinary pathologists in a blinded fashion according to a scoring scheme (Additional file 2).

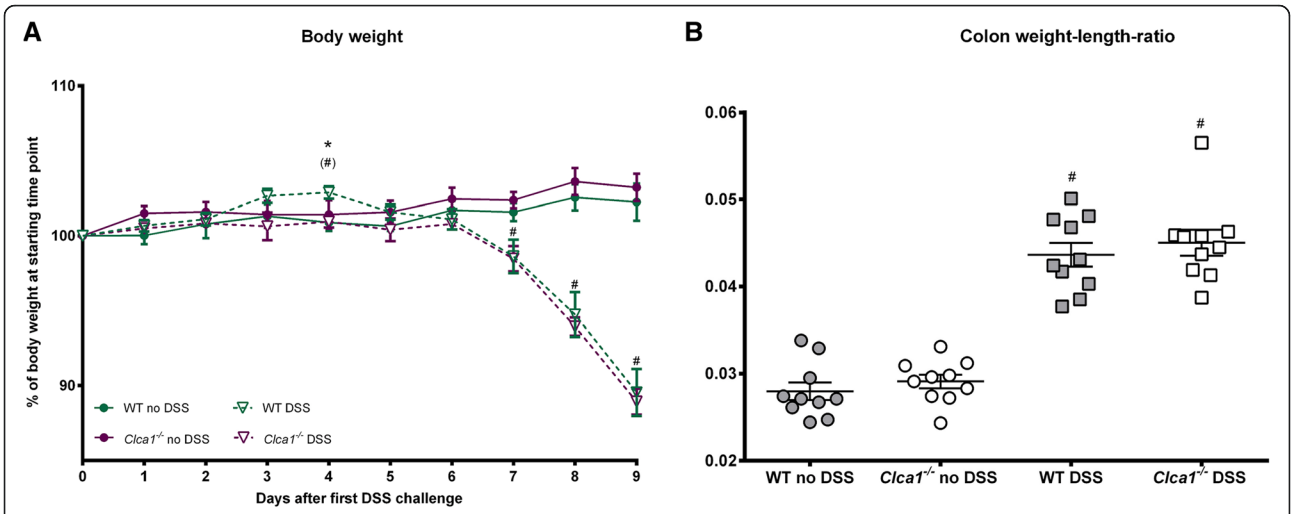

Fig. 1 Similar body weights and colon weight-length-ratios of *Clca1*$^{-/-}$ and WT mice. **a** From day 7 on, both DSS-treated genotypes showed a continuous weight loss to 88.9 ± 0.9 % and 89.5 ± 1.6 % at day 9 whereas the unchallenged mice continued a slight overall weight gain to 103.2 ± 0.9 % and 102.2 ± 1.2 % for *Clca1*$^{-/-}$ and WT mice, respectively. At day 4, DSS-treated WT mice had higher body weights than the WT controls and the DSS-challenged *Clca1*$^{-/-}$ group, the relevance of which, however, remains questionable. $n = 10 - 30$ per group. **b** The colon weight-length-ratios increased during DSS colitis compared to unchallenged controls from 0.027 ± 0.001 and 0.027 ± 0.001 to 0.044 ± 0.002 and 0.043 ± 0.001 for *Clca1*$^{-/-}$ and WT, respectively, however, with no difference between the genotypes. $n = 10$ per group. $^{\#}p < 0.05$ vs. the unchallenged control group. $^{*}p < 0.01$ between the genotypes

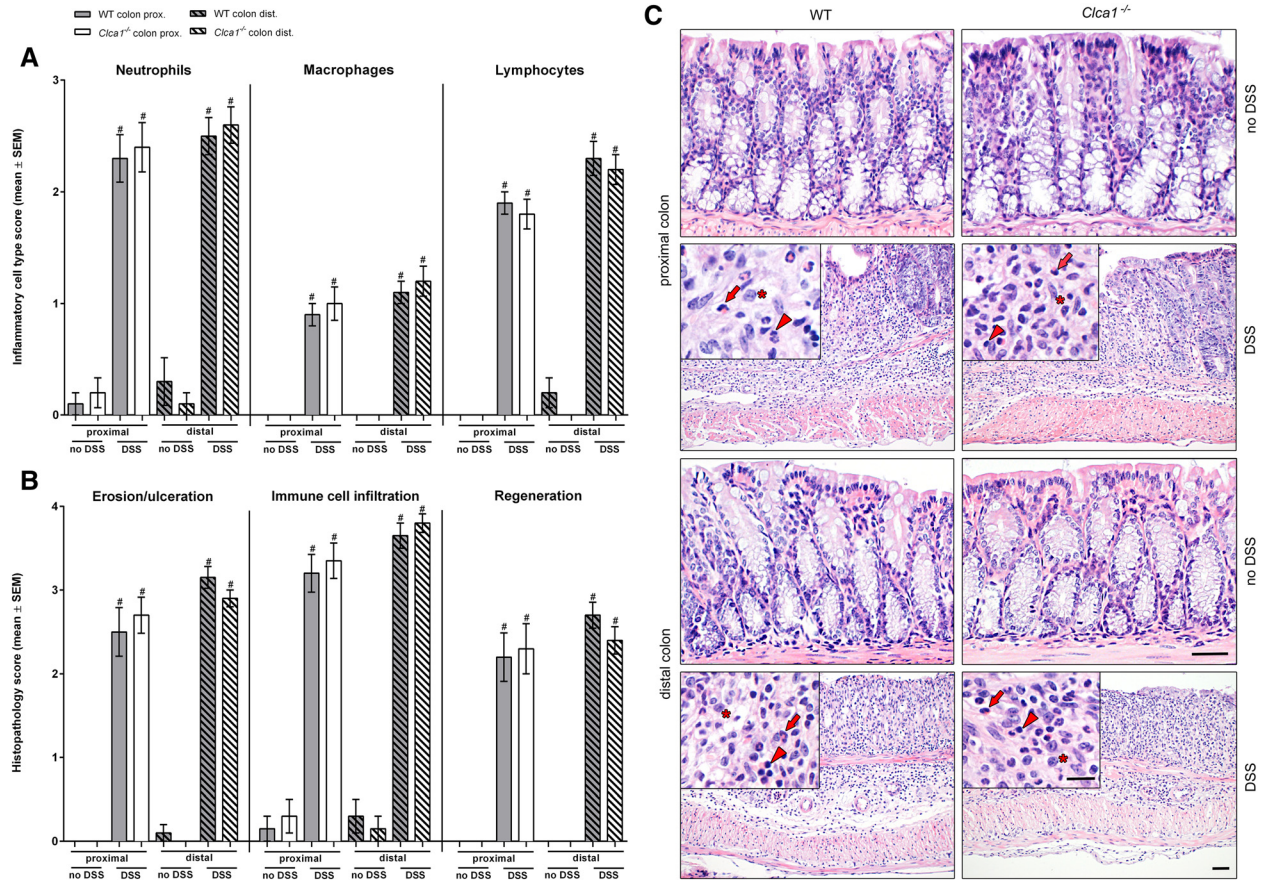

Fig. 2 Similar inflammation and regeneration in *Clca1*$^{-/-}$ and WT mice during DSS colitis. During DSS colitis, the **a** abundance of neutrophils, macrophages and lymphocytes, **b** the histopathology scores for erosion/ulceration and immune cell infiltration increased as well as regeneration in proximal and distal colon following a 7-day DSS colitis induction and administration of water for 2 consecutive days. No differences were observed between *Clca1*$^{-/-}$ and WT mice. **c** Representative histopathology of proximal and distal colon of DSS-treated *Clca1*$^{-/-}$ and WT mice and water controls; neutrophils (arrows), macrophages (asterisks) and lymphocytes (arrowheads). Bars: 20 μm (no DSS and insets) and 50 μm (DSS). $n = 10$ per group. $^{\#}p < 0.05$ vs. the unchallenged control group

RNA isolation and reverse transcriptase-qPCR

Total RNA isolation, primer and probe design, RT-qPCR and data analysis were performed as described [5, 10]. Transcript expression levels of *Cxcl-1*, *Cxcl-2*, *Il-17*, *Tnf*, *Ifnγ*, *Tff3* and *E-cad* were determined and normalized to the internal reference genes glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), elongation factor-1α (*Ef-1α*) and ß-2 microglobulin (*B2m*) as described [5]. Primers and probes are listed in Additional file 3.

Cytometric bead array

Colon cultures were established and supernatants processed as described [14] and stored at -80 °C until further analysis. Cytokine concentrations of CXCL-1, monocyte chemoattractant protein (MCP)-1, TNF, IFNγ, IL-1β, -2, -6, -13 and -17A were determined via cytometric bead array using a FACSCantoII and the FacsDiva software (all BD Biosciences, Heidelberg, Germany) as described [15, 16].

Statistics

Statistical analyses via Mann–Whitney-U test and graphical illustrations were performed using GraphPad PRISM 6 (GraphPad Software Inc., La Jolla, USA) and data are expressed as mean ± standard error of the mean (SEM) except for RT-qPCR data. Here, data are expressed as single value fold change of which a more than two-fold difference between the genotypes was considered relevant.

Results and discussion

The percentile body weights declined following day 6 of DSS application (Fig. 1a) and colon weight-length-ratios increased (Fig. 1b), without any difference between the genotypes (Fig. 1) which is in line with the genotype-independent decrease of stool consistency and increase of fecal blood content in the DSS colitis model described earlier [10]. At day 4, DSS-treated WT mice had higher body weights compared to the DSS-challenged *Clca1*$^{-/-}$

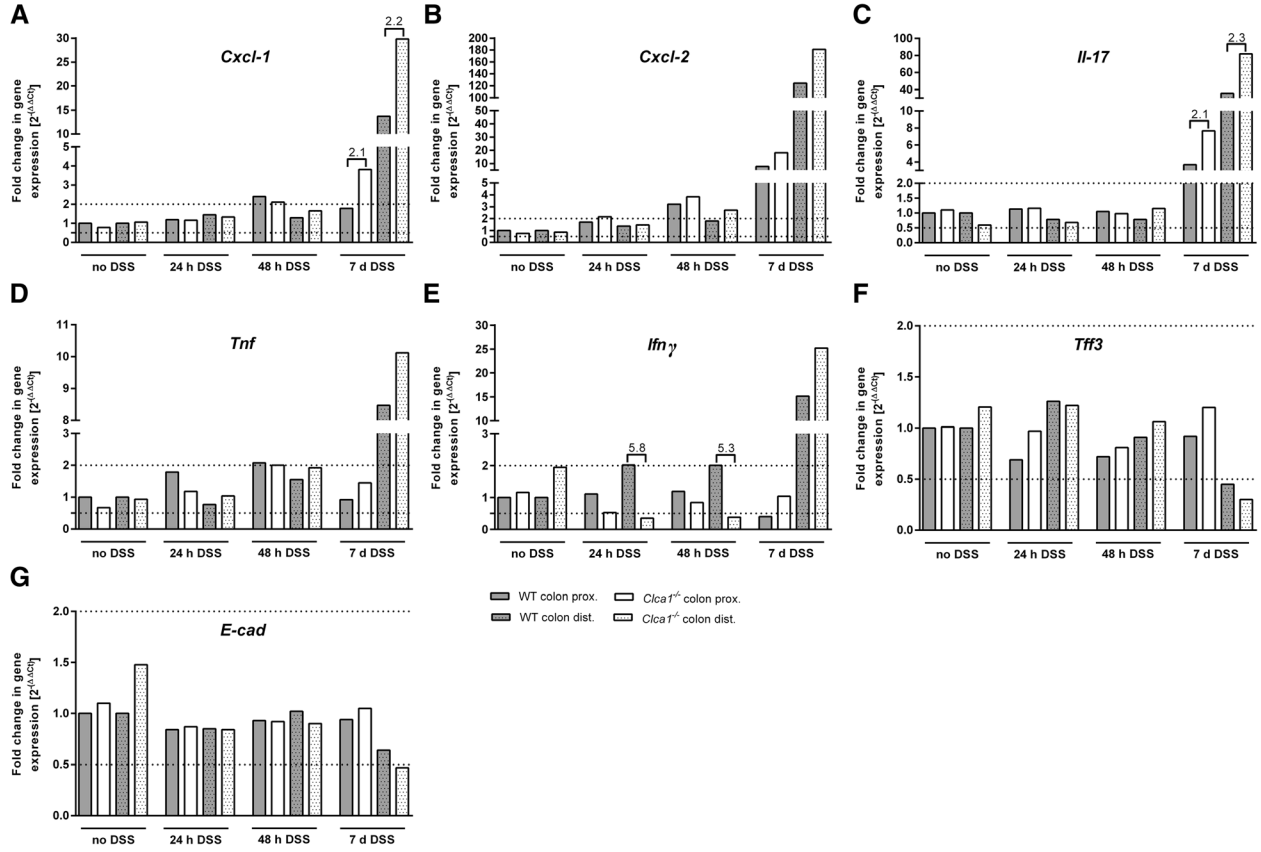

Fig. 3 Increase in *Cxcl-1*- and *Il-17*-mRNA expression in *Clca1*$^{-/-}$ mice during DSS-challenge. During DSS colitis, **a** *Cxcl-1*, **b** *Cxcl-2*, **c** *Il-17*, **d** *Tnf* and **e** *Ifnγ* were upregulated in the distal colon as the prime target site of DSS and sporadically also in the proximal colon. However, only *Cxcl-1* and *Il-17* showed a greater than two-fold increase of mRNA in the distal colon of *Clca1*$^{-/-}$ compared to WT. At earlier time points, *Cxcl-2* was upregulated at 24 h in *Clca1*$^{-/-}$ proximal colon and at 48 h in all except for WT distal colon. At 48 h, *Cxcl-1*, *Tnf* and *Ifnγ* were upregulated in proximal colon of *Clca1*$^{-/-}$ and WT, in proximal and in distal colon of WT mice, respectively. *Ifnγ* showed a more than five-fold decrease in the distal colon of DSS-challenged *Clca1*$^{-/-}$ compared to WT at 24 and also at 48 h. Expression of **f** *Tff3* in distal *Clca1*$^{-/-}$ and WT colon and of **g** *E-cad* in distal *Clca1*$^{-/-}$ colon was lower during colitis conditions, however, without difference between the genotypes. Dotted lines indicate a fold change of 0.5 and 2, respectively, as limits for valid statement of lowered or elevated expressions. A greater than two-fold difference in fold change was considered as relevant difference between the genotypes. Ct, cycle threshold. Relative quantification and comparison of groups were performed by the ΔΔCt method using unchallenged WT animals as controls (fold change = 1). *n* = 9–18 per group

mice but also to unchallenged WT controls. In this context and being only 2 % in difference between the genotypes, this single statistically significant data point is biologically questionable. Alternatively, in the context of *Ifnγ* mRNA decrease at early time points (see below) in *Clca1* $^{-/-}$ mice, this may indicate changes in metabolism in *Clca1*-deficient mice only. Importantly, Clca1-deficiency had no impact on any clinical parameter tested which is in line with the previous respiratory challenge model [5].

Histopathologically, Clca1-deficiency did not result in any effect on the extent and nature of inflammatory cells, epithelial defects and regeneration. During DSS colitis, neutrophils, macrophages and lymphocytes (Fig. 2a and c, insets) as well as erosion/ulceration, immune cell infiltration and regeneration (Fig. 2b and c) increased in proximal and distal colon, clearly reflecting the expected inflammation. At 24 und 48 h, no significant immune cell infiltrations or histopathologic alterations were observed (Additional file 4). In contrast to previous studies on airway inflammation in which neutrophil responses were either decreased in *Clca1*$^{-/-}$ mice after *S. aureus* infection [5] or increased after LPS challenge [6], no difference was observed histopathologically in the colitis model between the genotypes.

As expected, mRNA expressions of *Cxcl-1*, *Cxcl-2*, *Il-17*, *Tnf* and *Ifnγ* (Fig. 3a to e) were overall upregulated in distal and, sporadically, also in proximal colon during DSS colitis. These cytokines, except for *Il-17*, occasionally were slightly elevated at earlier time points.

However, *Cxcl-1* and *Il-17* (Fig. 3a and c) showed a greater than two-fold increase of mRNA copy numbers in distal colon of *Clca1*$^{-/-}$ compared to WT mice during colitis. The significance of the transiently lower *Ifnγ*

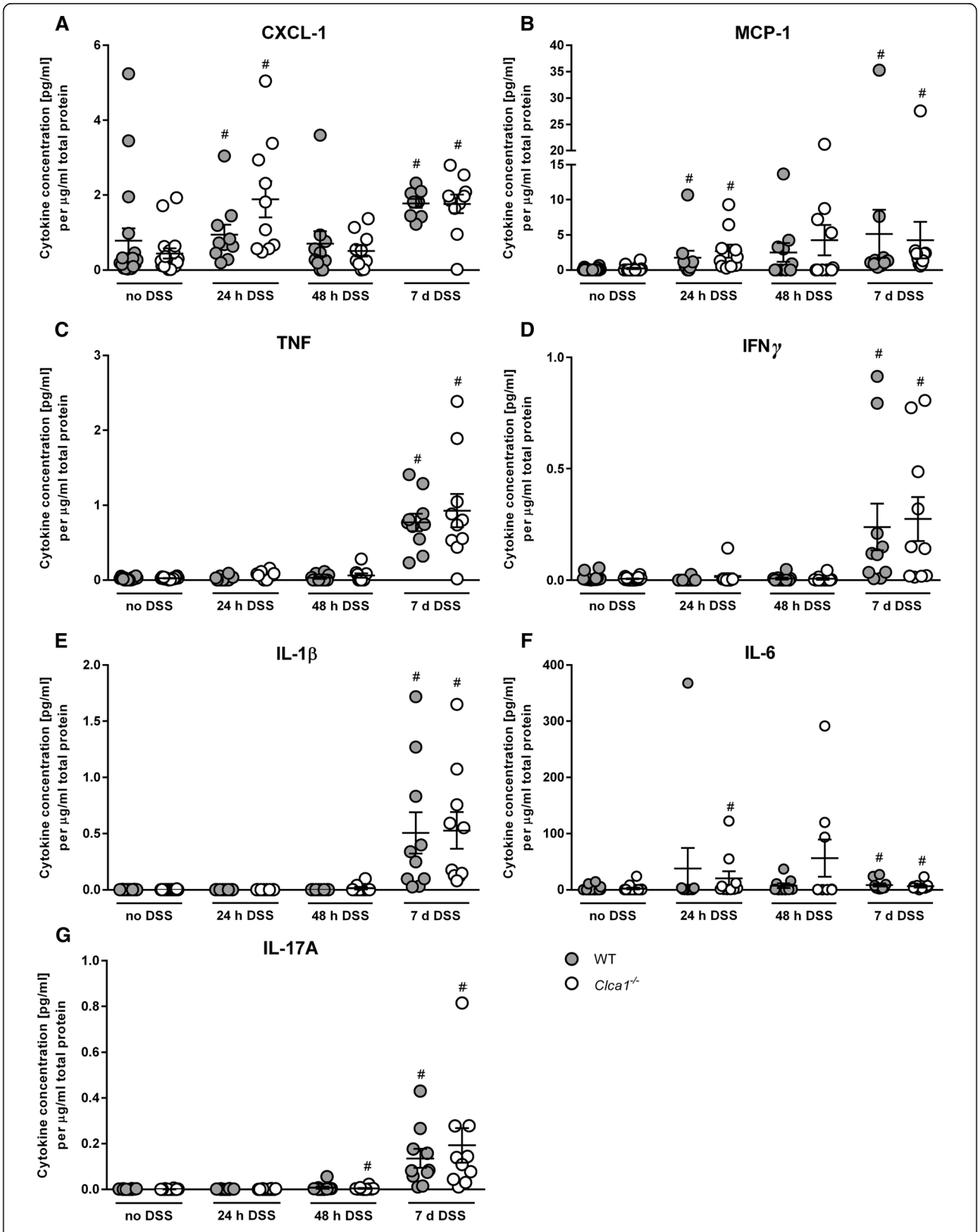

Fig. 4 (See legend on next page.)

(See figure on previous page.)
Fig. 4 Inflammatory cytokines are similarly upregulated on protein level in DSS colitis between the genotypes. Cytokines were determined on the protein level in colon supernatant via cytometric bead array and normalized to the respective total protein concentration. **a** CXCL-1, **b** MCP-1, **c** TNF, **d** IFNγ, **e** IL-1β, **f** IL-6 and **g** IL-17A were elevated during DSS colitis without any difference between the genotypes. CXCL-1, MCP-1 and IL-6, the latter only in *Clca1*$^{-/-}$, seemed also slightly elevated at 24 h as well as IL-17A in *Clca1*$^{-/-}$ at 48 h after DSS challenge, however, without statistical significance between the genotypes. $^{\#}p < 0.05$ vs. the unchallenged control group. $n = 9–18$ per group. Only if more than 5 mice had detectable protein values, the group was considered for valid statement of significance

expression levels in the distal colon of DSS-challenged *Clca1*$^{-/-}$ compared to WT at 24 and 48 h (Fig. 3e) is unclear due to lack of histologically evident immune cell infiltration at these time points (Additional file 4). Expression of *Tff3* mRNA in distal colon of *Clca1*$^{-/-}$ and WT mice and of *E-cad* in distal *Clca1*$^{-/-}$ colon (Fig. 3f and g) was lower during colitis, likely due to destruction of goblet and epithelial cells, respectively, without differences between the genotypes.

CXCL-1, MCP-1, TNF, IFNγ, IL-1β, -6 and -17A proteins (Fig. 4a to g) were similarly elevated during DSS colitis, CXCL-1 and MCP-1 also slightly at 24 h of DSS-challenge in both genotypes, IL-6 at 24 and IL-17A at 48 h in the *Clca1* $^{-/-}$ mice.

The cytokines IL-2 and -13 were below the detection limits at all time points.

Interestingly, differential expression of *Cxcl-1* mRNA has also been found in respiratory Clca1-deficient mouse models [5], consequentially with differences in CXCL-1 protein level and neutrophil recruitment [5, 6]. Additionally, *Il-17* was also differentially expressed in one model [5]. The prominently higher mRNA expression levels of *Cxcl-1* and *Il-17* in *Clca1*$^{-/-}$ DSS colitis mice may point towards initial regulatory events. This may become obvious on protein level at later time points as had been seen in the *S. aureus* pneumonia model [5]. The increase (1.4 fold) of IL-17A in the *Clca1* $^{-/-}$ mice of the 7-d group may confirm this notion.

Despite being opposite to the *S. aureus* pneumonia model in which *Cxcl-1* and *Il-17* were decreased in *Clca1*$^{-/-}$ mice [5], the DSS colitis data are in line with increased CXCL-1 responses following respiratory LPS-challenge [6]. Early Clca1-linked immune response modulation therefore seems to depend on the stimulus used. As solely LPS-mediated Toll-like receptor 4 signaling seems to be important for neutrophil recruitment, control of bacterial translocation and epithelial repair in acute DSS colitis [17], this model possibly shares similarities with the respiratory LPS challenge.

Our findings confirm the link of CLCA1 to early immune response modulation with a specific effect on *Cxcl-1* and *Il-17* albeit not decisive for clinical outcome. Potentially overlapping effects of CLCA1, including anion conductance and cellular differentiation, will have to be taken into account when further deciphering the interaction of Clca1 with *Cxcl-1* and *Il-17* in early immune responses.

Additional files

Additional file 1: Ethics statement, selection and treatment. (PDF 34 kb)

Additional file 2: Histopathological scoring scheme. (PDF 80 kb)

Additional file 3: Quantitative RT-PCR – primer and probe sequences. (PDF 108 kb)

Additional file 4: Results of histopathology scoring of 24 and 48 h-groups. (PDF 81 kb)

Abbreviations
CLCA: Chloride channel regulator, calcium-activated; gob-5: Goblet cell protein-5; DSS: Dextran sodium sulfate; *Clca1*$^{-/-}$: *Clca1*-deficient; Cxcl: Chemokine (C-X-C motif) ligand; IL: Interleukin; LPS: Lipopolysaccharide; WT: Wild type; RT-qPCR: Reverse transcriptase quantitative polymerase chain reaction; Tff: Trefoil factor; IFNγ: Interferone gamma; FELASA: Federation of Laboratory Animal Science Associations; h: Hours; d: Days; mRNA: Messenger ribonucleic acid; TNF: Tumor necrosis factor; Gapdh: Glyceraldehyde-3-phosphate dehydrogenase; Ef-1α: Elongation factor 1alpha; B2m: beta-2 microglobulin; MCP: Monocyte chemoattractant protein; SEM: Standard error of the mean; pg: Picograms; µg: Micrograms; ml: Milliliters; *S. aureus*: *Staphylococcus aureus*; KC: Keratinocyte chemoattractant; vs.: Versus; MIP-2α: Macrophage inflammatory protein 2-alpha; E-cad: E-cadherin (alias Cdh1).

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
NAE designed and executed the experiments, analyzed the data and wrote the paper. LM conceived of, designed and partially executed the experiments, provided critical analysis and reviewed the manuscript. SG partially executed the data analysis. RG partially executed the experiments and provided essential expertise. MMH provided essential expertise, participated in the study design and reviewed the manuscript. ADG conceived of and coordinated the study, participated in its design, provided critical expertise and reviewed the manuscript. All authors read and approved of the final manuscript.

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Additional file 1: Ethics statement, selection and treatment

All animal procedures were conducted at the ‘‘Research Institutes for Experimental Medicine”, Charité - Universitätsmedizin Berlin, Germany, in accordance with the FELASA guidelines [1] and approved by the local governmental authorities (State Office of Health and Social Affairs Berlin, approval IDs: T 0394/12; G 0170/12).

Weight- and age-matched female *Clca1*$^{-/-}$ and WT mice were given 2.5 % DSS (MW 36,000 - 50,000, 17.1 % sulfur substitution, no detectable free sulfate, pH 7.1, MP Biomedicals, LLC., Illkirch, France) for 24 (24 h-group) or 48 hours (48 h-group) to allow for the investigation of initial DSS effects, and for 7 days with 2 consecutive days of tap water only (7 d-group) for monitoring effects during acute colitis with beginning regenerative effects as described [2]. Solely female mice were used in the experiment due to ethical and animal welfare reasons. Using female mice reduces the risk of stress and rank fights with subsequent injuries and the possibility of stress-induced weight loss. Second, as females are more resistant to DSS compared to male mice [3], the risk of the animals losing weight too rapidly within the experiment is reduced. A too rapid weight loss could have lead to an exclusion of animals from the experiment in terms of humane end points which would have increased the overall number of animals used in the experiment.

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Additional file 2: Histopathological scoring scheme*

Cell type score for neutrophils, macrophages or lymphocytes	
0	Absent
1	Few
2	Moderate
3	Abundant

Erosion/ulceration			
Depth of erosions/ulcerations		Extent of erosions/ulcerations	
0	None	0	None (0)
1	*Lamina epithelialis*	1	Punctate (1)
2	+ *Lamina propria mucosae*	2	Minimal (2)
3	+ *Lamina muscularis propriae*	3	Moderate (3)
4	+ *Tela submucosa*	4	Widespread (> 3)

Immune cell infiltration			
Presence of immune cell infiltration		Extent of immune cell infiltration	
0	None	0	None
1	Minimal	1	Mucosal
2	Mild	2	Mucosal + submucosal
3	Moderate	3	Mucosal + submucosal + muscle
4	Severe	4	Mucosal + submucosal + muscle + serosa

Regeneration	
0	No reepithelization
1	Hyperplasia of crypt epithelium
2	Early reepithelization
3	Partial reepithelization
4	Complete reepithelization

*modified from Rachmilewitz D, Karmeli F, Takabayashi K, Hayashi T, Leider-Trejo L, Lee J, Leoni LM, Raz E: **Immunostimulatory DNA ameliorates experimental and spontaneous murine colitis.** *Gastroenterology* 2002, **122:**1428-1441.

Additional file 3: Quantitative RT-PCR – primer and probe sequences

Gene	Genbank Accession no.	Oligonucleotide Sequences (5'-3')	Reference
Cxcl1(KC)	NM_008176.3	Primer (upstream) GATGCTAAAAGGTGTCCCCA Primer (downstream)AGACTGCTCTGATGGCACCT TaqMan Probe FAM-GTCAGAAGCCAGCGTTCAC-BHQ	*
Cxcl2(MIP-2α)	NM_009140.2	Primer (upstream) CTGAACAAAGGCAAGGCTAACT Primer (downstream)ACCTGGAAAGGAGGAGCCT TaqMan Probe FAM-CTTTGGTTCTTCCGTTGAGG-BHQ	*
Il-17	NM_010552	Primer (upstream) GCTCCAGAAGGCCCTCAGA Primer (downstream)CTCTCCACCGCAATGAAGACCCTGA TaqMan Probe FAM-AGCTTTCCCTCCGCATTGA-BHQ	[1]
TNF	NM_013693.3	Primer (upstream) CATCTTCTCAAAATTCGAGTGACAA Primer (downstream)TGGGAGTAGACAAGGTACAACCC TaqMan Probe FAM-CACGTCGTAGCAAACCACCAAGTGGA-BHQ*	[2]
INFγ	NM_008337.3	Primer (upstream) GAACTGGCAAAAGGATGGTGAC Primer (downstream)GTTGCTGATGGCCTGATTGTC TaqMan Probe FAM-TGAAAATCCTGCAGAGCCAGA-BHQ*	ID 8139 [3]
Tff3	NM_011575.2	Primer (upstream) TAATGCTGTTGGTGGTCCTG Primer (downstream)CAGCCACGGTTGTTACACTG TaqMan Probe FAM-ATGTCAGAGTGGACTGTGGCTAC-BHQ	*
E-Cad (Cdh1)	NM_009864.2	Primer (upstream) GGCTTCAGTTCCGAGGTCTA Primer (downstream)AGTCTTCCGAAAAGAAGGCTG TaqMan Probe FAM-TCCTGGGCAGAGTGAGATTT -BHQ	*
Ef-1a	NM_010106.2	Primer (upstream) AAAAACGACCCACCAATGG Primer (downstream)GGCCTGGATGGTTCAGGATA TaqMan Probe FAM- AGCAGCTGGCTTCACGCTCAGGTG -BHQ	[4]
B2m	NM_009735.3	Primer (upstream) ATTCACCCCCACTGAGACTGA Primer (downstream)CTCGATCCCAGTAGACGGTC TaqMan Probe FAM-TGCAGAGTTAAGCATGACAGTATGGCCG -BHQ	[5]
Gapdh	NM_008084.2	Primer (upstream) TCACCACCATGGAGAAGG Primer (downstream)GCTAAGCAGTTGGTGGTGCA TaqMan Probe FAM-ATGCCCCCATGTTTGTGATGGGTGT-BHQ	[1]

*Primers and/or probes designed by authors.

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Additional file 4: Results of histopathology scoring of 24 and 48 h-groups

	Clca1$^{-/-}$ 24 h DSS proximal colon										*Clca1*$^{-/-}$ 24 h DSS distal colon									
	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10
Cell type score neutrophils	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cell type score macrophages	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cell type score lymphocytes	0	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Erosion/ulceration - depth	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Erosion/ulceration - extent	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Immune cell infiltration - presence	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Immune cell infiltration - extent	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Regeneration	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	WT 24 h DSS proximal colon										WT 24 h DSS distal colon									
	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10
Cell type score neutrophils	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cell type score macrophages	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Cell type score lymphocytes	0	0	0	1	0	0	0	1	1	1	0	0	0	1	1	0	0	0	0	1
Erosion/ulceration - depth	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Erosion/ulceration - extent	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Immune cell infiltration - presence	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Immune cell infiltration - extent	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Regeneration	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	Clca1$^{-/-}$ 48 h DSS proximal colon										*Clca1*$^{-/-}$ 48 h DSS distal colon									
	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10
Cell type score neutrophils	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cell type score macrophages	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cell type score lymphocytes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Erosion/ulceration - depth	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Erosion/ulceration - extent	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Immune cell infiltration - presence	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Immune cell infiltration - extent	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Regeneration	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	WT 48 h DSS proximal colon										WT 48 h DSS distal colon									
	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10
Cell type score neutrophils	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Cell type score macrophages	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cell type score lymphocytes	0	0	1	2	0	0	0	0	0	0	2	0	0	0	0	0	0	1	0	0
Erosion/ulceration - depth	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Erosion/ulceration - extent	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Immune cell infiltration - presence	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Immune cell infiltration - extent	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Regeneration	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

2.3 Soluble Mucus Component CLCA1 Modulates Expression of Leukotactic Cytokines and BPIFA1 in Murine Alveolar Macrophages But Not in Bone Marrow-Derived Macrophages

Authors: Erickson NA, Dietert K, Enders J, Glauben R, Nouailles G, Gruber AD, Mundhenk L

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Declaration of own portion of work in this research publication:

Contributions by N. A. Erickson: Independent design, preparation, completion, and evaluation of all experiments including cell culture, transfection, supernatant collection, determination of protein concentration, isolation of murine alveolar macrophages, isolation and differentiation of murine bone marrow-derived macrophages, stimulation of alveolar macrophages and activation of bone marrow-derived macrophages, cytometric bead array and multiplex assay analysis from macrophages, RNA isolation and RT-qPCR from macrophages as well as RT-qPCR from RNA of trachea and lung parenchyma from a previous study [110], immunohistochemical analysis of trachea and lung parenchyma from a previous study [110], statistical analysis, and interpretation. Subsequent preparation of the entire manuscript.

Contributions of other authors: All co-authors participated considerably to the study design, evaluation of experimental results and the creation of the manuscript.

Thomas Laufer, Mustafa Kahraman, and the staff of Hummingbird Diagnostics performed the microarray analyses.

Declaration on ethics: All applicable international, national, and institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted. Humane killing of mice with subsequent experiments were in accordance with the FELASA guidelines and approved by the local governmental authorities (State Office of Health and Social Affairs Berlin, approval ID: T 0104/06). Previous experiments [110] as source of select tissues re-examined here had been approved by the ethics committee of the Charité – University Medicine Berlin and local governmental authorities (approval ID: G 0358/11) and had also been conducted in strict accordance with the FELASA guidelines and recommendations for the care and use of laboratory animals [167].

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

You have to read this part online.

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3 Concluding Discussion

This study was performed to elucidate the function of CLCA1, focusing on its most conclusive roles, 1) as a structure-associated mucus component or 2) as signaling protein in innate immunity.

Due to its striking similarities in its abundance in the intestinal mucus and its expression profile compared to the main structural protein of the intestinal mucus layer, Muc2 [33, 84], it can firstly be hypothesized that CLCA1 may likewise function as a structural or structure-associated protein of this mucus barrier. Therefore, the intestinal mucus barrier of *Clca1*$^{-/-}$ and WT mice was investigated comparatively under naive conditions as well as during DSS-challenge.

As second most plausible function, CLCA1 is hypothesized to act as a signaling protein modulating cytokines in early innate immune responses. Numerous recent studies suggest a possible immune-modulatory function by cytokine expression modulation for human or murine CLCA1 in various respiratory cell cultures or *in vivo* inflammatory lung models [45, 109-111]. It is tempting to speculate that CLCA1 may have a similar effect in the intestine and therefore, the DSS colitis model was also analyzed regarding this specific effect.

In order to address presumable cytokine modulation in more detail, an exclusively murine *in vitro* model of CLCA1-activation of alveolar and bone marrow-derived macrophages was employed, since macrophages are the primary source of *Cxcl-1* [168], which had been found to be differentially regulated in murine respiratory *Clca1*$^{-/-}$ challenge models [45, 110]. Initial experiments regarding the human ortholog showed that CLCA1 is capable of inducing a pro-inflammatory cytokine response in macrophages [111].

3.1 CLCA1 as a Structure-associated Protein in the Mucus Barrier?

3.1.1 CLCA1 as a Structure-associated Protein in Naive Mice?

To date, the putative functional role of CLCA1 in mucus barrier structure, formation, and integrity has not been experimentally analyzed. Numerous publications have suggested that CLCA1 may function as a component of the mucus barrier, in particular in the intestinal tract [12, 13, 31, 33, 84]. Due to the conspicuous correlations of CLCA1 and Muc2 regarding their

expression pattern in the mucus, a similar phenotype of naive *Clca1*$^{-/-}$ mice as seen in *Muc2*$^{-/-}$ mice could be hypothesized.

Muc2$^{-/-}$ mice clinically showed growth retardation starting at around five weeks of age, followed by diarrhea at seven, occult blood in stool at eight, and gross bleeding with rectal prolapse at nine weeks [147]. In contrast, naive *Clca1*$^{-/-}$ mice investigated at a comparable age of six to eight weeks in this study were clinically inconspicuous. No differences in stool consistency or presence of blood in stool compared to WT controls were present (see 2.1). Furthermore, body weight, colon-weight length (see 2.2) and bacterial translocation into sentinel organs such as blood, mesenteric lymph nodes, liver, and spleen did not reveal any differences between naive *Clca1*$^{-/-}$ mice and WT controls (see 2.1).

Histologically, the colon of *Muc2*$^{-/-}$ mice showed crypt hyperplasia, loss of distinguishable goblet cells, mild immune cell infiltration, flattened epithelial cells, and lamina propria distortion at five weeks of age with loss of crypt architecture, increased lymphocyte infiltration, and erosions at later stages compared to naive WT animals [147]. In the present study, *Clca1*$^{-/-}$ mice were histologically assessed and compared to WT animals concerning immune cell infiltration as well as erosion or ulceration and regeneration without any observable difference between the genotypes (see 2.2). *Muc2*$^{-/-}$ mice develop adenoma at six months of age with progression to invasive adenocarcinoma at later points in time [147, 148]. In addition, two mouse strains with spontaneous *Muc2* mutations also develop spontaneous colitis by seven weeks of age [33, 169] and colon cancer after six to twelve months [147, 148]. These findings, however, were also not observable in *Clca1*$^{-/-}$ mice up to 24 months (pers. comm. K. Dietert).

An intestinal phenotype was also lacking in all genetically derived *Clca1*$^{-/-}$ models, which are based on different exon-specific knockouts, i.e. knockout of exon 7 [45], exons 7 and 8 [46], and exons 7 to 11 [105]. This underlines that CLCA1 does not seem to be a structure-associated protein in the intestinal mucus barrier under naive conditions.

As cytokines were found to be consecutively altered by a defective mucus barrier under naive conditions in *Muc2*$^{-/-}$ mice, specifically concerning the five- and four-fold increase of TNFa and IL-1b, respectively [147], the basic inflammatory cytokine levels were also analyzed in this study. In addition to these and the cytokines mentioned above, CXCL-1, CXCL-2, and IL-17, IL-6 and interferon gamma (IFNγ) were also analyzed on RNA and/or protein level in naive *Clca1*$^{-/-}$ and WT mice, however, without any difference between the genotypes (see 2.2). Hence, during naive conditions, the absence of CLCA1 also does not seem to have an impact on inflammatory parameters at molecular level.

In addition, the effect of CLCA1-deficiency on presumably differential or *de novo* mucin gene expression was also investigated in this study. Besides *Muc1*, *2*, *3*, and *4* as main intestinal mucins, *Muc5ac 5b*, and *6* were also analyzed (see 2.1). Muc5ac and 5b are the main respiratory mucins, but were also found to be expressed in the murine stomach [84] and human colon [170, 171], respectively. Muc5ac expression is induced by CLCA1 overexpression in airway cell culture systems and in the respiratory tract [25, 78]. In Crohn´s disease, MUC5AC expression is known to be induced in the intestine together with MUC5B and MUC6 [172] and also upon clearance of helminth infections [173]. Interestingly, a *de novo* expression of Muc6, which is normally restricted to the stomach and duodenum [174], had been observed in the distal colon of *Muc2*$^{-/-}$ mice [147]. No differential expression of *Muc1*, *2*, *3*, and *4* and no *de novo* expression of the other mucins investigated were found in *Clca1*$^{-/-}$ compared to WT mice (see 2.1). Thus, CLCA1 does not seem to have an influence on the expression of mucin genes in the naive mouse.

The onset of early, spontaneous colitis in *Muc2*$^{-/-}$ mice with its associated symptoms is due to their entire lack of a mucus barrier [33, 114, 147], rendering bacteria in direct contact with the colonic epithelial cells, deep down in the crypts, as well as intracellularly in some epithelial cells [33].

Notably, *Muc2*$^{-/-}$ mice experience bacterial overgrowth and develop spontaneous colitis, which may be ameliorated by antibiotics [142-144], pointing towards a reciprocal interaction between the mucus layer and the gut microbiota [136, 145]. Additionally, a change in intestinal microbiota composition gives rise to a significantly altered inner colonic mucus layer, perhaps facilitated by bacterial products such as LPS and SCFA. Nonetheless, the exact mechanisms of how bacteria affect the host epithelium and its mucus production are far from being understood [136]. Hence, the main intestinal bacterial groups - *Enterobacteriaceae*, enterococci, lactic acid bacteria, bifidobacteria, *Bacteroides/Prevotella subspecies* (*spp.*), *Clostridium leptum* group, *Clostridium coccoides* group, mouse intestinal bacteroidetes, and total eubacterial load - were analyzed in naive *Clca1*$^{-/-}$ and WT mice, the latter serving as reference for the genetic background and housing conditions. In the naive state, however, the microflora analysis of *Clca1*$^{-/-}$ and WT mice yielded no differences (see 2.1).

Apart from the *Muc2*$^{-/-}$ mice, various other murine models provide first in-depth insights into mucus layer formation and homeostasis, in which the colonic mucus layers show different degrees and facets of impairment depending on the genotype. In *glycan*-deficient *core 1*-deficient mice, Muc2 is less well glycosylated, exhibiting an impaired expression of intestinal O-glycans (mucin-type O-linked oligosaccharides), which has also been observed in UC

patients, rendering their mucus layer more easily degradable by bacteria [115]. Mice lacking the Nas1 sulfotransporter showed less sulfation of their mucins with increased susceptibility to colitis [159]. The inner mucus layer of sodium-hydrogen exchanger 3 (*Nhe3*)-deficient mice is, despite its stratified morphology and normal thickness, penetrable to bacteria, leading to spontaneous colitis [175, 176]. Its exact pathophysiology is not yet understood, but an optimal local ion milieu seems necessary for normal mucus expansion and organization [114]. Since mucus organization is also a result of additional cross-linking and processing, defects in these processes also contribute to inner layer penetrability [114].

Therefore, besides a probable function as a structural component itself, CLCA1 may have an impact on the mucus organization or during the transformation - such as in the cleavage process - from the inner to outer mucus layer. Proteolytic cleavage within the Muc2 amino- and carboxy-termini allow for volume expansion without breaking the covalent polymer structure [33]. The responsible proteases have not been identified, but have been hypothesized to be either of cellular or bacterial origin. Since germ-free mice also exhibit both mucus layers, cellular proteases are more likely [33].

Coinciding, CLCA1 has been hypothesized to function as zinc-dependent metalloproteinase (see also 1.3.2) [22, 23], which is known to alter mucus properties [83]. Structurally, CLCA1 possesses a VWA domain including a MIDAS, as well as a FnIII domain, which confer protein-protein-interaction [9, 24, 25]. Its capability of self-cleavage and cross-proteolysis was proven by induction of an E157Q mutation and co-transfection with WT CLCA1 [22]. It has been speculated that CLCA1 may be capable of directly altering mucus properties, i.e. its viscosity or rheology to facilitate clearance, since it is stored in mucin granules and secreted together with the mucus [83]. However, it is unknown to date if CLCA1 has other substrates, such as extracellular mucin glycoproteins or other components of the mucus layer, apart from itself [22].

In comparison to WT mice, *Clca1*$^{-/-}$ mice showed no different structural intestinal mucus phenotype when investigated by Muc2-immunofluorescent staining pattern analysis. Mucus penetrability, assessed using either bacteria-sized beads in an explant chamber or general bacterial FISH with Muc2 counterstain in confocal microscopy, and additional investigation of mucus layering and goblet cell filling revealed no difference between the genotypes. Moreover, mucus thickness and growth rate, i.e. in response to carbachol, a cholinergic agonist inducing intestinal epithelial ion and mucus secretion, did not differ between *Clca1*$^{-/-}$ and WT mice (see 2.1).

No difference in mucus composition concerning Muc2 and other important constituents of the mucus, such as Anterior gradient protein 2 homolog (Agr2), Fcgbp, Kallikrein-1 (Klk1), and

Zymogen granule protein 16 (Zg16), was observable by mass spectrometry proteomics or immunofluorescence (see 2.1). Due to the presence of various CLCA1 homologs in the same tissue, this redundancy may result in compensatory effects, which have been postulated especially for murine, but not for human CLCA1 and CLCA2 in the respiratory tract [9, 25, 102, 105]. Anyhow, no differential expression of CLCA4a and CLCA4b, which could account for compensatory function in the murine intestine, was observable (see 2.1).

3.1.2 CLCA1 as a Structure-associated Protein in DSS-challenged Mice?

No differences between *Clca1*$^{-/-}$ and WT controls were observed under unchallenged conditions. However, some phenotypes only become apparent during challenge, which had already been shown for *Clca1*-deficiency in the respiratory tract during *S. aureus* pneumonia, OVA- or LPS-challenge [45, 110]. Therefore, the effect of *Clca1*-deficiency was investigated in the colon under DSS-challenged conditions, which had never been performed before.

Utilization of the standardized and highly reproducible model of acute DSS colitis [156] allows for vast literature phenotype comparability. The entire investigation period was monitored clinically with in-depth analysis at three different points in time, i.e. during full colitis after seven days of DSS treatment as standard model and, additionally, at 24 and 48 hours of DSS treatment for investigation of early changes in the mucus layer (see 2.1) [123].

Clinically, DSS-treated *Clca1*$^{-/-}$ mice also did not show any genotype-specific alterations compared to WT animals whilst exhibiting a steady increase in weight loss, fecal blood content, and stool consistency scores with an increased colon weight-length-ratio at necropsy due to edema and colonic contraction during full colitis (see 2.1 and 2.2). Also, no difference in bacterial translocation into sentinel organs was observed (see 2.1). In mice with a thinner or no functional mucus layer, i.e. exhibiting the loss of an essential structural mucus protein, as in *Muc2*$^{-/-}$ mice, for example, DSS treatment immediately induces acute and massive bleeding long before any inflammation is observed [144, 158, 159]. They also exhibit a higher disease activity index (DAI, an index which is comprised of various colitis parameters) from the beginning of treatment with severe weight loss and gross bleeding toward day seven [147]. As *Clca1*$^{-/-}$ mice did not show such a phenotype after DSS treatment (see 2.1 and 2.2.), the CLCA1 protein does not seem to play a clinical role as a structure-associated mucus protein even under challenged conditions.

An overall significant mucus penetrability increase and loss of layering structure was observable both in *Clca1*$^{-/-}$ and WT mice from 24 to 48 h after initial DSS administration, which is in line with previous observations in WT mice [123], however, without a significant

difference between the genotypes (see 2.1). During acute colitis, the mucus layer is known to be completely dissolved [123]. Hence, it is impossible and superfluous to analyze structural aspects at this time point, which were therefore not investigated.

Concerning mucin gene expression, *MUC2* synthesis is decreased during pathological conditions such as UC [177], which could also be verified in this study for both genotypes, though without any genotype difference (see 2.1). Since a differential or *de novo* expression had been reported for other mucin genes under challenged conditions, *Muc5ac*, *-5b* and *-6* were also investigated after DSS administration. No changes in relative abundance or expression of these mucin genes were observed between the genotypes (see 2.1).

Similar to naive mice, the intestinal microbiota composition was investigated under DSS-challenged conditions with a focus on main gut bacterial groups, which are known to be altered during colitis development [165, 166] and also affect the mucus phenotype [136]. Following colitis induction, increases in the colonic *Clostridium leptum* and *coccoides* group as well as in bifidobacteria were observed, which is consistent with previous findings [165], yet without significant differences between the genotypes (see 2.1).

During DSS colitis, immune cell infiltration, erosion or ulceration, and regeneration increased similarly in both genotypes in the proximal and distal colon, clearly reflecting the expected inflammation, whilst at 24 and 48 hours no significant immune cell infiltrations or histopathologic alterations were observed in either genotype (see 2.2). In contrast to previous studies on airway inflammation, in which neutrophil responses were either decreased in *Clca1*$^{-/-}$ mice after *S. aureus* infection [110] or increased after LPS challenge [45], no difference was observed between the genotypes in the colitis model histopathologically, particularly in neutrophil abundancy (see 2.1). Nevertheless, it is important to note that the genotypic differences in the respiratory models were only present in one of the two distinct compartments analyzed, the conducting airways and the lung parenchyma. In contrast to the airways, which were analyzed by BAL, no differences were observed in the lung parenchyma, i.e. in severity, expansion of lung lesions, and particularly in neutrophil infiltration [110]. Therefore, a direct comparison of intestinal to respiratory challenge models is not possible due to the compartmental difference of these organs.

In short, clinical, macroscopic, histologic, microbiotic, and structural mucus parameters did not yield any difference between the genotypes under DSS-challenged conditions (see 2.1), which points to a function of CLCA1 other than in mucus structure, function, and barrier integrity.

Upon examination of the cytokine expression patterns during acute DSS colitis - during which virtually all cytokines measured were upregulated on mRNA and/or protein level in the distal colon as the prime target site of DSS - only *Cxcl-1* and *Il-17* surprisingly showed a differential mRNA expression pattern in the distal colon of *Clca1*$^{-/-}$ compared to WT mice (see 2.2).

This differential cytokine expression after DSS-challenge was the sole phenotype observed in *Clca1*$^{-/-}$ mice in this study. Since no genotype differences regarding the mucus barrier were found, this phenotype may not be considered a subsequent effect to an altered mucus barrier impairment of *Clca1*$^{-/-}$ mice, but may rather be in line with another hypothesized function: CLCA1 as a modulator of cytokine expression.

3.2 CLCA1 as a Modulator of Cytokine Expression

Numerous *in vivo* studies have recently suggested a possible cytokine regulatory function for CLCA proteins in various inflammatory lung models [45, 109, 110].

Similar to the cytokine expression profile in the DSS colitis model of this study, an increased CXCL-1 response was observed following respiratory LPS challenge of *Clca1*$^{-/-}$ mice [45], whereas the *S. aureus* pneumonia model contrarily yielded a decreased *Cxcl-1* and *Il-17* response [110] in *Clca1*$^{-/-}$ compared to WT mice.

The reason for this seemingly contradictory expression of cytokines in early CLCA1-linked immune response modulation may be its proposed dependency on the inducers of inflammation, a bacterial infection *vs.* challenge with isolated LPS, i.e. an acute bacterial infection with intact Gram-positive microorganisms instead of an isolated endotoxin as partial virulence factor [110]. Interestingly, important pathomechanisms such as neutrophil recruitment, control of bacterial translocation, and epithelial repair seem to be dependent on solely LPS-mediated Toll-like receptor (TLR) 4 signaling in acute DSS colitis models [178]. Such an overlapping mechanism could explain the similar phenotype in cytokine expression regulation in DSS colitis and the respiratory LPS challenge model in *Clca1*$^{-/-}$ mice.

In the above mentioned studies, *Cxcl-1* is the common denominator of CLCA1-associated differential regulation. For its further and isolated investigation, it is necessary to pinpoint the origin of *Cxcl-1* expression to a specific cell-type. *Cxcl-1* is known to be expressed by various cell-types such as macrophages, neutrophils, and epithelial cells [179, 180]. Since whole organ lysate or BAL was used for analysis [45, 110], the expressing cell-type cannot be

assessed. However, a more recent study identified the major tissue source of *Cxcl-1* as macrophages [168].

Recently, human CLCA1 was found to induce a pro-inflammatory cytokine response in a human monocyte cell line artificially differentiated into airway macrophage-like cells and also in primary porcine alveolar macrophages [111]. This finding opens a new functional perspective for CLCA1 and a first mechanistic approach towards understanding the phenotype of differential *Cxcl-1* regulation in *Clca1*$^{-/-}$ mice.

In order to establish if CLCA1 induces the same effect in the mouse and to elucidate similarities and possible differences between human and murine CLCA1 in immune functions, cytokine expression levels in alveolar macrophages stimulated with CLCA1 were investigated in a solely murine context (see 2.3).

Due to species-specific genomic differences within the CLCA family and differences in postulated functions of CLCA1 in humans and mice, such as the induction of mucus cell metaplasia [25, 102] and the modulation of a non-CFTR-mediated CaCC [105], the translation of functional observations between human and mouse CLCA proteins remains particularly challenging and must be carefully considered in further functional analyses. Mouse CLCA1 activated murine alveolar macrophages by inducing several early pro-inflammatory cytokines and chemokines such as IL-1, IL-6, and TNF-α, and the human IL-8 homologues CXCL-1 and CXCL-2 on the mRNA and protein levels (see 2.3) similar to the recently described activation of human and pig macrophages by human CLCA1 [111]. These findings point towards an evolutionarily conserved and thus probably substantial role of CLCA1 in early airway inflammation and are of particular importance considering previously established species-specific differences. Whilst functional differences of human and murine CLCA1 have been established concerning IL-13-induced airway mucus cell metaplasia [25, 102] and their modulation of a non-CFTR-mediated, calcium-dependent anion conductance [105], the activation of macrophages by human and murine CLCA1 seems to be coinciding and, hence, translatable. These findings underscore the necessity of murine model adequacy in studying the effect of CLCA1 in cytokine regulation.

Despite the fact that CLCA1 has never been reported to be expressed by macrophages or other immune cells [31], *Clca1*$^{-/-}$ alveolar macrophages were analyzed regarding a possible priming event in terms of previous CLCA1 contact in the local respiratory milieu. The stimulation of *Clca1*$^{-/-}$ alveolar macrophages with CLCA1 yielded virtually identical activation compared to WT cells (see 2.3). Hence, the activation of alveolar macrophages is completely independent of previous CLCA1 contact.

Since macrophage subtypes vary greatly in their phenotype, the capability of CLCA1 to elicit a pro-inflammatory cytokine response was also analyzed in bone marrow-derived monocytes differentiated to macrophages (BMDM) *ex vivo*. In contrast to alveolar macrophages, BMDM did not yield any cytokine expression induction after CLCA1 stimulation (see 2.3). This may indicate that non-airway macrophages may not serve as effector cells of this mucosa-associated secreted activator. Susceptibility to CLCA1 activation may be restricted to macrophages in mucosal environments where CLCA1 is normally present, such as the airways.

In the present study, murine CLCA1-conditioned HEK cell culture medium was employed (see 2.3) instead of purified CLCA1 protein since the main limitation of any *in vitro* study using purified CLCA1 protein as an additive is the potential functionality compromise or alteration by the purification processes. Ching *et al*. had shown that human CLCA1 purified by immunoprecipitation from CLCA1-cultured fetal bovine serum (FBS)-free medium and human CLCA1-cultured FBS-free medium itself yielded no difference in macrophage cytokine expression. Hence, the study verified that macrophage activation is not dependent on constituents that might potentially be induced and secreted by human CLCA1 [111]. Therefore, the effects seen in the present study (see 2.3) are to be attributed to CLCA1. Moreover, Ching *et al.* [111] argue that the use of CLCA1 cultured medium may be preferable over purified protein since CLCA1 needs to be capable of exerting its stimulatory effect in *in vivo* conditions, i.e. alongside a vast array of other factors and cytokines, as present in the BALF of inflamed airways [181]. Hence, CLCA1 cultured medium more closely resembles *in vivo* conditions and may therefore be considered a more robust model. Anyhow, it is important to note that these studies are based on HEK cell-cultured medium. Its composition cannot be readily compared to medium obtained from airway epithelial cells or even from inflamed airways, which would more closely resemble *in vivo* conditions.

Despite the fact that CLCA1 induced cytokine expression in alveolar macrophages, its receptor and intracellular pathway remain unknown. Interestingly, CLCA1-dependent stimulation of alveolar macrophages as well as the lack of BMDM activation shows similarities to the activation pattern of lipoteichoic acid (LTA), a TLR2 agonist [182], used as positive control in this study (see 2.3). Resident tissue macrophages are the source of the major neutrophil chemoattractants CXCL-1 and -2, which are regulated via TLR2, TLR3, and TLR4 depending on the pathogen and produced by signaling through myeloid differentiation primary response 88 (MyD88) [168]. From literature, activation of naive BMDM is known to be MyD88-dependent and to require cooperative signaling of both TLR2 and TLR4 [183]. In contrast, LPS, the primary ligand of TLR4 [184], alone is sufficient for their activation [185]. Hence, one could speculate that activation of TLR2 or 4 by LTA or LPS, respectively, is

sufficient for activation of airway macrophages as already shown [186-188], whereas naive BMDM are solely activated by LPS, but not by LTA, as seen in this study (see 2.3), due to lack of cooperative signaling of TLR2 and TLR4. It therefore remains to be established whether CLCA1 and LTA indeed share similar activation pathways.

In summary, this murine model of CLCA1 inducing cytokine expression in alveolar macrophages is translatable to its human counterpart (see 2.3). The results obtained here may fully explain all aspects of the phenotype of *Clca1*$^{-/-}$ mice infected with *S. aureus* via reduced stimulation of alveolar macrophages by the lack of CLCA1, but fail to explain the contrary phenotype seen in *Clca1*$^{-/-}$ mice during DSS colitis (see 2.3).

In order to elucidate this latter aspect, further experiments are warranted. Since epithelial cells have also been discussed as a source of *Cxcl-1* expression [180] and since BMDM, which were used as an unprimed reference macrophage population in this study, are an unspecific target due to their local milieu-dependent phenotype, further experiments could be based on stimulating either isolated intestinal epithelial cells or intestinal macrophages with CLCA1 *in vitro* (see also 3.4).

3.3 Unexpected Findings

Using global gene expression analyses to elucidate downstream effector pathways in early immune functions, other genes differentially regulated in alveolar macrophages upon activation by CLCA1 were identified. The most strongly down-regulated gene was the host-protective and immunomodulatory airway mucus component *BPI fold containing family A member 1* (*Bpifa1)*, also termed short palate, lung, and nasal epithelium clone 1 (SPLUNC1) protein in humans (see 2.3). BPIFA1 shares striking similarities with several functions hypothesized for CLCA1 in signaling, such as acting in immune defense mechanisms and liquid homeostasis in airway mucosal membranes [189] and regulating cytokine gene expression in macrophages [190], whilst genetic polymorphisms in the *Bpifa1* gene have recently been suggested to be associated with disease severity in CF [191].

To investigate a differential regulation of BPIFA1 *in vivo*, tissues from the pulmonary *S. aureus* challenge model [110] were re-examined (see 2.3). The trachea of *Clca1*$^{-/-}$ mice 24 hours post infection showed less reduced *Bpifa1* mRNA expression in comparison to WT animals with significantly higher numbers of BPIFA1-expressing respiratory epithelial cells, which confirms the macrophage *ex vivo* effect also *in vivo* (see 2.3).

Following *Pseudomonas* (*P.*) *aeruginosa* infection, *Bpifa1*$^{-/-}$ mice had significantly increased *Cxcl-1* and *-2* expression with subsequently increased neutrophils in BALF [191]. This phenotype might be secondary to the lack of BPIFA1 as an antimicrobial protein with increased bacterial burden [191]. However, since BPIFA1 is capable of regulating cytokine expression in alveolar macrophages [192, 193], it is conceivable that some aspects of the observed phenotype may also be due to the lack of BPIFA1 as a cytokine regulator. Interestingly, these findings are mirrored by the cytokine regulation observed in the *Clca1*$^{-/-}$ mouse model of acute *S. aureus pneumonia* [110]. This may be speculated to be partially due to the lack of CLCA1 regulating BPIFA1 and its down-stream alveolar macrophage cytokine expression.

These findings support the hypothesis that murine CLCA1 directly and specifically activates complex alveolar macrophage signaling and broaden the spectrum of regulatory effects of CLCA1 in downstream pathways by modulation of the multifunctional BPIFA1. They also shed new light on the alleged, partially species-specific and even contradictory role of CLCA1 in anion conductance in epithelial cells and hypothetic liquid homeostasis [6, 105]. BPIFA1 is, in addition to above-mentioned cytokine regulation, a known modifier of airway liquid homeostasis, i.e. of the epithelial sodium channel (ENaC) [194-196]. It remains to be investigated if the observed CLCA1-effects on anion conductance may be secondary to the CLCA1-induced modulation of BPIFA.

Moreover, BPIFA1 is known to be primarily expressed by tracheal and bronchial epithelial cells in mice and humans [189, 197-199]. Its expression in neutrophils is controversial, whilst it has never been reported in other hematopoietic cells [189]. Specifically, co-localization of BPIFA1 with monocytes, resp. macrophages in human CF lungs has failed to date [200]. This study confirmed that airway epithelial cells represent the majority of BPIFA1 expressing cells, but, as a second interesting finding, it also clearly establishes that mouse airway macrophages do express this protein (see 2.3). These findings further argue that both cell types - alveolar macrophages and respiratory epithelial cells - may be involved in CLCA1-mediated BPIFA1 expression.

The mechanisms of altered BPIFA1 modulation in *Clca1*$^{-/-}$ mice and a possibly direct regulatory role of CLCA1 on airway epithelial cells needs to be further elucidated, which may give rise to a better understanding of the BPIFA1-CLCA1 interplay and connection, possibly as components of a signaling cascade.

3.4 Conclusions and Outlook

CLCA1 does not seem to function as a structural component of the intestinal mucus barrier despite its abundant expression and colocalization with Muc2, neither in naive nor during DSS-challenged conditions. During acute DSS colitis, CLCA1 does not have an impact on the clinical symptoms and outcome, nor on the degree of severity of intestinal inflammation (see 2.1 and 2.2).

However, despite no decisive effect on clinical outcome and pathophenotype (see 2.1), a distinct modulation of cytokine expression with a specific effect on *Cxcl-1* and *Il-17* was observable during DSS colitis in *Clca1*$^{-/-}$ mice (see 2.2). This finding coincides with previous respiratory challenge models [45, 110], which, however, point towards the direction, i.e. up- or downregulation of CLCA1-mediated cytokine modulation being dependent on the stimulus used [110].

Apart from the CLCA1-mediated differential cytokine expression pattern in DSS colitis, particularly of *Cxcl-1*, *in vitro* analysis of isolated macrophages, which are known to be the prime source of *Cxcl-1* [168], showed that murine CLCA1 can induce cytokine expression (see 2.3) similarly to the reported effects caused by the human CLCA1 [111].

The results obtained here from *in vitro* macrophage studies may fully explain all aspects of the previously reported phenotype of *Clca1*$^{-/-}$ mice infected with *S. aureus* [110] via reduced stimulation of alveolar macrophages by the lack of CLCA1, but fail to explain the contrary phenotype in cytokine expression seen in *Clca1*$^{-/-}$ mice during DSS colitis (see 2.2 and 2.3). This differential cytokine regulation in the DSS colitis model underlines not only the previously hypothesized role of CLCA1 as signaling molecule in early immune response modulation, but also the stimulus-dependency of cytokine regulation.

Numerous questions yet remain to be addressed under careful consideration of current study limitations. The exact mechanisms of how CLCA1 may modulate the innate immune response in other gram-negative or -positive lung or intestinal infectious models remain to be established.

Since stimulation of alveolar macrophages as well as the lack of BMDM activation were shown to closely resemble the activation pattern of LTA, a TLR2 agonist [182], further studies are needed to establish whether CLCA1 and LTA indeed share similar activation pathways and also whether the activation of the respective TLR – TLR2 in the pulmonary *S. aureus*- and airway macrophage CLCA1-challenge and TLR4 in the intestinal DSS challenge – determines the modulatory effect of CLCA1-dependent cytokine release and cellular

recruitment. This could be further addressed by CLCA1-stimulation of e.g. HEK 293/CD14 or Chinese hamster ovary (CHO) cells rendered specifically responsive to LTA after transfection with TLR-2 [182]. Alternatively, TLR2-deficient alveolar macrophages derived from commercially available TLR2-deficient mice [201] could be employed for such cytokine expression profile analyses.

This study also shed new light on possible CLCA1 downstream pathways, specifically by revealing a CLCA1-dependent modulation of the multifunctional BPIFA1, which was also shown to be expressed in mouse airway macrophages for the first time. However, the mechanisms of altered BPIFA1 modulation in *Clca1*$^{-/-}$ mice and a possibly direct regulatory role of CLCA1 on airway epithelial cells need to be further elucidated, which may give rise to a better understanding of the hypothetic CLCA1-BPIFA1 signaling cascade. This question could be further addressed by incubating isolated tracheal epithelia, the major source of BPIFA1 expression [189, 197-199], with CLCA1 and subsequent analysis of BPIFA1 and cytokine expression pattern.

Since only alveolar macrophages, but not BMDM were activated by CLCA1, it may be assumed that CLCA1-susceptibility is restricted to macrophages in mucosal environments where CLCA1 is normally present, such as the airways. In order to elucidate this latter aspect more precisely, further experiments are warranted whether tissue-specific macrophages on other mucosal linings may also be activated by CLCA1, such as in the intestinal tract. Intestinal macrophages differ greatly from alveolar macrophages due to their derivation from the bone marrow and their maturation occurring in close dependency on the local milieu of the intestine [202, 203].

In regard to the DSS colitis model, it would be intriguing to specifically test intestinal macrophages in a further *in vitro* study. Contrary to all other tissue macrophages, such as lung alveolar macrophages, which are derived from fetal precursors and self-renew *in situ* [204, 205], intestinal macrophages have very poor proliferative capacity. Since *in situ* self-renewal of intestinal macrophages plays hardly any role under homeostatic conditions [206, 207], they must be replenished continually by blood monocytes originating from the bone marrow and, hence, may be more closely related to BMDM. Therefore, it is tempting to speculate that these cell types share a similar reaction pattern. Nonetheless, intestinal macrophages locally differentiate in close dependency on numerous local environmental factors [202, 203, 208-210], the exact function of which, however, needs yet to be determined [203]. Consequently, the local milieu, such as the intestinal tissue in which the macrophages reside, could render intestinal macrophages susceptible to CLCA1 activation. Anyhow, caution is warranted in this extrapolation and it remains to be elucidated which

reaction pattern intestinal macrophages display upon CLCA1-stimulation. At this point it could be speculated, however, that the influence of local factors could explain the contrary cytokine regulation in *Clca1*$^{-/-}$ mice infected with *S. aureus* - perhaps due to reduced stimulation of alveolar macrophages - compared to the phenotype seen in *Clca1*$^{-/-}$ mice during DSS colitis.

Besides macrophages, epithelial cells have also been discussed as a source of *Cxcl-1* expression [180]. Therefore, this cell population needs to be investigated isolated from the colonic tissue. These experiments could be performed by isolation and *in vitro* cultivation of mouse intestinal macrophages via established protocols [211-214] or of intestinal epithelial cells by a protocol specifically designed for innate immune function analysis [215]. The isolated cell populations could then be stimulated by CLCA1 (see 2.3). These *in vitro* basic analyses are preferential over *in vivo* studies since the effect of CLCA1 can be segregated from other possibly interfering mechanisms and, even more importantly, contribute to the reduction of animal experiments necessary for investigation and replace further murine CLCA1-based challenge studies for very basic mechanistic analyses. Laser capture microdissection (LCM) could be employed on sections of murine *Clca1*$^{-/-}$ and WT colon obtained from this study with consecutive RT-qPCR-based cytokine expression analysis, in order to analyze any difference caused by the presence or absence of CLCA1 as an internal control in comparison to CLCA1-stimulation of intestinal epithelial cells in culture.

In this study, *Clca1*$^{-/-}$ mice did not show any phenotype concerning mucus barrier structure. This lack of phenotype has also been present in respiratory *Clca1*$^{-/-}$ mouse models, in which compensatory effects of CLCA1 homologs have been discussed [25, 102, 105]. Anyhow, no differential CLCA homolog expression, particularly of the homologs CLCA4a and –b present in the intestinal tract, was evident (see 2.1). This may suggest that compensatory effects could be excluded. It could be speculated though that CLCA4a or 4b may already compensate at physiological levels since other murine CLCA members - particularly CLCA2 and 4a - were shown to be sufficient, but not necessary to produce mucus cell metaplasia in the respiratory tract [25]. Since the mouse, in contrast to humans, has multiple CLCA duplications with a total of up to eight family members, the only model sufficiently answering this question would be a complete knockout of all CLCA members present in the intestinal tract, i.e. CLCA1, 4a, and -4b. To date, such a knockout has not been established. More importantly, however, there are no means of excluding the possibility that other, non-CLCA proteins may compensate for the loss of CLCA1. Conceivable at this point would be, for instance, other enzymes operating synergistically with CLCA1 and taking over its function in the *Clca1*$^{-/-}$ model.

In the mouse, tracheal instillation of IL-13 failed to induce CaCC activity [105] or mirror the human IL-13-dependency of CLCA1-mediated airway mucus production [102] and mucus cell metaplasia [25, 105]. Nevertheless, the effect of human CLCA1 on cytokine expression in macrophages was verified in the murine model here (see 2.3). Hence, this study does not only underline the function of CLCA1 in cytokine regulation, but also reveals a function-specific interspecies translatability. In this regard, CLCA1 in cytokine regulation may serve as a valuable tool for further investigation. This study also emphasizes the need for careful mouse model assessment regarding translatability and extrapolation to other species.

Concluding, this study supports the alleged role of CLCA1 as a signaling molecule of innate immunity modulating inflammatory responses, particularly via cytokine regulation.

4 Summary

The CLCA1 Protein in Innate Immunity – A Mucus Barrier Component or Signaling Molecule?

Nancy Ann Erickson

The CLCA (chloride channel regulators, calcium-activated) family comprises highly-conserved proteins with broad tissue expression patterns in many species, which have been shown to possess pleiotropic, yet not exactly determined functions, particularly in inflammatory diseases. Specifically CLCA1, which is secreted by mucus-producing cells e.g. of the respiratory and intestinal tract, is differentially expressed in diseases such as cystic fibrosis (mucoviscidosis), asthma, or chronic obstructive pulmonary disease, serving as a potential therapeutic target.

Of all organ systems, CLCA1 is most highly expressed and secreted by mucus cells in the colon and has striking similarities in abundance and expression profile compared to the main structural mucin protein of the intestinal mucus barrier, Muc2. Apart from acting as a presumably structural component, CLCA1 may also function as an extracellular structure-modulating protein, i.e. as zinc-dependent metallohydrolase or as a calcium-dependent chloride channel regulator.

Hence, CLCA1 as a structure-associated component of the mucus barrier is one of the most substantiated hypothetic functions, which has not been addressed to date. Here, *Clca1*-deficient and wild type mice were compared clinically and pathologically under unchallenged conditions as well as at early points in time of dextran sodium sulfate challenge and during fulminant colitis. Expression profiles of select mucin and *Clca* genes in colon tissue and the fecal microbiota composition were analyzed by reverse transcriptase quantitative polymerase chain reaction. The intestinal mucus barrier was comparatively assessed in terms of mucus penetrability, layering, and goblet cell filling by fluorescence-*in situ*-hybridization. In addition, the barrier integrity was determined by culture analysis of bacterial translocation into sentinel organs.

Nonetheless, no differences between the genotypes were detected in naive or during dextran sodium sulfate-challenged conditions in any of the parameters analyzed. Therefore, CLCA1 does not seem to function as a structural component of the intestinal mucus barrier.

Since the second most promising function of CLCA1 includes it being a signaling molecule modulating early inflammatory responses on the cytokine level, the dextran sodium sulfate challenge model was also analyzed regarding this specific effect. Despite no decisive effects

on the clinical outcome, a distinct CLCA1 modulation of cytokine expression with a specific effect on chemokine (C-X-C motif) ligand 1 and interleukin 17 was observed during colitis. These findings coincide with previous respiratory challenge models, in which certain cytokines, particularly chemokine (C-X-C motif) ligand 1 and interleukin 17, were also differentially expressed between the genotypes, depending, however, on the stimuli used.

This differential cytokine regulation in the dextran sodium sulfate colitis model underlines the previously hypothesized role of CLCA1 as a signaling molecule regulating cytokine expression in early innate immune response. Initial experiments regarding the human ortholog had shown a pro-inflammatory cytokine response in macrophages. Due to species-specific CLCA1 differences between humans and mice, caution is warranted in translatability and extrapolation of data between the species. Differences of presumable functions have been established in humans and mice, e.g. the induction of mucus cell metaplasia and the modulation of anion conductance. Therefore, murine CLCA1-mediated activation of murine alveolar and bone marrow-derived monocytes differentiated to macrophages (bone marrow-derived macrophages) was analyzed. Lipopolysaccharide, the primary ligand of Toll-like receptor 4 and lipoteichoic acid, a Toll-like receptor 2 agonist, served as positive controls. Murine CLCA1 activated murine alveolar macrophages by inducing several early pro-inflammatory cytokines and chemokines - similar to the recently described activation of human and porcine macrophages by human CLCA1 - but failed to activate the bone marrow-derived macrophages.

Hence, these results may fully explain all aspects of the phenotype reported in *Clca1*-deficient mice infected with *Staphylococcus aureus* via reduced stimulation of alveolar macrophages by the lack of CLCA1, but fail to explain the contrary phenotype reported in *Clca1*-deficient mice during DSS colitis. Since bone marrow-derived macrophages were not activated by CLCA1 in this study, susceptibility to CLCA1 activation may be restricted to macrophages in mucosal environments where CLCA1 is normally present, such as the airways.

Global gene expression analysis of CLCA1-stimulated alveolar macrophages shed new light on possible CLCA1 downstream pathways. The most strongly down-regulated gene in CLCA1-stimulated alveolar macrophages identified was the host-protective and immunomodulatory airway mucus component *BPI fold containing family A member 1* (*Bpifa1*). BPIFA1 shares striking similarities with several functions hypothesized for CLCA1 in signaling, such as acting in immune defense mechanisms and liquid homeostasis in airway mucosal membranes, regulating cytokine gene expression in macrophages. Furthermore, BPIFA1 expression was also immunohistochemically located in mouse airway macrophages

for the first time. Additionally, an *in vivo Staphylococcus aureus* pneumonia mouse model was analyzed regarding BPIFA1 expression, which suggests that CLCA1 may also modify BPIFA1 in airway epithelial cells.

This study showed that CLCA1 may modulate early immune function not as a structural component of the mucus barrier, but as a modulator of cytokine expression in airway macrophages. The exact pathway including its putative receptor remains to be elucidated. Additionally, it should be investigated if CLCA1 may act on other effector cells than airway macrophages. The identification of BPIFA1 expression modulated by CLCA1 may be a link to explain complex downstream pathways associated with CLCA1, which remains to be addressed in the future.

5 Zusammenfassung

Das CLCA1 Protein in der angeborenen Immunität – Komponente der Mukusbarriere oder Signalmolekül?

Nancy Ann Erickson

Die CLCA (engl. *chloride channel regulators, calcium-activated*) Familie besteht aus hochkonservierten Proteinen mit einem breiten, Spezies-übergreifenden Gewebsexpressionsmuster, welche pleiotrope aber bislang nicht näher definierte Funktionen vor allem in inflammatorischen Erkrankungen besitzen. Speziell CLCA1, welches von Mukus-produzierenden Zellen, beispielsweise des Respirations- oder Intestinaltraktes, sezerniert wird, ist in Mukus-assoziierten, respiratorischen Erkrankungen wie zystischer Fibrose (Mukoviszidose), Asthma oder chronisch obstruktiver Lungenerkrankung (*engl.* Chronic Obstructive Pulmonary Disease) differenziell exprimiert und wird als potentielles therapeutisches Ziel diskutiert.

Von allen Organsystemen ist CLCA1 am stärksten im Kolon exprimiert und wird von Becherzellen des Kolons sezerniert. Es besitzt hinsichtlich Menge und Expressionsprofil bemerkenswerte Ähnlichkeiten mit dem Hauptmuzin der intestinalen Mukusbarriere, Muc2. Abgesehen von seiner Funktion als ausschließlich strukturelle Komponente könnte CLCA1 auch als ein extrazelluläres, die Mukusstruktur modulierendes Protein agieren, beispielsweise als Zink-abhängige Metallohydrolase oder als Kalzium-abhängiger Chloridkanal-Regulator.

Eine der belastbarsten hypothetischen Funktionen von CLCA1 ist demnach die einer Struktur-assoziierten Komponente der Mukusbarriere, welche jedoch bislang nicht untersucht wurde. *Clca1*-defiziente und Wildtyp-Mäuse wurden daher unter naiven Bedingungen als auch unter einer Dextran Natrium Sulfat-induzierten Kolitis zu verschiedenen Zeitpunkten hinsichtlich klinischer und pathologischer Parameter vergleichend untersucht. Expressionsprofile spezifischer Muzin- und *Clca*-Gene im Kolongewebe, als auch die fäkale Mikrobiota-Zusammensetzung wurden mittels Reverser Transkriptase Polymerase Kettenreaktion bestimmt. Des Weiteren wurde die intestinale Mukusbarriere im Hinblick auf bakterielle Penetrabilität, Mukusschichtung und Becherzellfüllung mittels Fluoreszenz-*in situ*-Hybridisierung vergleichend untersucht. Darüber hinaus wurde die Integrität der Mukusbarriere hinsichtlich bakterieller Translokation in Sentinelorgane mittels Kultur analysiert.

Es konnten jedoch in sämtlichen untersuchten Parametern keine Unterschiede zwischen den Genotypen unter naiven Bedingungen oder Dextran Natrium Sulfat-induzierter Barrierestörung gefunden werden. Daher spielt CLCA1 als strukturelle Komponente der intestinalen Mukusbarriere keine Rolle.

Das vorliegende Modell wurde demnach auch hinsichtlich einer weiteren, vielversprechenden Funktion von CLCA1 - als modulierendes Signalmolekül der Zytokinexpression - analysiert. Obwohl keine Effekte auf klinische Parameter beobachtet wurden, konnte eine deutliche Zytokinexpressionsmodulation durch CLCA1 mit einem spezifischen Effekt auf Chemokin (C-X-C motif) Ligand 1 and Interleukin 17 während der Dextran Natrium Sulfat-Kolitis beobachtet werden. Diese Resultate stimmen mit Ergebnissen respiratorischer Modelle überein, bei denen ebenfalls Chemokin (C-X-C motif) Ligand 1 und Interleukin 17 zwischen den Genotypen differenziell exprimiert waren. Jedoch wurde, abhängig vom eingesetzten Stimulus, eine gegensätzliche Zytokinregulation beobachtet.

Diese differenzielle Zytokinregulation im Dextran Natrium Sulfat-Kolitis-Modell unterstreicht die hypothetisierte Rolle von CLCA1 als Signalmolekül der frühen, angeborenen Immunantwort. Initiale Experimente hinsichtlich des humanen orthologen Vertreters hatten eine pro-inflammatorische Zytokinantwort in Makrophagen gezeigt. Aufgrund spezies-spezifischer Unterschiede von CLCA1 zwischen Mensch und Maus ist Vorsicht bei der Übertragung und Extrapolation von Daten zwischen den Spezies geboten. Beispielsweise wurden Unterschiede bezüglich der Induktion einer Mukuszellmetaplasie in den Luftwegen und der Modulation einer Anionenleitfähigkeit zwischen Mensch und Maus gefunden. Daher wurde die Aktivierung muriner Alveolarmakrophagen und zu Makrophagen ausdifferenzierter Monozyten des Knochenmarks (Knochenmarksmakrophagen) durch ausschließlich murines CLCA1 in dieser Arbeit analysiert. Lipoploysaccharid, der primäre Ligand des Toll-like Rezeptors 4, und Lipoteichonsäure, ein Toll-like Rezeptor 2 Agonist, dienten als Positivkontrolle. Murines CLCA1 induzierte mehrere proinflammatorische Zytokine und Chemokine in Alveolarmakrophagen, ähnlich der kürzlich beschriebenen Aktivierung humaner und porziner Makrophagen durch seinen humanen Orthologen. Knochenmarksmakrophagen konnten hingegen nicht aktiviert werden.

Die Ergebnisse können alle Aspekte des Phänotyps früherer Studien mit *Staphylococcus aureus*-infizierten, *Clca1*-defizienten Mäuse über eine durch das Fehlen von CLCA1 reduzierte Alveolarmakrophagenstimulation erklären. Unklar bleibt aber die Zytokinregulation in *Clca1*-defizienten Mäusen während der Dextran Natrium Sulfat-Kolitis, welche einen gegensätzlichen Verlauf zeigte. Da Knochenmarksmakrophagen nicht durch CLCA1 aktiviert wurden, ist die Empfänglichkeit gegenüber der CLCA1-Aktivierung vermutlich auf

Makrophagen mukosaler Kompartimente, in denen CLCA1 normalerweise präsent ist, wie beispielsweise dem Respirationstrakt, begrenzt.

Eine globale Genexpressionsanalyse der mit CLCA1 stimulierten Alveolarmakrophagen hat neue Erkenntnisse bezüglich möglicher *downstream pathways* von CLCA1 erbracht. Das in CLCA1-stimulierten Alveolarmakrophagen am stärksten herunterregulierte Gen war eine protektive und immunomodulatorische Komponente des Mukus des Respirationstraktes, *BPI fold containing family A member 1* (*Bpifa1*). BPIFA1 zeigt bemerkenswerte Ähnlichkeiten zu den für CLCA1 hypothetisierten Funktionen, wie beispielsweise die Funktion in immunologischen Abwehrmechanismen und der Flüssigkeitshomöostase respiratorischer, mukosaler Oberflächen und in der Regulation von Zytokin-Genexpression in Makrophagen. Darüber hinaus wurde die Expression von BPIFA1 in murinen Alveolarmarkophagen erstmals per Immunhistochemie nachgewiesen. Weiterhin konnte die Analyse eines *Staphylococcus aureus*-Lungenmodells der Maus zeigen, dass CLCA1 die Expression von BPIFA1 auch in respiratorischen Epithelzellen zu modulieren scheint.

CLCA1 scheint die frühe Immunantwort nicht als struktureller Bestandteil des Mukus, aber als Modulator der Zytokinexpression von Alveolarmakrophagen zu beeinflussen. Weitere Studien sollten die genauen Aktivierungswege einschließlich möglicher Oberflächenrezeptoren aufschlüsseln und mögliche andere Effektorzellen von CLCA1 identifizieren. Die hier identifizierte Verknüpfung von CLCA1 mit BPIFA1 und ihren Funktionswegen sollte zentraler Punkt der weiteren Forschung sein.

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7 Publications Related to this Research Thesis

Research publications in scientific journals:

- **Erickson NA**, Nyström EEL, Mundhenk L, Arike L, Glauben R, Heimesaat MMH, Fischer A, Bereswill S, Birchenough GMH, Gruber AD, Johansson MEV: The Goblet Cell Protein Clca1 (Alias mClca3 or Gob-5) Is Not Required for Intestinal Mucus Synthesis, Structure and Barrier Function in Naive or DSS-Challenged Mice. PLoS ONE (2015). DOI 10.1371/journal.pone.0131991
- **Erickson NA**, Mundhenk L, Giovannini S, Glauben R, Gruber AD: Role of goblet cell protein CLCA1 in murine DSS colitis. Journal of Inflammation (2016). DOI 10.1186/s12950-016-0113-8
- **Erickson NA**, Dietert K, Enders J, Glauben R, Nouailles G, Gruber AD, Mundhenk L: Soluble Mucus Component CLCA1 Modulates Expression of Leukotactic Cytokines and BPIFA1 in Murine Alveolar Macrophages But Not in Bone Marrow-Derived Macrophages. Histochemistry and Cell Biology (2018). DOI 10.1007/s00418-018-1664-y

Oral presentations:

- **Erickson NA**: Functional relevance of the mCLCA3 protein as a mucus layer element during intestinal barrier disruptions. Institute of Biomedicine, Department of Medical Biochemistry and Cell Biology, University of Gothenburg, Sweden (26.11.2013)
- **Erickson NA**, Nyström EEL, Mundhenk L, Johansson MEV, Gruber AD: Das Mukusprotein CLCA1 ist entbehrlich für die murine intestinale Mukussynthese, -struktur und –barrierefunktion. 58th Annual Conference of the German Veterinary Medical Society, Section Veterinary Pathology, Fulda, Germany (07.-08.03.2015) Tierärztliche Praxis / Ausgabe K, Kleintiere, Heimtiere (2015). 43(3) — S. A20, ISSN: 1434-1239

Poster presentations:

- **Erickson NA,** Nyström EEL, Mundhenk L, Heimesaat MM, Fischer A, Bereswill S, Johansson MEV, Gruber AD: Does lack of the goblet cell protein CLCA1 have an impact on the intestinal mucus barrier and microflora composition? Dahlem Research School - Spring School Symposium, Freie Universität Berlin, Germany (20.03.2014)

PUBLICATIONS RELATED TO THIS RESEARCH THESIS

- **Erickson NA**, Mundhenk L, Glauben R, Giovannini S, Nyström EEL, Johansson MEV, Gruber AD: Does Clca1 Modulate the Innate Immune Response During DSS Challenge In The Murine Colon? The 33rd Annual Meeting of The European Society of Veterinary Pathology & The 26th Annual Meeting of The European College of Veterinary Pathologists, Helsinki, Finland (03.09.2015). Journal of Comparative Pathology (2016). 154(1): 58-123
- **Erickson NA**, Dietert K, Enders J, Mundhenk L, Glauben R, Gruber AD: The Goblet Cell-Derived Soluble Mucus Component CLCA1 Activates Airway Macrophages in the Mouse. The 34th Annual Meeting of The European Society of Veterinary Pathology & The 27th Annual Meeting of The European College of Veterinary Pathologists, Bologna, Italy (08.09.2016). Journal of Comparative Pathology (2017). 156(1):135

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9 Conflict of Interest

The author of this thesis declares no conflict of interest.

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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.

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