Do leukocytes have a predisposition for an altered immune response in cystic fibrosis? – a pilot study in the *CFTR*$^{-/-}$ pig

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„Alles in allem kommt es ja nicht darauf an, recht viel gelesen zu haben und zu wissen, sondern in allem (...) stets unbefangen sich an das zu halten, wovon man Bereicherung und Freude gewinnt. Der Eine liest täglich Lessing, der andre schmeißt ihn an die Wand, und beide können recht haben.“ – *Hermann Hesse*

Stephan Gerdes † 3. August 2019

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-	negative
µg	Microgram
µl	Microliter
µm	Micrometer
+	positive
A	Area
ABC	ATP-binding cassette
AM	Alveolar macrophage
APC	Antigen presenting cell
Aq. bidest.	Bidistilled water
ASL	Airway surface liquid
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
CCL-2	Monocyte chemoattractant protein 1
CD	Cluster of differentiation
CF	Cystic fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CpG	Cytosin-phosphate-guanin
CR	Complement receptor
DAMP	Damage associated molecular pattern
dATP	Deoxyadenosine triphosphate
DC	Dendritic cell
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DIOS	Distal intestinal obstructive syndrome
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
dTTP	Deoxythymidine triphosphate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ENAC	Epithelial sodium channel
FBS	Fetal bovine serum

Fc	Fragment crystallizable
FITC	Fluorescein isothiocyanate
Fl	Fluorescence
FMIA	Fluorescent microsphere assay
FSC	Forward scatter
FU	Freie Universität
g	Units of gravity
GM-CSF	Granulocyte-macrophage colony stimulating factor
H	Height
HCL	Hypochlorous acid
i.e.	It est
i.m.	Intramuscular
IFN	Interferone
Ig	Immunoglobulin
IL	Interleukin
IM	Interstitial macrophage
kb	Kilobase
Kg	Kilogram
l	Liter
LMU	Ludwig-Maximilians-University of Munich
LPS	Lipopolysaccharides
LTA	Lipoteichoic acid
M-CSF	Macrophage colony stimulating factor
MΦ	Macrophage
M0	Unpolarized macrophages
M1	Classically activated macrophages
M2	Alternatively activated macrophages
mab	Monoclonal antibody
MFI	Median fluorescence intensity
MFI FL-I	Median fluorescence intensity of green
mg	Milligram
MHC	Major histocompatibility complex
min	Minutes
ml	Milliliter
mm	Millimeter

mM	Millimolar
MNL	Mononuclear leukocyte
MP	Mononuclear phagocyte
MPO	Myeloperoxidase
N.a.	Not available
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NE	Neutrophil elastase
NET	Neutrophil extracellular trap
NETosis	Formation of neutrophil extracellular traps
NK cells	Natural killer lymphocytes
nm	Nanometer
ODN	Oligodeoxynucleotides
Pam3Cys	Pam3Cys-SKKKK lipopeptide
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
pH	Negative log of hydrogen ion concentration in a water-based solution
PMA/Iono	Phorbol-12-myristat-13-acetat + ionomycin salt
PML	Polymorphonuclear leukocyte
PRR	Pattern recognition receptor
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SSC	Side scatter
TAE	Tris acetate buffer
TCR	T cell receptor
Th1	Type 1 T cell response
Th2	Type 2 T cell response
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TRIS	Tris (hydroxymethyl)-aminomethane
V	Volt
vs	Versus
WT	Wildtype

I. Introduction

Cystic fibrosis (CF) is a common lethal genetic disorder in people of European descent. Mutations in the anion channel called “Cystic Fibrosis Transmembrane Conductance Regulator” (CFTR) result into a multisystemic disease with severe intestinal, pancreatic and respiratory morbidities. Efforts in symptomatic treatment have improved lifespan of patients, however the destructive airway disease leads still unavoidably to premature death (Davis, 2006).

New treatment options can partially restore CFTR function and become available for increasing numbers of CF individuals, but these drugs are not available for all mutations and surprisingly not all patients improve in treatment who should genetically benefit from the CFTR modulator therapy (Ferkol, 2019). More than that, the course of the destructive lung disease is only reduced, not prevented (Bose et al., 2019; Grasemann and Ratjen, 2013). Therefore, additional potential intervention sites and treatment regimens are needed and since CF lungs are histologically normal at birth, early treatment could make a great difference in progression, severity and disease burden later in life (reviewed in Stoltz et al., 2015). In order to evaluate potential beneficial effects of existing treatment options before symptomatic disease has established, relevant biomarkers and profound knowledge of constituting principles are essential.

So far this is not the case for CF, although the cause and the consequences of the disease are known for years (Ferkol, 2019). It is known that hallmark features of the CF airway disease including mucus plugging, inflammation and infection are detectable already shortly after birth (Mott et al., 2012; Sly et al., 2013). Abnormal mucus production has been postulated to be the initiating factor of CF, as reflected by the term “mucoviscidiosis” to describe the syndrome (Ehre et al., 2014; Esther et al., 2019). But it remains intriguing, how an ineffective apical epithelial chloride channel results in such a fatal respiratory failure, while other congenital diseases of ion channels or primary ciliary dyskinesia with reduced mucociliary clearance as well have a substantially less severe course of disease, without inflammatory excess and loss of airway host defense to certain opportunistic pathogens including *Staphylococcus aureus, Pseudomonas aeruginosa* and fungal colonization (Cohen and Prince, 2012; Cohen-Cymberknoh et al., 2014).

Therefore, further constituting factors have been suggested. There is evidence for an initial role of immune cells, however it is controversially discussed, whether alterations are directly caused by loss of CFTR in the immune cells or whether abnormalities are secondary and acquired by the inflammatory environment (reviewed in Bragonzi et al., 2017; Hodson, 1980). Due to the limitations of clinical studies such as restricted sample availability, lack of appropriate controls, variable treatments and secondary changes of the early established disease, and the fact that many animal models failed to recapitulate CF properly (reviewed in Grubb and Boucher, 1999; Stoltz et al., 2015), examination of potentially aberrant immune mechanisms in CF was challenging. Examinations of further models are necessary to gain better perspectives of the disease constituting factors and target research efforts to accelerate progress of translational research in CF (reviewed in Meyerholz, 2016). As a result, the *CFTR*$^{-/-}$ pig has been developed (Klymiuk et al., 2012; Rogers et al., 2008b) and revealed valuable insight into constituting principles of CF in view of mucus release, reduced mucociliary clearance, ASL composition and altered innate immunity of epithelial cells (Bartlett et al., 2016; Ermund et al., 2018; Pezzulo et al., 2012; Stoltz et al., 2010). Although CFTR expression in leukocytes is known for years (Yoshimura et al., 1991) and further examination in the *CFTR*$^{-/-}$ pig was highly recommended (reviewed in Hartl et al., 2012; Rieber et al., 2014), it has not been carried out comprehensively yet.

The objective of this thesis was to explore a potential predisposition of immune cells from newborn *CFTR*$^{-/-}$ piglets for an altered innate or adaptive immune response. To this end, offspring were produced by breeding of *CFTR*$^{+/-}$ animals. Subsequently, phenotypical and functional properties of leukocytes from newborn *CFTR*$^{-/-}$ piglets and their wildtype (*CFTR*$^{+/+}$) littermates were examined to reveal potential alterations. Therefore, the frequency and activation status of major immune cell subpopulations, cytokine profiles from *in-vitro* stimulated mononuclear leukocytes and the phagocytic and oxidative potential of leukocytes were analyzed. So far, this is the first comprehensive analysis of innate and adaptive immune cells from peripheral blood, the spleen and most importantly the lungs in the CF pig model.

II. Review of literature

1. Cystic fibrosis

Cystic fibrosis is the most frequent lethal monogenic disease in people of Caucasian origin. More than 70 000 people are affected worldwide and highest incidence occurs with 1 in 3000 births in Northern Europe (reviewed in Collins, 1992; 2019). Although there was no further characterization for CF until the 20th century, it was already paraphrased for centuries as a fatal childhood disease. In the Almanac of Children's Songs and Games of Switzerland (Rochholz, 1857) we can read: "The child will soon die whose brow tastes salty when kissed".

In 1938 Anderson connected intestinal disorders with respiratory complications of young patients. As a result of characteristic histology of the pancreas with cyst-like lesions and surrounding fibrosis the disease was named Cystic Fibrosis (Anderson, 1938). In the following the detection of disturbed sweat electrolyte composition led to the still predictive diagnostic "sweat test" (Di Sant'Agnese et al., 1953; Gibson and Cooke, 1959). At that time, life expectancy of affected children was a few months. Meconium ileus and malabsorption were the dominant causes of death (reviewed in Davis, 2006). The etiology of cystic fibrosis was finally identified in 1989. Mutations in the gene coding for the anion channel CFTR are responsible for the disease (Kerem et al., 1989; Riordan et al., 1989; Rommens et al., 1989). For the last 30 years in parts of the world the fatal childhood disease has become a chronic disease of adulthood. In developed countries, this was achieved by improved detection and symptomatic treatment of neonatal intestinal obstruction, nutrition deficits, airway clearance and occurring infections (reviewed in Elborn, 2016). More recently, new treatment strategies have become available. Small molecules can restore defective CFTR protein expression or function. Nearly 90% of persons with CF could benefit from the latest combination of three modulators (Heijerman et al., 2019; Middleton et al., 2019). The extent of clinical benefit is limited and thus, there is still no live-saving treatment. Indeed, CF remains the most common inherited life limiting lung disease (Bose et al., 2019; Mall and Hartl, 2014).

1.1. Cystic fibrosis transmembrane conductance regulator

Cystic fibrosis is a Mendelian autosomal recessive disorder. The *CFTR* gene is located on chromosome 7 with a length of about 250 kb including 27 exons (Rommens et al., 1989). The encoded CFTR protein comprises 1,480 amino acids (Riordan et al., 1989) and is a membrane transport protein that belongs to the ATP-binding cassette (ABC) family. The molecular protein structure consists of two transmembrane domains forming the translocation channel and two cytoplasmic nucleotide binding domains that hydrolyze ATP. The activity of the channel is controlled by the phosphorylation status of the additional regulatory domain (reviewed in Liu et al., 2017; Riordan, 2008). Despite its architectural similarity to other members of the ABC transporter superfamily, CFTR is functionally unique in acting as an anion channel (Gadsby et al., 2006). CFTR is primarily expressed in the apical membrane of epithelial cells of various sites. Expression patterns are seen within the airways, pancreas, sweat glands, liver, intestine and epididymis. In addition, CFTR has been detected in non-polarized cells of the immune system (Plog et al., 2010; Yoshimura et al., 1991).

More than 1500 different mutations have been reported in the *CFTR* gene and many of them are associated with disease. The most common mutation leads to the absence of phenylalanine at position 508 (F508del), which accounts for approximately 70% of the mutated alleles (Bell et al., 2015). Mutations are divided into classes according to their functional consequences (Welsh and Smith, 1993). Within Class I mutations, like premature stop codons, frameshifts or nonsense mutations, CFTR protein is absent. Class II mutations including the F508del variant, lead to deficient protein processing and destruction of misfolded CFTR protein. Mutations that result in faulty channel regulation are classified into class III. Thus, class I - III mutations lack CFTR function and are associated with a more severe phenotype, whereas further classes generally show a milder phenotype due to residual CFTR function. There, limited CFTR function is caused by defective conductance (class IV), reduced mRNA stability (class V) or decreased membrane stability of the mature CFTR protein (class VI) (reviewed in Elborn, 2016; Ratjen et al., 2015). Apart from the basic gene defect, modifier genes and environmental factors contribute strongly to variable disease severity in CF individuals (reviewed in Cutting, 2015).

1.2. Clinical manifestations of cystic fibrosis

According to the expression pattern of *CFTR*, CF is a multisystemic disease. Clinical manifestations appear throughout life of affected people with strong variations in onset and characteristics (reviewed in Cutting, 2015). Already at the beginning of life, about 10-20% of CF newborns suffer from meconium ileus (MI). In early days being mostly a fatal appearance, the intestinal obstruction is nowadays overcome either by oral or rectal application of laxatives or by surgical intervention. Occurrence of MI normally correlates with severe phenotype of CF, is significantly affected by modifier genes, but only very limitedly influenced by environmental factors (Dupuis et al., 2016). Later in life, patients develop the distal intestinal obstruction syndrome (DIOS), which has similar prevalence rates as MI. In contrast to MI, DIOS is highly influenced by environmental factors, which impair gastrointestinal motility by chronic inflammation as a result of the abnormal intestinal milieu with thick mucus, low intestinal pH and dysbiosis (reviewed in Ooi and Durie, 2016). The latter is caused by the basic defect of CFTR and also by the functional loss of the hepatobiliary system and the pancreas. The most common manifestations of CF liver disease are hepatic steatosis, focal biliary cirrhosis, a micro-gallbladder or cholestasis. Treatment approaches include supplementation of oral bile salt ursodeoxycholic acid or liver transplantation in case of portal hypertension. Exocrine pancreas insufficiency occurs in 85% of CF patients and the onset of disease is strongly associated with the genotype. Defective CFTR protein leads to obstructions in pancreatic ducts and acini, resulting in extensive structural damage. Malnutrition, growth retardation and malabsorption of fat-soluble vitamins are symptomatic. Substitutional treatment with vitamins and pancreatic enzymes is well established (reviewed in Haller et al., 2014; Ledder et al., 2014). The destructive pancreatic disease can eventually also result in CF-related diabetes mellitus (CFRD) and if untreated, prognosis for CF patients is poorer. Another metabolic consequence of defective CFTR is osteopenia and the sweat defect predisposes patients for heat prostration and metabolic alkalosis (reviewed in Elborn, 2016). Nearly all males with CF suffer from congenital absence of the vas deferens and as a result from azoospermia (Jarvi et al., 1995), whereas females with CF are not affected in reproduction (McMullen et al., 2006). Although the previously mentioned manifestations are serious complications on their own, the ultimately life-span limiting part is mostly the respiratory manifestation of CF.

Destructive airways disease is associated with at least 80% of CF-related deaths (CF Foundation, 2019).

At birth, pulmonary disease in CF has not been recognized as clinically apparent, there were neither signs of mucus plugging nor of infection or inflammation (Bedrossian et al., 1976). Nevertheless, structural abnormalities of the airways are present already in newborns (reviewed in Grasemann and Ratjen, 2013) and, very significantly, the onset of disease is in early infancy (Armstrong et al., 2005; Mott et al., 2012). Chronic cycles of mucus accumulation, infection and inflammation initiate and perpetuate destructive pulmonary disease. Development of first bronchiectasis is associated with the colonization of the airways by bacterial pathogens like *S. aureus* and *Haemophilus influenzae*. Later in the course of progressive structural damage and reduction of oxygen tension *P. aeruginosa* becomes the predominant pathogen in the CF lungs. High concentrations of neutrophils appear in the luminal space of the airways and release high amounts of their harmful antimicrobial content without clearing infection (reviewed in O'Sullivan and Freedman, 2009; Ratjen et al., 2015). Inflammatory mediators like neutrophil elastase (NE), interleukin (IL)-6, IL-8 and tumor necrosis factor α (TNFα) from bronchoalveolar lavage fluid (BAL) indicate progression of an irreversible lung disease already early in life (Sly et al., 2013). So far, application of antibiotics, mucolytics, physical airway clearance and even CFTR modulators only delay deterioration of lung function and treatment fails to prevent recurrent exacerbations (Kleizen et al., 2019; Pittman and Ferkol, 2015; VanDevanter and Mayer-Hamblett, 2019). Finally, the progressive decline of lung function in CF results in a fatal respiratory failure, leaving only lung transplantation as optional treatment (Corey et al., 1997).

1.3. Pathophysiology of cystic fibrosis lung disease

Although the trigger of the disease, defective CFTR, and its clinical consequences have been well known for decades, the constituting principles of how an ineffective apical epithelial anion channel results in the described phenotypic manifestations of the airways are unknown. Many hypotheses have been controversially discussed (Esther et al., 2019). Without any doubt, impaired chloride and bicarbonate transport across epithelia is correlated with abnormal mucus function (Ehre et al., 2014; Quinton, 1983; 2008). Abnormal secretion of mucins in small and large

airways initiates impaired mucociliary clearance (Ermund et al., 2018; Esther et al., 2019). However, direct and indirect regulatory properties of CFTR on other ion channels or interactions of CFTR with immunological cellular pathways might contribute to disease development as well (reviewed in Stoltz et al., 2015). Specifically, the "low volume" hypothesis postulates excessive sodium reabsorption from the airway surface liquid (ASL) by an increased activity of an epithelial sodium channel (ENAC), accompanied by influx of H_2O into epithelial cells which results in mucus dehydration and impaired mucociliary clearance (Boucher, 2007). Contrary to mouse models, hyperactivity of ENAC and increased sodium reabsorption has not been observed in human, pig and ferrets (reviewed in Stoltz et al., 2015). In contrast to the "low volume" hypothesis, the "high salt" theory argues with increased sodium reabsorption. There it is suggested that high concentrations of NaCl in the ASL inhibit antimicrobial proteins, disrupt the periciliary layer and elicit a predisposition for airway infections (Goldman et al., 1997; Smith et al., 1996). Another theory for impaired bacterial killing by antimicrobial peptides was proposed on the basis of a reduced pH in CF lungs due to disturbed bicarbonate transport (Shah et al., 2016), but in recent studies no abnormal acidification has been detected in CF patients (Schultz et al., 2017).

Another fundamental matter of debate is whether airway inflammation in CF is a result of infection or if it occurs independently, either caused by disturbed innate or adaptive immunity (reviewed in Nichols et al., 2008; Roesch et al., 2018). Importantly, babies with CF who died from neonatal meconium ileus have no signs of inflammation at birth (Khan et al., 1995). After birth, inflammatory markers are increased in children with CF within months without pathogen detection (Esther et al., 2019). Congruently, *CFTR*-knockout ferrets develop inflammatory lung disease without infection (Rosen et al., 2018). Furthermore, bacterial challenge in mice, which lack CFTR in the myeloid lineage only, resulted in similar inflammatory abnormalities as mice which lack CFTR in the entire body (Bonfield et al., 2012). It is obvious that finally a vicious circle of infection and massive inflammation leads to permanent structural damage already in the lungs of very young CF patients (Pillarisetti et al., 2011; Ramsey et al., 2014). For dissecting fundamental processes, it is necessary to identify the essential players of mucosal immunity and their complex interaction.

2. Mucosal immunity in cystic fibrosis

With every breath, the influx of air into the airways is accompanied by the exposure of the pulmonary surface to exogenous particles. A highly regulated defense network of mucosal immunity is needed to maintain respiratory function and prevent continuous activation or manifestation of pathology (reviewed in Guilliams et al., 2013b; Weitnauer et al., 2016). For years, researchers have focused primarily on the first barrier of airway defense to understand the failure of mucosal immunity in CF (reviewed in Nichols et al., 2008), mainly due to the significant expression levels of CFTR in epithelial cells. However, CFTR has also been detected in granulocytes, mononuclear phagocytes and lymphocytes (reviewed in Bonfield and Chmiel, 2017; Ratner and Mueller, 2012).

2.1. Impaired airway barrier function

Under physiological conditions, the primary barrier function of the lung tissue towards the lumen of the airways is formed by a continuous layer of epithelial cells, constituting paracellular integrity, the secretion of mucus and antimicrobial proteins into the ASL as well as mechanical clearance (reviewed in Weitnauer et al., 2016).

In CF, the primary host defense is dysregulated in multiple ways. Besides structural alterations such as the shape, diameter and smooth muscle orientation of larger airways in CF, abnormal integrity of the tight junctions in epithelial cell is described (Molina et al., 2015). The reduced epithelial integrity results in an increased paracellular permeability of bacterial products and therefore supports induction of inflammation. Whether this is a primary effect of defective CFTR or secondary due to inflammation is argued by the finding that anti-inflammatory drugs in patients with CF augment airway epithelial junctions and integrity (Asgrimsson et al., 2006). Mucociliary clearance, removing foreign material entrapped in mucus by ciliary beating, is dramatically reduced in the absence of CFTR (Ermund et al., 2018). This has been ascribed to the imperfect expansion of mucin proteins that are secreted into the ASL, their potentially impaired release from gland openings and the abnormal attachment on the epithelial surface. As a consequence of impaired clearance, bacteria are accumulated in the airways shortly after birth (reviewed in Stoltz et al., 2015).

Other components of the ASL appear compromised in CF as well. The levels of

usually abundant antimicrobial peptides seems to be partially downregulated in CF and further diminished by increased activity of proteases secreted from granulocytes in the ASL (reviewed in Bruscia and Bonfield, 2016b). In addition, the proteases also cleave extracellular matrix into small fragments and the degradation product prolin-glycin-prolin (PGP) is a potent damage associated molecular pattern (DAMP). High mobility group protein-1 (HMGB1), a nuclear protein which is released by necrotic cells is another DAMP. In the airways of CF individuals both, PGP as well as HGMB1, are highly abundant and by fueling the leukocyte influx connects the activity of the airway barrier to other components of the mucosal immunity (reviewed in Hartl et al., 2012). Oxidants contribute to pulmonary damage as well. Abnormally high amounts of reactive oxygen species (ROS) are detected in BAL fluid from CF patients. Airway epithelial cells are partially responsible for the high oxidative environment by increased production of ROS in CF. Even worse, lack of CFTR downregulates the transport of antioxidants across the luminal membrane of epithelial cells. Therefore, protection by antioxidative metabolites like glutathione and thiocynate is blunted in the ASL of CF patients. Dysregulated induction of transcription factors for the antioxidant response in CF may further contribute to altered metabolism of antioxidants (reviewed in Cohen and Prince, 2012; Galli et al., 2012).

Pathogen associated molecular patterns (PAMPs) are highly present in the lungs of CF patients. In early stages of the disease an increased exposure time to bacterial products including lipoteichoic acid (LTA) from gram positive bacteria or lipopolysaccharides (LPS) and flagellin from gram negative bacteria are caused by the reduced mucociliary clearance. In later stages, the permanent pulmonary colonization by bacteria is a persistent stimulus for pathogen recognition receptor (PRR) pathways (reviewed in Cohen and Prince, 2012). High amounts of PAMPs are even present in the circulation of CF individuals (del Campo et al., 2011).

Furthermore, lipid abnormalities in the airways are related to defective CFTR and contribute to the pro-inflammatory immune environment (reviewed in Cantin et al., 2015; Roesch et al., 2018). A high ratio of arachidonic acid to docosahexaenoic acid is detected in airway epithelial cells of CF patients, causing a cascade of detrimental effects. First the main arachidonic acid metabolite is the potent neutrophil chemoattractant leukotriene B4 (LTB4). Due to a further impairment of a regulatory loop, high concentrations of LTB4 can be detected in BAL fluid of CF

patients (Konstan et al., 1994). In the counterpart, anti-inflammatory resolvins, a product of docosahexaenoic acid, are markedly decreased in CF (Ringholz et al., 2018). The reason for the imbalance of fatty acids is not fully clear, but might be caused by pancreas insufficiency. The consequence of increased pro-inflammatory and decreased anti-inflammatory lipid metabolites is, however, evident and favors the hyperinflammatory state of the CF airways. The relevance of imbalanced fatty acid composition is documented by decreased lung inflammation of individuals under substitution treatment with docosahexaenoic acid (Leggieri et al., 2013).

As a consequence of impaired primary barrier functions, multiple infectious (PAMPs) as well as non-infectious (DAMPs) stimuli are activating pro-inflammatory pathways in airway epithelial cells (reviewed in Cohen and Prince, 2012). On top of that, it is hypothesized that the immune response itself in epithelial cells is dysregulated as well by defective CFTR modulation. Alterations begin with distinct expression of PRRs, but also include alterations in the downstream signaling pathways. It is suggested that in CF airway epithelial cells pro-inflammatory transcription factors have an increased activity after stimulation, whereas transcription factors for termination of inflammation show a decreased activity, resulting in an excess of chemoattractants (reviewed in Nichols and Chmiel, 2015). However, hyperinflammation has not been universally observed in airway epithelial cells *in-vitro*. Moreover, before the chronic environment in the CF lungs is established, CF airway epithelial cells might have an intrinsically blunted immune response (Parker et al., 2012; Ratner and Mueller, 2012; Zheng et al., 2003). Nevertheless, the ultimate fate of CF airways is not dictated by the aberrant airway barrier alone, but involves attraction of high leukocyte numbers, predominantly neutrophilic granulocytes (reviewed in Brennan, 2008).

2.2. Polymorphonuclear leukocytes

Neutrophils are bone marrow derived polymorphonuclear leukocytes (PMLs), that circulate with the bloodstream and are recruited to sites of infection by chemotaxis. The short living neutrophils deploy a wide range of antimicrobial functions and play an important role for primary host defense. They eliminate microorganisms by phagocytosis, release antimicrobial peptides and secrete neutrophil extracellular traps (NETs) to capture bacteria (reviewed in Borregaard, 2010). Recent findings suggest that the functional repertoire of different PML phenotypes consists of

additional functional properties such as modulating the innate and adaptive immune response via cytokines and receptors or inhibit T cell activity via amino acid deprivation (reviewed in Margaroli and Tirouvanziam, 2016). Expression of CFTR is less abundant in neutrophils than in epithelial cells, but it is detectable on the transcript level (Painter et al., 2006) and it seems to be localized at protein level in the plasma membrane, as well as in secretory vesicles and phagocytic vacuoles in humans and mice (Ng et al., 2014; Zhou et al., 2013).

Massive recruitment and accumulation of neutrophils is one of the major hallmarks of the CF lung disease. Migration through the airway wall across epithelial and endothelial monolayers seems to be unaffected by CFTR dysfunction (Pizurki et al., 2000; Sorio et al., 2016), but altered responsiveness to chemoattractants is being debated (reviewed in Hayes et al., 2011; Laval et al., 2016). Undeniable are the high amounts of neutrophil activators and chemoattractants found in BAL and circulation of CF patients (Mackerness et al., 2008). Combined, the massive influx of neutrophils into the airways, their increased lifespan due to delayed apoptosis (Gray et al., 2018) as well as reduced efferocytosis and mucocilary clearance, result in early accumulation and plugging of neutrophils in the bronchioles of CF patients (Regamey et al., 2012).

The major and most rapid killing mechanism of neutrophils against bacteria is pathogen uptake into phagosomes. Subsequent intracellular killing is done in the phagolysosome which results from the fusion of the phagosome with granules, filled with proteolytic enzymes and microbicidal oxidants (reviewed in Reeves et al., 2015; Underhill and Ozinsky, 2002). The phagocytic host defense of CF neutrophils is supposed to be impaired in several aspects. Distinct expression pattern of the phagocytosis initiating receptors and compromised phagocytosis of neutrophils have been reported as reasons for decreased microbicidal activity in CF (reviewed in Hayes et al., 2011; Laval et al., 2016; Roesch et al., 2018). Interestingly, *ex-vivo* treatment of PMLs from CF individuals with CFTR potentiator ivacaftor restored altered receptor expression pattern (Bratcher et al., 2016). But not all studies were able to detect impaired phagocytosis of neutrophils (reviewed in Cohen and Prince, 2012). In contrast to the controversies about phagocytosis, abnormal intracellular killing in CF neutrophils has been clearly demonstrated in many studies. It has been suggested that reduced intracellular microbicidal activity is caused by decreased ability to generate hypochlorous acid

(HCL), the most potent oxidant in PMLs (Ng et al., 2014; Painter et al., 2008) and by impaired intracellular generation of further ROS (Houston et al., 2013; Painter et al., 2006; Zhou et al., 2013).

Lack of CFTR is also related with abnormal granule trafficking in CF neutrophils by disturbed intracellular ion homeostasis. Abnormal granule trafficking is assumed to have negative impact on both, intracellular as well as extracellular bacterial killing. On the one hand, primary granules containing proteases like NE, cathepsins and myeloperoxidase (MPO) are released in excess to the lung environment, on the other hand, secondary and tertiary degranulation containing antimicrobial peptides such as lactoferrin and human cathelicidin are decreased in CF (reviewed in Reeves et al., 2015). Again, these effects can be reversed by CFTR modulators, suggesting that dysregulated degranulation in PMLs is immediately associated with defective CFTR (Pohl et al., 2014). Another potentially dysregulated extracellular antimicrobial pathway with harmful consequences in the CF airways, especially in stages of chronic bacterial infection, is the formation of NETs (NETosis) and indeed large quantities of extracellular DNA augment the viscosity of airway secretions (reviewed in Law and Gray, 2017). CF neutrophils show delayed apoptosis with "vital NETosis" and the preference of necrosis rather than apoptosis (Gray et al., 2018) and are therefore assumed to be the major source of extracellular DNA in the CF lungs. The high numbers of neutrophils in the CF airways are further fueling the oxidative airway environment by production of extracellular ROS under chronic exposure to bacteria (reviewed in Laval et al., 2016). As a consequence of the divergent activities of PMLs in CF, ASL composition is directly modified, including high amounts of self-damaging proteases and oxidases, low levels of antimicrobial peptides, NET formation and reduced phagocytosis (reviewed in Bruscia and Bonfield, 2016b; Pohl et al., 2014).

Beyond the failure of direct pathogen killing mechanisms, CF neutrophils perpetuate the vicious circle of inflammation by the release of pro-inflammatory cytokines IL-8 and IL-17 (reviewed in Roesch et al., 2018). The contribution of eosinophilic and basophilic granulocytes to the disease development is unclear at the moment, although CF individuals suffer from fungal colonization and have higher prevalence of allergic symptoms (Koller et al., 1994; Ratner and Mueller, 2012; Tracy and Moss, 2018). Last but not least, the role of PMLs in modeling adaptive immune cells in CF is even less understood than their intrinsic alterations

of microbicidal activity. While the PML phenotype of myeloid suppressor cells is generally known to modulate the adaptive immune response, inhibition of T-cell activation by the programmed-death ligand 1 of myeloid suppressor cells is hypothesized to play a minor role in the CF environment (Ingersoll et al., 2015).

2.3. Mononuclear phagocytes

Macrophages (MΦs), monocytes and dendritic cells (DCs) form the mononuclear phagocyte system (reviewed in Hume et al., 2019). The functional role of mononuclear phagocytes (MPs) in the airways is the surveillance of homeostasis and regulation of the immune response against viral, bacterial and fungal pathogens. In the lungs there are two resident types of MΦs, alveolar macrophages (AMs) and interstitial macrophages (IMs). Although CFTR expression is lower than in epithelial cells, and neutrophils have gained more attraction in CF research, resident AMs are the first immune cells being confronted with particles that have not been removed mechanically from the airways. Thus, CF-related consequences for the mononuclear phagocyte system might be substantial for the management of infection and inflammation in the lungs (Bonfield and Chmiel, 2017; Di et al., 2006).

Normally, invasive pathogens are phagocytized and eliminated within endocytic vacuoles (reviewed in Hussell and Bell, 2014). Similar to granulocytes, pathogens are killed in MPs in the phagolysosome by oxygen metabolites, lysozymes and microbicidal peptides (reviewed in Cohen and Prince, 2012). In the case pathogens cannot be sufficiently cleared by resident MΦs and homeostasis is overwhelmed, MΦs initiate the immune response by the release of cytokines (reviewed in Allard et al., 2018). After initiation, neutrophils resemble the first line of the invading defense brigade, while later predominantly additional monocytes are acquired from the circulation and MΦs become the predominant cell type and, first fueling, but finally resolving inflammation (reviewed in Soehnlein and Lindbom, 2010). Therefore, MΦs require a broad plasticity in their activation states to fulfill their regulatory role in health and disease. Undifferentiated MΦs (M0) are polarized into classically activated MΦs (M1) by cytokines like IFNγ, GM-CSF, or by PAMPs such as LTA or LPS. Alternatively activated MΦs (M2) are derived from M0 by IL-4, IL-10, IL-13 or M-CSF. M1 stimulate inflammation by secreting elevated

amounts of pro-inflammatory mediators. Furthermore, bacteria are killed by phagocytosis and intracellular killing. In contrast, M2 polarization is associated with efferocytosis and resolution of the acute immune response. Effective balance between the different MΦ-polarization states is an essential criterion for maintaining health (reviewed in Murray and Wynn, 2011).

In CF, estimations on intrinsically altered or secondarily acquired MΦ-polarization have been inconclusive so far (Cory et al., 2014; Murphy et al., 2010). Large variations between M1 or M2 polarization occur in CF individuals, because MΦs dynamically adapt to the state of the disease, the bacterial burden and the specific treatment regimen of each patient (reviewed in Bruscia and Bonfield, 2016a), suggesting a strong component of acquired polarization. Besides, data from animal models propose a tendency towards the M1 phenotype in the case of CF (Meyer et al., 2009). *In-vitro* studies suggest that fewer numbers of M0 polarize into M2 and that these M2 have even abnormal functional properties including endocytosis for removing cell debris, while M1 polarization and function turn out normal (Tarique et al., 2017). Due to their heterogenous origin, the diversity of MPs is even broader than the plasticity of the M1 and M2 phenotypes (Byrne et al., 2020; Guilliams et al., 2013a). Interestingly, elevated numbers of MΦs have been detected already in the lung tissue from CF fetuses (Hubeau et al., 2001b). Moreover, the absolute number of AM in BAL fluid from CF infants is highly increased even in the absence of detectable pathogens, but positively correlated with the concentration of the monocyte chemoattractant protein 1 (CCL-2)(Brennan et al., 2009).

Beyond the changes that MΦs underwent in the CF pro-inflammatory environment, the lack of CFTR seems to result also in intrinsic hyperinflammatory MΦs. Indeed, *in-vitro* cultivation of MΦs from CF individuals and animal models have revealed higher basal production and exaggerated release of pro-inflammatory cytokines like TNFα, IL-1β, IL-6, IL-8, CCL-2 and IFNγ after bacterial stimulation (reviewed in Hartl et al., 2012). Thus, MΦs might resemble an essential source of the inflammatory environment in the CF airways. Exaggerated release of anti-inflammatory IL-10 from MΦs has been described *in-vitro*, but BAL fluid from CF individuals contained less IL-10 than normal (reviewed in Lévêque et al., 2017).

Another component of MPs hyperinflammation is the impaired formation and trafficking of endosomes presumably as a result of alterations of microtubule

processing and trafficking proteins (Rymut et al., 2015; Zhang et al., 2013). A major source of excessively released cytokines and chemokines in MPs from CF children has been seen in the altered expression of toll like receptors (TLR)-4 and TLR-5, membrane bound PRRs that result in long-lasting activation of downstream signaling pathways and robust transcription of pro-inflammatory cytokines (Simonin-Le Jeune et al., 2013; Sturges et al., 2010). Specifically, TLR-4 is activated by LPS and DAMPs and disturbed degeneration processes are suggested to cause hyperproduction of cytokines in CF (reviewed in Akira et al., 2006; Bruscia et al., 2011; Ratner and Mueller, 2012). The degradation of PRRs is mediated by altered membrane lipid composition of cholesterol and other sphingolipids by changing the formation of lipid rafts; in CF MPs these processes appear to be impaired (Hamai et al., 2009; Zhang et al., 2013). Moreover, negative feedback loops of pro-inflammatory signaling are disturbed by abnormal fatty acid metabolism in CF MPs as well (Andersson et al., 2008).

Apart from changes in cytokine release of MPs, modified receptor function has also consequences for phagocytosis and elimination of bacteria by MPs (reviewed in Roesch et al., 2018; Zhang et al., 2018). In addition to PRRs, effective uptake of bacteria requires fragment crystallizable receptors (FcRs) and complement receptors (CRs). FcRs recognize the constant region of immunoglobulins (Ig) and CRs the activated complement factors on the surface of opsonized bacteria. Although distinct expression patterns of surface receptors in CF MPs have been directly associated with missing CFTR (Van de Weert-van Leeuwen et al., 2013), the abnormal occurrence of FcRs and CRs has been related to proteolytic cleavage in the CF airway milieu as well (reviewed in Lévêque et al., 2017). Besides reduced bacterial uptake, studies propagated also reduced bactericidal activity (Del Porto et al., 2011) as a result of defective acidification of the phagolysosome in MPs (Barasch et al., 1991; Di et al., 2006). It is important, to consider that CFTR does not directly contribute to the abnormal acidification of the phagosome (Barriere et al., 2009; Haggie and Verkman, 2007). Still, both the reduced bactericidal activity and increased production of cytokines are supposed to be a result of disturbed autophagy in CF MΦs (Abdulrahman et al., 2011; 2013).

Besides the impaired phagocytosis and exaggerated proinflammatory signaling, CF MΦs are also affected in their ability to clear the high numbers of dead neutrophils from the airways to prevent clogging of the bronchioles. Efferocytosis seems to be

impaired in the case of CF as a result of combined intrinsic and environmental factors (Vandivier et al., 2002; 2009). Normally the scavenger receptor CD206 and the macrophage receptor with collagenous structure mediate efferocytosis and are upregulated in presence of apoptotic cells (reviewed in Soehnlein and Lindbom, 2010), in sputum and BAL from CF individuals the expression of both scavenger receptors are decreased on MΦs (Garratt et al., 2012; Wright et al., 2009).

It is of note that despite the great research efforts, it is still not clear which functional alterations of CF MPs are due to defective CFTR function and which are consequences of the abnormal airway environment. Particularly, incongruent results are likely due to the use of different model systems, stimuli and experimental conditions (reviewed in Cohen-Cymberknoh et al., 2013; Roesch et al., 2018).

2.4. Lymphocytes

The high abundance of myeloid cells in the bronchoalveolar compartment and the low numbers of lymphocytes in BAL fluid have driven research predominantly towards defective first line host defense in CF immunity (reviewed in Hartl et al., 2012). Accumulating lymphocytes in the sub-epithelial tissue of bronchioles of CF children suggest, however, a relevant role even in early CF pathogenesis (Hubeau et al., 2001a; Regamey et al., 2012). In fact, CFTR expression of lymphocytes has been described a long time ago (Moss et al., 1996; Yoshimura et al., 1991). Nuclear factors of activated T cells, which support T-cell differentiation, are interfered by abnormal CFTR function as well, suggesting inappropriate cytokine expression of CF lymphocytes as an intrinsic result of defective CFTR. In addition, the higher susceptibility of CF individuals for allergic reactions, asthma, dermatitis and hyperinflammatory immune responses are intriguing arguments for a substantial role of the adaptive immune response in CF (reviewed in Ratner and Mueller, 2012).

The balance between $CD4^+$ T-cell subpopulations appears as a critical issue in CF. This is illustrated by the predisposition in CF for the type 2 T-cell response (Th2), which is sufficient for fighting parasites and is proallergic, but is insufficient for fighting many bacteria (Hartl et al., 2006). The clearance of normally less common pathogens like *P. aeruginosa* or representatives of the *Burkholderia cepacia* complex, requires Th1 and increased levels of IL-12 (Moser et al., 2002). In the CF mouse, the Th2 predilection of lymphocytes with a hyperinflammatory immune

response has been recapitulated and directly related to the lack of CFTR. As a consequence of the Th2 derived cytokine IL-4, B cells produce large quantities of IgE and thus support proallergic dysregulation (Mueller et al., 2011).

Another subset of particular interest in the exaggerated immune response are lymphocytes that release IL-17, because it plays a crucial role in eliminating gram negative bacteria and neutrophil recruitment (reviewed in Bruscia and Bonfield, 2016b). Excessive amounts of IL-17 are detected in the BAL and moreover, higher quantities of IL-17 producing cells are present in the airways of CF patients (Tan et al., 2011). Adaptive immune responses are normally tightly controlled by regulatory T cells to prevent excessive inflammation (reviewed in Josefowicz et al., 2012). The decreased numbers of regulatory T cells in the circulation and the airways from CF patients are supposed as a main reason for the imbalance of T-cell activation (Hector et al., 2015). Alterations in regulatory T cells are also thought to shift the CF airway environment from IL-10 based anti-inflammatory status towards a pro-inflammatory neutrophil attracting environment (Moss et al., 1996). From a functional point of view, altered abundance of regulatory T cells is suggested to be a consequence of inhibiting effects of the pro-inflammatory IL-6 and abnormal tryptophan catabolism in CF patients (reviewed in Ratner and Mueller, 2012; Roesch et al., 2018).

It is evident that aberrant immune function is not restricted to defined cell populations. Rather, an extensive crosstalk between constituents is taking place. A typical example are γδ T cells, proposed mediators between innate and adaptive immune system (reviewed in Shiromizu and Jancic, 2018). In respect of their intra-mucosal localization, the γδ T cells should be of outstanding interest in CF. Surprisingly, investigation on the role of γδ T cells is limited and their role in CF and other airway pathologies remains puzzling (reviewed in Cheng and Hu, 2017). At least it is known that sub-bronchial located γδ T cells contribute with IL-17 production to the inflammation in the CF lungs (Tan et al., 2011) and their release of TNFα and IFNγ against *P. aeruginosa* is higher than from conventional T cells in CF patients (Raga et al., 2003).

Pro-inflammatory cytokines are also released by the innate lymphocyte subset of natural killer (NK) cells (Tan et al., 2011). In CF patients the proportion and phenotype of NK cells seem to be altered (Mulcahy et al., 2019). In addition, the

lymphocyte subset of invariant NKT cells, which express the T cell receptor as well as the NK cell receptor, accumulate, trigger cell death and recruit neutrophils in CF mouse models. These effects were diminished by deletion of the invariant NKT cells (Siegmann et al., 2014).

Collaborative interaction is also taking place between lymphocytes and antigen presenting cell (APCs) like MΦs and DCs and are necessary in the host response to infections (reviewed in Guilliams et al., 2013b). In the case of CF, this is relevant for pulmonary exacerbations which do not only comprise bacteria, but also respiratory viruses (reviewed in Cohen-Cymberknoh et al., 2013). Resolution of viral infection in CF airways is impaired due to impaired interferon mediated activation of DCs (Parker et al., 2012; Zheng et al., 2003). Furthermore, antigen presentation by the major histocompatibility complex (MHC) activates the adaptive immune response, but in CF patients the surface molecule is downregulated on circulating MPs (Hofer et al., 2014). In this context it is noteworthy that genome wide association studies linked MHC II variants to CF lung disease severity (Wright et al., 2011). Additionally, impaired expression of the co-stimulatory molecules CD80 and CD86 of MPs and delayed maturation of DCs are thought to impair co-stimulation of T cell activation (reviewed in Hartl et al., 2012; Soltys et al., 2002). To sum up, it is postulated that defective CFTR in MPs intrinsically downregulates the antigen presentation pathway and therefore affects the adaptive immune system (Hampton and Stanton, 2010), similar to other chronic inflammatory diseases such as asthma or allergic bronchopulmonary aspergillosis (reviewed in Bruscia and Bonfield, 2016a).

3. The pig as an immunological model for cystic fibrosis

Clinical examination of patients and histopathological analysis of tissue samples have been essential to improve survival and the quality of life of CF individuals. But patient derived data have also limitations for understanding the pathogenesis of CF. This is specifically true for the dysregulated immune system in CF. Nearly all observations are obtained after the onset of the disease. At an early stage of disease, examination often cannot be done in humans and later in the course, secondary changes due to infection and inflammation may overlay potential initiating factors. Detailed analysis is restricted to local examination and can only be done in biopsies at rare occasions. Lack of appropriate control groups and variations in pathogenesis

due to modifier genes, environmental influences and individual treatment further compromise the informative value of these studies. (Meyerholz, 2016; Stoltz et al., 2015; Welsh et al., 2009). *In-vitro* models are popular, but their usage is limited in remodeling the complex consequences of defective CFTR for the organism. *In-vitro* cultures are vulnerable for artefacts as consequences of clonal selection, differentiation of cells as well as the insufficient reflection of anatomical details or impaired communication between interacting cells and tissues. The danger of insufficient conclusions is illustrated by the variety of results from distinct *in-vitro* models, which appear inconclusive about CF disease initiation (reviewed in Matarese et al., 2012; Ratner and Mueller, 2012; Roesch et al., 2018).

To circumvent these limitations, various CF animal models including mouse, rat, zebrafish, sheep, ferret and pig have been developed. Significantly, not all models revealed the major hallmarks of CF and especially, CF mouse models fail to develop airway disease spontaneously (reviewed in Semaniakou et al., 2019). It remains hypothetic, whether the failure of CF airway disease manifestation in mouse models is caused by major differences in genetics, anatomy, physiology or immunology, but the poor predictive value of rodents used in biomedical research for pathophysiological research in inflammatory diseases appears to be a general feature (Seok et al., 2013). So far, the porcine CF models show the greatest resemblance to the human phenotype (reviewed in Cutting, 2015). The value of the pig for evaluating metabolic, cardiovascular, infectious and genetic diseases has been proven in multiple ways (reviewed in Aigner et al., 2010; Meurens et al., 2012). Furthermore, the progress in xenotransplantation demonstrates organ function is highly comparable between human and pigs (Ibrahim et al., 2006; Längin et al., 2018).

Due to the high translational capacity in other context, expectations are rising for the in-depth immunological characterization of the CF pig model. This is also based on the profound knowledge that has been gained on the immune system of the pig. Following mouse and humans, pigs have the best characterized immune system (Fairbairn et al., 2011; Summerfield, 2009). Understanding the pig immunity is considered as highly relevant for gaining inter-species insights of the mechanistic principles of CF. In addition to the understanding of pathophysiology, more appropriate translation of therapies in the clinical setting is urgent as well (reviewed in Bragonzi et al., 2017; Dhooghe et al., 2014; Rieber et al., 2014). In the following,

advantages and peculiarities of pigs as an immunological model in respect of CF are referred to man and mice.

3.1. Genetic, anatomic and physiologic considerations

In terms of genetics, the porcine genome is comparable to the human genome in size and composition (reviewed in Bassols et al., 2014). Despite mice and human have closer common ancestor than human and pig, the genetic distance between pig and human is smaller than between human and mouse. This is normally explained by the much shorter generation time of rodents and their evolutionary adaption to specific niches. The high evolutionary conservation between human and pig is illustrated by functionally conserved genes, such as *CFTR*. In fact, the ortholog in pig has the closest similarity compared to the human CFTR of all existing CF models (reviewed in Semaniakou et al., 2019). From the evolutionary point of view, the immune system has, due to the permanent challenge by pathogens, a specifically high evolutionary turnover. The strong positive selection resulted in a faster genetic drift of immune related genes than others (Ellegren, 2008). Given these specific properties, the immune system is among the most diverse between species, and therefore genetic difference between human and mouse is even more pronounced (Dawson et al., 2013; 2017; Fairbairn et al., 2011). As a consequence, the immune system of pig is of significant value for CF. Furthermore, the outbred pig population is more representative for the heterogeneity in patient's population than inbred mouse strains (reviewed in Groenen et al., 2012; Seok et al., 2013).

Similar to the pure genetic aspects, pigs share remarkable physiological similarities with humans. Both are omnivorous species, therefore their organs share generally common anatomic and metabolic features. In terms of anatomy and size, pigs allow human-like clinical monitoring. Especially, anatomy and morphology of the airways are supposed to be advantageous for investigation (reviewed in Rogers et al., 2008a). The number of lung lobes of pigs differs from the human counterpart. Whereas in contrast to mice, pigs have similar to humans structural inter- and intra-lobular connective tissue in the lungs. Moreover, lymphoid tissue in the nasopharynx and tonsils is present in pig's upper respiratory tract like in humans. In mice these lymphoid organs are missing (reviewed in Meurens et al., 2012). The inverted lymph nodes are a structural particularity of pigs. However, no resulting functional difference is known from that. Furthermore, two types of intestinal

lymph follicle areas are described in pigs, the jejunal and ileal Peyer's patches. Unlike in chicken or sheep, there is no putative function known for porcine Peyer's patches (reviewed in Rothkötter, 2009).

Regarding microscopic airway anatomy, pigs recapitulate the pattern of submucosal glands in small and large airways of human, whereas mice have only poor numbers of submucosal glands in the proximal trachea (reviewed in Rogers et al., 2008a). Besides that, in mice the use of ion channels to maintain the electrophysiological properties of the lungs is thought to be different from humans and pigs. Especially, high sodium absorption by the epithelial sodium channel ENAC in absence of CFTR seems to be a particularity of mouse epithelial cells (Chen et al., 2010).

A certain anatomical particularity of pigs, the epitheliochorial placenta turns out as an interesting feature of early immunological investigations in newborn piglets. Unlike in the hemochorial placenta of humans or hemotrichorial placenta of mice, no immunity transfer in the uterus is observed in piglets. Moreover, each pig fetus is separated from its littermates in the uterus, thus there is no circulation between different genotypes. Passive transfer of secreted Igs is dominant in intestine, milk and mucosal sites of pigs like in humans (reviewed in Mair et al., 2014; Salmon et al., 2009; Sinkora and Butler, 2016). Similar to human infants, newborn piglets are immunocompetent at birth and capable for immunological priming of adaptive immunity (Nguyen et al., 2016; Talker et al., 2013; Vreman et al., 2018). In contrast to the high standard of hygiene in most mouse holdings, pigs are exposed to infectious, nutritional and environmental antigens comparable to humans. Indeed, shared pathogens like influenza, methicillin resistant *S. aureus* or swine erysipelas are feared because of their easy transmission between swine and human. As a consequence, the pig has been used widely to study viral and bacterial respiratory infectious diseases of humans including *P. aeruginosa* (reviewed in Meurens et al., 2012).

3.2. Immune system of pigs

Like men and mice, pigs have the full set of innate and adaptive immune effectors of mammals. For the most part, the pathogen recognition and the humoral innate response are conserved grossly between human, pig and mouse. PRRs including TLRs, nucleotide-binding oligomerization domain like receptors, retinoic acid-inducible gene-I like receptors, DNA sensors and lectin receptors in pig are nearly

as in humans (reviewed in Mair et al., 2014), whereas a specific expansion of PRRs has been described for mice (Dawson et al., 2017). The expression pattern of TLRs in human is recapitulated by porcine immune cells, while mice show exceptional higher expression of the endosomal TLR 9 within MΦs, which is responsible for recognizing non-methylated cytosine-phosphate-guanin (CpG) motifs of bacterial and viral DNA. Regarding the humoral system, differences between species are found as well. Within antimicrobial peptides, defensin composition of mice appears different from human (reviewed in Fairbairn et al., 2011), but a pig-specific peculiarity is assumed as well: loss of α-defensins, while β-defensins occur functionally similar to humans in the airways (reviewed in Mair et al., 2014).

Overall, however, all immune cell subpopulations characterized in humans and mice are also present in pigs, although different expression profiles of the characteristic cluster of differentiation (CD) markers appear (reviewed in Auray et al., 2016; Meurens et al., 2012). Thus, for identification of immune cell subsets species specific characterization by CD markers is necessary (reviewed in Gerner et al., 2009). It is of note that 359 orthologues of the human CD markers have been detected in pigs (Dawson and Lunney, 2018; Piriou-Guzylack and Salmon, 2008).

3.2.1. Polymorphonuclear and mononuclear phagocytes

Similar to humans, pigs have relatively high percentages (50-70%) of granulocytes within peripheral blood leukocytes, while granulocyte population of rodents represents only 10-25% of peripheral blood leukocytes. (reviewed in Fairbairn et al., 2011). Significantly, like in humans and unlike in rodents, there is a direct homologue of the important neutrophil chemoattractant IL-8 in pigs (Hol et al., 2010; Kapetanovic et al., 2012). In addition, porcine neutrophils have proven their valuable use for evaluating their functional properties as a model for CF relevant pathogens in the airways including *S. aureus* and *P. aeruginosa* (Bréa et al., 2012; Chevaleyre et al., 2016; Gray et al., 2018).

Congruent with the findings in mice and human, circulating and tissue resident porcine MPs can be divided into at least two major subpopulations, in pigs by their expression of the haptoglobin scavenger receptor CD163 (reviewed in 2013; Fairbairn et al., 2011) and further characterization of monocyte-, MΦ- and DC-subsets into more detail is predominantly characterized as well (Auray et al., 2016;

Fernández-Caballero et al., 2018). Based on influenza, respiratory mononuclear phagocyte network reveals close similarities between pigs and the human counterpart (Guilliams et al., 2014; Maisonnasse et al., 2016a; 2016b). In view of CF it is an interesting fact that the immune response of porcine MΦs against LPS showed the same effect as humans, whereby murine MΦs follow different activation pathways (Kapetanovic et al., 2012). At the moment it remains unclear, whether the described pulmonary intravascular MΦs are really a special feature of the pig lungs or whether the identification in human lungs has not yet been achieved, according to the situation of interstitial lung MΦs (Bordet et al., 2018; Liegeois et al., 2018).

3.2.2. Lymphocytes

Globally, only little peculiarities have been described for pigs regarding innate and adaptive lymphocyte subsets and effector functions. Differentiation of T cells via Th1/Th2, IL-17 production and regulatory T-cell function is also traceable in lymphocytes from pigs (reviewed in Gerner et al., 2009; Käser et al., 2012; Sassu et al., 2017). Intracellular agents induce Th1 immunity with IL-2 and IFNγ release and subsequently, Th1 immunity activates cytotoxic T cells and MΦs, while Th2 polarization stimulates antibody production by B cells and control of extracellular pathogens. Like in humans and mice, T cells with IL-17 synthesis are seen to act on extracellular pathogens. Regulatory T cells control the immune response to prevent harmful dysregulation. Functional cytokine orthologs and corresponding cells of the T-cell paradigm have been described (reviewed in Meurens et al., 2012). Moreover, B cells from piglets are a suitable model for B-cell development as well (Butler et al., 2009). Another lymphocyte subset, the γδ T cells, is present at higher frequencies in the circulation of pigs compared to humans and mice, but otherwise, porcine γδ T cells share intraepithelial localization at various sites including the lungs. Their functional plasticity ranges from protective immunity against extra- and intracellular pathogens, tumor surveillance, modulation of innate and adaptive immune responses, tissue healing to maintaining structural organ integrity similar to human and mouse (Hammer et al., 2020; Rodríguez-Gómez et al., 2019).

Like in human and mouse, porcine NK cells are found in many anatomical sites at varying frequency. Their functional properties consist of comparable cytolytic activity and production of cytokines like IFNγ and TNFα (reviewed in Mair et al.,

2014). Significantly, NK cells seem to be functional competent very early in life of piglets (Talker et al., 2013), but characterization of further innate lymphocyte subpopulations is limited in pigs due to the lack of established markers (Dawson and Lunney, 2018).

3.3. Defective host defense of cystic fibrosis pigs

In general, newborn *CFTR*$^{-/-}$ piglets (CF piglets) recapitulate major early malformations of CF with intestinal, hepatobiliary, male reproductive and pancreatic lesions and their malfunctions at birth (Klymiuk et al., 2012; Pierucci-Alves et al., 2011; Rogers et al., 2008b). Several congenital respiratory defects are observed in newborn CF piglets: Similar to CF infants, tracheal shape is abnormal and the tracheal caliber is reduced. In addition, variable thickening of the posterior wall in the caudal trachea and in the large bronchi are observed due to abnormal smooth muscle cell orientation. Submucosal glands occur in CF piglets at similar frequency as in control animals, but CF glands are hypoplastic (Klymiuk et al., 2012; Meyerholz et al., 2010; Rogers et al., 2008b). Lack of CFTR thus disrupts the fetal development of the airways (Meyerholz et al., 2018), but functional consequences of structural alterations of larger airways are unclear (reviewed in Stoltz et al., 2015). Apart from larger airways, lungs of newborn CF piglets appear morphologically normal (Rogers et al., 2008b).

Very importantly, lungs of CF piglets lack inflammation at birth. Even at the transcriptome level hierarchical clustering of unchallenged newborn CF airway revealed no specific differences of inflammatory pathways. No differences between CF piglets and controls have been observed in total cell number, relative abundance of neutrophils and IL-8 concentration in BAL (Bartlett et al., 2016; Stoltz et al., 2010). Although unchallenged newborn CF piglets have no signs of acute infection, the airways seem to be colonized with bacteria more often. After intrapulmonary instillation of *S. aureus,* the ability to eradicate bacteria from the lungs is reduced in newborn *CFTR*$^{-/-}$ piglets (Stoltz et al., 2010). Then, within weeks, CF pigs spontaneously develop pulmonary characteristics, with a heterogenous picture and similar severity like in CF patients. Typical CF hallmarks, including mucus accumulation, chronic infection, inflammation and tissue remodeling are observed. Leukocyte infiltration varied from moderate in the airway walls to neutrophil accumulation in the luminal space (Ostedgaard et al., 2011; Stoltz et al., 2010).

Typical as well is the unclarity of which pathophysiologic mechanisms specifically impair the host defense of neonatal CF airways. There are clear indications of slower mechanical transport and disturbed mucin processing in the trachea of newborn *CFTR*$^{-/-}$ piglets (Ermund et al., 2018). Congruently, CF pig airways exhibit a suppression of ciliary and flagellar biosynthetic pathways after instillation of *S. aureus*. In addition, apoptotic pathways appear increased (Bartlett et al., 2016). Interestingly, the bacterial eradication from the smaller airways is even less effective than in the trachea (Stoltz et al., 2010), consistent with different host defense in distinct compartment of the airways. Pezzulo et al. (2012) assumed that abnormal acidification impairs functionality of antimicrobial peptides in the ASL.

All mentioned examples suggest influence on pathogen clearance and infection, but so far immune cells in the *CFTR*$^{-/-}$ piglet lungs have not been examined comprehensively, despite the evidence of impaired function of leukocytes (Gray et al., 2018; Paemka et al., 2017). The aim of this thesis was studying immune cells directly *ex-vivo* from neonatal *CFTR*$^{-/-}$ piglets in comparison to *CFTR*$^{+/+}$ littermate controls in order to detect potential predilections of *CFTR*$^{-/-}$ leukocytes for an altered immune response.

III. Animals, Materials and Methods

1. Animals

Heterozygous *CFTR*$^{+/-}$ pigs had been generated by gene targeting with a bacterial artificial chromosome vector. The large regions of homology of the vector led to insertion of a STOP box into the start codon of the *CFTR* exon 1 in primary porcine kidney cells. Correctly monoallelic modified single-cell clones had been used for somatic cell nuclear transfer (Klymiuk et al., 2012). Subsequently, breeding with the offspring had provided male and female heterozygous *CFTR*$^{+/-}$ pigs. In the current thesis, piglets generated by breeding were sacrificed for scientific purposes and the present work does not include animal experiments according to the German Welfare Act.

2. Materials

2.1. Apparatuses

5424 R centrifuge	Eppendorf, Hamburg
5804 R centrifuge	Eppendorf, Hamburg
Accu-jet® pro pipette controller	Brand, Wertheim
Agarose gel electrophoresis chamber	Owl Inc., USA
Autoclav Varioklav 400	H+P Labortechnik, Oberschleißheim
Cell counting chamber Neubauer improved	Assistent, Sondheim von der Rhön
CO_2 cell incubator	Binder, Tuttlingen
CoolCell	BioCision, USA
DMi1 inverted microscope	Leica Microsystems, Wetzlar
Flow cytometer BD FACScan	BD Biosciences, Heidelberg
Flow cytometer BD FACSCanto II	BD Biosciences, Heidelberg
Gel documentation system	BioRad, Munich
Incubator B15	Heraeus, Hanau
Labcycler thermocycler	SensQuest, Göttingen
Lamina flow HB 2448K	Heraeus, Hanau
Lamina flow Hera Safe KS 12	Heraeus, Hanau

Microwave	DAEWOO, Korea
Multipipette E3	Eppendorf, Hamburg
Multitip pipet (300 µl)	Eppendorf, Hamburg
Pipettes (1000 µl, 200 µl, 100 µl, 20 µl, 10 µl, 2,5 µl)	Eppendorf, Hamburg
Pipettes (1000 µl, 200 µl, 20 µl, 2µl)	Gilson Inc, USA
Power Pac 300 gel electrophoresis unit	BioRad, Munich
Select vortexer	Select BioProducts, USA
Tecan Sunrise Elisa reader	Tecan, Austria
Thermo-Shaker TS-100	bioSan, Latvia
Vibrating Platform Shaker Titramax 100	Heidolph, Schwabach
Waterbath JB Nova 5	Grant Instruments, UK

2.2. Software

CellQuest™ Pro	BD Biosciences, Heidelberg
ClustVis web tool	http://biit.cs.ut.ee/clustvis/ (Metsalu and Vilo, 2015)
FACSDiva 6.1.3	BD Biosciences, Heidelberg
Flowjo™ 10	BD Biosciences, Heidelberg
G*Power 3.1.9.4	HHU Düsseldorf; (Faul et al., 2007)
Magellan	Tecan, Austria
Prism 8.3.1	GraphPad Software, USA

2.3. Consumables

Cell strainers 70 µm	ThermoFisher Scientific
Centrifuge tubes Cellstar® (15 ml, 50 ml)	Greiner-Bio One, Frickenhausen
Cotton wool	Household, autoclaved
Cryovials (1.5 ml)	TPP, Switzerland
Li-Hep Monovette® collection system	Sarstedt, Nürmbrecht
Microplate U-bottom 96 well	Eppendorf, Hamburg
PCR reaction tubes (0.2 ml)	Brand, Wertheim
Pipette tips (1000 µl, 250 µl)	Eppendorf, Hamburg
Pipette tips with filter (1000 µl, 200 µl, 20 µl, 10 µl)	Kisker Biotech, Steinfurt
Safe-Lock reaction tubes (1.5 ml, 0.5 ml)	Eppendorf, Hamburg

Serological pipettes Cellstar (25 ml, 10 ml, 5 ml)	Greiner-BioOne, Frickenhausen
Tissue culture dish (100 mm)	Sarstedt, Nürmbrecht
Tissue culture plate 96 well	Eppendorf, Hamburg
Tubes Falcon (12x75 mm)	BD Biosciences, Heidelberg

2.4. Chemicals and other reagents

10x LongRange PCR Buffer	Qiagen
Bromophenol blue	Carl Roth, Karlsruhe
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich
dNTPs (dATP, dCTP, dGTP, dTTP)	ThermoFisher Scientific
Ethanol	Carl Roth, Karlsruhe
Ethylenediaminetetraacetic acid (EDTA)	Carl Roth, Karlsruhe
Flagellin from *P. aeruginosa*	Invivogen, France
GelRed® nucleic acid gel strain	Biotium, USA
GeneRuler 1-kb Plus DNA Ladder	ThermoFisher Scientific
HEPES buffer (1 M) Gibco™	ThermoFisher Scientific
Ionomycin calcium salt	Merck, Darmstadt
Lipopolysaccharide from *P. aeruginosa*	ThermoFisher Scientific
ODN 2216	Invivogen, France
ODN 2243	Invivogen, France
Pam3Cys-SKKKK	EMC Microcollections, Tübingen
Pancoll human, density 1.077 g/ml	PAN-Biotech, Aidenbach
Paraformaldehyde (PFA)	Sigma-Aldrich
PBS without Ca^{2+} and Mg^{2+}	PAN-Biotech, Aidenbach
Penicillin / Streptomycin Gibco™	ThermoFisher Scientific
Phorbol-12-myristat-13-acetat (PMA)	Merck, Darmstadt
Porcine plasma	In-house, University of Veterinary Medicine Vienna
RPMI 1640 with stable glutamine	PAN-Biotech, Aidenbach
Sera Pro FBS, low endotoxin	PAN-Biotech, Aidenbach
Tris-(hydroxymethyl)-aminomethane (Tris)	Carl Roth, Karlsruhe
Türk's solution	Merck, Darmstadt
Ultra-pure LPS from *E. coli* O111:B4	Invivogen, France
Universal Agarose	Bio&Sell, Nürnberg

2.5. Drugs, enzymes and oligonucleotides

Drugs

Altrenogest (Regumate®)	MSD, Unterschleißheim
Azaperon (Stresnil®)	Elanco, Bad Homburg
Choriogonadotopine (Ovogest®)	MSD, Unterschleißheim
Cloprostenol (Estrumate®)	MSD, Unterschleißheim
Embutramid, Mebezonium, Tetracain (T61®)	MSD, Unterschleißheim
Ketamine hydrochlorid (Ursotamin®)	Serumwerk Bernburg, Bernburg
Perforelin (Maprelin®)	Veyx-Pharma, Schwarzenborn

Enzymes

Collagenase type I	ThermoFisher Scientific
Deoxyribonuclease I	ThermoFisher Scientific
HotStarTaq DNA Polymerase	Qiagen, Hilden

Oligonucleotides

All oligonucleotides were purchased from ThermoFisher Scientific.

Cg2f 5’ AGA AGA GTA GGG CCT TTG GCA T 3’

Cg1r 5’ TGG CTG AAC TGA GCG AAC AAG T 3’

Cg5r 5’ AGC ACA TGT GGG TCT TAG AGT ACG 3’

2.6. Buffers, media and solutions

Water deionized in a Millipore device (EASYpure® II) and termed as aq. bidest. was used as solvent when necessary.

TAE-Buffer

Tris	2	M
EDTA	50	mM

dNTP-mix

dATP, dCTP, dGTP, dTTP	2	mM

4% PFA solution

4% PFA

PBS (1x) buffer

Digestion solution

250 Units/ml DNASE I

300 Units/ml Collagenase I

1% Penicillin/Streptomycin

2% FBS

2% HEPES

RPMI 1640

Wash medium

5% FBS

RPMI 1640

Culture medium

10% FBS

1% Penicillin/Streptomycin

RPMI 1640

Freezing medium

10% DMSO

40% FBS

RPMI 1640

Staining buffer

10% porcine plasma

PBS without Ca^{2+} and Mg^{2+}

2.7. Kits

All kits were used according to the manufacturers' protocols.

DuoSet® Ancillary Reagent Kit 2	R&D Systems, USA
Intracellular Fixation & Permeabilization Buffer Set eBioscience™	ThermoFisher Scientific
Nexttec™ Genomic DNA Isolation Kit	Nexttec, Leverkusen
Phagoburst™	BD Biosciences, Heidelberg
Phagotest™	BD Biosciences, Heidelberg
Porcine TNFα DuoSet®	R&D Systems, USA

2.8. Antibodies and staining reagents

All non-commercially acquired antibodies were kindly provided by the Institute of Immunology, Department for Pathobiology, University of Veterinary Medicine Vienna (UVV), Vienna.

Table 1. Reagents and antibodies for flow cytometry staining

Reagent/Antibody	Clone	Manufacturer
Mouse anti-pig CD45-Alexa Fluor® 488	K252.1E4	BioRad
Mouse anti-pig CD8α-Biotin	11/295/33	UVV[1]
Mouse anti-pig CD4	74-12-4	UVV
Mouse anti-pig CD8α	11/295/33	UVV
Mouse anti-pig CD25-Alexa Fluor® 647	3B2	UVV[2]
Mouse anti-pig TCR γδ	PPT16	UVV
Mouse anti-pig CD2	MSA4	UVV
Mouse anti-pig CD27-Alexa Fluor® 647	B30c7	UVV[2]
Mouse anti-pig CD8β-Biotin	PPT23	UVV[1,3]
Mouse anti-bovine CD14	CAM36A	BioRad
Mouse anti-pig CD172a	74-22-15A	UVV
hCTLA4 / Fc chimera for CD80/86 detection	N.a.	Sigma-Aldrich
Mouse anti-pig CD11b	MIL4	BioRad
Mouse anti-pig CD163-PE	2A10/11	BioRad
Mouse anti-pig CD16-FITC	G7	BioRad
Mouse anti-pig NKp46-Alexa Fluor® 647	VIV-KM1	BioRad[2]
Mouse anti-pig CD79α-PE	HM57	Dako
Mouse anti-pig Ki-67-Brilliant Violet 421™	B56	BD Biosciences
Mouse anti-pig MHCII	MSA3	Kingfisher Biotech
Goat anti-pig IgG1-Biotin	N.a.	Southern Biotech
Goat anti-mouse IgG2a-PE-Cy7	N.a.	Southern Biotech
Goat anti-mouse IgG2b-A488	N.a.	Jackson
Goat anti-mouse IgG2b-Biotin	N.a.	Southern Biotech
Goat anti-mouse IgG1-Alexa Fluor® 647	N.a.	ThermoFisher Scientific
Fixable Viability Dye eFluor™ 780	-	ThermoFisher Scientific
Streptavidin-PE-Cy7	-	ThermoFisher Scientific
Streptavidin-Brilliant Violet 605™	-	BioLegend
Streptavidin-PE	-	ThermoFisher Scientific
Anti-mouse IgG ChromePure™	-	Jackson
Mouse anti-pig CD3-PerCP - Cy™ 5.5	BB23-8E-8C8	BD Biosciences

[1] biotinylation with EZ-Link™ Sulfo-NHS-LC-Biotin (ThermoFisher Scientific)

[2] conjugation with Alexa Fluor® 647 Protein Labeling Kit (ThermoFisher Scientific)

[3] non-biotinylated mAb commercially available from BioRad

3. Methods

3.1. Generating and collecting samples of *CFTR*$^{-/-}$ and *CFTR*$^{+/+}$ piglets

CFTR$^{-/-}$ piglets and wildtype (WT) *CFTR*$^{+/+}$ littermate controls were produced by mating of heterozygous *CFTR*$^{+/-}$ animals. Samples were taken within the first day of life to minimize the environmental influence. Furthermore, at least a pair of one CF piglet and one WT piglet were sacrificed per litter. For selection of relevant animals from a litter, piglets were genotyped immediately after birth.

Breeding with heterozygous *CFTR*$^{+/-}$ pigs

Female *CFTR*$^{+/-}$ pigs were routinely estrus synchronized for breeding, either according to the protocol for gilts or the protocol for sows (Figure 1). Treatment was carried out according to manufacturer's instructions.

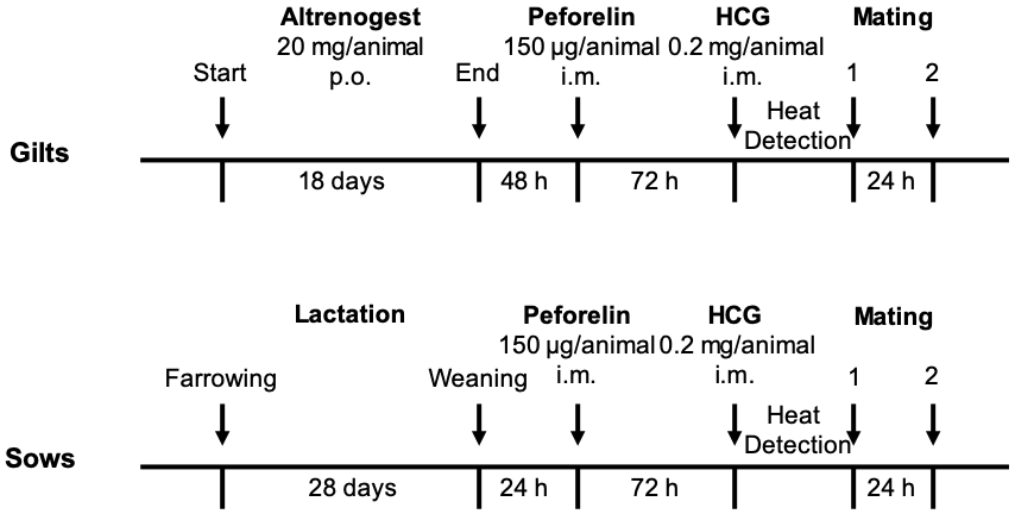


Figure 1. Estrus synchronization protocols.

Farrowing was introduced by administration of cloprostenol (175 µg/animal) i.m. after 113 days of gestation and birth was given the day after. Within statistical ranges, a litter contained *CFTR*$^{-/-}$, heterozygous *CFTR*$^{+/-}$ and wildtype *CFTR*$^{+/+}$ littermates, according to the Mendelian rules of inheritance.

Genotyping of newborn piglets

The genotypes of newborn piglets were directly identified by polymerase chain reaction (PCR). One set of primers detected WT sequence and another set the transgenic sequence of the *CFTR* locus.

For that purpose, a fast and potent PCR has been established previously (thesis of Michaela Désirée Dmochewitz, Chair for Molecular Animal Breeding and Biotechnology, LMU Munich). Genomic DNA was isolated from pig tails. The resulting DNA eluate was immediately ready to use for PCR reaction and the components of PCR were mixed on ice to a final volume of 20 µl in 0.2 ml reaction tubes. Aq. bidest. served as negative template control and in addition, previously isolated genomic DNA samples from the three occurring genotypes were used as controls. Further details of composition and conditions of the PCR are listed in Table 2 and 3. Following PCR, presence and size of the PCR product was verified by gel-electrophoresis. Therefore, 2 µl of a 1:250 mixture of GelRed® with bromophenol blue was added to each sample. In a next step, 20 µl of each sample was inserted in individual gel slots of a 1% agarose gel in an electrophoresis chamber filled with TAE-buffer. 6 µl of GeneRuler™ 1-kb standard was added to an additional gel slot for determination of the fragment size of the PCR product. An electric field (130 V) was applicated for 30 mins and afterwards DNA band were visualized under UV light.

Table 2. Composition of PCR for CFTR loci

10x PCR buffer	2.0 µl
dNTPs (2 mM)	2.0 µl
Primer f (10 µM)	0.4 µl
Primer r (10 µM)	0.4 µl
Taq Polymerase (5 U/µL)	0.2 µl
Aq. Dest.	13 µl
DNA template	2 µl

Table 3. Cycler protocol of PCR for CFTR loci

Denaturation	95 °C	5 min	
Denaturation	95 °C	20 s	
Annealing	56 °C	20 s	35x
Elongation	72 °C	30 s	
Final	72 °C	5 min	
Termination	4 °C	5 min	

Collecting samples

Blood, spleen and lungs were sampled within the first 21 hours after birth. The newborn piglets were anesthetized with intramuscular injection of ketamine hydrochloride (2 ml/ kg bodyweight) and azaperone (0.5 ml/kg bodyweight) and subsequently euthanized via intracardial injection of embutramide, mebezonium, tetracain (0.1 ml/kg bodyweight). In the following, 20-30 ml of blood were collected by intracardiac puncture with an 18-gauge needle into heparinized tubes and kept at room temperature. During necropsy, the entire spleen was collected and stored shortly in a 50-ml tube filled with PBS with 1% Penicillin/Streptomycin on ice. The lungs were explanted including trachea and either likewise stored in PBS with 1% Penicillin/Streptomycin on ice or randomly sampled from the different lung lobes and stored in 4% PFA solution at room temperature for histopathological investigation, which was performed by Lars Mundhenk, Institute of Veterinary Pathology, FU Berlin.

3.2. Isolation of mononuclear leukocytes from blood, spleen and lungs

Mononuclear leukocytes (MNLs) were isolated under sterile conditions immediately after necropsy by density gradient centrifugation. Therefore, samples were prepared as single cell suspensions:

Blood: Heparinized blood was diluted with PBS (20°C) in the ratio 1:2.

Spleen: The spleen was cut into small pieces and then strained with cold PBS through a 70-µm cell strainer with help of a plunger from a sterile 10-ml syringe. The spleen cell suspension was centrifuged (10 min, 470 g, 4°C) and resuspended in 25 ml PBS (20°C) per sample.

Lungs: Left and right lung tissue was chopped with scissors into small pieces (3x4x3 mm) and rinsed with cold PBS to remove cells of blood-origin. Subsequently, lung tissue was minced with 50 ml digestion solution for 60 minutes at 37°C in an incubator with magnetic stirrer. After enzymatic digestion, remaining tissue was removed from the suspension by decanting through a sterile metal tea sieve and thereafter dead cells were removed by filtration through sterile cotton wool. The resulting cell suspension was centrifuged (10 min, 420 g, 4°C) in 50-ml tubes and resuspended in 25 ml PBS (20°C) per sample.

In order to improve leukocyte separation from erythrocytes in newborn animals the commercially available leukocyte separation medium with 1.077 g/ml was diluted with PBS to 1.075 g/ml according to the manufacturer's formula:

$$V\% = \frac{(D' - D\%) \times 100}{D'' - D\%}$$

D' *...required final density (g/ml)*

D'' *...high starting density (g/ml)*

D% *...density of the iso-osmotic dilution solution (g/ml)*

V% *...volume per cent of the starting solution with high density*

For performing density gradient centrifugation, 25 ml of cell suspension were gently layered above 15 ml of leukocyte separation medium in 50-ml tubes. After a centrifugation step (30 min, 900 g, acceleration 4, deceleration 1, 20°C), the MNLs accumulating within the interphase were collected and transferred to a new 50-ml tube. Subsequently, the isolated cells were washed twice with cold PBS and once with cold wash medium. Washing consisted of centrifugation (10 min, 420 g, 4°C), discarding supernatant and resuspending cells. Thereafter, isolated MNLs were resuspended in 5 ml cold culture medium and 10 µl of the cell suspension were mixed with 90 µl of Türk's solution for counting. The number of isolated MNLs was counted manually in a Neubauer improved cell counting chamber. The MNLs were either used freshly for cultivation or were cryopreserved in cryovials with freezing medium and cooled slowly in freezing containers to −80°C overnight. The next day, samples were stored in liquid nitrogen prior to use.

3.3. Phenotyping mononuclear leukocytes

The relative abundance of major leukocyte subpopulations was determined by multicolor flow cytometry. In addition, the activation status of each subset was determined by analyzing the relative abundance of proliferative cells within the subset. Moreover, expression of several surface receptors from mononuclear phagocytes was quantified by flow cytometry.

3.3.1. Flow cytometry staining

The cryopreserved MNLs were defrosted, transferred to 15-ml tubes filled with staining buffer (37°C) and centrifuged (10 min, 470g, 20°C). The supernatant was

discarded and the remaining cells were resuspended in two ml of staining buffer (4°C). Leukocytes were counted and $1x10^6$ cells per panel were stained in microtiter plates. Details of staining panels for phenotyping are provided in Table 4. Cell surface antigens were stained in three incubation steps. Free binding sites of secondary antibodies were blocked with anti-mouse IgG antibodies and intracellular antigens were stained after fixation and permeabilization. All incubation steps took place for 20 min at 4°C and were followed by two washing steps. A washing step comprised the addition of 200 µl staining buffer, centrifugation (4 min, 470 g, 4°C), discard of supernatant and the use of a plate shaker. After blocking, cells were washed with PBS (4°C), in order to prevent interference of the staining buffer with the fixable viability dye stain eFluor™ 780 of the third extracellular labelling. Thereafter, cells were fixed and permeabilized with the eBioscience™ Intracellular Fixation & Permeabilization Buffer Set. Intracellular staining, washing and final resuspending in 250 µl was performed with the provided buffer from the kit. Finally, the stained leukocytes were transferred to 5-ml tubes for analysis by flow cytometry.

Table 4. Staining panels used for flow cytometry.

Antigen	**Fluorochrome**	**Labeling strategy**
Isolated cells control for leukocytes		
CD45	Alexa647	Directly conjugated
Natural Killer cells		
CD3	PerCP-Cy5.5	Directly conjugated
CD8α	PE	Two step biotin-streptavidin
NKp46	Alexa647	Directly conjugated
CD16	FITC	Directly conjugated
Ki-67	Brilliant Violet 421	Directly conjugated
γδ T cell and cytotoxic T cell staining		
TCR-γδ	Alexa488	Secondary antibody[a]
CD2	PE-Cy7	Secondary antibody[b]
CD27	Alexa647	Directly conjugated
CD8β	PE	Two step biotin-streptavidin
Ki-67	Brilliant Violet 421	Directly conjugated
B cell, regulatory T cell and activated & memory T cell staining		
CD4	Alexa488	Secondary antibody[a]
CD8α	PE-Cy7	Secondary antibody[b]
CD25	Alexa647	Directly conjugated
CD79	PE	Directly conjugated
Ki-67	Brilliant Violet 421	Directly conjugated

Staining of mononuclear phagocytes for expression of CD14 and CD80/86		
CD14	Alexa647	Secondary antibody[c]
CD80/86	PE-Cy7	Secondary antibody[b]
CD163	PE	Directly conjugated
CD172a	Alexa488	Secondary antibody[d]
Ki-67	Brilliant Violet 421	Directly conjugated
Staining of mononuclear phagocytes for expression of CD11b and CD16		
CD11b	Alexa647	Secondary antibody[c]
CD16	FITC	Directly conjugated
CD163	PE	Directly conjugated
CD172a	PE-Cy7	Secondary antibody[e]
Ki-67	Brilliant Violet 421	Directly conjugated
Respiratory mononuclear phagocyte network staining		
MHC-II	PE-Cy7	Secondary antibody[b]
CD14	BrilliantViolet605	Secondary antibody[f]
CD45	Alexa647	Directly conjugated
CD163	PE	Directly conjugated
CD172a	Alexa488	Secondary antibody[d]
Ki-67	Brilliant Violet 421	Directly conjugated

[a] anti-IgG2b-Alexa488
[b] anti-IgG2a-PE-Cy7
[c] anti-IgG1-Alexa647
[d] anti-IgG2b-biotin; Streptavidin - Alexa488
[e] anti-IgG2b-biotin; Streptavidin - PE-Cy7
[f] anti-IgG1-biotin; Streptavidin - Brilliant Violet 605

3.3.2. Flow cytometry analysis

Multicolor stained leukocytes were analyzed with a BDFACSCanto™ II flow cytometer. The flow cytometer was equipped with three lasers (405, 488 and 633 nm). Per sample between $5x10^4$ to $2x10^5$ leukocytes were acquired by the FACSDiva™ software. Leukocytes were discriminated by their light scatter properties and then hierarchically gated towards the relevant subpopulations. Flow cytometry data was analyzed by FlowJo™ software. Gating strategies for lymphocyte subsets are provided in Figure 2 and for MPs subsets in Figure 3. The activation status of each subset was determined by the percentage of cells, which expressed the proliferation marker Ki-67 (Figure 2 H and 3 D).

Lymphocytes were initially identified based on their forward and side scatter (FSC/SSC) (Figure 2 A). Single cells were discriminated from cell aggregates by plotting FSC-area (FSC-A) against FSC-height (FSC-H). Dead cells were excluded by a viability dye. Cells with high autofluorescence were also excluded from analysis by investigation of their autofluorescence within 510±25 nm (designated

as AmCyan) following excitation with the violet laser (Figure 2 A).

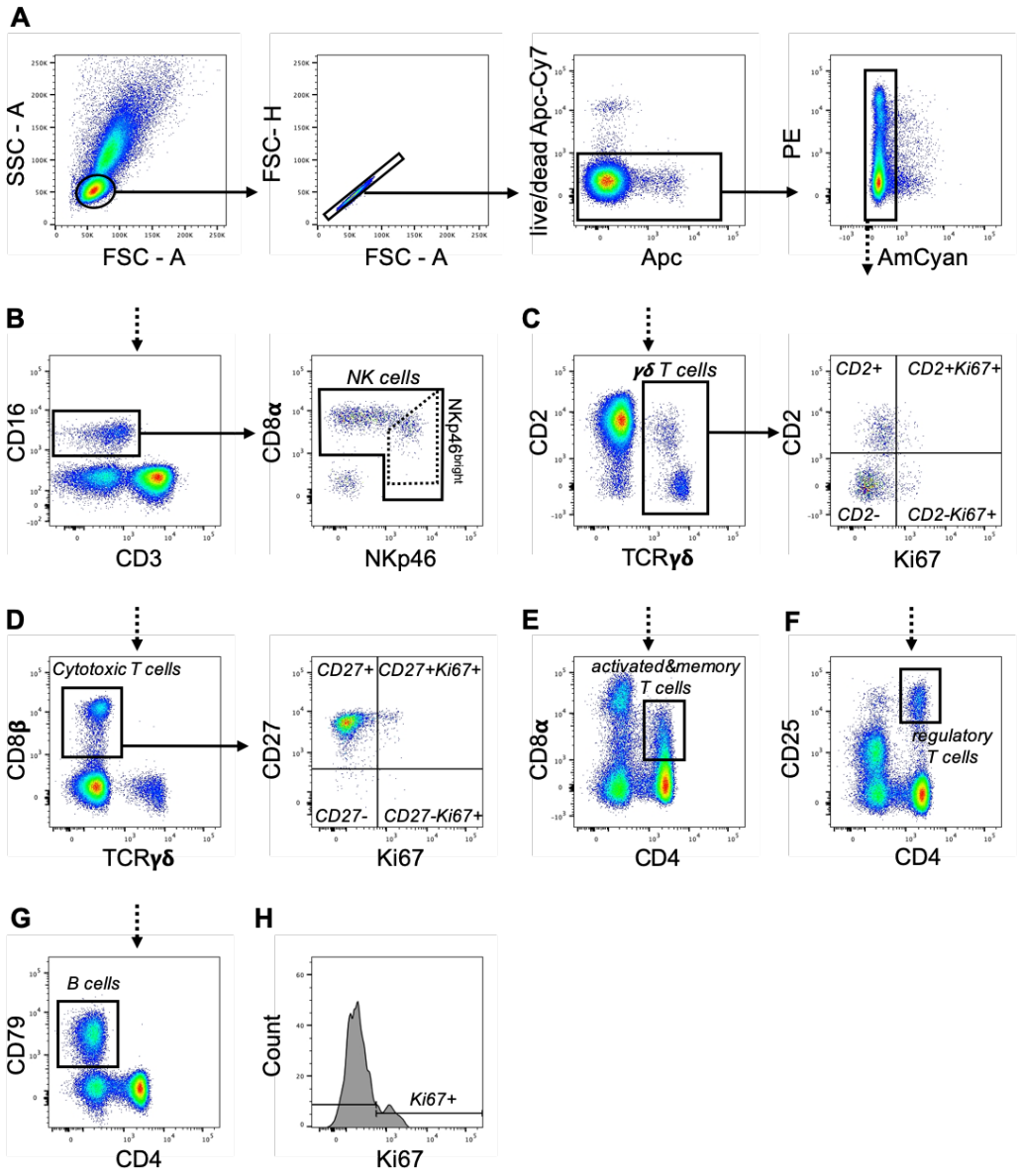


Figure 2. Gating strategies for major lymphocyte subsets. (A) Exclusion of cell aggregates, dead cells and cells with high autofluorescence from lymphocytes. (B) NK cells. (C) γδ T cells. (D) Cytotoxic T cells. (E) Activated and memory T cells (F) Regulatory T cells. (G) B cells. (H) Ki-67+ cells.

NK cells, identified by a $CD3^{-}CD16^{+}$ phenotype (i.e. non-T cells and non-B cells), were discriminated on the basis of the expression of CD8α and NKp46 or both (Figure 2 B). Furthermore, the frequency of the $NKp46^{bright}$ subset within NK cells was determined. The prominent lymphocyte subset of γδ T cells in pigs was identified by their expression of the γδ T cell receptor (TCR) and further phenotypically divided by their CD2 expression (Figure 2 C). In addition, the frequencies of major cell subsets of the adaptive immune system were determined.

Cytotoxic T cells were characterized by CD8β expression and lack of γδ TCR and further divided by their CD27 expression (Figure 2 D). Activated and memory T helper cells were identified as $CD4^{+}CD8\alpha^{+}$ cells (Figure 2 E). Regulatory T cells were characterized by a co-expression of CD4 and high expression of CD25 (Figure 2 F). B cells were determined as $CD4^{-}CD79\alpha^{+}$ lymphocytes (Figure 2 G).

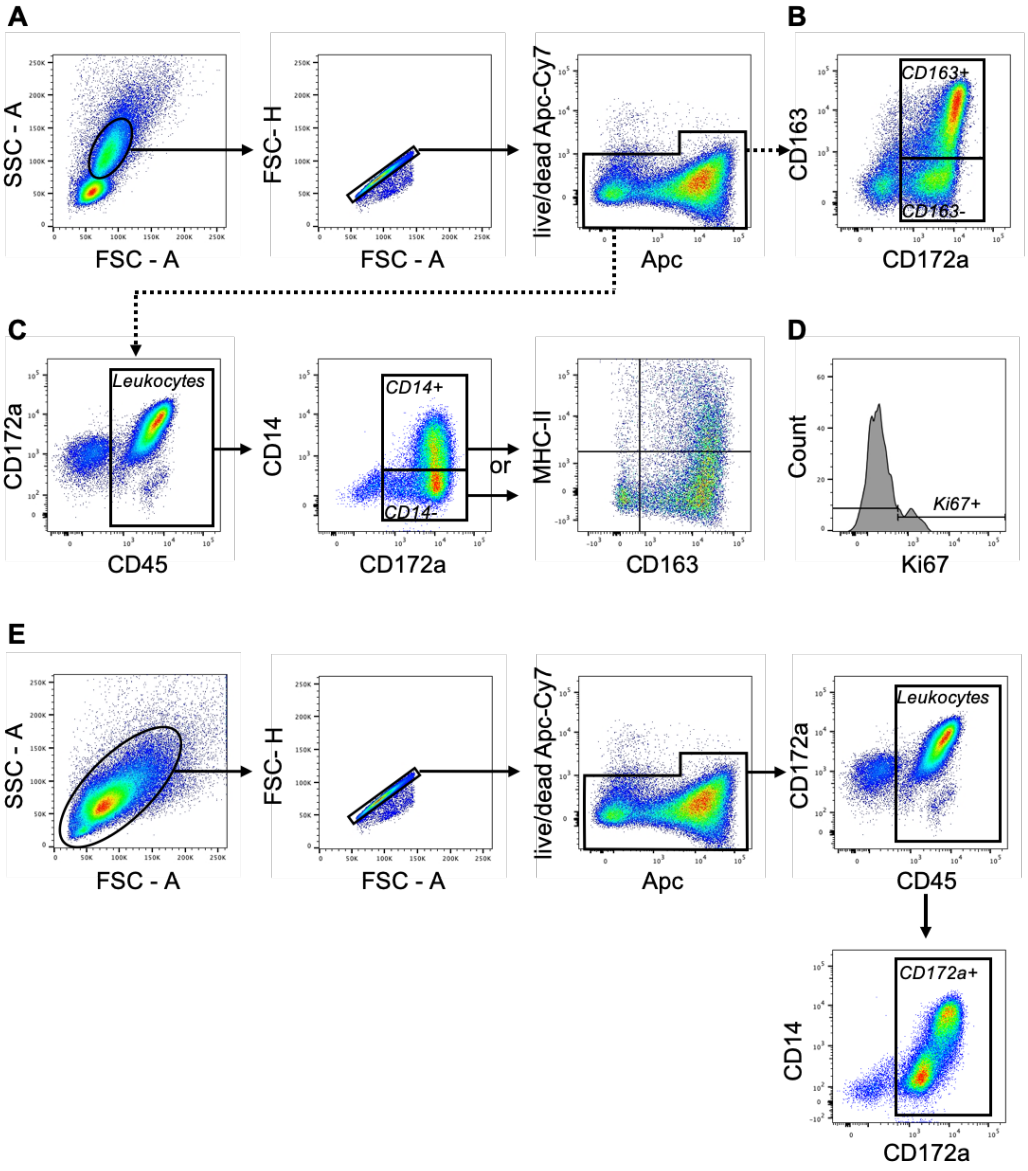


Figure 3. Gating strategies for mononuclear phagocytes analyses. (A) Exclusion of cell aggregates and dead cells from MPs. (B) "$CD163^{+}$" and "$CD163^{-}$" MPs. (C) Respiratory mononuclear phagocyte network. (E) Frequency of MPs within respiratory leukocytes.

For all flow cytometry analysis of mononuclear phagocytes, large mononuclear cells were identified based on their light scatter properties. Cell aggregates were removed by plotting FSC-A against FSC-H. Moreover, dead cells were also

excluded by a viability dye (Figure 3 A). MPs were identified by their expression of CD172a and were further discriminated into CD163 positive and negative cells (Figure 3 B). Furthermore, the expression of CD14, CD80/86, CD11b and CD16 was determined by their median fluorescence intensity (MFI) in $CD163^+$ MPs and $CD163^-$ MPs. In addition, the respiratory mononuclear phagocyte network of the lungs was examined in more detail. First, non-leukocytes and debris were excluded from MPs based on expression of the common leukocyte antigen CD45. Further hierarchical gating was applied after flow cytometry data was visualized with dimensionality data reduction by principle component analysis with the ClustVis web tool (Metsalu and Vilo, 2015) and by using t-distributed stochastic neighbor embedding algorithm with FlowJo™ software. In a next step, $CD172a^+$ MPs from the lungs were divided into a CD14 positive and a negative subset and then divided into four groups based by their expression of MHC II and CD163 (Figure 3 C). As well, the relative abundance of proliferating MPs within these subsets was determined (Figure 3 D). The relative abundance of total MPs within respiratory leukocytes was determined as well. Leukocytes were identified based on their light scatter properties. After excluding cell aggregates, dead cells and non-leukocytes, total MPs were identified by their CD172a expression (Figure 3 E).

3.4. Functional analysis of phagocytes

Functional properties of immune cells with phagocytic potential from peripheral blood were examined immediately after necropsy. For that purpose, two commercially available whole blood assays were used and measured by flow cytometry.

3.4.1. Phagocytosis assay

Leukocyte phagocytosis was examined by the Phagotest™ kit. Opsonized bacteria (*Escherichia coli*) labeled with fluorescein isothiocyanate (FITC) were added to 100 µl heparinized whole blood. Subsequently, one sample per animal was incubated at 37°C and another sample served as negative control by incubating at 0°C. In a next step, the FITC signal of not ingested bacteria was quenched. Thereafter leukocytes were washed and fixed. For discriminating eukaryotic cells from bacteria, genomic DNA was stained. Leukocytes were analyzed by flow cytometry (BD FACScan) and 1×10^4 leukocytes were acquired by live gating in the

CellQuest™ software for diploid DNA content in the red fluorescence (FL-Red) histogram. Flow cytometry data was analyzed with the FlowJo™ software. In a first gating step, eukaryotic cells were identified by high signal in the FL-Red histogram. Thereafter, either granulocytes or monocytes were identified by their scatter light properties. Cells that had performed phagocytosis were positive for green fluorescence (FL-Green) and the median fluorescence intensity (MFI FL-I) correlated with the number of ingested bacteria per individual leukocyte (Figure 4).

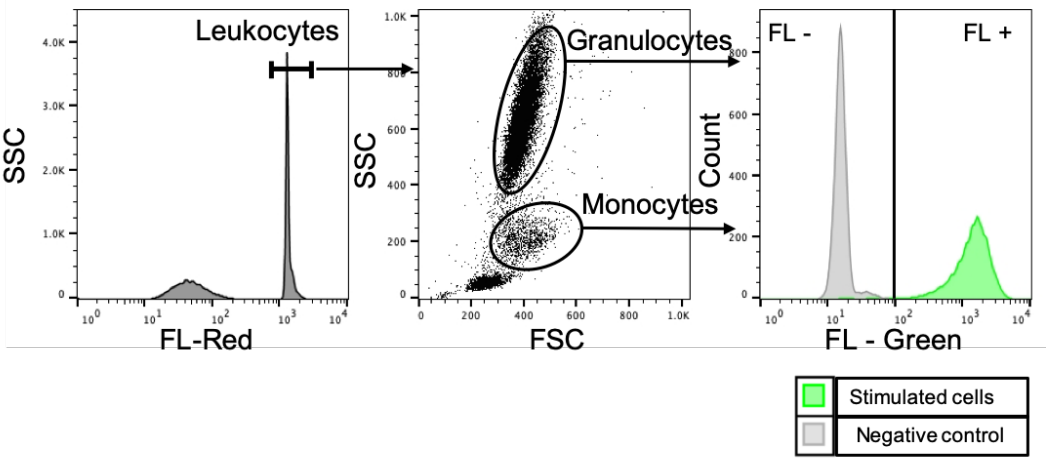


Figure 4. Gating strategies for phagocytosis and oxidative burst assays. Leukocytes were identified by their high DNA staining capacities. Subsequently granulocytes and monocytes were discriminated by their scatter light properties. Either granulocytes or monocytes were analyzed for their green fluorescence properties, allowing the identification of phagocytosed bacteria.

3.4.2. Oxidative Burst assay

Oxidative Burst of leukocytes was explored by the Phagoburst™ kit. Leukocytes of 100 µl heparinized whole blood were stimulated with opsonized bacteria (*E. coli*) and another sample without stimulus served as negative control. Generation of the reactive oxygen species was measured by the reduction of the fluorogenic substrate dihydrorhodamine to rhodamine. Additionally, cells were fixed and DNA was stained after 20 mins of stimulation. Consistent to the phagocytosis assay, leukocytes were analyzed by flow cytometry and 1×10^4 leukocytes were acquired. Analysis determined the percentage of oxidative burst positive leukocytes by green fluorescence and MFI FL-I correlated with the oxidation quantity per individual leukocyte (Figure 4).

3.5. Cytokine profile of *in-vitro* stimulated leukocytes

Freshly isolated MNLs from blood and lungs were stimulated *in-vitro* by a broad panel of PRRs-activating as well as non-PRR activating molecules. The induced response of leukocytes from *CFTR*$^{-/-}$ piglets in comparison to WT piglets were investigated by examination of the resulting cytokine release.

3.5.1. *In-vitro* stimulation

Isolated mononuclear leukocytes were cultivated in 96-well culture plates for 18 hours at 37°C in 5% CO_2 at $2x10^5$ cells per well in a volume of 200µl culture medium. For stimulation, ligands for TLR-2, TLR-4, TLR-5 and TLR-9 were used (Table 5). In addition, the freshly isolated MNLs were cultivated with medium as negative control and with PMA+Ionomycin (PMA/Iono) PRR independent cytokine production was induced. For each stimulus quadruplicates of wells were prepared and un-stimulated cells served as control. The following day, supernatant and cells were harvested and transferred to 1.5 ml tubes. Quadruplicates of each stimulus were pooled. Cells were removed by centrifugation (400 g, 5 min) and subsequently two supernatant aliquots were prepared per stimulus. Supernatants were stored frozen in 1.5 ml tubes at −80°C prior to cytokine quantification.

Table 5. Stimuli for in vitro cultivation of mononuclear leukocytes

Stimulus	PRR	Final concentration
PMA+Ionomycin	independent	5 ng/ml / 500 ng/ml
Pam3Cys-SKKK	TLR-2	0.75 µg/ml
LPS *P. aeruginosa*	TLR-4	1 µg/ml
LPS *E. coli*	TLR-4	1 µg/ml
Flagellin from *P. aeruginosa*	TLR-5	1 µg/ml
ODN 2216	TLR-9	5 µg/ml
ODN 2243	*ODN* 2216 control	5 µg/ml

3.5.2. Cytokine quantification

Supernatants were analyzed for IL-1β, IL-8, CCL-2, IFNα, IFNγ, IL-10, IL-12 and IL-4 by a multiplex fluorescent microsphere assay (FMIA). The FMIA was performed by Lisa Reiter at the University Clinic for Swine, University of Veterinary Medicine (UVV), Austria as described elsewhere (Ladinig et al., 2014).

In addition to FMIA, concentration of TNFα was determined by Porcine TNF-α DuoSet® enzyme-linked immunosorbent assay (ELISA). 100 µL of thawed supernatant was used for analysis. In case of PMA/Iono the supernatants were diluted 1:5 with PBS prior of use. All materials were provided by the DuoSet® Ancillary Reagent Kit 2. Readings of color development were made directly at 450 nm with an ELISA reader. The TNFα concentration of samples was calculated by Magellan software. Standard dilution served as reference. Both methods of cytokine measurement, FMIA and ELISA, were performed for doublets per sample.

3.6. Statistical analysis

Data sets were statistically analyzed with GraphPad Prism software. Each symbol in scatter dot plots represents results from one animal. For descriptive statistics median and interquartile range (±) are indicated for genotype specific groups. Furthermore, differences between CF and WT animals were assessed using the Wilcoxon matched sign ranked test for paired analysis unless otherwise stated. Alternatively, Mann-Whitney U test was performed for independent analysis. Accounting multiple comparisons, appropriate Bonferroni correction was applied. Significance threshold for corrected p-values was set at 0.05.

To prevent misinterpretation of statistical testing, sensitivity of each analysis was calculated by the G*Power 3.1.9.4 tool. The characteristic required effective size parameters dz for Wilcoxon sign ranked test or qd for Mann-Whitney U were calculated two sided with a power of 80% and the corresponding smallest group size of the analysis.

Due to limited amount of supernatant from stimulation assays, out-of-range cytokine concentrations were imputed, either by the lowest detected respective cytokine concentration, if values were below the minimum or by the highest detected respective cytokine concentration, if values were above the maximum. Thus, for cytokine profiles only descriptive statistics were deployed.

IV. Results

1. Pathological examination of the airways

Before euthanasia and during necropsy all animals were macroscopically examined. None of the 34 used piglets had visible signs of mucus plugging, acute infection or inflammation. As described earlier, malformed trachea with triangular shape and reduced diameter was observed as a typical sign of airway pathology in CF piglets. Another hallmark of CF, the meconium ileus, was noticed in the abdominal cavity of *CFTR*$^{-/-}$ piglets as well (Figure 5 A-C).

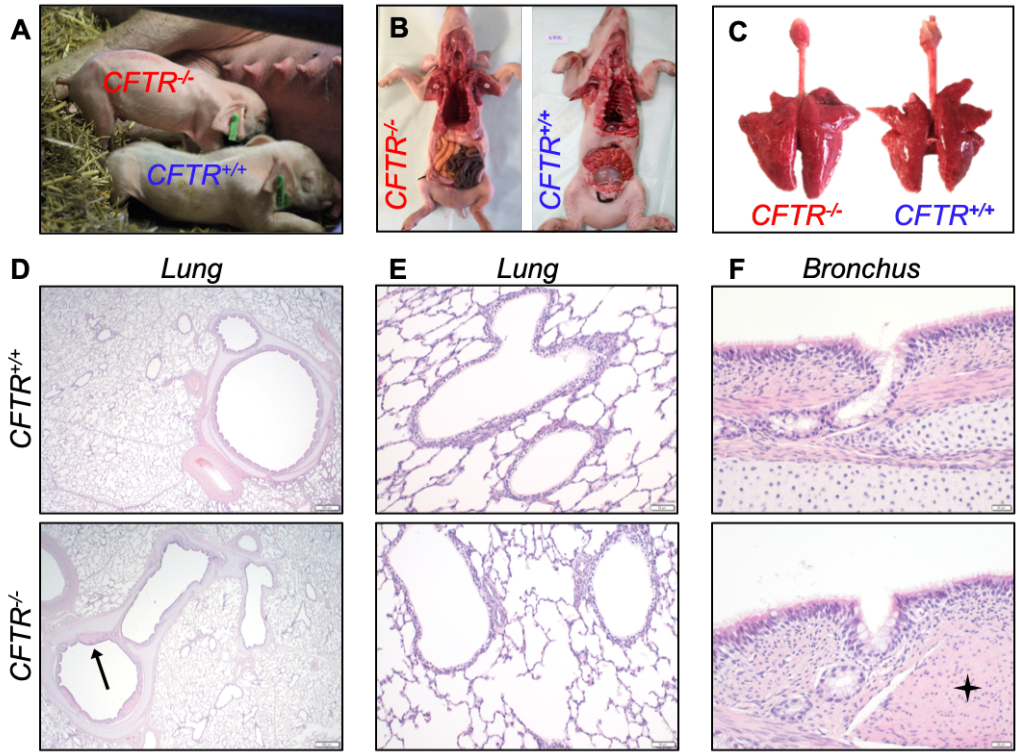


Figure 5. Necropsy and histology of airways. (A) Piglets were kept for 18 hours with the sow, while the genotype was determined by PCR. (B) Piglets after lung and spleen explantation with visible meconium ileus in CF piglets. (C) CF lungs, with malformed trachea, and WT lungs. (D)-(E) Lack of visible mucus plugging, inflammation or infection. Histology of the airways, hematoxylin and eosin stain. (D) Malformed smooth muscles in large bronchi of CF airways (black arrow). No signs of mucus plugging or inflammation. 500µm indicated. (E) Lung parenchyma of piglets appears normal. 50 µm indicated. (F) Bronchial epithelia and submucosal glands. No signs of mucus plugging and typical smooth muscle cell bundles seen in newborn CF piglets (star). 20 µm indicated. All data is representative. Data kindly provided by Lars Mundhenk from the Institute of Veterinary Pathology, FU Berlin.

For systematic comparison with existing studies, histopathological exploration of the airways was performed by Lars Mundhenk (Institute of Veterinary Pathology, FU Berlin). Randomly taken samples were stained with hematoxylin and eosin (HE). Congruent with the gross pathological analysis and the literature no signs of acute infection, inflammation or mucus plugging were observed (Figure 5 D and E). Typical smooth muscle bundles of the bronchioles were seen in the CF airways (Figure 5 F). Furthermore, tracheae from CF animals were regularly examined by Anna Ermund (Mucus Biology group, University of Gothenburg, Sweden) for velocity and showed typical reduced mucociliary transport with altered mucus properties in the large airways. Thus, *CFTR*$^{-/-}$ piglets showed major hallmarks of CF and the phenotype was in accordance with the literature.

2. Isolated mononuclear leukocytes

In different laboratories, several protocols exist for isolating immune cells from blood, spleen and lung tissue from juvenile and adult pigs. The focus of this thesis was on mononuclear leukocytes (MNLs). Isolation by density gradient centrifugation was used to derive a highly pure immune cell population from blood, spleen and lungs. For optimizing the isolation procedure for newborn animals, the focus was on the density gradient centrifugation process, because it was the most critical part of isolation. For that purpose, MNLs from neonatal porcine blood were isolated with different parameters including densities of 1.074/1.075/1.076/1.077 g/ml for the separation medium and centrifugation was performed at three different accelerations, 700/800/900 g. Different constellations were performed two times. Due to an improved separation of erythrocytes from MNLs and in accordance to Talker et al. (2013), a density of 1.075 g/ml and acceleration of 900 g was adopted for further isolation. After modification, protocols proved robust in further isolation experiments.

From 17 CF piglets and 17 age-matched littermate controls 25±4 ml blood, the spleen and the lungs were collected. The numbers of isolated MNLs were counted from at least 10 littermate pairs from blood, spleen and lungs. No genotype-based differences have been observed in isolating immune cells from blood and spleen. However, absolute number of isolated mononuclear leukocytes from total lung tissue was significantly increased in CF piglets ($7.7x10^7$ cells) compared to WT control animals ($3.3x10^7$ cells) (Figure 6 A).

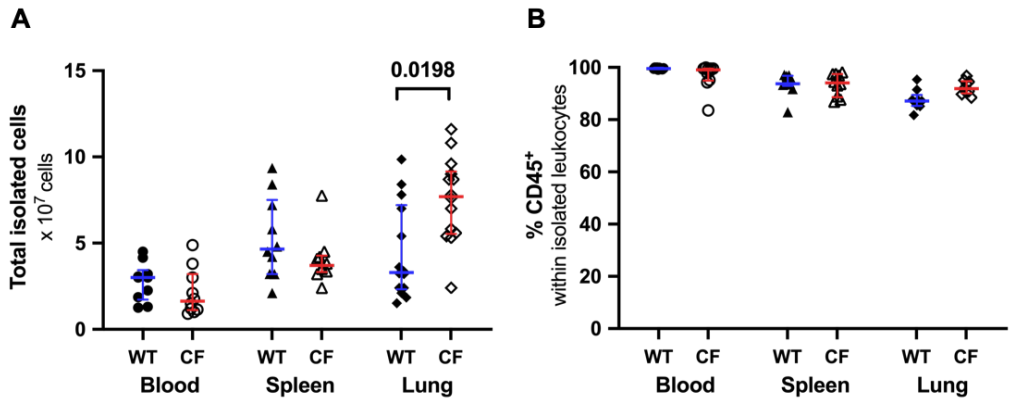


Figure 6. Isolated immune cells. (A) Total amount of isolated MNLs from blood (n=10), spleen (n=10) and lungs (n=14). (B) Frequency of CD45⁺ leukocytes within isolated cells (n=9).

Furthermore, the purity of isolated cells had been proven by determining the abundance of the common leukocyte antigen CD45 positive cells within the leukocyte specific light scatter gate. After staining samples from 9 different littermate pairs, isolated cells from blood, spleen and lungs had similar rates about 90% $CD45^+$ cells (Figure 6 B). Thus, the isolation procedure has proven to be robust.

In spite of a robust isolation process, the broad panel of performed analyses required high quantities of cells to generate accurate results. Due to the restricted amounts of lymphocytes and mononuclear phagocytes in newborn piglets, not all assays could be performed with samples of each individual. In order to minimize environmental influences on the data, all analysis, except the analysis of the respiratory mononuclear phagocyte network, were based exclusively on the comparison of pairwise derived data from CF and WT littermates.

3. Frequency and activation status of major leukocyte subsets

Phenotypical characterization of subpopulations followed established marker protocols for reflecting major subpopulations within MNLs. First, relative abundancy of subpopulations within live lymphocytes or live MPs respectively were compared (Figure 7). Second, the frequencies of proliferating Ki-67^+ cells within the respective leukocyte subpopulation were determined to reflect the activation status of the subset (Figure 8). Additionally, in some leukocyte subsets further activation states and functional properties were phenotypically discriminated (Figure 9 and 10).

Remarkably high variation between individuals as well as between litters was observed for both, the frequency and the proliferation of major immune cell subpopulations from blood, spleen and lungs (Figure 7 and 8). In spite of the high variability and multiple comparisons in statistical testing, γδ T cells were significantly decreased in the circulation and in the lungs of CF piglets (blood: p=0.024; lung: p=0.0024). The median of relative γδ T cells abundance in CF piglets was only the half compared to WT piglets in blood (CF: 5.4% vs WT: 10.8%) and in the lungs (CF: 3.5% vs WT: 8.3%). The proliferation status of γδ T cells, however, appeared similar in CF and WT animals (Figure 8). Furthermore, we investigated the frequency of $CD2^+$ cells within γδ T cells, because $CD2^+$ cells are completely functional, while no functional role has been related to $CD2^-$ γδ T cells so far. Surprisingly, the frequency of $CD2^+$ γδ T cells from CF piglets was not significantly different compared to γδ T cells derived from WT (Figure 9 A). There was a tendency towards a decreased frequency of γδ T cells in the spleen, but this difference did not reach statistical significance.

In CF piglets, the proportion of NK cells in blood was as a tendency increased (CF: 4.5% vs WT: 1.6%). However, this apparent rise might be only a mathematical consequence of the decrease of γδ T cells in CF lymphocytes. In contrast to the frequency, the proliferative activity of NK cells was comparable in CF and WT piglets (Figure 8). Within NK cells, $NKp46^{bright}$ cells have the highest capacity of cytotoxicity and cytokine production, therefore also their relative appearance was investigated (Figure 9 B). The proportion of $NKp46^{bright}$ cells within NK cells was dramatically reduced in the blood of CF (CF: 18.9 % vs WT: 37.5%; p=0.0204), while no significant difference in $NKp46^{bright}$ cells between CF and WT piglets was found in the lungs. Similar to innate NK cells, the adaptive immunity representative of killer cells, the cytotoxic T cell appeared not different in frequency or proliferation, although functional terminally differentiated $CD27^-$ cytotoxic T cells showed the tendency of higher appearance (CF: 8% vs WT: 4.7%) in the cytotoxic T cell subpopulation from CF airways (Figure 9 C). One lymphocyte subpopulation, the regulatory T cells showed statistically significant differences between CF and WT piglets in their abundance in the spleen (p=0.048), albeit at a low level (Figure 7). Additionally, similar proliferation status of CF and WT regulatory T cells was observed in the spleen (Figure 8).

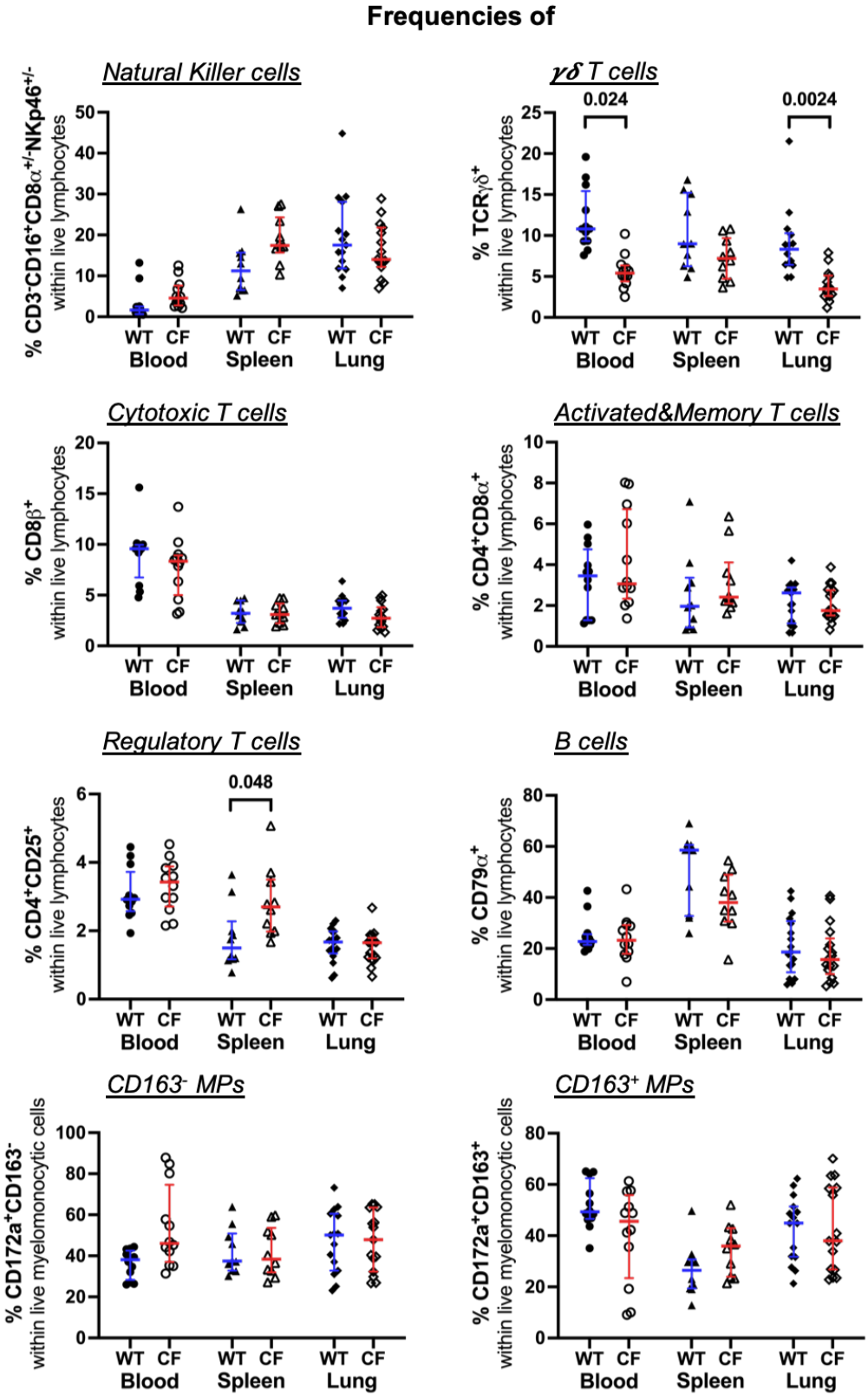


Figure 7. Frequencies of major immune cell subpopulations. (Blood: n=12; Spleen: n=10; Lungs: n=14).

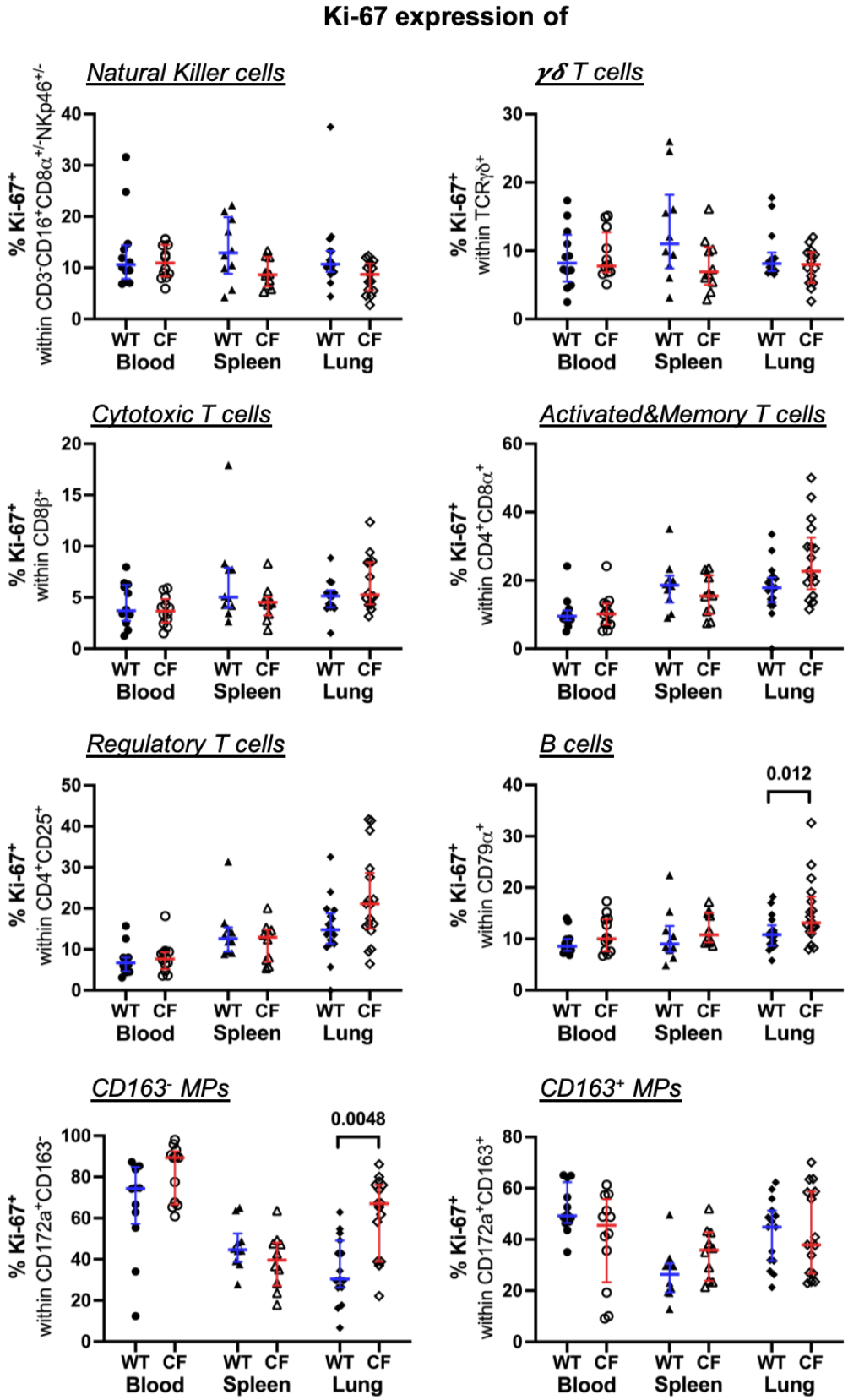


Figure 8. Ki-67 expression of major immune cell subpopulations. (Blood: n=12; Spleen: n=10; Lungs: n=14).

Regarding the proliferation status of lymphocyte subsets in general, representatives of adaptive immunity, such as regulatory, activated and memory T cells and B cells tended to be more proliferative in CF airways (Figure 8), but the difference appeared moderate when comparing the medians of CF piglets with WT piglets (regulatory T cells: 1.5 fold; activated & memory T cells: 1.3 fold; B cells: 1.2 fold) and only in B cells the proliferative activity of lymphocytes was significantly different (p=0.012).

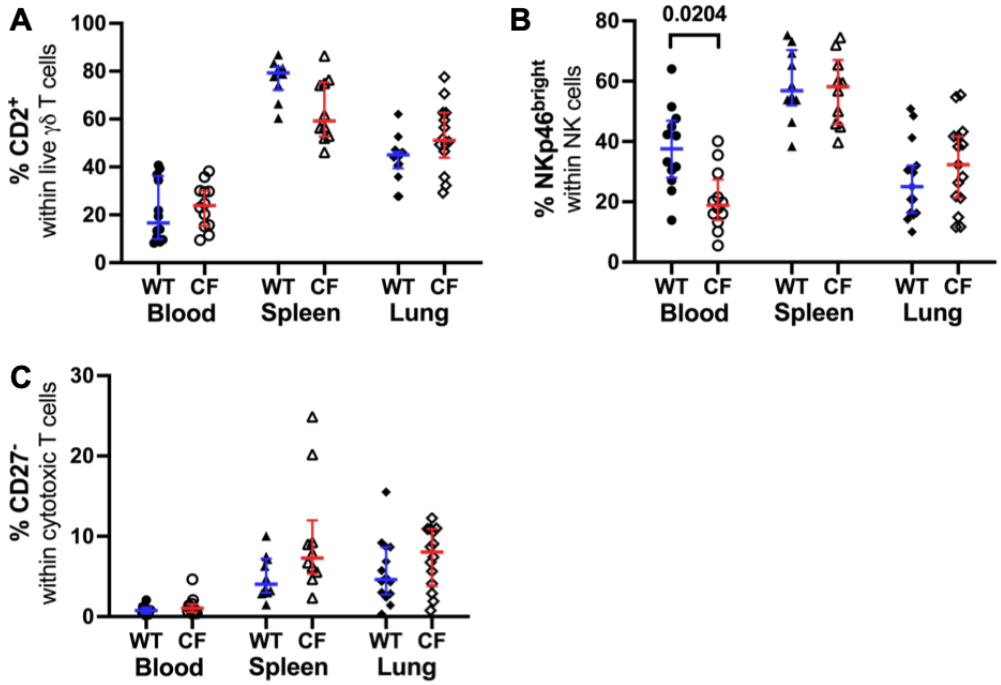


Figure 9. Detailed characterization of γδ T cells, NK cells and cytotoxic T cells. (A) Frequency of CD2$^+$ within γδ T cells. (B) Frequency of NKp46^{bright} within NK cells. (C) Frequency of terminally differentiated CD27$^-$ within cytotoxic T cells. (Blood: n=12; Spleen: n=10; Lungs: n=14).

Besides lymphocytes, the phenotype of mononuclear phagocytes was characterized as well. For that purpose, the relative abundance of two functional MP subpopulations discriminated by CD163 were determined. Generally, it is suggested that in systemic or local inflammation a shift towards CD163$^+$ MPs is recognized. In the current analysis, CD163$^+$ cells from blood, spleen and lungs appeared without significant statistical differences within myeloid CD172a$^+$ cells (Figure 7). However, the subset of CD163$^-$ MPs from CF airways turned out as two-fold more proliferative (p=0.0048). 67% of CD172$^+$CD163$^-$ cells from CF lungs were positive for the proliferation marker Ki-67, while from WT lungs only 30.4% of CD163$^-$ MPs were positive for Ki-67 (Figure 8).

Furthermore, we investigated the expression levels of two functional surface receptors on CD163$^+$ MPs. Higher median fluorescence intensity (MFI) of the LPS sensing CD14 was suggested on CD163$^+$ MPs from CF lungs. The CD14 MFI of CD172a$^+$CD163$^+$ cells from CF piglets was with 8980 significantly (p=0.009) increased compared to the CD14 MFI of 5962 on WT CD163$^+$ MPs (Figure 10 A). It is remarkable that the MFI of CD14 on CD163$^-$ MPs appeared highly reduced (CF: 3533 vs WT: 8245) within the circulation (Figure 10 B) and that circulating CF CD172a$^+$CD163$^+$cells showed higher MFI of the co-stimulatory molecule CD80/86 than CD163$^+$ cells derived from WT blood (Figure 10). Apart from the mentioned observations, statistical testing resulted in no further significant differences between CF and WT immune cell subpopulations. It has also to be mentioned, that further discrimination by the markers did not correlate to general differences in the MPs subpopulations.

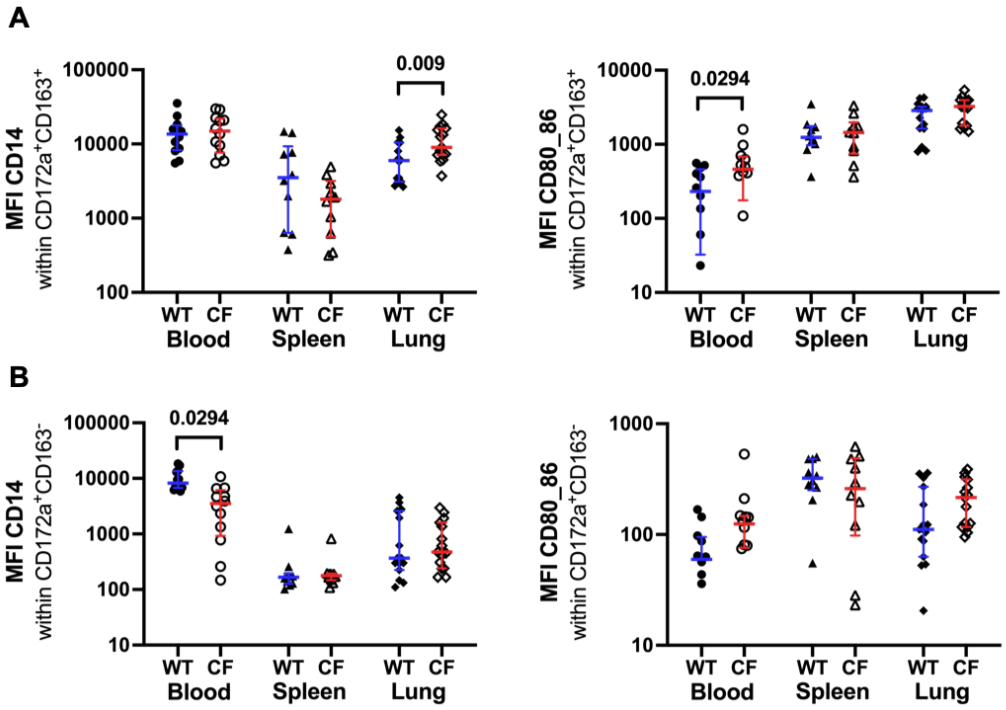


Figure 10. Expression of CD14 and CD80/86 receptors within (A) CD163$^+$ mononuclear phagocytes and within (B) CD163$^-$ mononuclear phagocytes. MFI indicated the expression level of respective receptor. (Blood: n=12; Spleen: n=10; Lungs: n=14).

4. Phagocytic and oxidative potential of phagocytes

As uptake and intracellular killing of bacteria by ROS is an essential pathway of host defense against bacterial pathogens in the airways, another focus of this thesis was on immune cells with phagocytic potential. Whole-blood assays were used for evaluating the functional properties of circulating phagocytes. Therefore, both subsets of professional phagocytes were represented either by granulocytes or by monocytes. Phagocytosis assays were performed from 12 pairs of CF and WT littermates on 12 different days. Oxidative burst assays were performed in the same way with 10 littermate pairs.

4.1. Granulocytes

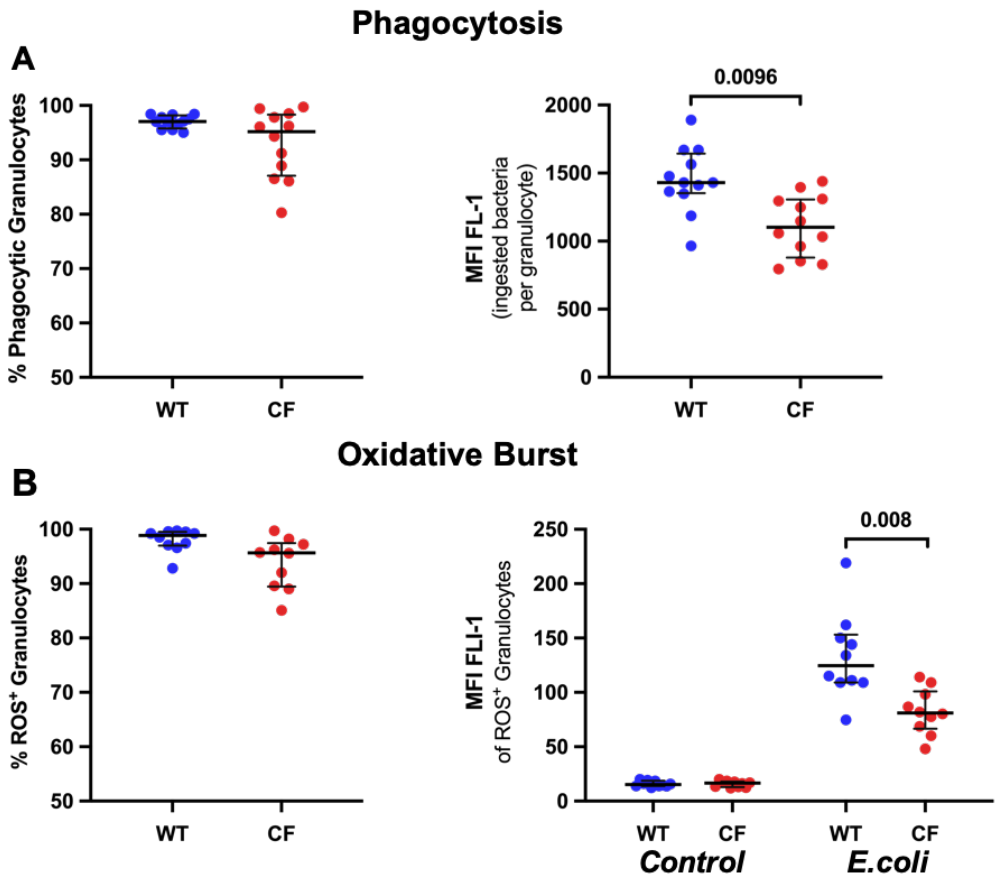


Figure 11. Phagocytic and oxidative potential of granulocytes from blood. (A) In the phagocytosis assay the phagocytic rate of blood granulocytes (left panel) and their phagocytic capacity per granulocyte (right panel) were examined. (n=12). (B) Furthermore, in the oxidative burst assay the rate of ROS producing granulocytes (left panel) and their oxidative burst capacity per granulocyte were determined (right panel). (n=10).

The phagocytic rate of granulocytes was calculated as proportion of granulocytes exerting uptake of opsonized FITC-labeled *E. coli*. Granulocytes from CF piglets had a similar rate of phagocytosing *E. coli* like granulocytes from WT animals (CF: 95.2 % vs WT: 97.1 %). The number of ingested bacteria per granulocyte was indicated by the green median fluorescence intensity (MFI FL-I). CF granulocytes had a phagocytic capacity per single cell that was declined to 80% of the rate in WT granulocytes (CF: 1103 vs WT: 1430; p=0.0096) (Figure 11 A).

In addition, the production of ROS, indicated by the reduction of the fluorescent substrate dihydro-rhodamine to rhodamine, upon stimulation with opsonized unlabeled *E. coli* was examined in blood granulocytes as a parameter for the oxidative burst dependent potential of intracellular killing. Consistent with the phagocytic activity of granulocytes, the rate of ROS producing granulocytes was similar in blood from CF (95.7%) and WT (98.9 %) piglets. Furthermore, ROS production per granulocyte was examined by the MFI FL-I. Whereby the ROS production per single CF granulocyte in presence of bacteria was declined to 65% of a WT granulocyte (CF: 81.1 vs WT: 124.5; p=0.008). MFI FL-I of unstimulated granulocytes appeared similar between CF and WT animals (Figure 11 B). Thus, granulocytes of CF piglets from the circulation had a reduced phagocytic and oxidative burst capacity against bacterial stimulation.

4.2. Monocytes

Like in granulocytes, the phagocytic rate of blood monocytes from CF piglets was similar to monocytes from blood of WT piglets (CF: 92.3% vs WT: 89.8%). Likewise, the number of ingested bacteria per monocyte from CF piglets was reduced with the same ratio as observed in granulocytes (CF: 786.5 vs WT: 998.5) (Figure 12 A). Strikingly contrary to the phagocytic rate, the percentage of ROS producing CF monocytes was declined to half of the number compared to WT monocytes (CF: 30.6 % vs WT: 64%; p=0.008). In addition, monocytes from CF piglets produced even less ROS per monocyte (CF: 64.5 vs WT: 77.1; p=0.0156). Green MFI in unstimulated monocytes of both genotypes remained similar (Figure 12 B). Congruently with granulocytes circulating monocytes had a reduced phagocytic capacity as well. However, monocytes from CF blood showed lower proportion of ROS producing cells and had lower intracellular ROS levels after bacterial stimulation.

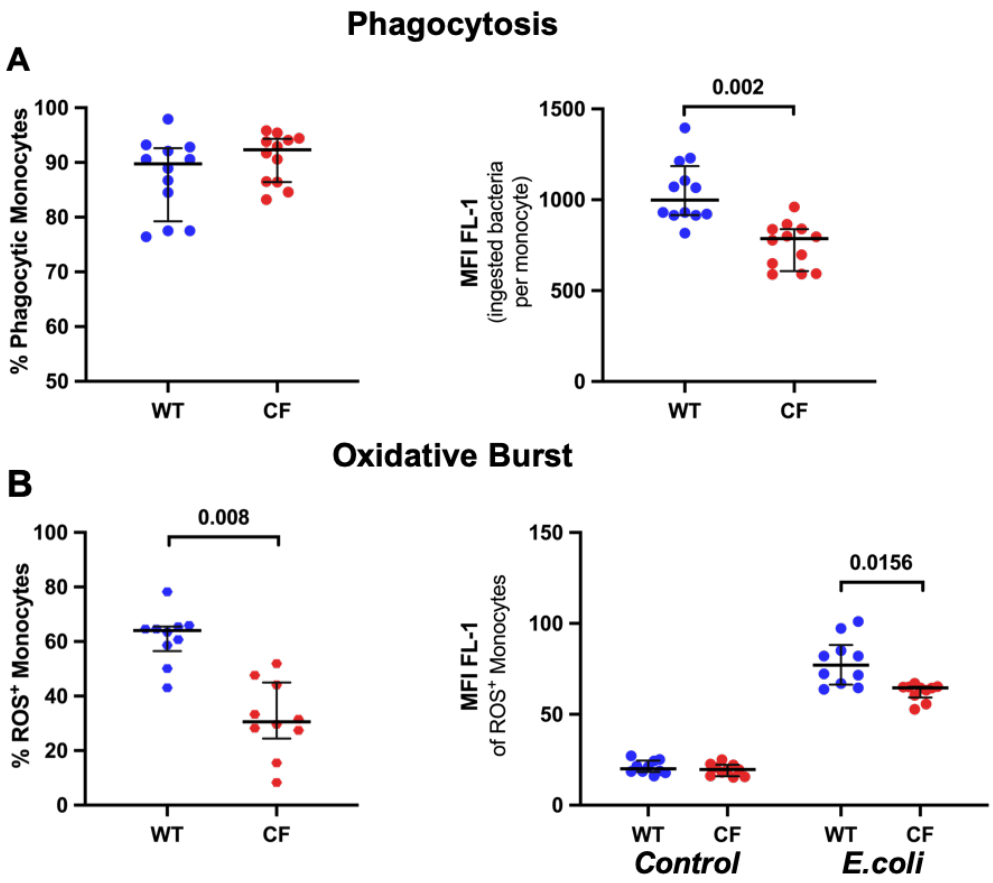


Figure 12. Phagocytic and oxidative potential of monocytes from blood. (A) In the phagocytosis assay the phagocytic rate of blood monocytes (left panel) and their phagocytic capacity per monocyte (right panel) were examined as well. (n=12). (B) In the oxidative burst assay the rate of ROS producing monocytes (left panel) and their oxidative burst capacity per monocytes were determined (right panel). (n=10).

4.3. Expression of phagocytic receptors on mononuclear phagocytes

In order to explore potential sources for the attenuated phagocytic potential of monocytes from CF piglets, the expression of two phagocytosis initiating receptors within MPs was investigated.

An obviously decreased expression of the type III Fc-γ receptors (FcR) on $CD163^+$ MPs from blood, spleen and lungs of CF animals was determined. The mean expression level, indicated by MFI, showed that $CD172a^+CD163^+$ cells from CF piglets express only about the half of the FcR compared to MPs from WT piglets (Figure 13 A). Furthermore, subset of $CD163^-$ MPs had a similar decline in CD16 expression in the blood, but not in the spleen or in the lungs (Figure 13 B). In accordance with the literature, also all neonatal porcine MPs expressed CD16 in the analysis (Figure 13 C).

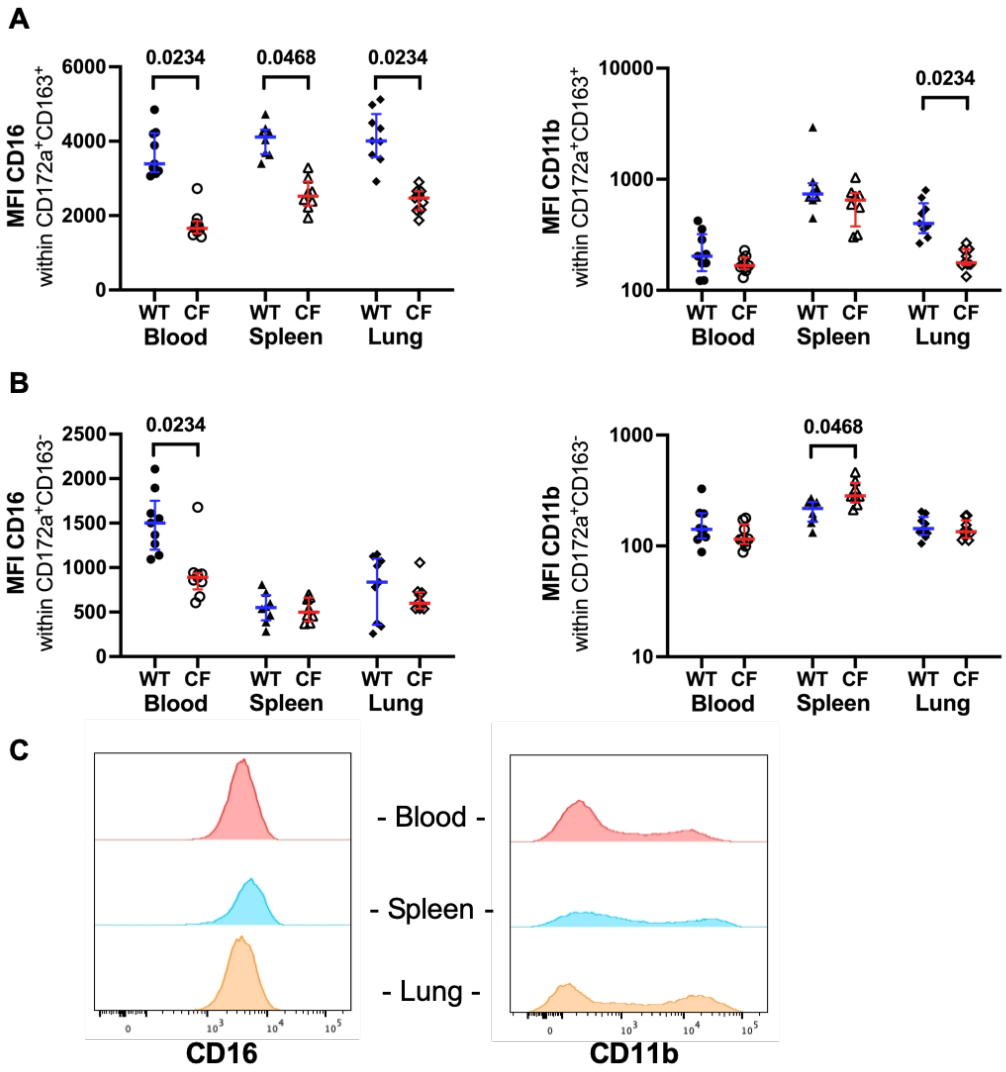


Figure 13. Expression of phagocytic receptors in mononuclear phagocytes. The expression level of CD16 and CD11b on (A) CD163$^+$ MPs and on (B) CD163$^-$ MPs was determined by the MFI in flow cytometry. (C) Representative histograms of CD16 and CD11b expression within CD172a$^+$ cells (Blood: n=9; Spleen: n=8; Lungs: n=9).

Regarding the complement receptor CD11b on the subset of CD163$^+$ MPs from CF lungs, there was a significant reduction of the expression, but only in the lungs. The MFI within the CD172a$^+$CD163$^+$ subset from CF airways was 177±58 compared to WT CD11b MFI of 400±283 (Figure 13 A). As another significant difference appeared in the higher MFI within CD163$^-$ MPs from the CF spleen (Figure 13 B), but the difference appeared small and at a low significant degree (p=0.0468). Of note, there was the limitation that CD11b was only partially expressed on MPs from blood, spleen and lungs (Figure 13 C).

5. Cytokine profile of *in-vitro* stimulated leukocytes

An effective immune response requires cross-talk between different leukocyte subpopulations, which can be directly mediated through receptors such as MHC, TCR or the co-stimulatory CD80/86 receptors. Another mechanism of the communication between cells is mediated by messenger substances such as cytokines. Therefore, the potential for cytokine production of mixed leukocyte populations were investigated after *in-vitro* stimulation. Mixed leukocyte stimulation assays were performed with freshly isolated MNLs from blood and lungs of 6 littermate pairs of CF and WT piglets and produced cytokines were analyzed in the supernatant.

Regarding the heat map of relative cytokine release, the results of this pilot analysis delivered an ambiguous picture at the first sight. For some cytokines, which are mainly produced by dendritic cells and γδ T cells such as IFNα, IFNγ and IL-12 decreased production appeared in blood and lung MNLs from CF piglets. Contrary, release of cytokines such as IL-10, IL-8, CCL-2, TNFα and IL-1β, which are primarily produced by MΦs, showed a general trend towards increased production in the lungs (Figure 14).

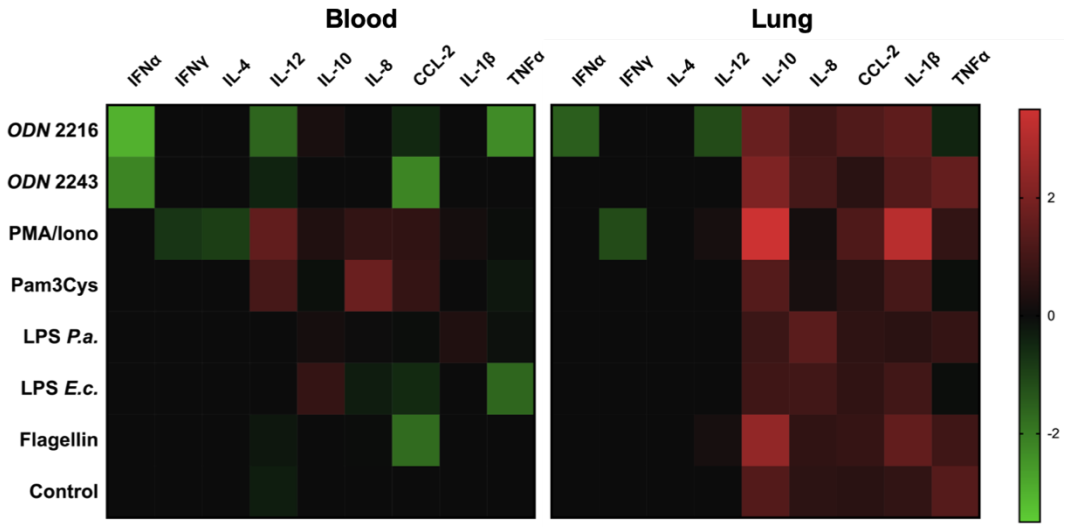


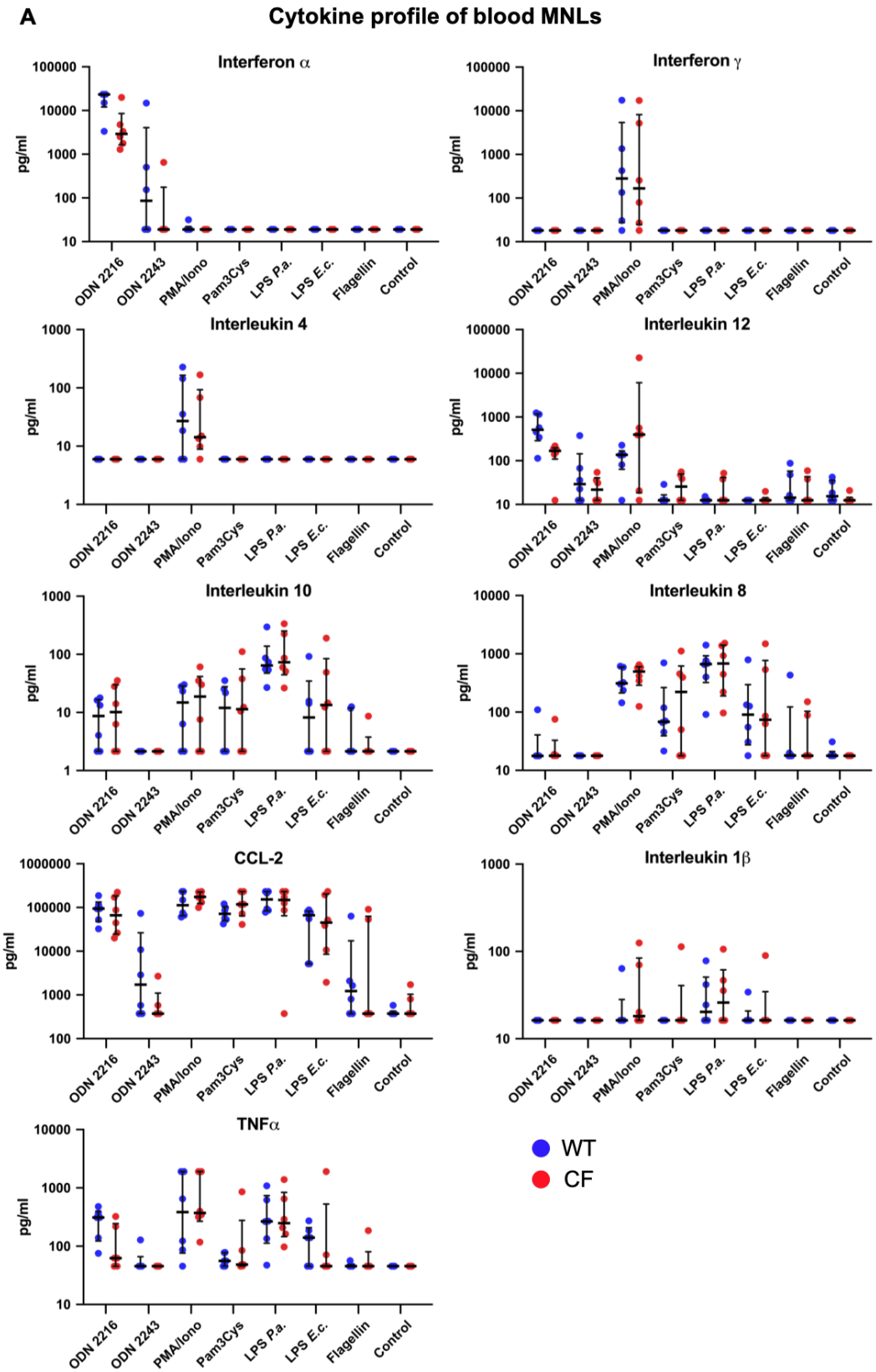
Figure 14. Relative cytokine release of CF mononuclear leukocytes. Relation was determined by dividing the median of the cytokine concentrations from CF piglets with the median of WT cytokine concentrations. In a next step, fold change was transformed with log_2 in order to visualize the relative cytokine production in a heatmap (n=6).

In a detailed view, for some of the cytokine profiles a consistent trend was observed, whereas for the majority pattern, the high variation of cytokine, as indicated by the large interquartile range, prevented a senseful interpretation. Globally, cytokine profiles of neonatal MNLs from blood and lungs showed similar ability of both genotypes to produce cytokine against various stimuli (Figure 15). Although some differences between CF and WT littermates in supernatant cytokine levels were suggested by the cytokine profiles. In view of stimulating blood MNLs with the CPG motif rich oligonucleotide *ODN* 2216, MNLs from CF piglets produced in the median approximately 10 times less IFNα than WT MNLs (CF: 2915±2436 pg/ml vs WT: 23379±6337 pg/ml). MNLs from lungs showed a less prominent difference in IFNα production (Figure 15 B). Supernatants of blood MNLs stimulated with *ODN* 2216 showed decreased concentrations of IL-12 (CF: 168±44 pg/ml vs WT: 531±637 pg/ml) and TNFα (CF: 62±129 vs WT: 310±173 pg/ml). Similar to the findings for IFNα, the difference of IL-12 and TNFα levels between the genotypes appeared less prominent in supernatants from lung leukocytes (Figure 15 B). Interestingly, in most supernatants of MNLs from CF airways no IFNγ production at all was detected after PMA/Iono stimulation (Figure 15 B), while in the circulation MNLs of both CF and WT piglets showed similar rate and capacity of IFNγ production (Figure 15 A).

On the other hand, there were several cytokines which showed a tendency of increased production in mixed leukocytes assays from lungs of CF piglets. In supernatants from MNLs of CF lungs the median concentration of the leukocyte attractants CCL-2 and IL-8 were increased with all stimuli and also in the control. For example, IL-8 seemed to be increased after stimulation with LPS from *E. coli* (CF: 2331±2497 pg/ml vs WT: 1210±662 pg/ml) or LPS from *P. aeruginosa* (CF: 4477±1514 pg/ml vs WT: 1691±991 pg/ml) (Figure 12 B). Kind of surprising was the consistent and genotype-independent high basal production of CCL-2, a major monocytes/MΦs attractant, in supernatants from neonatal lung leukocytes (Figure 15 B).

IL-10 production capacity of CF lung leukocytes appeared increased after PMA/Iono stimulation (CF: 93±84 pg/ml vs WT 2±18 pg/ml). Overall, supernatants of lung leukocytes from CF piglets tended to higher levels of IL-10 independent of the stimulus (Figure 15 B). Moreover, already basal production of IL-10 (control)

was observed in some of the CF lung MNLs supernatants, whereas IL-10 was not in supernatants of unstimulated blood leukocytes from CF piglets or from WT MNLs (Figure 15 A).



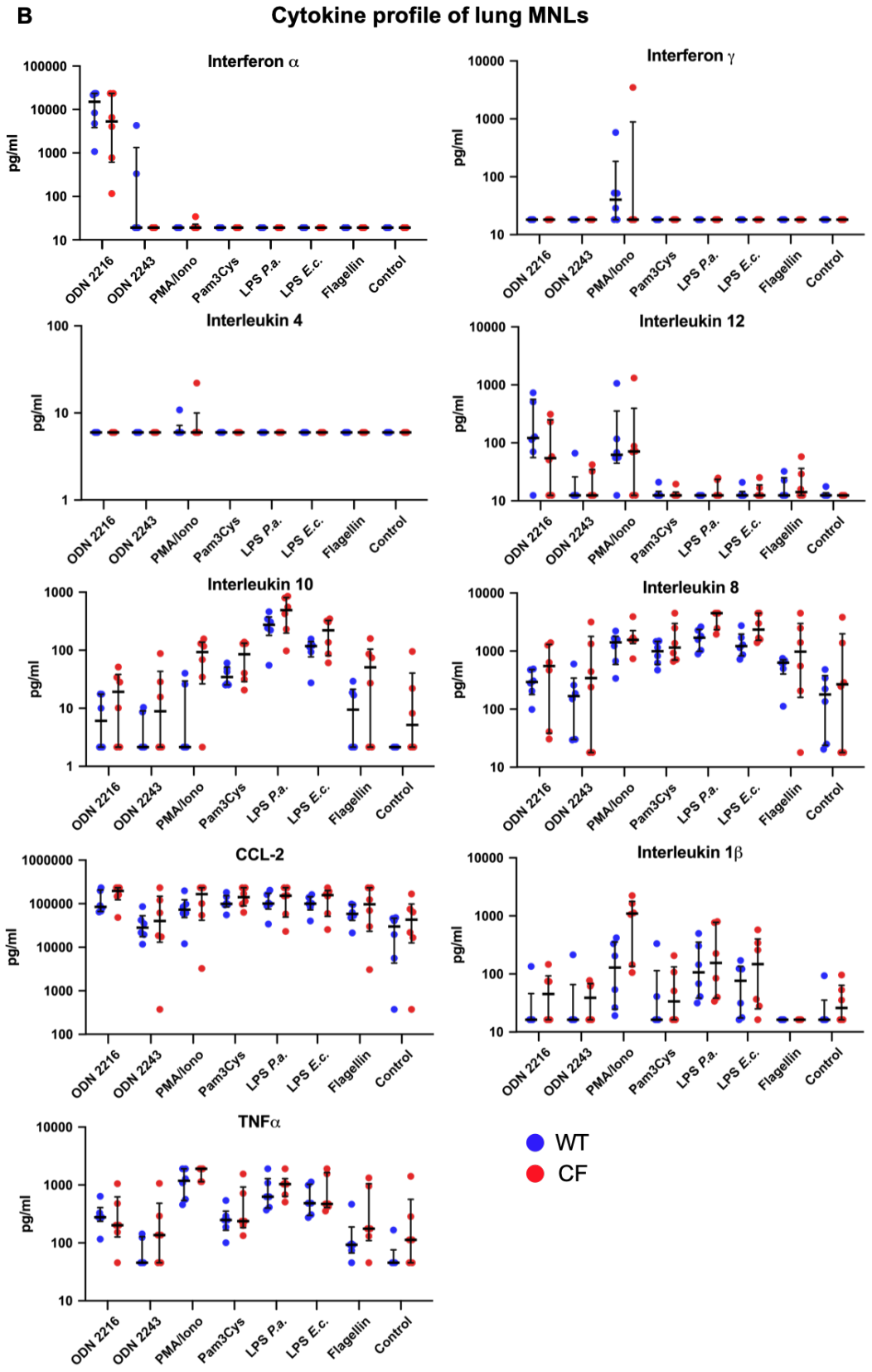


Figure 15. Cytokine profile of mononuclear leukocytes. Freshly isolated MNLs from (A) blood and (B) lungs of six litters were stimulated in-vitro and supernatants were analyzed for cytokine production (n=6).

Surprisingly and according to the anti-inflammatory IL-10, the pro-inflammatory cytokines TNFα and IL-1β had the tendency of higher concentrations in supernatants of mixed leukocytes assays from CF airways (Figure 15 B). Especially, IL-1β production capacity appeared to be increased after PMA/Iono stimulation (CF: 1092±1128 vs WT: 129±266). As well interesting is the tendency of CF lung MNLs to produce more likely pro-inflammatory cytokines like IL-1β and TNFα in the control without stimulation (Figure 15 B).

6. Respiratory mononuclear phagocyte network

The findings of functional alterations of phagocytic activity, altered receptor expression and altered relative cytokine release of MPs stimulated a further examination of the respiratory network of myeloid cells. Moreover, while phenotyping major leukocyte subpopulations increased frequencies of $FSC^{high}SSC^{high}$ cells indicated also a relative increase in myeloid cell numbers within lung leukocytes (Figure 3 A), but there was no appropriate marker constellation in the staining panel to quantify this observation appropriately. Therefore, a new staining panel was designed on the basis of the common leukocyte antigen CD45 marker combined with several myeloid markers, which had been previously described to separate the respiratory DC/MΦ-network into five different subpopulations including conventional DCs, monocyte-derived DCs, monocyte-derived MΦs and resident MΦs (Maisonnasse et al., 2016b). For flow cytometry analysis, isolated lung MNLs (n=6 per genotype) from 5 litters were used and hierarchical gating strategy provided the rate of MPs ($CD172a^+$) within total leukocytes ($CD45^+$) (Figure 16 A). Indeed, the impression from the scatter-based gating was confirmed: the relative abundance of MPs among leukocytes from CF piglets was significantly increased compared to WT littermates (Figure 16 A).

Two methods were used for dimensionality reduction of flow cytometry data to focus further gating. According principal component analysis (PCA) with the MFI of the panel specific myeloid markers including CD172a, CD14, CD163, MHC II and the proliferation marker Ki-67 (Table 5), a distinct plasticity of the mononuclear phagocyte system was suggested in the airways from CF piglets (Figure 16 B). In addition, dimensionality reduction by t-SNE algorithm of FlowJo™ revealed differences in the markers CD14 and Ki-67 (Figure 16 B) in accordance with the previous screening approach (Figure 7 and 8).

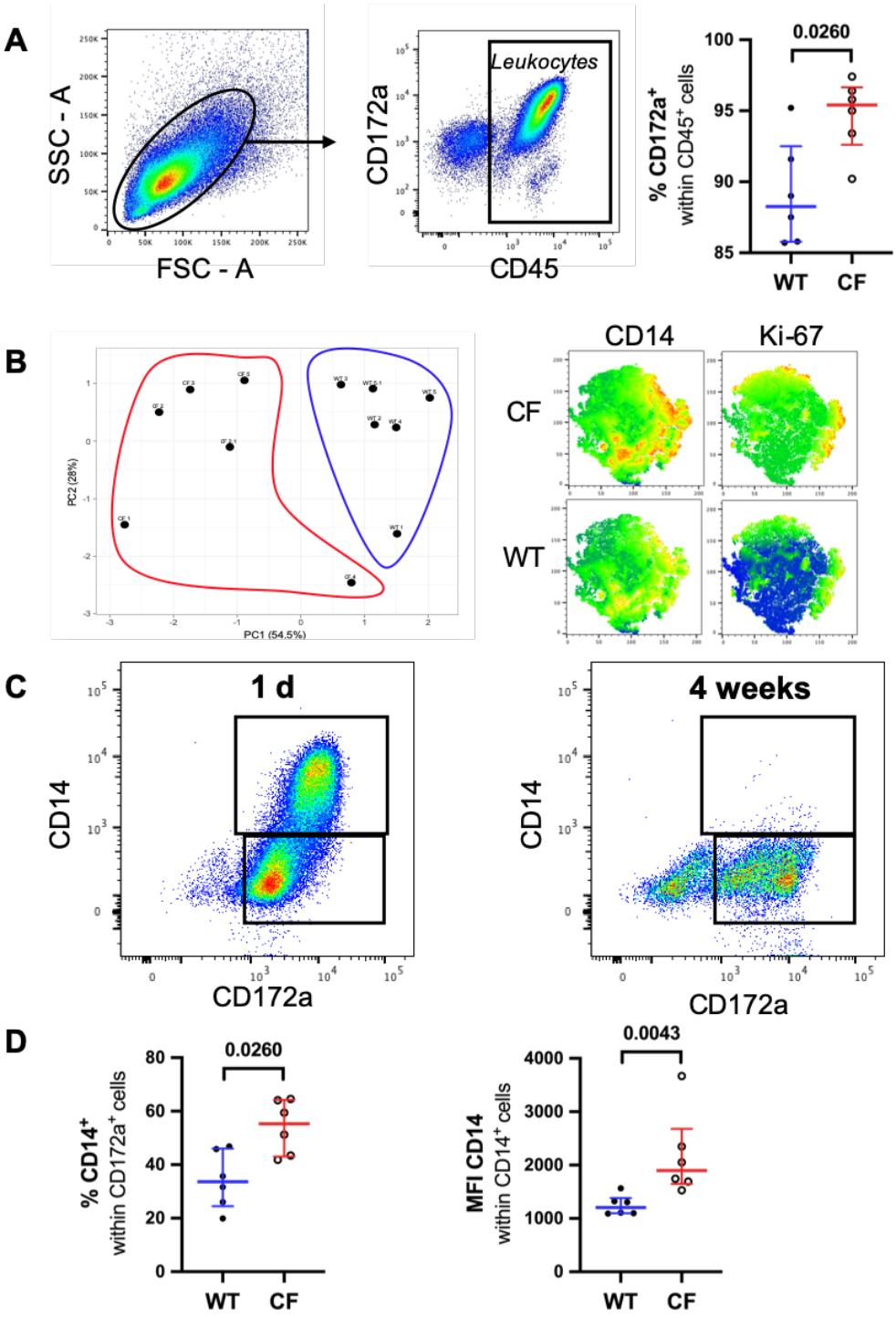


Figure 16. Respiratory mononuclear phagocyte network. (A) Relative abundance of MPs within leukocytes (completely seen in figure 4 E). (B) Principal component analysis of myeloid markers indicated genotype specific differences and dimensionality reduction by t-SNE algorithm revealed different distribution of the markers CD14 and Ki-67. (C) Discriminating respiratory MPs by their CD14 expression. High proportion of $CD14^+$ in newborn piglets, while $CD14^+$ were nearly absent in the lungs of older animals. (D) Higher frequencies of $CD14^+$ within CF MPs and increased expression of CD14. The significance of differences between CF and WT populations was assessed using Mann-Whitney U test (n=6).

Monocyte-derived cells in the lungs were identified by their CD14 expression (Figure 16 C), and interestingly in newborn piglets this subset was highly prominent, while in healthy 4 weeks old pigs it was nearly absent. In MPs from CF airways the proportion of $CD14^+$ cells appeared more abundant (CF: 55.3±21.3 % vs WT: 33.65%±21.57 %; p=0.026). In accordance with the screening, MFI of CD14 within $CD14^+$ MPs from CF lungs was higher than from WT lungs (Figure 16 D).

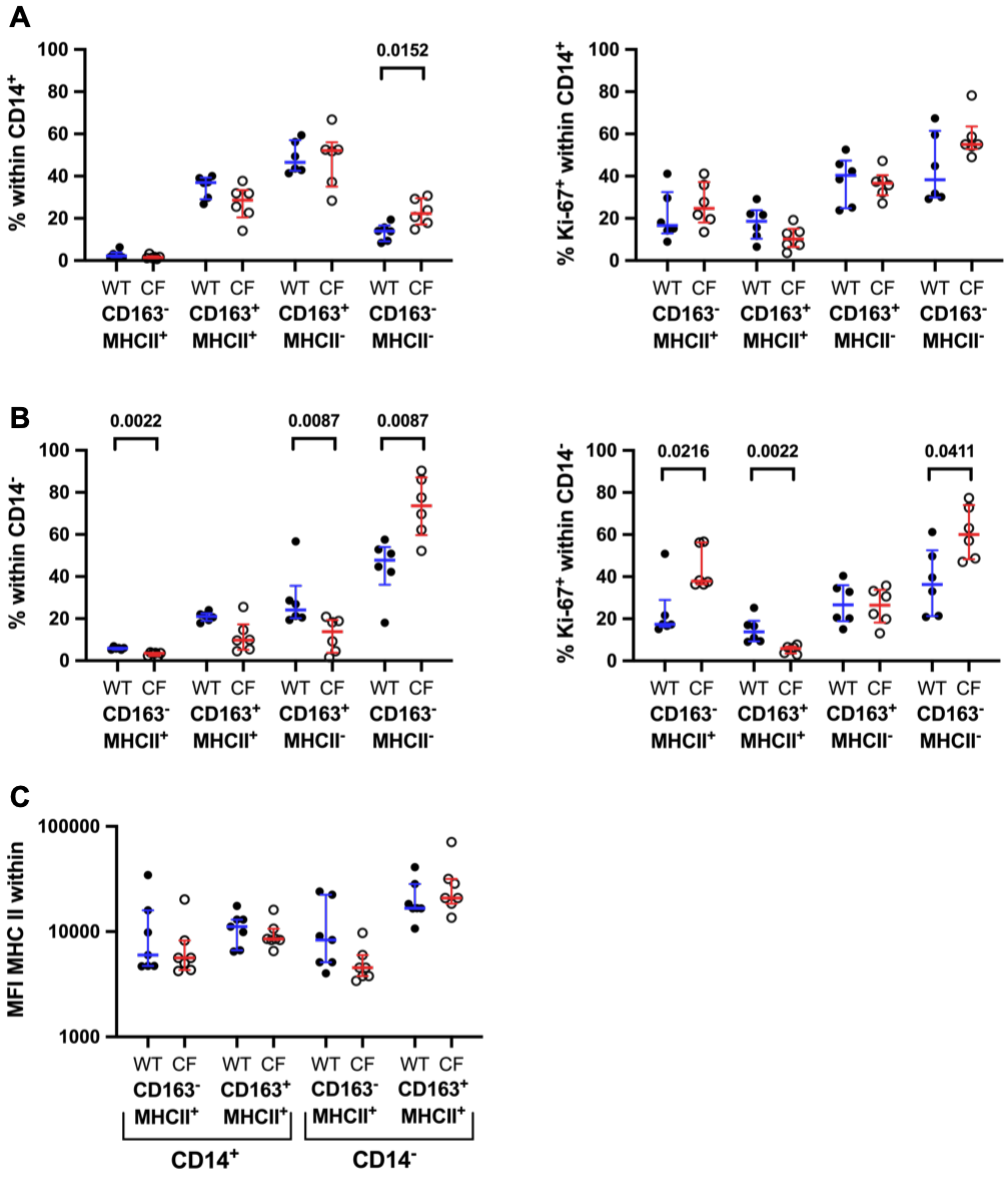


Figure 17. Plasticity of respiratory mononuclear phagocytes. (A) Subsets of $CD14^+$ MPs from lungs and their proliferation status. (B) Subsets of $CD14^-$ MPs and their proliferation status. Mann-Whitney U test without correction was used (n=6).

The phenotype of immature $CD172a^+MHC^-CD163^-$ MPs seemed to be present with higher frequencies in the $CD14^+$ subset in CF airways. Despite of that finding,

further characterization of the $CD14^+$ subset showed a similar phenotype and proliferation status in CF and WT (Figure 17 A).

In contrast, $CD14^-$ mononuclear phagocytes from CF airways showed a different status. The frequency of the dominating and immature phenotype $CD172a^+MHCII^-$ $CD163^-$ subset was higher and their proliferative activity was increased compared to WT MPs (Figure 17 B). Thus, the mononuclear phagocyte network from neonatal CF airways differed from the WT lungs in several ways. Additionally, the expression of MHC II on airway MPs were investigated due to the assumed dysregulated cytokine production in the airways. However, similar expression levels were observed in CF and WT MPs (Figure 17 C).

7. Statistical challenges

In the current pilot study, multiple statistical tests were used to identify significant differences between two genotypes, but statistics give only probabilities and within limited sample sizes false positive or false negative assumptions might easily occur. In order to reduce the probability of false positive assumptions Bonferroni correction was applied and because statistics are a matter of discretion, also the number of tests, which were used to adjust critical p-values, are indicated in Table 6. Due to the limitations of characterizing effects on the basis of their significance, observations should normally be characterized by effect sizes, as low p-values might not always ensure practical relevance. On the other hand, it would be highly questionable to assume that the sample effect size resembled population effect size appropriately in the current thesis. In order to prevent misinterpretation of statistical testing, the maximum sensitivities of the major analyses were calculated by the G*Power tool and are indicated in Table 6. This was useful to evaluate for which minimum effect size the respective test was sufficiently sensitive. The characteristic required effective size parameters dz for Wilcoxon sign ranked test or qd for Mann-Whitney U account the smallest effect that could have been detected with the statistical power of 80% in the respective test.

Cohen's definitions of *small* (0.2), *medium* (0.4), *large* effects (0.8) were helpful to assess such specifications and suggested that only substantially large effects could have been detected with high probability in the examined population, as the minimal required effect size dz was 1.3.

Table 6. Sensitivity of the analyses.

Analysis	**Number of statistical tests**	**Required effect size *dz* or *qd***
Isolated immune cells (Figure 6 A)	3	1.3
Frequencies of major immune cell subpopulations (Figure 7)	24	1.7
KI-67 expression of major immune cell subpopulations (Figure 8)	24	1.7
Frequency of $CD2^+$ γδ T cells (Figure 9 A)	3	1.4
Frequency of $NKp46^{bright}$ NK cells (Figure 9 B)	3	1.4
Frequency of $CD27^-$ cytotoxic T cells (Figure 9 C)	3	1.4
Expression of CD14 and CD80/86 within $CD163^+$ MPs (Figure 10 A)	6	1.4
Expression of CD14 and CD80/86 within $CD163^-$MPs (Figure 10 B)	6	1.4
Phagocytic and oxidative potential of granulocytes from blood (Figure 11)	4	1.3
Phagocytic and oxidative potential of monocytes from blood (Figure 12)	4	1.3
Expression of phagocytic receptors within $CD163^+$ MPs (Figure 13 A)	6	1.7
Expression of phagocytic receptors within $CD163^-$ MPs (Figure 13 B)	6	1.7
Relative abundance of MPs within leukocytes (Figure 16 A)	-	1.8
Frequency of $CD14^+$ within MPs (Figure 16 D)	-	1.8
Expression of CD14 (Figure 16 D)	-	1.8
Plasticity of respiratory mononuclear phagocytes (Figure 17)	-	2.3

V. Discussion

In order to identify the main contributors to chronic airway damage in CF, it is essential to understand the role of immune cells in airway host defense failure and in establishing the chronic inflammatory respiratory environment. This thesis reveals relevant aspects for an intrinsically affected immune system in a CF pig model already at birth and therefore recommends to focus further research on initiating factors rather than on secondary consequences of the disease hallmarks. In a long-term perspective, the work might also contribute to the effective prevention of pulmonary exacerbations and progressive loss of respiratory function in CF.

In newborn CF piglets we did neither observe visible signs of mucus plugging, infection nor inflammation in the airways, which resembles the findings in Bartlett et al. (2016). If maintained for longer periods of time, the CF pigs would have developed spontaneously major hallmarks of the CF lung disease within weeks (Stoltz et al., 2010). The CF pig model is thus highly comparable to CF babies in respect of immune system and the challenges it faces due to the permanent stimulation of inhaled exogenous particles and pathogens (Butler et al., 2009; Nguyen et al., 2016). Major limitations of environmental influences such as timely changing pathogen pressure in pig facilities and occurrence of secondary malformations in clinical studies were circumvented by examination of neonatal CF piglets. In this context it might be worth mentioning that the occurrence of fatal meconium ileus in CF piglets (Klymiuk et al., 2012; Stoltz et al., 2010) was not a limitation of the study, but rather forced us to focus on a virgin-like status that truly revealed the intrinsic predisposition of the CF immune system to aberrant activity.

Besides the advantages of closely reflecting the pathogenesis of CF patients, the significant efforts for producing CF pigs in terms of time and resources need consideration. The limited capacity of animal production therefore compromised the statistical power of this study by the sample size, although the extent of partially 14 examined animals per cohort really resemble a substantially high number, compared to globally practiced large animal studies (reviewed in Fairbairn et al., 2011; Ricci et al., 2020; Summerfield, 2009). Specifically, in a systematic study in CF pigs on the pancreas disease the mean sample size was only half of ours (Abu-

El-Haija et al., 2011). The statistical power was increased by pairwise comparison of CF and WT littermates, which excludes substantial influence of genetic diversity. As indicated, the smallest required effect sizes were 1.3, suggesting that only very large effects could have been detected with high probability as statistically significant, whereas smaller effects might have been overseen false-negatively, albeit resembling significant differences as well (Faul et al., 2007). Moreover, sensitivity was strongly reduced by the number of statistical tests applied in each analysis. Another common pitfall of multiple testing, the increased probability of false-positive findings, was accounted by the Bonferroni correction. Although this approach has been criticized as being too conservative (Ranganathan et al., 2016), we used it as a commonly accepted and widely distributed method in scientific studies.

In spite of the natural limitations of this pilot study due to substantially varying parameters and limited sample size, the comprehensive evaluation of multiple phenotypical and functional parameters gave substantial insight into CF-specific aberrations of the immune system. As effects of genetic diversity were reduced by pairwise testing and environmental influences were diminished by examination of newborn piglets, the frequency and the activation status of major mononuclear leukocyte subpopulations, cytokine profiles after *in-vitro* stimulation and phagocytic potentials suggested a substantial *CFTR* genotype-based predisposition of the immune cells.

Increased numbers of isolated MNLs from CF lungs

The absolute quantification of immune cells from whole organs is definitely a critical ambition, when potentially disturbing factors such as the isolation efficiency or different organ sizes are considered. However, no genotype specific deviation has been observed for organ weight or volume between CF and WT piglets (Meyerholz et al., 2010; Rogers et al., 2008b). Furthermore, isolation of MNLs by density gradient centrifugation is well established since many years (Boyum, 1964). Albeit isolation of immune cells from neonatal tissue seems difficult and a rapid increase in blood MNL count has been observed over the first days (Talker et al., 2013), the adaptations of isolation protocols developed in this thesis turned out to be robust and the recovery of immune cells from blood, spleen and lungs appeared quite efficient compared to another study using blood from four days old piglets (Vreman et al., 2018). When trying to recover immune cells from BAL of newborn

piglets, hardly any leukocytes could be found, which is in accordance to a report from the US (Paemka et al., 2017). Both findings are consistent with data from murine models, which showed that the luminal space of the airways is primarily colonized during the first week of life (Tan and Krasnow, 2016). Moreover, the number of AMs in BAL of piglets was found to rise over the first weeks (Dickie et al., 2009). Overall, for blood, spleen and lungs, similar isolation efficiencies can be assumed. Albeit the immune system develops rapidly after birth, the numbers of examined animals, the high numbers of cells in the isolates and the different experimental examinations suggest that artefacts caused by the isolation procedure itself are unlikely. As this was not true for BAL, no further evaluation of this specimen was carried out.

Phenotypical analysis revealed that predominantly MPs could be isolated from the lungs (Figure 16 A). Interestingly, Hubeau et al. (2001b) observed considerably increased numbers of MΦs in lung tissue from late developmental stages of human CF fetuses without changes of inflammatory markers or increased occurrence of neutrophils. Interestingly as well, the increased numbers of MNLs in the neonatal airways can be linked to a hypothesis of accumulating CFTR-deficient monocytes in the airway wall. Sorio et al. (2016) proposed that loss of CFTR leads to a decreased expression of β_1 and β_2 integrins in monocytes and a deficient selective adhesion. As migration of monocytes from the blood vessels into lung parenchyma does not rely on integrins, whereas their migration into alveolar space is dependent on integrin mediated adhesion, this would lead to their accumulation in the pulmonary interstitium. Very much in line, the reduced expression of CD11b in pulmonary MPs (Figure 13 A) might be involved in this process as it does not only act as complement receptor, but - as a member of the β_2 integrin subfamily - it is also associated with cellular attachment (Piriou-Guzylack and Salmon, 2008). Although it is rather unlikely that downregulated CD11b is solely responsible for impaired transmigration and interstitial accumulation of MPs, its role deserves further investigations. On the other hand, comprehensive examination of the transmigration process of MPs in CF lung tissue is advised for future investigations.

First, the precise localization of MPs in lung tissue by stereometric immunohistochemical analysis has been initiated in a collaborating group (Lars Mundhenk, Institute of Veterinary Pathology, Berlin, Germany). Unfortunately, the availability of reliable markers for porcine MPs in paraffin embedded tissue is

limited (reviewed in Ezquerra et al., 2009; Meyerholz et al., 2016). Antibodies against CD68 or MPO are used, but they are not sufficient to localize all MP subsets precisely, thus further markers have to be established (Piriou-Guzylack and Salmon, 2008). Furthermore, comprehensive expression analysis of migration mediating selectins in airway epithelial cells and immune cells would be of interest. Reports from CF lungs which have been examined after lung transplantation proposed increased expression of the selectin ICAM-1 in epithelial cells (Hubeau et al., 2001a), but its role in early stages of the disease is not clear.

Altered plasticity of respiratory mononuclear phagocytes

The absolute higher numbers of MNLs in lungs (Figure 6 A), the shift towards an increased proportion of CD172a$^+$ MPs in this population (Figure 16 A), as well as general changes in the plasticity of the respiratory mononuclear phagocyte network (Figure 16 D) demanded a closer examination in newborn piglets. The examination was unfortunately compromised by the diverse origin of MPs and their dynamic regulation of activity. Mechanistic work in murine models proposes that during early prenatal development lung MΦs invade from the liver or yolk sac (reviewed in Ginhoux and Guilliams, 2016), while around birth fetal hematopoietic monocytes colonize the lungs and differentiate into long living resident tissue MΦs (Guilliams et al., 2013a; Tan and Krasnow, 2016). Importantly, these perinatal derived cells comprise the major proportion of pulmonary MΦs in homeostasis (Hashimoto et al., 2013), whereas in disease, monocyte-derived MΦs are additionally acquired from the circulation and are activated (reviewed in Hussell and Bell, 2014).

Thus, we were confronted with a great diversity of MPs from various sources in the airways of CF piglets. In pigs, monocyte-derived MPs are highly CD14$^+$ when they invade the airways at birth (Figure 16 D) (Maisonnasse et al., 2016b; Ondrackova et al., 2013) and then loose CD14$^-$ expression until it nearly disappears in lung samples at an age of 4 weeks (Figure 16 C). Therefore, the increased frequency of CD14$^+$ cells in airways of newborn CF piglets (Figure 16 D) suggests an increased influx of monocytes and the high expression level of CD14 on these cells, as well as the increased abundance of less differentiated CD163$^-$MHCII$^-$ (Figure 17 A), a characteristic marker constellation of immature MPs, on CD14 cells indicates their naive status (Chamorro et al., 2005). Importantly, there was no association, within this CD14$^+$ (Figure 17 A), with an inflammatory CD163$^+$MHCII$^-$ phenotype

(Ondrackova et al., 2010). This was further supported by similar concentrations of inflammatory cytokines such as TNFα, IL-1α and IL-8 in studies using BAL from CF and WT piglets (Bartlett et al., 2016). Furthermore, there were also phenotypical suggestions from the circulation, which would support increased extravasation of monocytes into the lungs (Figure 10). As the decline of CD14 expression on $CD163^-$ MPs and upregulation of CD80/86 on the subset of $CD163^+$ MPs in the CF circulation suggested more differentiated monocyte cell states. This is observed in porcine monocytes before extravasation (reviewed in Ezquerra et al., 2009).

In contrast to the $CD14^+$ MPs, which mainly descend from freshly invading monocytes, the $CD14^-$ status is much less indicative for a certain source of progenitors, as it predominantly comprises primary DCs, resident tissue MΦs as well as their progenitors (Auray et al., 2013; Maisonnasse et al., 2016b). Similar to the $CD14^+$ subset, immature $CD163^-MHCII^-$ cells occur at higher frequencies and appear more proliferative within $CD14^-$ cells in CF vs WT samples (Figure 17 B). This is very much in line with the increased proliferative activity of $CD163^-$ MPs in the screening approach (Figure 8). Theoretically, monocyte-derived MPs might also contribute to the $CD14^-$ population, as they lose CD14 expression in the maturation process (Chamorro et al., 2005; Ezquerra et al., 2009). This is not likely, because of the conflicting dominance of immature $CD163^-MHC^-$ status of $CD14^-$ MPs.

When referring to the picture of early stages of human CF lung disease, where thickened airway walls and increased numbers of AM and IM are observed coincidently (Brennan et al., 2009; Mott et al., 2012; Regamey et al., 2012), the data from CF piglets support the key role of MPs initiating the lung disease whereas the grossly normal airway anatomy and unsuspicious appearance of airway walls suggest their thickening as a later hallmark. Moreover, the aberrant respiratory mononuclear phagocyte system occurring in a non-inflammatory environment suggests a potential primary defect of resident pulmonary MPs which could further contribute to the failure of local mucosal immunity, while a global fatal immunodeficiency is not observed in CF.

Independent of their origin, it is unclear whether the $CD14^-$ MPs participate in attracting monocyte-derived cells into the pulmonary interstitium or if monocyte-derived cells only fail to transmigrate through the airway walls on their way to

colonize the luminal space. Furthermore, it remains elusive whether the increased proliferative activity of $CD14^-$ MPs in the lungs is an intermediate result of CFTR deficiency or whether it occurs due to aberrant cytokine signaling in the CF lungs (Jenkins et al., 2011).

It is important to consider that phenotypic characterization of immune cell populations is a general, fast and popular way to identify alterations in the case of specific circumstances. For estimating the consequences of these proposed alterations, it is, however, essential to prove changes also at a functional and physiological level.

Reduced phagocytic and oxidative potential of monocytes and granulocytes

By using a whole-blood assay for studying phagocytosis, isolation artefacts are excluded and robust diagnosis is facilitated as it has been proven for congenital immunodeficiencies such as chronic granulomatous disease (CGD) and myeloperoxidase (MPO) deficiency (Richardson et al., 1998). Although only one bacterial stimulus can be used at a time and an influence of CF plasma on immune cells cannot be excluded (Zhang et al., 2019), the altered functional properties of professional phagocytes and their phenotypic pattern suggest that absence of functional CFTR attenuates an essential pathway of host defense. Very importantly, the observed alterations were detected in circulating phagocytes, suggesting that their aberrant phagocytic potential is not induced by early changes in the airways such as proteolytic airway environment (Alexis et al., 2006; Hartl et al., 2007). Furthermore, the proportions of monocytes subsets in the circulation are, congruent with patient data (Mulcahy et al., 2019), not altered (Figure 7). Interestingly, the expression of CD14 was declined in the $CD163^-$ MPs in blood. Normally the decline of CD14 is associated with more differentiated MPs, which are more effective in phagocytosis (Chamorro et al., 2005; Fairbairn et al., 2013; Fernández-Caballero et al., 2018).

Finally, phagocytosis is impaired by the reduced uptake of bacteria per cell, but not due to reduced numbers of phagocytic granulocytes or monocytes (Figures 11 and 12). Together, these findings clearly reject the idea that altered phagocytic potential is caused by substantial changes of circulating immune cells and supports the hypothesis of an attenuated phagocytic host defense by loss of CFTR (Barnaby et al., 2018; Ng et al., 2014; Robledo-Avila et al., 2018).

From a mechanistic point of view, reduced phagocytosis by monocytes might be a consequence of reduced bacterial uptake or impaired bacterial killing (Di et al., 2006; Zhang et al., 2018). Surprisingly, this work suggests that both steps are impaired in CF piglets. Although porcine monocytes have similar to CF monocytes and MΦs the broad expression pattern of phagocytic receptors, which mediate uptake of opsonized particles (reviewed in Fairbairn et al., 2011), the detailed characterization of bacterial uptake is challenging. Still, the phenotyping of phagocytic mediating receptors indicated like in CF patients (reviewed in Sallenave, 2014) a substantial downregulation of the FcR CD16 in blood, spleen and lungs, whereas the complement receptor CD11b appeared reduced only in the lungs (Figure 13). Although the expression pattern of CD11b on porcine MPs is not completely characterized so far (Auray et al., 2016), these results are congruent with data from human CF patients (Simonin-Le Jeune et al., 2013; Van de Weert-van Leeuwen et al., 2013). In contrast, a down-regulation of CD16 has so far not been reported in human CF samples (Van de Weert-van Leeuwen et al., 2013). In humans, $CD16^+$ characterizes only the subset of non-classical monocytes, whereas in the pig CD16 is abundant on all MPs (Figure 16). This divergence might be caused by species-specific peculiarities (Fairbairn et al., 2013). Of note, CD16 and CD11b are not representative for all receptors, as the co-stimulatory molecule CD80/86 appeared moderately up-regulated in $CD163^+$ MPs in the circulation of CF piglets (Figure 10).

In addition to the impaired bacterial uptake, bacterial killing, as indicated by ROS production, was reduced as well. In granulocytes, finding the same proportion of cells which produced less ROS was in perfect concordance to the bacterial uptake in these cells (Figure 11). In monocytes, however, even the proportion of ROS-producing cells was dramatically diminished and those cells did also produce less ROS than WT cells (Figure 12). Based on the performed experiments, it remains unclear, whether the oxidative potential of phagocytes is reduced as a consequence of the lower number of ingested bacteria or whether the reduced oxidative activity is downregulating bacteria uptake to deal with manageable amount of bacteria. On the other hand, the reduced frequency of ROS producing monocytes, while at the same time the frequency of phagocytic monocytes was unaffected, clearly indicates, that a substantial proportion of monocytes is not able to activate the oxidative burst cascade although they internalize bacteria. It remains elusive, how those cells deal

with phagocytized bacteria, but a closer look at the above mentioned other congenital immunodeficiencies CDG and MPO might be helpful. Both diseases are characterized by impaired oxidative burst. In mild versions of CGD and generally in MPO deficiency no increased occurrence of granulomas has been observed (Kuhns et al., 2010; Schwenkenbecher et al., 2018; Yu et al., 2018). Similar to CF, however, both diseases are instead characterized with recurrent infections by pathogens such as *S. aureus, P. aeruginosa* or *Aspergillus* spp.. All these pathogens have in common that they are enabled to survive ineffective respiratory bursts due to their catalase activity. Even more important, symptomatic infections in MPO appear only as soon as a co-morbidity like diabetes is present (reviewed in Dinauer, 2014).

Regarding granulocytes, oxidative burst is also related with extracellular killing mechanisms such as NETosis and degranulation (Reeves et al., 2015) and, thus, changes at these stages might be immediately linked to alterations in extracellular killing mechanisms. Conclusively, it has to be stated that already at birth the phagocytic host defense is altered, which is most likely caused by the absence of CFTR, but further investigations will be necessary for clarifying details

Major lymphocyte subpopulations with lower frequencies of γδ T cells

Results from phenotypical exploration of major leukocyte subsets grossly match the findings of an earlier described study (Abu-El-Haija et al., 2011), concluding that no systemic inflammatory phenotype is observed in newborn CF piglets. As already mentioned, the conclusions of analyzing adaptive immune cell subsets might be impaired by the limited statistical power (i.e. the insufficient numbers of animals). This is of particular relevance for subsets of T and B cells, which appear mostly immature at birth and develop rapidly afterwards (Talker et al., 2013). Therefore, minor abnormalities of lymphocytes in CF (reviewed in Ratner and Mueller, 2012) could remain unnoticed. On the other hand, parameters that appear significantly different in such dynamic process deserve attention. A good example might be the slightly, but significantly increased proliferative activity of B cells from the CF lungs (Figure 8), which supports the theory of a CF predilection towards Th2 immunity (Hartl et al., 2006; Mueller et al., 2011) to a certain extent. On the other hand, increased Th2 activity would be accompanied by an increase in IL-4 production, an effect that we did not detect in our MNL stimulation assays

(Figure 15). A potential explanation for this might be that apparently low numbers of activated and memory T cells (Talker et al., 2013), being a pre-requisite for mixed lymphocyte activation in MNLs.

Similarly puzzling, the slight but significant increase in regulatory T cells in the spleen remains inconclusive (Figure 7). Interestingly, our analysis suggested neither any regulatory T cell deprivation nor lack of anti-inflammatory IL-10 signaling (Figure 15). Therefore, it might support an acquired decline of regulatory T cells in CF (Hector et al., 2015). Furthermore, the slightly increased NK cell population in the circulation (Figure 7), as well as the significantly reduced NKp46^{bright} subpopulation in the circulation (Figure 9 B) are in line with their migration into the pancreas (Abu-El-Haija et al., 2011), whereas the decreased frequencies of NK cells in blood from CF patients (Mulcahy et al., 2019) put these findings into question.

It should be noted, that the proportional increase of regulatory T cells and NK cells might be simply artificial consequences of the general decrease of γδ T cells in the circulation, spleen and lungs (Figure 7). Indeed, the study of Abu-El-Haija et al. (2011) showed the same tendency, but their small sample sizes might have impaired the statistical power to identify the significance of the findings. The uniquely high amount of γδ T cells in the pig will definitely increase the risk of misinterpreting the increase of other cell population as statistically significant changes, although in reality only the proportion of the dominant γδ T cells was decreased. The changes in this cell population were, however, so striking that statistical misinterpretations can be excluded and functional consequences are likely. The latter are difficult to estimate because γδ T cells are, although abundant in human, mouse and pig, often not included in the analysis of immune cells and knowledge about their functional properties is limited. Importantly, their proposed relevance in mediating communication between innate and adaptive immune system (Hammer et al., 2020) might be of relevance in CF (Raga et al., 2003; Tan et al., 2011). This is not only because the aberrantly regulated innate immune system might have further consequences at the adaptive side, but also due to the potential misinterpretation of signals due to impaired γδ T cell function. Besides these speculative excursions, the protective role of γδ T cell in airways have been shown in TCRδ$^{-/-}$ mice, where LPS challenge causes hyperinflammation and expansion of MPs in the airways (Wehrmann et al., 2016). Intriguingly, the increased numbers of MPs in the airways

(Figure 6 and 16 A) and the suggested proinflammatory cytokine pattern (Figure 14) in this study reflect also the finding in newborn CF piglets.

Cytokine profiles

In general, the cytokine profiles from blood and lung MNLs do not give substantial hints for broad inflammatory changes in CF. This reflects cytokine profiles from CF (Kosamo et al., 2019). It is important, to recognize the large variation between samples (Figure 15), which strongly indicates that the sample size of n=6 animals is far too small to draw reliable conclusions. Further, experiments were conducted with a specific stimulant, on a batch of multiple different subpopulations of immune cells. If the cytokine response was strongly altered in one or the other sub-population, this might have been diminished in the overall picture of entire cell population. The alternative examination of subpopulations is, on the other hand, also critical, because the isolation procedure might change transcriptional regulation, an effect which is highly relevant for the complexly and rapidly controlled expression of cytokines (Vieira Braga and Miragaia, 2019). In addition, the concentrations of stimulants in such assays were not in a physiologic range, but are widely used (Auray et al., 2016; Braun et al., 2017).Thus, interpretation of data from cytokine stimulation assays needs serious considerations.

Still, there is a remarkable tendency towards increased basal release of IL-1β, TNFα, CCl-2 and IL-10, which can be seen as evidence for a pro-inflammatory status of the lungs at birth. This would reflect results from *in-vitro* derived MΦs from CF patients (Simonin-Le Jeune et al., 2013) and the increased capacity of IL-1β production after PMA/Iono stimulation would be in line with alteration of cytokine signaling by MΦs (Bruscia et al., 2009; Paemka et al., 2017). At the present point of time it cannot reliably answered whether these data indicate a kind of hyperinflammatory status of the respiratory immune system. It remains also unclear, whether their increased production is a result of an intrinsically exaggerated cytokine production of certain MΦ subpopulations or whether it is the result of the altered composition of the mixed leukocyte population or caused by altered cellular crosstalk between the immune cells in the lungs.

Although in humans mainly acquired alterations are described in human DCs (Hartl et al., 2012), we observed a reduction of IFNα, IL-12 and TNFα production. These cytokines are primarily produced by plasmacytoid DC (pDC) upon stimulation of

TLR-9 in the circulation (Auray et al., 2016). On the other hand, the reduced numbers of γδ T cells might also directly contribute to the blunted TLR-9 response (Wen et al., 2012) or indirectly by modulating the immune response against CpG motifs. Interestingly, the effect of IFNα, IL-12 and TNFα production upon *ODN* 2216 stimulation was less pronounced in the supernatants from lung MNLs than in the circulation. Again, this might be a calculation artefact, as the lungs, but not the blood comprises a significant proportion of mature MΦs, which are sensitive for CpG motifs via TLR-9 and also produce IFNα, IL-12 and TNFα (Auray et al., 2010; Sautter et al., 2018) and MΦs might thus counterbalance a potentially impaired effect of pDCs. Evidently, the distinct MNL composition in lungs and blood is definitely a major source for differences in cytokine pattern between these specimens.

To summarize we have no clear evidence for systemic predisposition of hyperinflammation, but we have some indications of a blunted induction of the adaptive immune response by DC against viral and bacterial pathogens via the TLR-9 pathway and independently from a potential higher production capacity of inflammatory cytokines, the total increased numbers of MPs in the CF lungs have the potential to contribute significantly to the inflammatory environment in the CF airways already at birth.

Conclusion

In general terms, the evaluation of immune cells from newborn *CFTR*$^{-/-}$ piglets revealed substantial novel aspects of the CF lung disease initiation. Already at birth and without any sign of infection and only very poor evidence of a pro-inflammatory environment, immune cells from CF piglets showed several characteristics of CF. The absolute number of MNLs was increased in the lungs, with a relative increase of MPs, while the expression of phagocytic receptors was reduced. This can be seen in line with a reduced phagocytic and oxidative potential of circulating monocytes and granulocytes. Among lymphocytes, γδ T cells were significantly decreased, suggesting that not only innate immunity is changed, but also the interaction with the adaptive immune system is disturbed. Many of the findings of the current evaluation are consistent with data from CF patients, suggesting that the observed aberrations are characteristic for the disease and that the CF pig represents an excellent model to gain further mechanistic insights.

VI. Summary

Do leukocytes have a predisposition for an altered immune response in cystic fibrosis? – a pilot study in the *CFTR*$^{-/-}$ pig

Cystic Fibrosis (CF) is a common lethal autosomal recessive genetic disorder within people from European descent. Mutations in the gene coding the chloride channel Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) lead to a fatal multisystemic disease affecting most severely the intestinal tract, the pancreas and the airways. Efforts in symptomatic treatment improve the lifespan of patients, but altered host-environmental interactions still lead to the destructive airway disease and ultimately to premature death. As reflected by the early term “mucoviscidosis”, abnormal mucus production and reduced mucociliary clearance clearly participate in the early pathogenesis. Further constituting factors have been postulated, including aberrant immunity, to contribute to the impaired host defense and the chronic hyperinflammation of the CF airways as well. Studying the pathogenesis in CF subjects is challenging due to the disease onset within babies. Thus, there is an immense yield of disparate findings and it remains particularly unclear whether observed abnormalities within CF immune cells are acquired by the early established inflammatory environment or whether CF leukocytes are causing exaggerated inflammation and failure of the airway host defense.

In contrast to many other animal models of CF, the porcine model has proven its close similarities to human. The aim of this thesis was to explore a potential predisposition of CF leukocytes for an altered immune response in the pig model. To this end CF and wildtype (WT) control animals were produced by breeding with the established heterozygous *CFTR*$^{+/-}$ pig herd at the Chair for Molecular Animal Breeding and Biotechnology. In order to reduce the influence of genetic diversity and environment upon analysis, littermate pairs of CF and WT genotypes were used within the first day of life for isolating naïve mononuclear leukocytes (MNLs). Furthermore, a histological examination ensured that no signs of mucus plugging, inflammation or infection was present in newborn piglets.

The focus of this thesis was on MNLs, which were isolated by density gradient centrifugation from blood, spleen and lungs. Surprisingly, the CF lungs comprised significant higher numbers of MNLs and within this increased absolute number of

leukocytes the proportion of mononuclear phagocytes (MPs) was significantly increased. Moreover, the plasticity of respiratory MPs differed in CF and further analysis of the major leukocyte subpopulations and their activation status were carried out. The comprehensive analysis revealed that MPs had an altered phagocytic receptor expression, which coincided with a reduced uptake of bacteria and a decreased oxidative potential of circulating monocytes and granulocytes.

The subset of γδ T cells was significantly decreased among lymphocytes in the circulation and the airways. This suggested alterations in the interaction of innate and adaptive immunity. The analyses of cytokine profiles from *in-vitro* stimulated mixed MNLs were - due to their high variances - not conclusive.

Many findings of this thesis were consistent with data derived from CF patients, suggesting that several abnormalities in leukocyte function are consistent throughout the course of the disease.

VII. Zusammenfassung

Haben Leukozyten eine Veranlagung für eine veränderte Immunantwort bei Mukoviszidose? – eine Pilotstudie am Schweinemodell

Mukoviszidose, auch unter der Bezeichnung zystische Fibrose (ZF) bekannt, ist eine häufig auftretende tödliche autosomal-rezessive Erbkrankheit, die überwiegend bei Personen europäischer Herkunft auftritt. Mutationen im Gen des Chloridkanals *Cystic Fibrosis Transmembrane Conductance Regulator* (CFTR) führen zu einer tödlichen Multisystemerkrankung mit schwersten Krankheitsbildern im Darmtrakt, der Bauchspeicheldrüse und in der Lunge. Die Fortschritte in der symptomatischen Behandlung haben die Lebenserwartung von betroffenen Personen erhöht, aber dennoch führt ein Teufelskreis aus überschießender Entzündung und bakterieller Besiedlung zu irreversiblen Schäden der Atemwege, die letztlich zwangsläufig die Notwendigkeit einer Lungentransplantation oder den vorzeitigen Tod verursachen. Wie bereits der Begriff "Mukoviszidose" aufzeigt, sind eine beeinträchtigte Mukusbildung und eine reduzierte mukoziliäre Reinigung an der Entstehung der Krankheit beteiligt, jedoch wird angenommen, dass weitere Faktoren so wie eine veränderte Immunantwort zur Entstehung der klinischen Symptomatik beitragen. Die Untersuchung der pathologischen Mechanismen ist durch den Krankheitsbeginn im frühsten Kindesalter erschwert und es gibt eine Vielfalt an unterschiedlichen Befunden. Die Pathogenese ist nicht bekannt und es bleibt ungewiss, ob eine veränderte Immunantwort von ZF-Immunzellen die Folge der chronischen Lungenentzündung ist oder ob die Leukozyten selbst die Ursache für die überschießende Entzündung und das Versagen des lokalen Immunsystems der Lunge sind.

Im Gegensatz zu vielen anderen Tiermodellen hat sich das Schweinemodell für die Mukoviszidose-Forschung bewährt. Das Ziel dieser Arbeit war es, die mögliche Immunzellenprädisposition für eine veränderte Immunantwort bei ZF im Schweinemodell zu untersuchen. Zu diesem Zweck wurden ZF-Ferkel und genetisch unveränderte Kontrolltiere (WT) durch Zucht mit der bereits etablierten heterozygoten $CFTR^{+/-}$-Schweineherde am Lehrstuhl für Molekulare Tierzucht und Biotechnologie erzeugt. Um die Einflüsse von Genetik und Umwelt in der Analyse der Immunzellen zu reduzieren, wurden die Proben paarweise von neugeborenen

ZF und WT Ferkeln aus einem Wurf entnommen. Bei Geburt zeigten die neugeborenen ZF-Ferkel keine sichtbaren Krankheitsmerkmale, wie festsitzenden Schleim, entzündliche Prozesse oder Infektionen der Lunge.

Der Schwerpunkt dieser Arbeit lag auf den mononukleären Leukozyten (MNL), die mit Hilfe der Dichtegradienten-Zentrifugation aus Blut, Milz und Lunge isoliert wurden. Überraschenderweise enthielten die ZF-Lungen eine signifikant höhere Anzahl von MNL und innerhalb dieser absolut erhöhten Zahl war der Anteil der mononukleären Phagozyten (MP) signifikant erhöht. Auch war die Zusammensetzung der MP-Subpopulationen in der Lunge verändert und in der weiteren Analyse der Leukozyten-Subpopulationen waren die Rezeptoren in ZF, die für die Phagozytose relevant sind, signifikant geringer exprimiert. Dies ging mit einer signifikant reduzierten Aufnahme von Bakterien in Monozyten und Granulozyten aus dem Blut einher und die Produktion von Sauerstoffradikalen, die für die intrazelluläre Abtötung der Bakterien verantwortlich sind, war signifikant gemindert.

Innerhalb der Lymphozyten waren die γδ T-Zellen in der Blutzirkulation und in der Lunge signifikant vermindert, was auf eine Fehlregulation zwischen angeborener und adaptiver Immunität hinweist. Die genaue Funktionsweise von γδ T-Lymphozyten ist nicht bekannt und die Analyse der Zytokinprofile von *in-vitro* stimulierten gemischten MNL Populationen war aufgrund der hohen Streuung nur eingeschränkt interpretierbar.

Viele Untersuchungsergebnisse dieser Arbeit stimmen mit Daten von ZF-Patienten überein, was darauf hindeutet, dass funktionelle Abweichungen der Leukozyten über den gesamten Krankheitsverlauf gleichmäßig auftreten.

VIII. Index of Figures

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X. Literature

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