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Characterization of *Aspergillus fumigatus* specific T helper cell responses and protein targets in cystic fibrosis patients

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I. Abbreviations

A. fumigatus	*Aspergillus fumigatus*
ABPA	Acute bronchopulmonary aspergillosis
APC	Antigen-presenting cell
ARTE	Antigen-reactive T cell enrichment
BEC	Bronchial epithelial cell
CF	Cystic fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CPA	Chronic pulmonary aspergillosis
DC	Dendritic cell
EC50	Half maximal effective concentration
IFNγ	Interferon gamma
IgE	Immunoglobulin E
IL	Interleukin
LPS	Lipopolysaccharide
MALT	Mucosa-associated lymphoid tissue
MHC	Major histocompatibility complex
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PI	Propidium iodide
PRR	Pathogen recognition receptor
pTreg	Peripheral induced regulatory T cells
SAFS	Severe asthma with fungal sensitization
STAT	Signal transducer and activator of transcription
Tcon	Conventional T cell
TCR	T cell receptor
Tfh	Follicular T helper cell
Th	T helper cell
Th1	T helper cell 1
Th2	T helper cell 2
Th17	T helper cell 17
Treg	Regulatory T cell
tTreg	Thymus derived regulatory T cell

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1 Introduction

1.1 Immune system

The human body is constantly confronted with a variety of organisms. It is one of the major tasks of the immune system to recognize these organisms and to discriminate between self and non-self as well as harmless and dangerous. These classifications are necessary to elicit appropriate reactions, like the elimination of certain microbes while maintaining the co-existence with others or the acquisition of tolerance towards self and harmless organisms and molecules. The immune system can be divided into an innate and an adaptive system. Both are closely linked, influence and interact with each other. The fast but rather unspecific reactions of the humoral and cellular factors that form the innate immune system are completed by the defense mechanisms of the specific and acquired immune system. The latter is also responsible for the development of the immunological memory that allows a fast and strong immune reaction in a subsequent exposure to the same pathogen.

1.1.1 Innate immunity

When an invading pathogen is able to conquer the physical and chemical barriers of the body, it is confronted with the innate immune system. It contains a variety of cell types and humoral components with different effector functions like phagocytosis (macrophages), the release of cytotoxic granules (eosinophils, basophils, neutrophils, mast cells, natural killer cells) and opsonization or cell lysis (complement) (Delves Peter J. and Roitt Ivan M. 2000b). Another important function is the interaction and activation of the adaptive immune system through antigen presentation (dendritic cells (DCs), macrophages) and the release of proinflammatory molecules. The cells recognize typical pathogenic structures like lipopolysaccharides (LPS) of gram-negative bacteria with pattern-recognition receptors (PRR) (Medzhitov and Janeway Jr 1997). Thus, the identification of pathogens directly induces the various defense reactions of the innate immune system, but as they remain unspecific the action of the adaptive immune system is often required.

1.1.2 Adaptive immune system

The response of the adaptive immune system is antigen specific and precise, but the initiation needs some time. The cellular components are the lymphocytes which can be divided into B cells and T cells. Unlike the cells of the innate immune system that

recognize frequent pathogen patterns, lymphocytes possess surface receptors that identify only one specific antigen. These receptors are the result of complex processes during the maturation and selection of the cells in the bone marrow (B cells) and the thymus (T cells) to ensure specificity of the cells and prohibit reactivity to harmless antigens (Parkin and Cohen 2001). The selected lymphocytes leave the bone marrow and the thymus as naïve cells and begin to circulate between the peripheral blood and secondary lymphoid organs, like the spleen, lymph nodes or mucosal-associated lymphoid tissue (MALT). Once a lymphocyte is confronted with its specific antigen, a process of activation, differentiation and clonal expansion is induced, leading to the development of effector lymphocytes who all share the same specificity. Effector B cells, so-called plasma cells, produce antibodies as a specific humoral defense mechanism (Delves Peter J. and Roitt Ivan M. 2000a). After the elimination of the pathogen, most of the effector lymphocytes die through apoptosis but some survive and create the immunological memory. These memory cells are another important characteristic of the adaptive immunity and allow the organism to react faster and stronger against the same pathogen in a following encounter.

1.2 T cells

T cells are part of the adaptive immune system. They can be divided into two major groups: the cytotoxic CD8+ cell and the so-called T helper (Th) cells which are CD4 positive. The expression of the co-receptors CD8 and CD4 is part of the T cell maturation in the thymus. All T cells recognize peptide fragments of antigens with highly specialized receptors that are bound to the membrane: The T cell receptor (TCR). Each TCR is composed of two polypeptide chains with most T cells expressing the α/β heterodimer and a minority of T cells bearing the γ/δ TCR (Delves Peter J. and Roitt Ivan M. 2000a). Importantly, TCRs do not recognize antigens directly, but only when they are presented to them on special glycoproteins, the major histocompatibility complexes (MHC). MHC class I is expressed on all nucleated cells and presents peptides of intracellular pathogens to CD8+ T cells, leading to the killing of the pathogen presenting cell in case of antigen recognition. The antigen recognition of CD4+ T cells is restricted to MHC class II which is only expressed by specialized antigen presenting cells (APCs) like DCs, macrophages and B cells (Rudolph et al. 2006). This first step of T cell activation must be followed by other co-stimulatory signals. The binding of CD28 which is expressed by all T cells to the B7 complex of the

corresponding APC is crucial for survival and proliferation of the T cells (June et al. 1987). Shortly after the activation, CD4+ T cells express CD154+ (CD40L) on the surface which is important for the interaction with other cell types, especially B cells (Grewal and Flavell 1996; Frentsch et al. 2005). Further differentiation into the Th cell subgroups is strongly influenced by the cytokine environment. The different effector CD4+ T cells have a variety of tasks and functions in the immune response by interacting with many other cell types of the adaptive and the innate immune system. The subgroups can be characterized by the production of cytokines and the expression of certain surface molecules (Zhu and Paul 2008). Apart from effector function, the cells can be classified according to their phenotype. Naïve CD4+ T cells express the surface marker CD45RA and cells of the memory compartment upregulate CD45RO. Depending on the stage of maturation, the profile of memory T cells shows some special characteristics, like the expression of the lymph node homing factor CCR7 in central memory cells which is not present in effector memory cells that are circulating outside the lymphatic organs (Sallusto et al. 2004).

1.2.1 Th cell subgroups

The first differentiation into subsets in the CD4+ T cell compartment was the description of T helper 1 and 2 (Th1 and Th2) cells in mice (Mosmann et al. 1986). As of today, three other major groups are defined: T helper 17 (Th17) cells, regulatory T cells (Tregs) and follicular helper cells (Tfh) (Zhu et al. 2010; Breifeld et al. 2000). Each lineage plays a distinct role in the immune response. Th1, Th2 and Th17 cells are protecting the host against different types of pathogens. In contrast, Tregs have suppressive capacities and can suppress unwanted immune reactions and thereby generate a balance between inflammation and tolerance. Tfh are essential to assist B cells during their maturation and the antibody class-switch. They can be characterized via the surface marker CXCR5 which allows them to migrate to the B cell follicles (Breifeld et al. 2000), the transcription factors Bcl6 as the driving transcriptional factor (Nurieva et al. 2009), the upregulation of genes for PD-1 and ICOS (Kim et al. 2004; Dorfman et al. 2006) and the effector cytokines TGFβ, IL-12 and IL-23 which are described to be important in humans (Schmitt et al. 2014; Vinuesa et al. 2016).

The predominant cytokine milieu, provided by cells of the innate immune system, initiates signal transducer and activator of transcription (STAT) pathways which lead to the expression of specific transcription factors. These so-called master regulators and

the STAT factors determine the further differentiation program of the naïve Th cell by the upregulation of lineage defining genes and active repression of factors that would lead into an alternative fate (Zhu et al. 2010). The introduced pathways do not always follow the strict rules that are described here and many processes are not yet fully understood. The co-expression of different master-regulators is reported (Yu et al. 2015; Mirlekar 2020) as well as the simultaneous production of lineage-defining cytokines (Hegazy et al. 2010). It is also unclear if the fate decision of a naïve CD4+ T cell is really ultimate once the differentiation process started or if the cells are able to change their phenotype due to changing environment (Yang et al. 2008; Gagliani et al. 2015). Furthermore, there might be more CD4+ T cell subsets that can be distinguished from the four groups that are presented in the following (Kaplan et al. 2015; Duhen et al. 2009).

1.2.1.1 Th 1 cells

The Th1 cells are mainly involved in the host defense against intracellular pathogens (Paul and Seder 1994). The differentiation program is induced by interferon gamma (IFNγ) and interleukin (IL) 12 which activate STAT1 and STAT4 (Hsieh et al. 1993; Afkarian et al. 2002). Both factors lead to enhanced expression of T-bet, the master regulator of the Th1 lineage (Szabo et al. 2000). The differentiated Th1 cells produce IFNγ, resulting in a positive feedback mechanism for the differentiation of more Th1 cells and the suppression of Th2 and Th17 cells (Zhu et al. 2010). An exaggerated Th1 response is associated with the induction of autoimmune diseases and chronic inflammation (Zhu and Paul 2008).

1.2.1.2 Th2 cells

Physiologically, Th2 cells mediate the immune response against extracellular pathogens, especially helminths (Paul and Seder 1994). They are of particular clinical interest as they are involved in the development of pathologies such as asthma and allergies (Walker and McKenzie 2018; Murphy and Reiner 2002). Similar to the Th1 cell differentiation, a signature cytokine also serves as an important signal to induce the Th2 lineage: IL-4 activates STAT6 leading to the upregulation of GATA3 (Kaplan et al. 1996; Zheng and Flavell 1997), the master regulator of Th2 cells, which together with STAT5 results in the expression of IL-4, IL-5, IL-9 and IL-13 (Zhu et al. 2003). These cytokines have extensive effects in the immune response as a variety of cells is affected. Interaction with B cells leads to antibody class-switch to IgE, macrophages,

eosinophils and mast cells get activated and goblet cell hyperplasia and smooth muscle construction are initiated (Walker and McKenzie 2018). Additionally, IL-4 intensifies the Th2 reaction by stimulating the differentiation of more Th2 cells and suppressing the other Th cell lineages (Zhu et al. 2010).

1.2.1.3 Th17 cells

Th17 cells extended the CD4+ T cell subsets as a distinct cell population in 2005 (Harrington et al. 2005; Park et al. 2005) and since this first description, many characteristics of Th17 cells were reported. Different combinations of cytokines, i.e. IL-23, IL-1β or IL-6 and TGFβ have been described to induce the activation of the transcriptional factors STAT3 (Harris et al. 2007) and RORγt (Ivanov et al. 2006) and thus the Th17 differentiation program (Bettelli et al. 2006; Harrington et al. 2005) . The differentiated effector cells produce the signature cytokines IL-17A, IL-17F and IL-22 which mainly interact with neutrophils and epithelial cells (Onishi and Gaffen 2010). Th17 cells seem to play important roles in different immunological states of an individual: on the one hand they are described to be important for the homeostatic balance with microflora, especially in the gut and the host defense against extracellular bacteria and fungi (Conti and Gaffen 2015) but on the other hand they are potential drivers of autoimmune diseases like inflammatory bowel disease (McGeachy et al. 2019; Ouyang et al. 2008). The processes and factors which determine the protective or pathogenic pathways of the Th17 cells are still under debate (Peters et al. 2011; Stockinger and Omenetti 2017).

1.2.1.4 Regulatory T cells

Another subset of CD4+ T cells are the Tregs which differ from the other effector Th cells as their main function is not the defense against pathogens, but the control and regulation of immune responses. The signature molecule of all Tregs is Foxp3. Other characteristics are the high expression of CD25 (the alpha chain of the IL-2 receptor) and CTLA-4, both serving as important molecular mediators to suppress the other T cell lineages (Wing et al. 2008; Boehmer 2005). In mice, activated Tregs produce immunomodulating cytokines like IL-10, TGFβ and granzymes (Josefowicz et al. 2012). Thymus-derived Tregs (tTregs) are responsible for tolerance against self-antigens which are presented to them on MHC class II molecules in the thymus in the presence of IL-2, IL-15 and IL-7, leading to the upregulation of Foxp3 (Josefowicz et al. 2012; Lio and Hsieh 2008). In mice it has been shown that another population of Tregs

leaves the thymus as naïve CD4+ cells and gets activated in the periphery (pTregs) to mediate tolerance to non-self antigens, including commensal microbiota, dietary antigens and potential allergens (Plitas and Rudensky 2016). The differentiation is induced after TCR activation and co-stimulatory signals through TGFβ, IL-2 and retinoid acid (Chen et al. 2003; Davidson et al. 2007; Xiao et al. 2008). The existence and role of pTregs in the human immune system remains poorly understood. The importance of Treg function becomes dramatically clear in case of their deficiency which leads to autoimmune and inflammatory disorders, allergies, infections and cancer (Plitas and Rudensky 2016).

1.3 Cystic fibrosis

Cystic fibrosis (CF) is a multisystemic autosomal-recessive disorder caused by mutations in the CFTR gene which is encoding for the Cystic Fibrosis Transmembrane Conductance Regulator, an ion channel protein (Riordan et al. 1989). The resulting misbalance of the water salt homeostasis leads to viscous mucus production in several body compartments. The gastrointestinal affection often leads to endocrine and exocrine pancreatic insufficiency, resulting in diabetes mellitus and malabsorption. An involvement of the biliary conduct system can induce hepatic cirrhosis which requires liver transplant in the end stage of disease (Elborn 2016). Particularly strongly affected is the lung: the thickened mucus and impaired mucociliary clearance cause bronchial obstruction and create a solid medium for undisturbed settlement and proliferation of a complex pathogen flora. Additionally, direct influence of the CFTR defect on innate immune defense mechanisms is described. Dysregulated epithelial function, mucociliary transport defect, change of pH and impaired antimicrobial effect could support a proinflammatory environment and prevent appropriate killing of pathogens (Cohen and Prince 2012; Stoltz et al. 2015). The structural reorganization of the chronically challenged lung leads to a progressive loss of lung function. Advanced medical care in specialized CF centers and the development of new therapeutic agents contributed to a drastic improvement in life expectancy over the last decades from only a few month to a median survival age of nearly 50 years in countries with developed health care systems (Scotet et al. 2020). Special attention should be paid to respiratory development as today respiratory failure is the main life limiting factor in CF. Therefore, respiratory monitoring, accurate supporting management of the lung and targeted pathogen treatment in case of exacerbation are key factors in CF patient care (Smyth

et al. 2014). The most common pathogens are *Pseudomonas aeruginosa* and other gram-negative bacteria, *Burkholderia cepacia spp*. and non-tuberculous mycobacteria (Parkins and Floto 2015). In addition to the different bacteria, fungal pathogens are frequently identified in respiratory specimens of CF patients (Schwarz et al. 2018; Pihet et al. 2009; Poore et al. 2021). While the role of bacteria in CF pathogenesis is well established, the relevance of the fungal airway colonization for CF pathogenesis is still a matter of debate (Pihet et al. 2009; Williams et al. 2016)

1.4 Aspergillus fumigatus

Aspergillus fumigatus (*A. fumigatus*) is a saprotrophic airborne fungus with high abundance indoors and outdoors. Its asexual reproduction cycle starts with the release of small spores or conidia with only 2µm in diameter. Once these resting conidia arrive in a suitable environment, they start to swell and germinate further into germ tubes and hyphae (Park and Mehrad 2009). Even though humans are exposed to spores of *A. fumigatus* on a daily basis, the encounter has no serious consequences for most people. On the other hand, *A. fumigatus* is causing a wide range of diseases in susceptible individuals. The type and severity of the disease is depending on the immune status of the host. Immunocompetent individuals can develop chronic pulmonary aspergillosis (CPA) without invasive fungal growth, mainly after surviving pulmonary tuberculosis or another cavitary lung disease (Alastruey-Izquierdo et al. 2018). This rather mild condition is in strong contrast with the invasive forms of aspergillosis which occur in immunocompromised patients with high rates in mortality and morbidity (Patterson et al. 2000; Neofytos et al. 2009). The third group of *A. fumigatus* related diseases are the hypersensitivity reactions severe asthma with fungal sensitization (SAFS) and acute bronchopulmonary aspergillosis (ABPA) which present mostly in individuals with pre-existing chronic lung diseases like asthma or CF (Antunes et al. 2010; Moss 2014). In healthy individuals, the inhaled conidia is mostly cleared out by the respiratory epithelial cells with their ciliary action (Park and Mehrad 2009). The conidia that can escape the mucociliary clearance are confronted with lung resident immune cells like alveolar macrophages. Dysfunction of these mechanisms leads to an impaired innate anti-fungal defense, allowing the germination of the fungus and requires activation of the adaptive immune system (Park and Mehrad 2009). CD4+ T cells have been shown to be important mediators of anti-fungal immune responses (Hohl et al. 2009; Beck et al. 2006; Scheffold et al. 2018; Bacher et al. 2015).

In healthy humans is has been shown that *A. fumigatus* generates strong Treg responses that prevent inappropriate immune reactions, such as Th2 mediated allergies (Bacher et al. 2014a; Bacher et al. 2016). In addition, a small subset of *A. fumigatus*-specific T cells produces IFNγ, indicating a differentiated Th1 phenotype whereas the majority of conventional T cells (Tcons) are still in the naïve state (Bacher et al. 2014a). Strikingly, their response is influenced by the morphological form of the fungus as it is expressing different proteins in the stages of germination (Bacher et al. 2014b; Bozza et al. 2002). The search of immunogenic antigens is still ongoing as their determination would allow more specific diagnosis of *A. fumigatus* related diseases (Kurup 2005; Latgé and Chamilos 2019). In CF patients, *A. fumigatus* is the most frequent filamentous fungus found in sputum samples (Masoud-Landgraf et al. 2014). Clinical consequences of colonization alone are still unclear, but approximately 39,1% of all CF patients develop *A. fumigatus* sensitization which is associated with poorer lung function (Wojnarowski et al. 1997; Maturu and Agarwal 2015) and can lead to ABPA in susceptible individuals. Notably, Bacher et al. could show that *A. fumigatus* specific CD4+ T cells from CF patients with severe allergic reactions against the fungus have a phenotype different from that of healthy individuals. They present with a dominant Tcon reaction and show a strong Th2 effector response, indicated by elevated levels of IL-4, IL-5 and IL-13 (Bacher et al. 2014a).

1.4.1 Acute bronchopulmonary aspergillosis

ABPA is a hypersensitivity pulmonary disorder that can occur in individuals with prior sensitization to *A. fumigatus* (Patel and Greenberger 2019; Agarwal et al. 2013). Particularly affected are patients with CF with a prevalence of 8,9% (Maturu and Agarwal 2015). Historically, Greenberger and Patterson classified five stages of the disease with stage I as the acute phase, presenting with deterioration of the general clinical condition, wheezing and malaise. Stage II was defined as remission of the acute disease whereas patients in stage III re-exacerbated. Corticosteroid-dependent asthma (stage IV) is present when a lowering of the corticosteroid leads to worsening of the clinical condition or re-elevation of immunoglobulin E (IgE) and finally stage V is the fibrotic stage, the dramatic long-term complication of ABPA (Patterson et al. 1982; Patel and Greenberger 2019). As the clinical presentation is variable and symptoms of ABPA are partly overlapping with conditions of CF, the clinical diagnosis of APBA in CF patients is especially challenging. Different diagnostic criteria have been proposed

over the years, using a mix of serological, immunological and radiological findings. The minor criteria of the CF Foundation Group are shown in table 1 (Stevens et al. 2003). Still, ABPA is considered to be under-diagnosed and new diagnostic tools are required as the early diagnosis is the key factor in preventing loss of lung function. CD4+ T cells could represent an interesting diagnostic target as they are important mediators of the immune response against fungi (Scheffold et al. 2018; Bacher et al. 2015). Th2 cells are considered to be crucial in the pathogenesis of ABPA regarding the hypersensitivity component of the disease. It could be shown that patients with ABPA have an increased Th2/Th1 ratio (Becker et al. 2015) and elevated levels of Th2 cytokines after stimulation with *A. fumigatus* (Knutsen and Slavin 2011; Skov et al. 1999; Bacher et al. 2019). Probably other Th cell subsets like Tregs and Th17 cells are involved as well (Bacher et al. 2019), but the pathological pathways are not yet fully understood. After diagnosis, treating management includes suppression of the over-reacting immune system with glucocorticoids and anti-fungal drugs, mostly azoles. Immunomodulatory approaches in form of monoclonal antibodies against IgE or Th2 cytokines can be other options with the most experience in using the anti-IgE antibody omalizumab (Patel et al. 2019; Sunman et al. 2020).

Table 1: Minimal diagnostic criteria (Stevens et al. 2003)

1. Clinical condition	Acute or subacute clinical deterioration (cough, wheeze, exercise intolerance, exercise-induced asthma, change in pulmonary function, or increased sputum production) not attributable to another etiology.
2. Total IgE concentration	Total serum IgE concentration of1500 IU/mL (1200 ng/ mL). If ABPA is suspected and the total IgE level is 200–500 IU/mL, repeat testing in 1–3 months is recommended. If patient is taking steroids, repeat when steroid treatment is discontinued.
3. Prick skin test	Immediate cutaneous reactivity to *Aspergillus* (prick skin test wheal of 13 mm in diameter with surrounding erythema, while the patient is not being treated with systemic antihistamines) or in vitro demonstration of IgE antibody to *A. fumigatus.*
4. IgG concentration or radiographical signs	One of the following: (a) precipitins to *A. fumigatus* or in vitro demonstration of IgG antibody to *A. fumigatus;* or (b) new or recent abnormalities on chest radiography (infiltrates or mucus plugging) or chest CT (bronchiectasis) that have not cleared with antibiotics and standard physiotherapy.

1.5 Detection of antigen-specific CD4+ T cells

Antigen-specific CD4+ T cells are important mediators of the adaptive immune system. The frequency in the peripheral blood varies widely from 1 cell in 10^4 to 10^7 regarding naïve Th cells and 10^{-5} - 5% in the memory pool, depending on the individual's immune status and the type of antigen (Bacher et al. 2013). These low cell numbers make their analysis challenging due to technical limitations. Bacher et al. developed a method that allows the direct *ex vivo* analysis of these rare cells for further characterization of phenotype and effector function called antigen-reactive T cell enrichment (ARTE) (Bacher et al. 2013; Bacher et al. 2014b). Activated CD4+ T cells upregulate the surface marker CD154 (Grewal and Flavell 1996; Frentsch et al. 2005). The ARTE technology utilizes the expression of this activation marker by indirect magnetic labelling after short-term stimulation with the antigen of interest. This is followed by a magnetic separation process of the cells. Further processing includes surface staining, fixing and permeabilization of the cells which is followed by intracellular staining. The final step is performed with multiparameter FACS analysis allowing the characterization of multiple Th cell features, like markers for naïve and memory Th cells and the expression of phenotype markers or effector cytokines (Bacher et al. 2013). Thus, ARTE allows the detailed analysis of antigen-specific Th cells responses of individuals, independent of knowledge of their MHC alleles or the immunogenic antigen or peptides. Even whole antigen lysates can be used for stimulation that are naturally processed by the individual antigen presenting cells during the short stimulation time. This is of particular importance, since for many clinically relevant antigens the precise T cell target epitopes remain unknown.

By using the ARTE technology, a characterization of differences in antigen-specific T cells against disease-relevant antigens can be performed, e.g. between healthy individuals and patients with allergies, leading to new insights regarding physiological and pathological Th cell responses. Due to the short time of stimulation, ARTE bypasses the confounding factors of long *in vitro* cultures, but allows direct conclusions about the *in vivo* situation. Together with the fast sample processing and the possibility to analyze Th responses to different antigens in one donor at the same time, this technology offers broad advantages and possibilities for clinical applications in screening, diagnosis and follow-up studies.

1.6 Aim of the project

Patients with CF suffer from a wide variety of different pathogen interactions and infections. *A. fumigatus* is found to be one of the major allergens in these patients (Antunes et al. 2010). The immune reactions towards *A. fumigatus* and the different immunologic procedures that might be triggered by the fungus are not yet understood. To approach these open questions, the aim of this study was to investigate the T helper cell responses against *A. fumigatus* in CF patients. For the analysis of these rare antigen specific cells, the magnetic enrichment of CD154+ Th cells (ARTE) was used (Bacher et al. 2013) to characterize the phenotype and effector functions of *A. fumigatus*-specific Th cells. The Th cell reaction was correlated with clinical data such as the disease status of the patient and *A. fumigatus* specific IgE levels.

A major problem for the targeted treatment of CF patients against fungal pathogens is the lack of specific and sensitive diagnostic methods for the different fungal-mediated diseases. Currently, diagnostics are based solely on the detection of fungal pathogens, which does not provide any information about the status of the infection. Therefore, a particular question of this study was to characterize *A. fumigatus*-specific T cells in patients who are sensitized to *A. fumigatus* or who are suffering from ABPA. The diagnosis of ABPA is clinically challenging and currently used methods are limited in their prediction, not always available and sometimes hard to interpret (Knutsen and Slavin 2011). We hypothesized that via the analysis of the CD4+ T cell reaction against *A. fumigatus* the actual status of host-pathogen interaction can be more accurately determined.

Finally, the antigenic T cell target proteins of *A. fumigatus*-specific T cells in CF patients remain poorly defined. Therefore, another focus of this thesis was to define potential protein T cell targets, focusing on allergenic proteins that elicit pathogenic Th2 responses in CF. The identification of such proteins could contribute to a better understanding of the immune processes induced by *A. fumigatus* in CF patients and improve the sensitivity of a T cell based diagnostic assay for *A. fumigatus*-mediated diseases in comparison to a whole *A. fumigatus* lysate.

Material

1.7 Samples

For the antigen reactive T cell enrichment, blood samples of 98 CF patients were provided by PD Dr. med. Carsten Schwarz and Dr. med. Patience Eschenhagen (Charité Berlin). After giving their informed consent, 50 ml of blood were taken. 12 healthy people were recruited as a control cohort. The antigen specific T cell expansion was performed with 5 CF patients and 6 healthy donors. All patients and controls contributed voluntary to this study and signed informed consent (ethics committee CAU Kiel D578/18, ethics committee Charité Berlin EA2/058/20 and ethics committee Klinikum Westbrandenburg AS 48(bB)/2021). The detailed data table can be found in the appendix (supplementary data 2).

1.8 Chemicals, buffers and media

Table 2: Chemicals, buffers and media

Name	Trade name	Produced by
AB-serum	Human serum	Sigma-Aldrich, Saint Louis, MO, USA
Brefeldin A	Brefeldin A from *Penicillium brefeldianum*	Sigma-Aldrich, Saint Louis, MO, USA
CD14-Beads	CD14 MicroBeads, human	Miltenyi Biotec, Bergisch Gladbach, Germany
DMSO	Dimethylsulfoxide	Sigma-Aldrich, Saint Louis, MO, USA
EDTA	UltraPure™ 0.5M EDTA, pH 8.0	Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania
Ficoll	Ficoll-Paque™ PLUS	GE Healthcare Bio-Sciences AB, Uppsala, Sweden
Interleukin 2	Mouse IL-2 IS, premium grade	Miltenyi Biotec, Bergisch Gladbach, Germany
Ionomycin	Ionomycin, Calcium salt from *Streptomyces conglobatus*	Sigma-Aldrich, Saint Louis, MO, USA
Life-Death-Dye	Viobility 405/520 Fixable Dye	Miltenyi Biotec, Bergisch Gladbach, Germany
PBS	Phosphate Buffered Saline	Lonza, Wakersville, MD, USA
Penicillin-Streptomycin	Penicillin-Streptomycin	Sigma Aldrich, Rehovot, Israel
PMA (Phorbol-12-myristat-13-acetat)	PMA	Sigma-Aldrich, Saint Louis, MO, USA
Propidium Iodide	Propidium Iodide Solution	Miltenyi Biotec, Bergisch Gladbach, Germany
RPMI	Gibco™ RPMI 1640 Medium	Life Technologies, Paisley, United Kingdom
TexMACS	TexMACS™ Medium	Miltenyi Biotec, Bergisch Gladbach, Germany
X-Vivo	X-Vivo™ 15	Lonza, Verviers, Belgium

Table 3: Composition of buffers and media

Name	Content
Expansion medium for cells in culture	TexMACS + human AB-serum (1:20) + Penicillin-Streptomycin (1:100) + Interleukin 2 (1:500) + 30 ng OKT3 on the first day of expansion
Freezing medium	Human AB-serum + X-Vivo + DMSO 2:2:1
PBS	2,683 mM KCl, 1,470 mM KH2PO4, 136,893 mM NaCl, 8,058 mM Na2HPO4 · 7 H2O, without magnesium or calcium
PEB	PBS + 2mM EDTA + 0,5% AB-serum
RPMI	RPMI 1640 with L-Glutamine and 25mM HEPES
TexMACS	Glutamine, human albumin, phenol red, pH: 6,5-7,9
X-Vivo	L-glutamine, gentamicin, phenol red

1.9 Kits

Table 4: Kits

Trade name	Content	Produced by
CD154 MicroBead Kit, human	1 ml anti-human CD154-Biotin, 2 ml anti-Biotin-MicroBeads	Miltenyi Biotec, Bergisch Gladbach, Germany
Inside Stain Kit	25 ml Inside Fix, 2 x 55 ml Inside Perm	Miltenyi Biotec, Bergisch Gladbach, Germany

1.10 Antigens

Table 5: Antigens

Antigen	Concentration	Produced by
C. albicans (whole lysate)	1 mg/ml (40 µl/ml)	Greer Laboratories, Cambridge, MA, USA
A. fumigatus (whole lysate)	2 mg/ml (40 µl/ml)	provided by O. Kniemeyer/A. Brakhage HKI Jena
PMA/ionomycin	20 ng/ml	Sigma Aldrich
Inomycin	1 µg/ml	Sigma Aldrich
Peptide pools of A. fumigatus (Th2 targets and Th2 non targets)	10µg/protein/ml (rec. proteins) 1µg/ peptide/ml (peptide pools)	Generation as previously described (Bacher et al. 2019)
Aspf4 (single protein *A. fumigatus*)	10 µg/ml (2,3 µl/ml)	provided by O. Kniemeyer/A. Brakhage HKI Jena
CsnB (single protein *A. fumigatus*)	10 µg/ml (6,5 µl/ml)	provided by O. Kniemeyer/A. Brakhage HKI Jena
ScwA (single protein *A. fumigatus*)	10 µg/ml (6,5 µl/ml)	provided by O. Kniemeyer/A. Brakhage HKI Jena

1.11 Antibodies

Table 6: Antibodies

Target	Fluorochrome	Produced by
CD3, Clone OKT3		Miltenyi Biotec, Bergisch Gladbach, Germany

CD4	VioBlue	Miltenyi Biotec, Bergisch Gladbach, Germany
CD4	BV421	BioLegend, San Diego, CA, USA
CD4, Clone MTT466	APC-Vio-770	Miltenyi Biotec, Bergisch Gladbach, Germany
CD4, Clone MTT466	Antibody pure	Miltenyi Biotec, Bergisch Gladbach, Germany
CD8	PerCP	Miltenyi Biotec, Bergisch Gladbach, Germany
CD14	PerCP	Miltenyi Biotec, Bergisch Gladbach, Germany
CD20	PerCP	Miltenyi Biotec, Bergisch Gladbach, Germany
CD25	PE	Miltenyi Biotec, Bergisch Gladbach, Germany
CD28	Antibody pure	Miltenyi Biotec, Bergisch Gladbach, Germany
CD40	Antibody pure	Miltenyi Biotec, Bergisch Gladbach, Germany
CD45 RA	PECy5	BioLegend, San Diego, CA, USA
CD45 RO	PE-Vio770	Miltenyi Biotec, Bergisch Gladbach, Germany
CD45 RO	APC	Miltenyi Biotec, Bergisch Gladbach, Germany
CD69	PE	Miltenyi Biotec, Bergisch Gladbach, Germany
CD127	APC	Miltenyi Biotec, Bergisch Gladbach, Germany
CD154	FITC	Miltenyi Biotec, Bergisch Gladbach, Germany
CD154	APC	Miltenyi Biotec, Bergisch Gladbach, Germany
CCR7	FITC	Miltenyi Biotec, Bergisch Gladbach, Germany
CCR7	PE-Vio770	Miltenyi Biotec, Bergisch Gladbach, Germany
GMCSF	PE-CF594	BD Biosciences, San Jose, CA, USA
IL-4	PerCPCy5	BioLegend, San Diego, CA, USA
IL-5	Vio667	Miltenyi Biotec, Bergisch Gladbach, Germany
IL-17A	PEVio770	Miltenyi Biotec, Bergisch Gladbach, Germany
IL-13	PE	Miltenyi Biotec, Bergisch Gladbach, Germany
IFNγ	BV650	BD Biosciences, San Jose, CA, USA
Ki67	AF700	BD Biosciences, San Jose, CA, USA
TNFα	APC PE-770	Miltenyi Biotec, Bergisch Gladbach, Germany
IFNγ	APC Vio-770	Miltenyi Biotec, Bergisch Gladbach, Germany
IL-17A	FITC	Miltenyi Biotec, Bergisch Gladbach, Germany
IL-4	PE	Miltenyi Biotec, Bergisch Gladbach, Germany

1.12 Usage material

Table 7: Usage material

Name	Trade name	Produced by
1,5 ml and 2 ml microtubes	Microtubes 1,5 ml/ 2 ml	Sarstedt, Nümbrecht, Germany
5 ml Eppendorf tube	Eppendorf Tubes® 5,0 ml	Eppendorf, Hamburg, Germany
15 ml and 50 ml tubes	15 ml/ 50 ml Cellstar® Polypropylen Tubes	Greiner Bio-One, Frickenhausen, Germany
Cryotubes, conic, 1 ml	Cryotube 1,0 ml SI	Thermo Fisher Scientific, Jiangsu, China
Cell culture multiwell plate with 6, 12, 24 or 48 wells	CELLSTAR® Cell Culture Multiwell Plates	Greiner Bio-One, Frickenhausen, Germany
96 well plate, with u-shaped or flat bottom	Nunc™ MicroWell™ 96-Well, Nunclon Delta-treated, U-Shaped-Bottom/ Flat-Bottom Microplate	Thermo Fisher Scientific, Roskilde, Denmark
384 well plate	384 Well Microplate	Corning, Kennebunk, ME, USA
Tubes for taking blood	S-Monovette 9ml K3E	Sarstedt, Nümbrecht, Germany
Butterfly canula	Safety-Multifly ® 21 G, 200 mm long	Sarstedt, Nümbrecht, Germany
Disposable tips for serologic pipettes 50 ml, 25 ml, 10 ml, 5 ml	CELLSTAR® Serologic Pipettes	Greiner Bio-One, Frickenhausen, Germany
Disposable tips for multistepper	Combitips Advanced 10ml, 2,5ml, 0,5ml	Eppendorf, Hamburg, Germany
Pipette tip 10 µl 20 µl, 200 µl, 1000 µl	Pipette Tip 10 µl 20 µl, 200 µl, 1000 µl	Sarstedt, Nümbrecht, Germany
MS and LS separation column	MS Columns/ LS Columns/ LD Columns	Miltenyi Biotech, Bergisch Gladbach, Germany

1.13 Devices

Table 8: Devices

Name	Trade name	Produced by
Bench	LaminAir	Heraeus, Hanau, Deutschland
Centrifuge	Megastar 3.0R	VWR, Leuven, Belgium
Centrifuge (small)	Micro Star 17R	VWR, Leuven, Belgium
FACSAria	BD FACSAria™ Fusion Cell Sorter	BD Life Sciences, San Jose, CA, USA
Incubator	Heracell™ 150i CO_2 Incubator	Heraeus, Hanau, Germany
MACS magnet holder	MACS® Multi Stand	Miltenyi Biotec, Bergisch Gladbach, Germany
MACSQuant 10	MACSQuant® Analyzer 10 Flow Cytometer	Miltenyi Biotec, Bergisch Gladbach, Germany
MACSQuant 16	MACSQuant® Analyzer 16 Flow Cytometer	Miltenyi Biotec, Bergisch Gladbach, Germany
Multi-channel pipette 5-50 µl	Finnpipette F1	Thermo Fisher Scientific Oy, Vantaa, Finland
Multistepper	Multipipette® M4	Eppendorf, Hamburg, Germany
Magnet for cell separation	QuadroMACS™ Separator, OctoMACS™ Separator	Miltenyi Biotec, Bergisch Gladbach, Germany
Serological pipette	Accu-Jet® pro	Brand, Wertheim, Deutschland
Shaker	Titramax 101	Heidolph, Schwabach, Germany
Vortex	MS2 Minishaker	IKA Werke, Staufen, Germany

1.14 Data analysis programs

Table 9: Programs for data analysis

Name	Produced by
FlowJo 10.6	BD Biosciences, San Jose, CA, USA
GraphPad Prism 9	GraphPad Software, La Jolla, CA, USA
MACSQuantify® Software	Miltenyi Biotec, Bergisch Gladbach, Germany
Microsoft Office 2019 Standard	Microsoft, Redmond, WA, USA
Microsoft Excel	Microsoft, Redmond, WA, USA

2 Methods

2.1 Antigen-reactive T cell Enrichment

ARTE allowed the direct *ex vivo* analysis and characterization of Th cells. After contact with their specific antigen, Th cells expressed CD154 on the surface, as an activation marker. These activated cells were labelled in two steps: First, anti-CD154-biotin marked the activated T cells and in a second step anti-biotin microbeads were linked to the biotin-carrying cells. The indirect magnetic labelling of CD154+ cells was followed by separation via magnetic columns. Surface staining was performed on the first

column, followed by permeabilization, fixation and intracellular staining on a second column before flow cytometric analysis (Bacher et al. 2013). Thereby, phenotype and effector function of these rare cells could be analyzed. The detailed protocol is described in the following.

2.1.1 Isolation of PBMCs

The isolation of peripheral mononuclear cells (PBMCs) was performed with the Ficoll-Hypaque density gradient centrifugation. The different blood components were separated by their various densities. Because platelets have a similar density like PBMCs, multiple washing steps needed to be performed after the first centrifugation step. The cell count was done with the MACS Quant 16® flow cytometer: 10 µl of the cell suspension were diluted with 90 µl PEB. To exclude dead cells 1 µl of propidium iodide (PI) was added. The cells were resuspended in RPMI + 5% human AB-serum and then plated depending on the cell number into multiwell plates. Of the five prepared wells, four were used for different antigens and one served as an unstimulated control to check for of background expression of CD154 of the Th cells. $1*10^6$ cells were stored separately for the original staining.

2.1.2 Original staining (Ori)

The original staining determined the percentages of CD4+ T cells and memory Th cells within the PBMCs of a donor. That knowledge allowed to calculate the frequencies of specific activated Th cells and memory Th cells among CD4+ cells. After washing, the cell pellet was incubated with the staining mix (table 10) for 15 minutes at 4°C. After another washing step, the cells were resuspended in fresh PEB buffer and PI was added in a ratio of 1:100 before the flow cytometric analysis on the MACSQuant 16®. The gating strategy is shown in figure 1.

Table 10: Staining Ori

Surface target	Fluorochrome	Dilution
CD8/CD14/CD20	PerCP	1:10
CD4	VioBlue	1:10
CD45RO	PE-Vio770	1:10
CCR7	FITC	1:50
CD25	PE	1:10
CD127	APC	1:10
+ MACS buffer ad 50 µl	-	-

2.1.3 Stimulation of PBMCs

The cells were stimulated with a whole lysate or single proteins for 7 hours in total at 37 °C in the presence of CD28 and CD40 (1µg/ml). Thereby, the upregulation of CD154 was induced. The antigens which were used are listed in table 11. After 5,5 hours of stimulation, 1 µg/ml per well (ratio 1:1000) of Brefeldin A was added. Brefeldin A inhibited the secretion of intracellular proteins and cytokines and allowed an intracellular analysis later in the process. When the stimulation time was completed, the cells were harvested in 1,5 ml Eppendorf tubes.

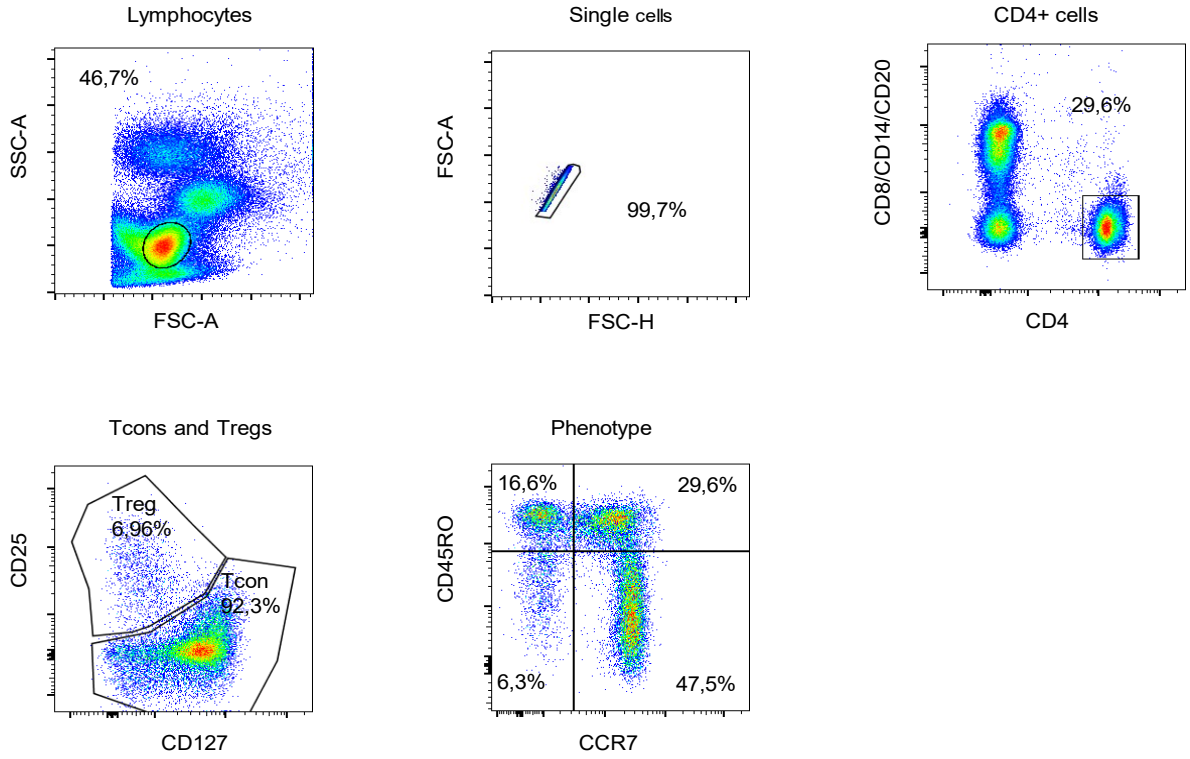

Figure 1: **Gating strategy original staining:** The first gate defines the lymphocyte population in the forward and sideward scatter. The next gates exclude doublets and lymphocytes other than CD4+ T cells. The surface markers CD25 and CD127 discriminate between conventional and regulatory Th cells. Next, both subpopulations are subdiveded in naïve Th cells and the different memory Th cell populations with the markers CCR7 and CD45RO. Shown here is an example for the distribution of conventional Th cells from a CF patient. The numbers in the plots indicate the percentage of the parental population.

Table 11: Antigens

Antigen	Concentration
C. albicans (whole lysate)	40 µg/ml
A. fumigatus (whole lysate)	40 µg/ml
Th2 targets (protein mix *A. fumigatus*: CatB, Asp f2, CpcB, Asp f3, Fg-gap)	10 µg/protein/ml for recombinant proteins 1µg/peptide/ml for peptide pools
Th2 non-targets (protein mix *A. fumigatus*: Crf1, Shm2, Sod3, Asp f22, Scw4, TpiA, GliT, Pst1, Gel1)	10 µg/protein/ml for recombinant proteins 1µg/peptide/ml for peptide pools
Aspf4 (single protein *A. fumigatus*)	10 µg/ml (2,3 µl/ml)
CsnB (single protein *A. fumigatus*)	10 µg/ml (6,5 µl/ml)
ScwA (single protein *A. fumigatus*)	10 µg/ml (6,5 µl/ml)

2.1.4 Enrichment of antigen-reactive T cells

After harvesting, the cells were washed with PEB buffer (400 xg for 5 minutes, 4°C) and afterwards incubated with varying concentrations of two different CD4 antibodies for 15 minutes at room temperature. Thus, the different stimulation conditions got labelled with a “matrix barcode”, allowing to merge all conditions of one donor from now on. Additionally, the matrix staining contained αCD154-biotin for the labelling of activated Th cells. After another washing step, αBiotin-microbeads were added to the cells for 15 minutes at 4°C, establishing the link with the activated Th cells which were labelled with the αCD154-biotin before. For the magnetic enrichment, the cells were added on an equilibrated MS column which was placed in an OctoMACS® magnet. As only the CD154+ cells were magnetically labelled, these cells stayed on the column, whereas the other PBMCs dropped out. Two washing steps were performed with 1 ml PEB buffer and 500 µl of PBS buffer.

2.1.5 Surface staining and intracellular staining

For further analysis, the cells were stained. The staining steps were performed directly on the column to prevent loss of cells during centrifugation. The columns should be covered during incubation to protect the fluorochromes from light. The antibodies which were used are listed in the tables 12 and 13. First, the surface staining was added on the columns for 15 minutes at room temperature. This was followed by a washing step with 500 µl PBS and the elution of each column with 800 µl of PEB. In preparation for the intracellular staining, 200 µl of Inside Fix were added for 10 minutes at room temperature to fix the cells. Afterwards, the cells were put on a second column. To allow the antibodies which were used in the intracellular staining to enter the cells, 200 µl of Inside Perm were added to start the permeabilization of the cells. The intracellular staining was left on the columns for 12 minutes at room temperature. Subsequently,

each column was washed with 200 µl of Inside Perm and then eluted in a 2 ml Eppendorf tube with 1 ml of PEB. 750 µl supernatant were carefully removed after centrifugation for 5 minutes at 400xg before starting the flowcytometric analysis.

Table 12: Surface staining

Surface target	Fluorochrome	Dilution
CD8/CD14/CD20	VioGreen	1:50
CD45-RA	PECy5	1:60
+ TSE (tandem signal enhancer)	-	1:10
+ life-dead-dye	-	1:60
+ Vitamin C ad 60 µl	-	-

Table 13: Intracellular staining

Intracellular target	Fluorochrome	Dilution
CD154	FITC	1:50
GMCSF	PE-CF594	1:50
IL-4	PerCPCy5	1:20
IL-5	Vio667	1:10
IL-17A	PEVio770	1:50
IL-13	PE	1:50
IFNγ	BV650	1:20
Ki67	AF700	1:50
+ TSE (tandem signal enhancer)	-	1:10
+ Vitamin C	-	1:10
+ Perm buffer ad 60 µl	-	-

2.1.6 Flow cytometry analysis

After processing the cells as described, a flow cytometric analysis with the MACS Quant 16® was performed. Therefore 200 µl of the sample's volume were taken up.

2.1.7 Gating strategies and evaluation

Further analysis was performed with FlowJow software. A multistep gating strategy was used to approximate the cell population of interest (Fig. 2). First, the population of lymphocytes had to be defined in the forward and sideward scatter, followed by the exclusion of doublets and dead cells. To differentiate between CD4+ cells and other lymphocytes, cells which expressed a surface marker of the dump channel (CD8, CD14 or CD20) were excluded. The different stimulations could then be separated by using the different "barcodes" which were created with the matrix staining. For each condition, the activated CD154 expressing cells were gated and checked for the distribution of memory and naïve Th cells. Next, it was looked for cytokine production among the memory Th cells.

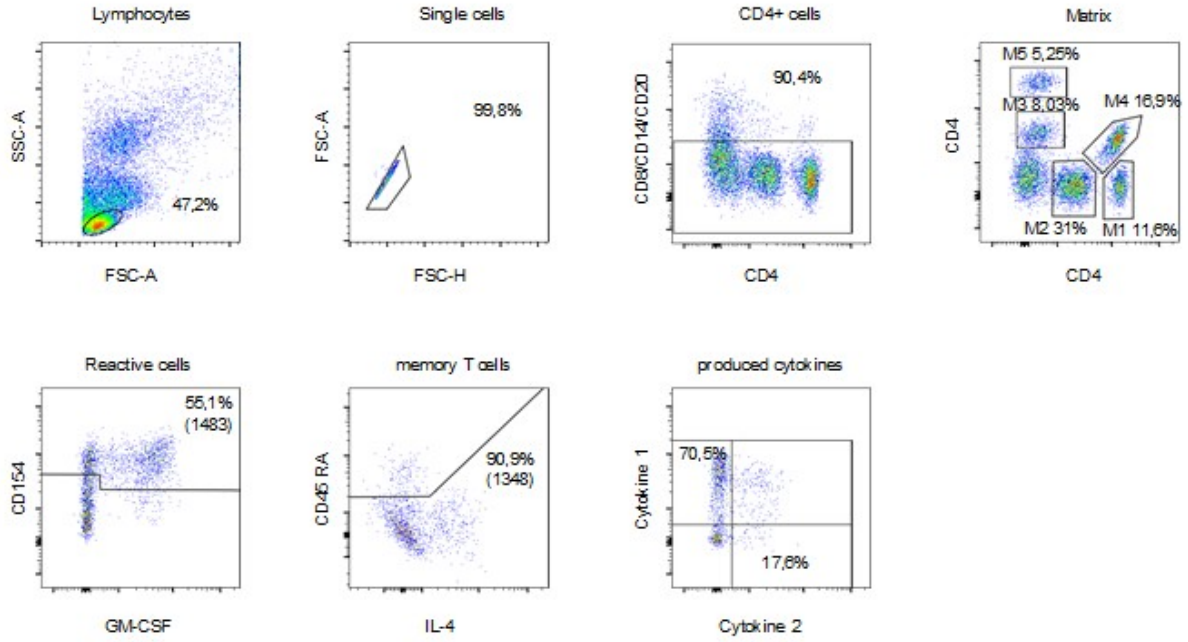

Figure 2: **Gating streategy ARTE**: First steps are the definition of the lymphocyte population and exclusion of doublets and CD8+/CD14+/CD20+ cells. The matrix staining allows the analysis of CD154 and memory T cell frequencies for each condition. In the last plot, memory Th cells are checked for production of cytokines. Written in the plots are the percentages of the parental population and cell numbers in brakets for CD154+ and memory Th cells

2.1.8 Calculation

2.1.8.1 Frequency of antigen specific cells

To calculate the frequency of antigen-specific Th cells, the number of enriched CD154 expressing Th cells was related to the total number of stimulated CD4+ cells. To calculate the frequency of memory T cells, the frequency is then multiplied by the percentage of memory cells of the CD154+ cells and divided by 100.

$$F = \frac{N\left(CD\,154 + Th\,cells\right)}{N\left(stimulated\,Th\,cells\right)} \times \frac{\%\,memory\,Th\,cells}{100}$$

2.1.8.2 Enrichment factor

The enrichment factor is an indirect measure for the *in vivo* interaction of the organism and a specific antigen. The amount of memory Th cells of an individual is influenced by multiple factors like age and the enrichment factor allows to bypass this impact by relating the antigen-specific Th cell response to the proportion of all memory Th cells. The enrichment factor sets the response of memory cells in the total CD4+ T cells

compartment and in the antigen-specific T cells into ratio. Thus, an enrichment factor > 1 likely indicates a prior encounter whereas an enrichment factor < 1 suggests that the antigen has probably not elicited a memory reaction before.

$$E = \frac{(\%\,antigen-specific\,memory\,Th\,cells \times \%\,all\,naive\,Th\,cells)}{(all\,memory\,Th\,cells \times antigen-specific\,naive\,Th\,cells)}$$

For example, given the proportion of antigen-specific memory Th cells is 85,4% and of for all memory Th cells 53,78%, the enrichment factor is calculated as follows:

$$E = \frac{(85{,}4 \times (100-53{,}78))}{(53{,}78 \times (100-85{,}4))} \frac{(85{,}4 \times 46{,}22)}{(53{,}78 \times 14{,}6)} = 5{,}03$$

, indicating a prior interaction of this individual with the antigen.

2.1.8.3 EC50 value

The half maximal effective concentration (EC50) can be calculated based on the results of titration experiments with expanded and re-stimulated antigen-specific Th cells. This value indicates the concentration of antigen which is needed to provoke a reaction in 50% of the Th cells. Thereby it is an indicator for the functional affinity of the expanded cells at which low EC50 values imply high affinity as only a low concentration of the antigen is needed to activate the Th cells. EC50 values were calculated from dose-response curves using GraphPad PRISM. These curves were plotted as a semi-logarithmic plot, where the amount of antigen is plotted (on the X axis) as the log of antigen concentration and the response is plotted (on the Y axis) using a linear scale. To compare the EC50 values of different donors, the bottom and top of the curve were defined as 0 and 100%, respectively.

2.1.8.4 Statistical analysis

Statistical analysis for analysis of significance levels was performed with ordinary ANOVA or the Kruskal-Wallis-test, depending on the results of normal distribution. Normal distribution was checked with Shapiro-Wilk-test. All statistical analysis was performed with GraphPad Prism.

2.2 Antigen-specific T cell expansion

For further characterization of antigen specific Th cells, expansions of 4 healthy donors and 3 CF patients were done. Activated memory Th cells were bulk sorted after short-term stimulation and CD154+ enrichment and kept in culture for 3-4 weeks. The following re-stimulation allowed the analysis of the functional affinity of the antigen-

specific cells towards their antigen and checking potential cross-reactivity after confrontation with other antigens.

2.2.1 Isolation of PBMC

The isolation of PBMCs was performed as described above. The cells were plated depending on the number of cells and conditions in RPMI + 5% AB-Serum and were incubated over night at 37 °C.

2.2.2 CD14 MACS

For the generation of APCs which were needed for the re-stimulation, the magnetic enrichment of CD14 positive cells was done. Freshly isolated PBMCs were incubated with 15µl CD14 micro beads per $1x10^7$ cells for 15 minutes at 4 °C and then washed with 15-20 ml PEB buffer. Depending on the number of cells an MS or LS column was equilibrated and the cells were added in 1 ml PEB buffer, followed by three washing steps with PEB buffer. The cells were eluted and transferred in cryovial tubes in ratio of 1:1 with freezing medium. The following freezing process down to -70°C was done gently to avoid unnecessary cell death.

2.2.3 Antigen stimulation of the PBMC

The PBMCs were stimulated with *A. fumigatus* and CsnB for 6 hours in presence of anti-CD40 (1µg/ml; 1:100) and anti-CD28 (1µg/ml; 1:2000). The concentrations are listed in table 14.

Table 14: Antigens T cell expansion

Antigen	Concentration
Aspergillus fumigatus	40 µl/ml; 40 µg/ml
CsnB	6,5 µl/ml; 10 µg/ml

2.2.4 Magnetic enrichment of CD154 positive T cells

The cells were carefully harvested in a 50 ml tube after 6 hours of stimulation. After a washing step with PEB, the cells were incubated for 15 minutes at 4 °C with an anti-CD154 biotin VioBright FITC conjugate (2.5 µl per $5x10^7$ cells) to label the activated cells. This was followed by another washing step and the magnetic labelling with biotin micro beads (2.5 µl per $5x10^7$ cells, incubation time again 15 minutes at 4°C). Subsequently, the cells were washed with 10-15 ml PEB and then taken into 1 ml PEB for the application on an equilibrated MS column. The negative fraction (CD154-) was collected for the generation of feeder cells. Further processing of the CD154+ cells

included two washing steps with PEB and a surface staining, allowing to distinguish the different CD154+ subgroups before the sorting process to only sort and expand the population of interest (table 15). The surface staining was added to the column for 15 minutes in the dark at room temperature.

Table 15: Surface staining T cell expansion

Surface target	Fluorochrome	Dilution
CD4	VioBlue	1:50
CD8/14/20	PerCP	1:50
CD69	PE	1:50
CD45RO	APC	1:50
CCR7	PEVio770	1:50
+ PEB ad 50 µl	-	-

The staining was followed by a washing step with 1 ml PEB buffer and afterwards the cells were eluded with 1ml RBMP + 5 % AB-Serum. After centrifugation for at 400 g for 5 minutes, 700 µl of the supernatant were carefully discarded and the remaining volume was put on ice.

2.2.5 Feeder cells

The negative fraction of the CD154 enrichment was used for the generation of feeder cells. After a cell count, the cells were irradiated for 20 minutes with 13 Gray. $3x10^5$ cells were plated in 50 µl of expansion medium plus 30 ng OKT3 (anti-CD3 antibody) per well in a 96 well plate (U-bottom). The remaining feeder cells were kept frozen at -70°C for a potential boost of the antigen specific T cells.

2.2.6 Fluorescence activated cell sorting (FACS):

The FACS-sort was performed for an increase of the cell's purity before the expansion. Only CD154+ cell with a memory phenotype were included. The cells were sorted directly in the well. Each well already contained feeder cells in expansion medium.

2.2.7 Expansion of antigen-specific Th cells

The antigen-specific cells were kept in culture for 3-4 weeks before the re-stimulation. During this time, medium change, feeding and splitting was performed as needed.

2.3 Re-stimulation of Antigen-specific T cells

After sufficient proliferation of the antigen specific T cells, the cell lines were stimulated with different antigens for further analysis. Thus, reactivity, functional affinity and cross reactivities could be determined.

2.3.1 Preparation for the re-stimulation - Plating APCs and resting T cells

On the first day of preparation for the re-stimulation APCs were plated in a 384 well plate. Therefore, APCs were thawed carefully or freshly generated via CD14 MACS (4.2.2). For each testing condition $0.5x10^5$ - $1x10^5$ APCs were plated in 50 µl X-Vivo medium per well. To suppress the activating signals of the expansion medium, the T cells should rest for at least 24 hours before further processing. Thus, as much expansion medium as possible was taken out of each well and replaced with RPMI + 5% of AB-serum.

2.3.2 Preparation for re-stimulation - Plating T cells:

The cells could be harvested after a sufficient resting period. 10 µl of the cell suspension was added to 90 µl of PEB to do a cell count. Afterwards, the Th cells were added in a ratio of 1:1 to the APCs in 20 µl RPMI+5% AB-serum per well.

2.3.3 Re-stimulation and further processing:

Each condition was re-stimulated with *A. fumigatus*, *C. albicans* and CsnB. As a negative control, one well was left unstimulated and as for positive control PMA/ionomycin was used. Additionally, the *A. fumigatus* and CsnB specific T cells were also tested with a protein titration (steps for CsnB: 10 µg/ml, 4 µg/ml, 1 µg/ml, 0.1 µg/ml, 0.01 µg/ml; steps for *A. fumigatus* 40 µg/ml, 10 µg/ml, 1 µg/ml, 0.1 µg/ml, 0.01 µg/ml). In total, the cells were stimulated for 6 hours and after 2 hours, Brefeldin A (1:1000) was added. In the following, 50 µl of supernatant per well were carefully discarded, leaving each well with 20 µl of volume. On the surface, only CD4 was stained (0.2 µl per well, CD4 VB calculated for 10 µl; table 16). To ensure that the antibody and the cells would be able to mix, the plate was shorty centrifuged after the staining was added. The incubation time was 15 minutes in darkness to protect the fluorochromes. For the first two minutes the plate should be placed on a shaker.

Table 16: Surface staining re-stimulation

Surface target	Fluorochrome	Dilution
CD4	VioBlue	1:50
+ PEB buffer ad 2 µl per well + 10% of total volume PEB buffer (error rate)	-	-

Next, the cells were fixed. Therefore, 5 µl of inside fix per well were added (centrifuge for 1 minute, 400g; incubation for 12 minutes, dark, first two minutes on shaker). After

the fixation, the intracellular staining could be added (centrifuge for 1 minute, 400 g; 15 minutes in the dark, first two minutes on shaker; table 17). The permeabilization was performed in the same step with perm buffer. It was stained for CD154 and TNFα as activation markers. Cytokine production was checked for IFNγ, IL-17A and IL-4. In the protein titrations, it was only looked for CD154 and TNFα.

Table 17: Intracellular staining re-stimulation

Surface target	Fluorochrome	Dilution
CD154	APC	1:50
TNFα	APC PE-770	1:50
IFNγ	APC Vio-770	1:50
IL-17A	FITC	1:10
IL-4	PE	1:10
+ 10X Perm ad 5 µl per well + 10% of total volume Perm (error rate)	-	-

To wash the cells after the intracellular staining, 40 µl of PEB buffer per well were added. After centrifugation for 5 minutes at 400 g, 50 µl of supernatant were discarded. Now 50 µl of PEB buffer were added and each well was re-suspended directly before the flow cytometry analysis. The analysis was performed with the MACSQuant 10®. The gating strategy is shown in figure 3.

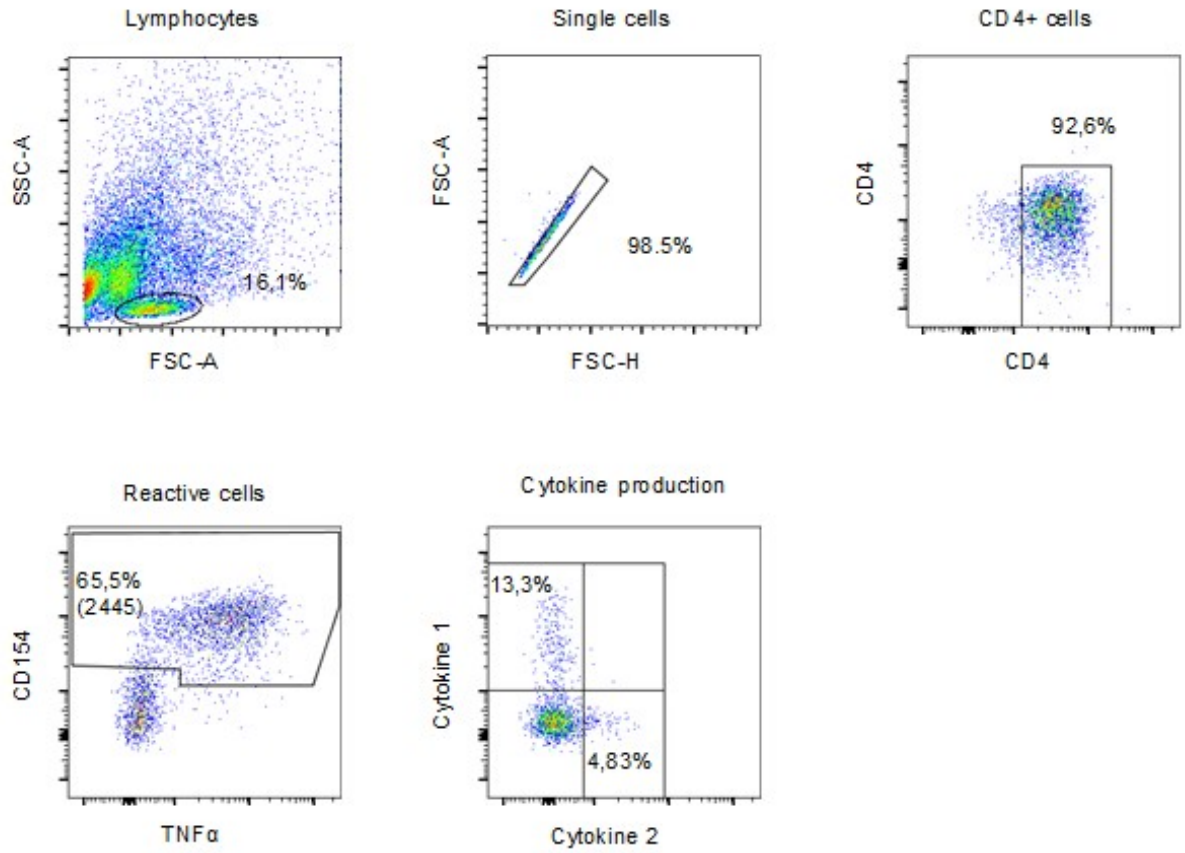

Figure 3: **Gating strategy re-stimulation:** After definition of lymphocytes, exclusion of doublets, CD4+ T cells are defined by using two CD4 antibodies. In the next steps, reactive cells, indicated by CD154+TNFα+, are analyzed for cytokine production. Percentages of the parental population are written in the graphs and cell number of CD154+ Th cells in brackets.

3 Results

A. fumigatus is one of the major allergens in cystic fibrosis patients (Warner et al. 1976). Fungal sensitization can lead to ABPA, which can cause irreversible loss of lung function due to lung fibrosis and bronchiectasis when left untreated. Clinical diagnosis is challenging, especially in CF patients which can result in delayed or false diagnosis (Knutsen and Slavin 2011). Symptoms are overlapping with general conditions in CF patients and serologic findings like IgE antibodies are not discriminating between sensitization and the acute disease and severity of ABPA. The description of *A. fumigatus* specific Th cells could provide new and more detailed insights in the immunological mechanisms and pathways in the host-fungus interaction. To detect and analyze these rare cells, Bacher et al. developed a method that allows direct *ex*

vivo characterization via indirect magnetic labelling of CD154, a surface marker for activated CD4+ T cells (Bacher et al. 2013).

The enrichment allows subsequent in-depth evaluation of the phenotype and effector functions of all Th cells which reacted to the antigen of interest with high sensitivity and specificity. With the characterization of different Th cell responses, induced by *A. fumigatus,* Th cell analysis could be used to define different host-pathogen interaction states and as a tool in diagnosis and follow up on *A. fumigatus* related diseases. For more precise information, fungal Th cell protein targets need to be defined with a special interest in Th2 targets as ABPA occurs mostly in individuals with prior sensitization.

3.1 Differences in T cell response regarding clinical characteristics

For a better understanding of the immune response against *A. fumigatus*, the enrichment of *A. fumigatus* specific CD4+ T cells was performed with PBMCs of CF patients and a healthy control group. To analyze Th cell characteristics reflecting sensitization and ABPA, the patients were divided into four groups depending on their *A. fumigatus* specific IgE level (cut-off 0,35 kU/l) and whether they were in an active form of disease (acute or chronic-recurrent/subacute ABPA) or had history of ABPA (table 18). Acute ABPA was defined according to the consensus guideline (Stevens et al. 2003). Chronic-recurrent/subacute ABPA covered patients with a relapse of ABPA within two months after the end of treatment and receiving an efficient prophylactic treatment with corticosteroids (0,5-1 mg/kg bodyweight) and anti-fungal medication for at least three months and history of ABPA defined patients without treatment for over 6 months and without recurrence. *A. fumigatus* specific CD4+ T cells were analyzed for memory or naïve phenotype and effector cytokine production among memory cells. The included parameters covered the major Th cell groups: IFNγ representing Th1 cells, IL-17A for Th17 cells and IL-4, IL-5 and IL-13 for the characterization of the Th2 response. Additionally, production of the inflammatory cytokine GM-CSF was analyzed as well as the frequency of Ki-67 as a marker of recently activated cells.

Table 18: Overview of patient groups

IgE <0,35 kU/l	IgE >0,5 kU/l	Acute/subacute ABPA	History of ABPA
49 patients	27 patients	16 patients	4 patients

3.1.1 Frequencies of *A. fumigatus* reactive CD4+ T cells and distribution of memory T cells

The activation marker CD154 is expressed by Th cells after activation of the TCR with their specific antigen. The CD154+ frequency and the fraction of memory and naïve cells within the CD4+ T cells can provide first insights about the immune status of an individual. As shown in Figure 4A neither CD154+ nor memory T cell frequencies are showing statistically significant differences between the groups, although there is a trend for higher memory Th cell frequencies in the CF patients compared to healthy controls. The general number of memory T cells is influenced by multiple factors, especially age and thereby differs inter-individually. To bypass these influences, the number of *A. fumigatus* specific memory cells was set in ratio to the general amount of memory Th cells of an individual. This so-called memory enrichment factor shows the relative enrichment of specific memory cells compared to the total CD4+ memory compartment (Fig. 4B). CF patients show a higher memory enrichment factor than healthy donors, with the highest enrichment in the CF patients with acute/subacute ABPA as an immunological sign for a strong immune reaction *in vivo*. The reactivity of these cells can also be seen in the increase of the Ki-67 frequency (Fig. 4C), as a marker for activation and proliferation.

3.1.2 Effector cytokine expression can be correlated to clinical parameters

For more detailed information about the Th cell reaction *in vivo*, the memory cells were analyzed for their cytokine release (Fig 5). CF patients without sensitization to *A. fumigatus* and the healthy controls show a similar cytokine pattern with a pre-dominant IFNγ response and rather low GM-CSF production. In contrast to healthy individuals, increased levels of IL-17A were detected in all CF patient groups, confirming previous results (Bacher et al Cell 2019). As *A. fumigatus* is not a typical Th17-inducing pathogen, the increase of the IL-17A production in the patient cohort is likely due to cross-reactivity to *C. albicans* and reflects an outgrowth of such cross-reactive T cells due to the constant pathogen confrontation in CF patients, as it has been recently shown (Bacher et al. 2019).

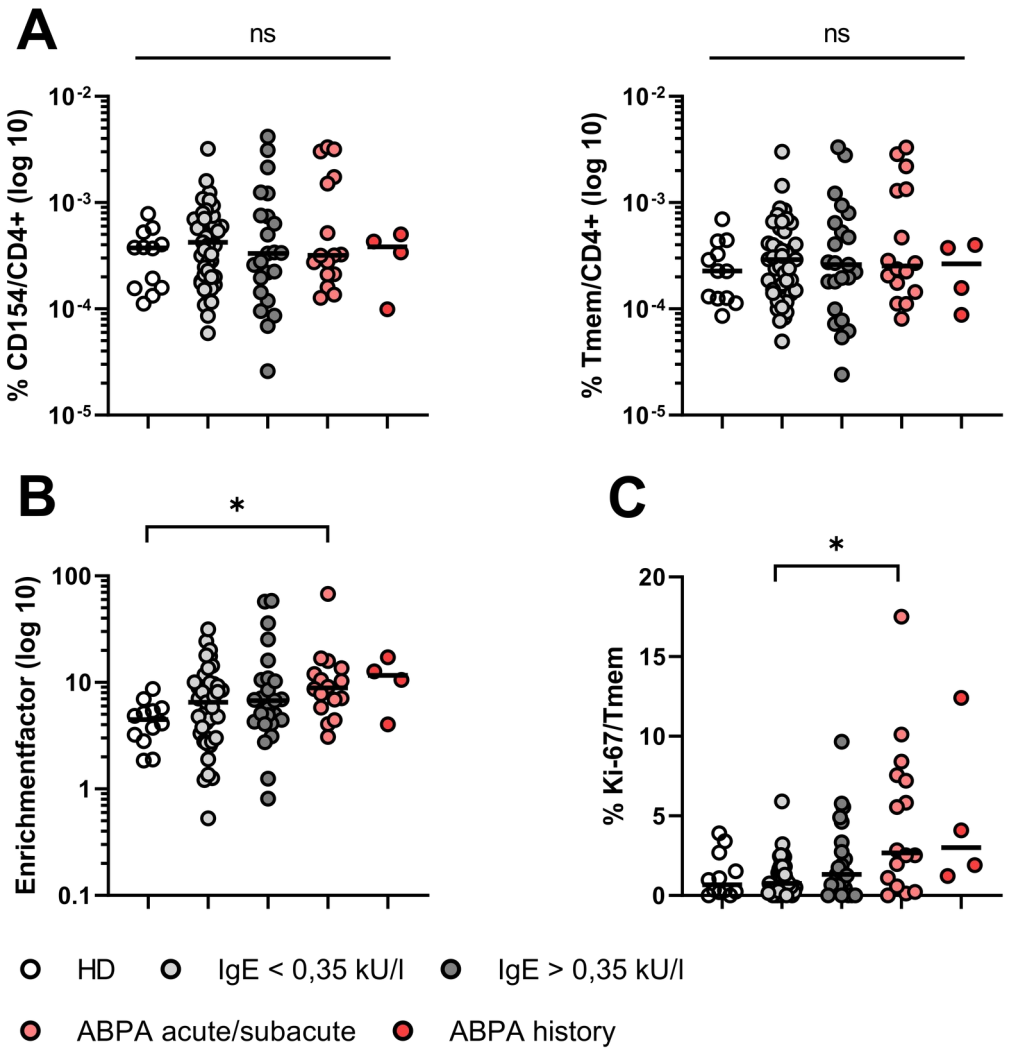

Figure 4: ***A. fumigatus* specific CD4+ T cell responses in healthy donors and CF patients.** Left to right: healthy donors and CF patients divided in the following 4 groups: patients with no sensitization to *A. fumigatus* (indicated by specific IgE levels below 0,35 kU/l), CF patients with sensitization to *A. fumigatus* (indicated by specific IgE levels above 0,35 kU/l), CF patients with acute or subacute ABPA and CF patients with history of ABPA. **A**: frequencies of CD154+ cells memory T cells among CD4+ T cells on a logarithmic scale **B**: enrichment factor of memory T cells on logarithmic scale **C**: frequency of Ki-67 among memory T cells

Horizontal bars are medians. Statistical analysis was performed with Kruskal-Wallis-Test ($p < 0,0001$ ****, $p < 0,001$ ***, $p < 0,01$ **, $p < 0,1$ *).

As expected, the frequencies for Th2 cytokines are very low in healthy controls and patients without sensitization. In contrast, *A. fumigatus* sensitization is reflected by a stronger Th2 cell response: CF patients with a specific IgE level > 0,35 kU/l or ABPA show increased levels of IL-4 in comparison to non-sensitized patients which is in line with previous results (Bacher et al. 2014a; Bacher et al. 2016). Furthermore, slightly higher levels of IL-5 and IL-13 are detected in individual patients (Fig. 5B). Interestingly, the acute/subacute ABPA group can be further distinguished from the

sensitized patients by taking the whole cytokine pattern into account. The Th2 reaction is strongly enhanced as shown by the increased frequencies of IL-4, IL-5 and IL-13 and the decrease of IFNγ production. Additionally, the highest GM-CSF levels can be found in this group. The patients with history of ABPA show heterogeneous results, which is likely due to the low number of analyzed patients.

In summary, the clinical status can be related to specific Th cell characteristics, like Th1 and Th2 cytokine production. Interestingly, there seems to be a connection between the expression of IL-4, IL-5 and IL-13 and GM-CSF that requires further analysis.

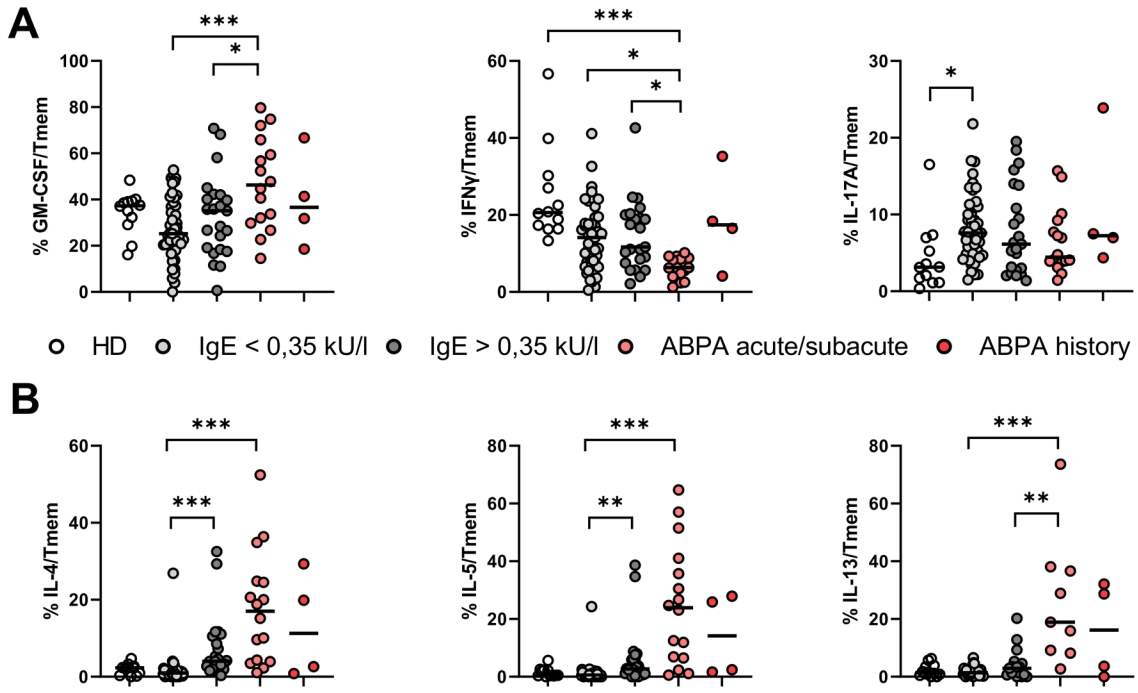

Figure 5: **Cytokine release of *A. fumigatus* specific CD4+ T cells in healthy donors and CF patients**. Left to right: healthy donors and CF patients divided in the following 4 groups: patients with no sensitization to *A. fumigatus* (indicated by specific IgE levels below 0,35 kU/l), CF patients with sensitization to *A. fumigatus* (indicated by specific IgE levels above 0,35 kU/l), CF patients with acute or subacute ABPA and CF patients with history of ABPA. **A**: frequencies of GM-CSF, IFNγ and IL-17A within the memory T cell compartment **B**: frequencies of the Th2 cytokines IL-4, IL-5 and IL-13 among memory T cells

Horizontal bars are medians. Statistical analysis was performed with ANOVA or Kruskal-Wallis-Test ($p < 0,0001$ ****, $p < 0,001$ ***, $p < 0,01$ **, $p < 0,1$ *).

3.2 A proposition of two immunological subgroups in cystic fibrosis patients

To get a better understanding about the possible connections and influences between the Th cell response and *A. fumigatus* associated diseases, a more detailed analysis of

these reactions was performed. Special interest was given to the Th2 response to analyze whether cytokine release patterns could be used for a more precise discrimination of the different patient groups with sensitization to *A. fumigatus* or ABPA.

3.2.1 IL-4 high and IL-4 low responders show distinct cytokine profiles

To analyze the Th2 response induced by *A. fumigatus*, patients were classified based on the production of IL-4, the signature cytokine of the Th2 response and marker for *A. fumigatus* sensitization and ABPA (Bacher et al. 2014a; Bacher et al. 2016). A frequency below 15% was considered to be low and a production of more than 15% IL-4 as high frequency (Bacher et al. 2019). Next, the expression of other cytokines in the IL-4 high and low responders was analyzed (Fig. 6A). Strikingly, high frequencies of IL-4 were associated with high GM-CSF production, especially in the ABPA patients. Interestingly direct co-staining of IL-4 and GM-CSF revealed that IL-4 was mainly co-produced to GM-CSF (Fig. 6B). In contrast, the production of IFNγ and IL-17A were elevated in the IL-4 low responders. The increase of GM-CSF in the group of IL-4 high responders suggests a connection between the intensity of the Th2 response and the release of GM-CSF. Additionally, it is raising the question if this phenotype indicates a more pathogenic Th2 response which contributes to the development of ABPA in *A. fumigatus* sensitized CF patients as most patients with acute/subacute ABPA show this pattern.

3.2.2 Description of Th2 vs Th1 phenotype in GM-CSF secreting *A. fumigatus* specific Th cells

To analyze the connections between the different Th cell fates and GM-CSF production, the cytokine expression profile of GM-CSF+ Th cells was analyzed. This characterization clearly revealed two subgroups of GM-CSF secreting cells (Fig. 6C and D). Whereas one group showed a pre-dominant IFNγ response and low levels of Th2 cytokines, the other group included patients that presented a clear Th2 cytokine profile. Patients were considered as Th2-GM-CSF+ when the frequencies of IL-4, IL-5 or IL-13 exceeded 15% (Bacher et al. 2019). Interestingly, all patients but one diagnosed with ABPA were contained in the second group, thereby including the majority of patients with ABPA, even those with low Th2 cytokine production among memory T cells. The other patients in the Th2-GM-CSF+ group were either sensitized to *A. fumigatus* or had history of ABPA. Only two patients in this group did not show serological signs of sensitization.

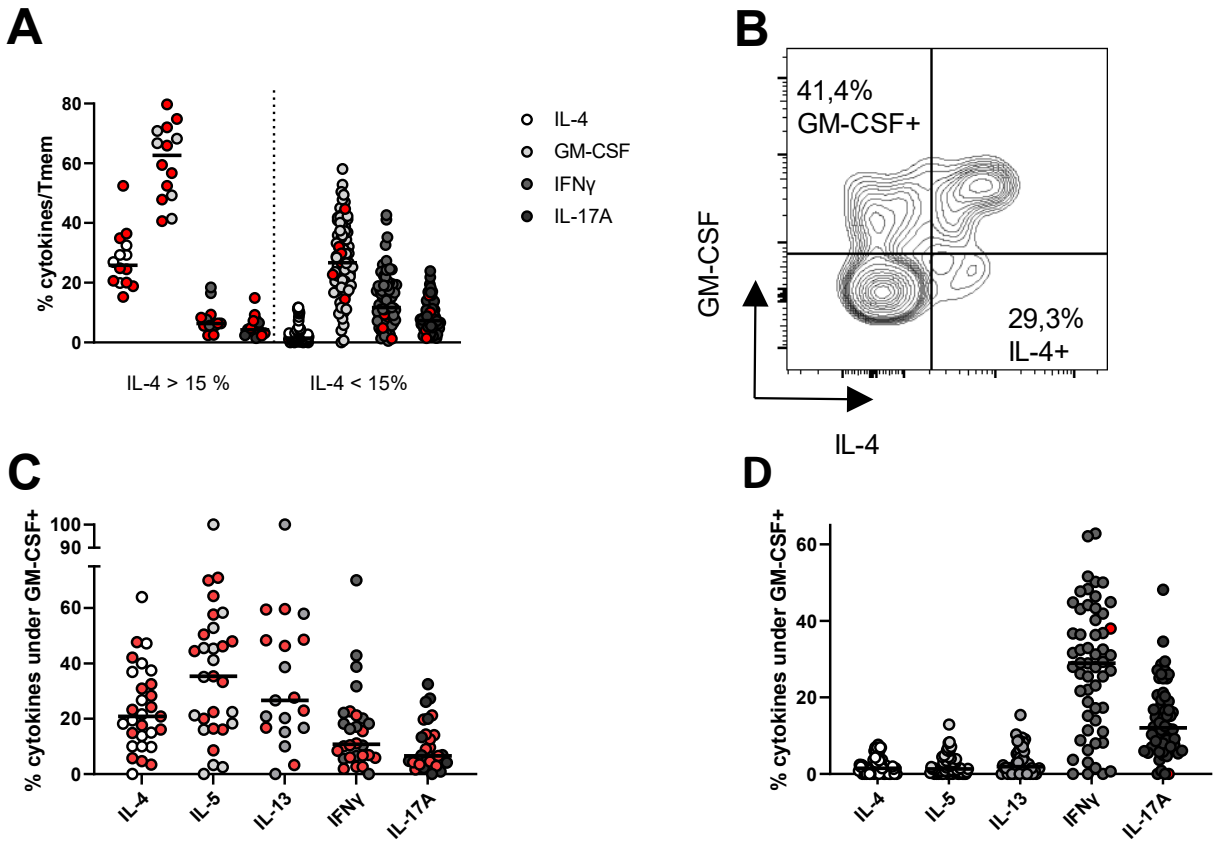

Figure 6: **Subgroups in CF patients according to GM-CSF and Th2 cytokine release. A**: frequencies of IL-4, GM-CSF, IFNγ and IL-17A among memory T cells depending on high (more than 15%) or low (less than 15%) IL-4 frequencies in cystic fibrosis patients. **B**: exemplary dot plot for co-production of GM-CSF and IL-4. **C** and **D**: cytokine expression profile of Th2-GM-CSF+ (C) and Th1-GM-CSF+ (D).

For figures 6 A, C, D: red dots indicate patients with acute/subacute ABPA.

In summary, CF patients can be classified according to the intensity of the Th2 response, indicated by the IL-4 frequencies. A strong Th2 reaction is associated with an increase of GM-CSF production. Analysis of GM-CSF secreting cells shows two subgroups with Th2 or Th1 phenotype, respectively. As most of the ABPA patients are contained in the Th2-GM-CSF+ group, these cells might be pathological Th2 cells although they can be found in patients without ABPA as well. A follow-up on these patients would be interesting, to see whether the Th cell phenotype is stable or if the patients are at risk of developing ABPA in the future. As GM-CSF is also produced by IFNγ expressing Th cells which are found in most patients without ABPA or sensitization to *A. fumigatus*, the role of GM-CSF might be depending on the immune status of the host and the pre-existing cytokine milieu.

3.3 Characterization of *A. fumigatus* Th2 targets

Another interest of this project was to find out whether it is possible to identify further proteins of *A. fumigatus* which serve as dominant Th cell targets, especially for Th2 cells. The complex proteome of the whole fungal lysate presents a great variety of proteins and therefore, immune-dominant Th cell targets of *A. fumigatus* are so far poorly characterized. Additionally, the use of a defined pool of fungal proteins would likely increase the reproducibility of the results inter- and intra-individually and might help to find more sensitive and specific immunological markers for diagnosis and treatment options as next steps. It could already be shown that proteins of the active growing fungus elicit stronger Th cell reactions than those of the resting conidia (Bacher et al. 2014b). Mycelia-derived proteins were subdivided into two pools, based on their previous described capability to reactivate expanded *A. fumigatus* specific Th2 cell lines from CF patients (Bacher et al. 2016). The “Th2 target” protein pool contained the proteins CatB, Asp f2, CpcB, Asp f3, Fg-gap and the “non Th2 target” pool consisted of Crf1, Shm2, Sod3, Asp f22, Scw4, TpiA, GliT, Pst1, Gel1. Patients were classified as “allergic” and “not allergic” depending to the *A. fumigatus* specific IgE levels (cut-off 0,35 kU/l) to see if the Th cell response would differentiate according to the prior host fungus interaction.

3.3.1 Th cell response depends on the protein pool and sensitization status of the patients

To get a first impression of the Th cell reaction towards the two protein pools, frequencies of CD154+ cells and memory Th cells within the CD4+ cells were analyzed. Moreover, the enrichment factor was calculated and the memory cells were analyzed for expression of the proliferation marker Ki-67 (Fig. 7). The distribution of activated and memory T cells was similar for the two protein pools in both patient cohorts, but overall lower frequencies were detected for the protein pools than for the whole fungal lysate. These findings show that both pools contain proteins that induce memory Th responses in CF patients, but do not cover all T cell target proteins contained in the *A. fumigatus* lysate. Interestingly, allergic CF patients also show a trend for higher enrichment factors compared to non-allergic patients in response to the “non Th2 target” protein pool. No differences in Ki-67 expression were seen between both protein pools.

Next, the phenotype of the memory Th cells was analyzed. Interestingly, the cytokine expression exhibited differences between the patient groups and the protein pools. For the “non-Th2 target” pool, both patient groups show a predominant IFNγ/IL-17A phenotype with low frequencies for the Th2 cytokines, supporting the idea that those proteins do not have allergenic potential. The responses to the “Th2 target” protein pool looks differently for the patient groups (Fig.8).

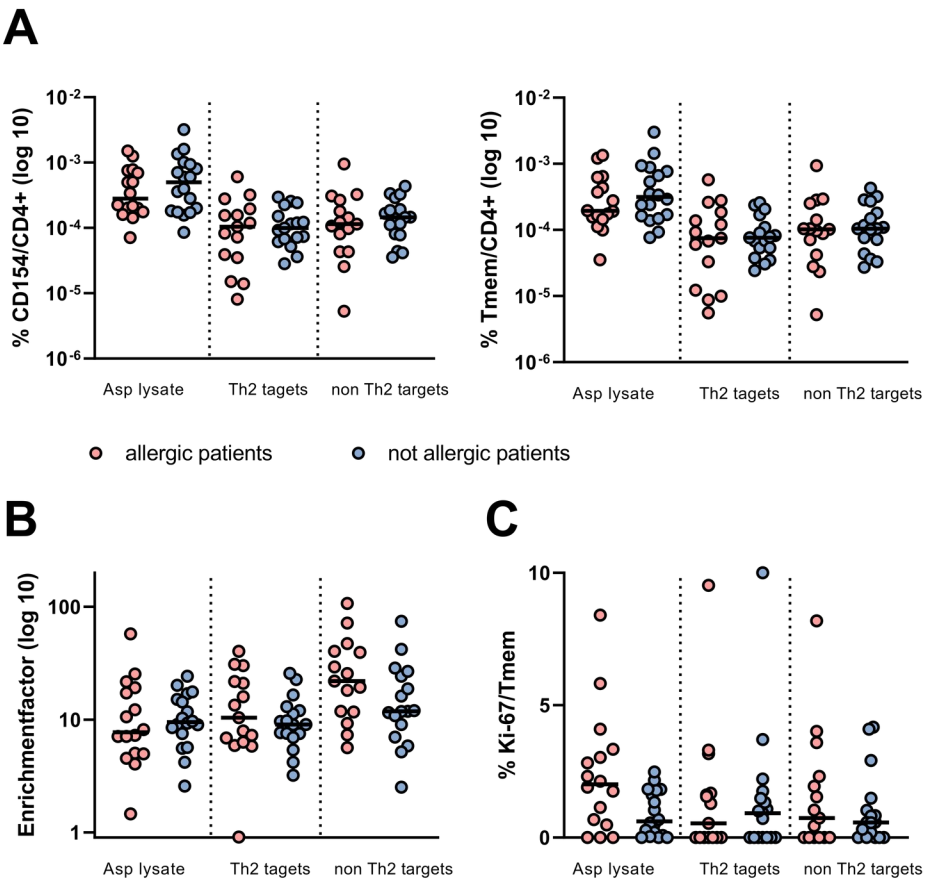

Figure 7: **CD4+ T cell responses against *A. fumigatus* lysate and two *A. fumigatus* protein pools.** The protein pools are defined as “Th2 targets” and “non Th2 targets”. Patients are grouped into “allergic” and “not allergic” depending on their *A. fumigatus* specific IgE level (cut-off 0,35 kU/l). **A**: frequencies of CD154+ cells memory T cells among CD4+ T cells on a logarithmic scale **B**: enrichment factor of memory T cells on logarithmic scale **C**: frequency of Ki-67 among memory T cells

Whereas the cytokine release of the patients without sensitization is similar to the response towards the “non Th2 target” pool, the allergic cohort shows a clear Th2 phenotype against the “Th2 target” proteins. High frequencies for IL-4, IL-5 and IL-13 are accompanied by a decrease in the IFNγ and IL-17A production as well as an increase for GM-CSF producing memory cells. These reactions are reflecting the response of the allergic patients towards the whole fungal lysate. Importantly, whereas

only 50% of the allergic patients react with a Th2 response to the whole lysate, 12 out of 15 allergic patients (80%) are responding with increased IL-4, IL-5 and IL-13 frequencies to the “Th2 target” pool (Fig. 8B). The three remaining patients present increased IFNγ/IL-17A production to the “Th2-target” pool, demonstrating that the ongoing search for other potential Th2 targets is necessary. Furthermore, some allergic patients show a Th2 response towards the “non Th2 targets”, emphasizing the complexity of the immune response in general and indicating that the classification of “Th2 target” and “non Th2 target” proteins might not be true for all patients due to inter-individual differences.

It could be shown that the previously described proteins are memory Th cell targets in CF patients, irrespectively of their sensitization status. The increase of Th2 cytokines in most of the allergic patients as reaction towards the potential “Th2 target” pool supports the idea of a few proteins which serve as major allergens in *A. fumigatus*. Some allergic patients show only a Th2 response towards the proteins, but not the whole lysate, indicating a more accurate imaging of the processes *in vivo* when using the proteins. Thus, the investigation of more proteins with allergic potential is required to detect even more patients.

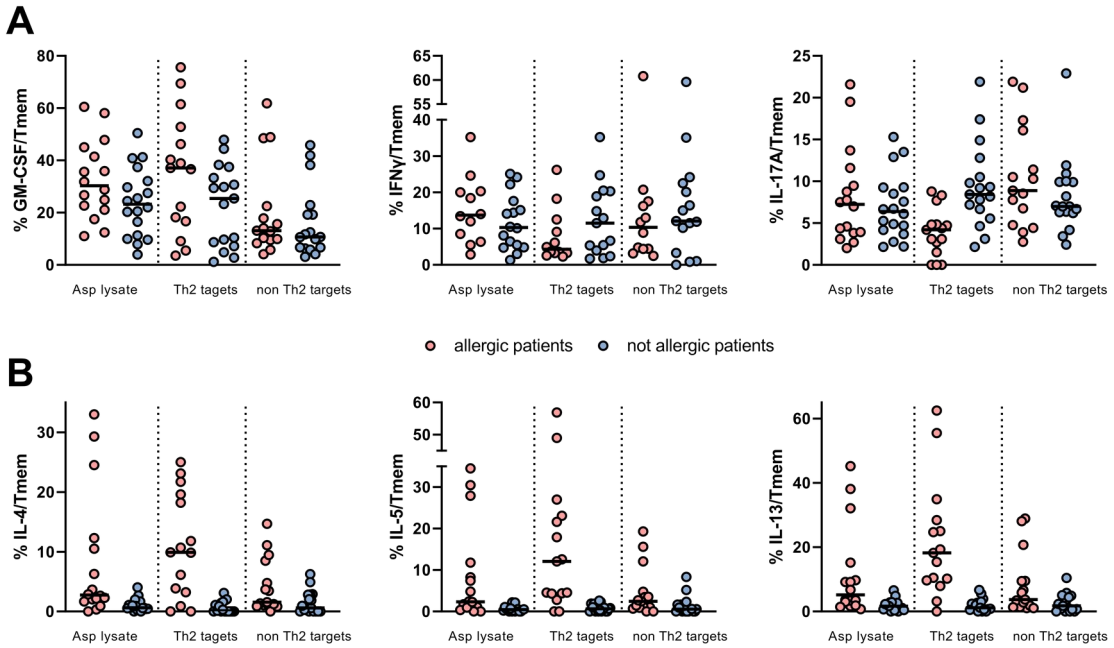

Figure 8: **Cytokine release of CD4+ T cells in response to *A. fumigatus* lysate and two *A. fumigatus* protein pools.** The protein pools are defined as “Th2 targets” and “non-Th2 targets”. Patients are grouped into “allergic” and “not allergic” depending on their *A. fumigatus* specific IgE level (cut-off 0,35 kU/l). **A**: frequencies of GM-CSF, IFNγ and IL-17A within the T memory cell compartment **B**: frequencies of the Th2 cytokines IL-4, IL-5 and IL-13 among memory T cells

3.4 Screening for possible new Th cell targets

To identify potential further *A. fumigatus* T cell target proteins, the Th cell reaction of healthy donors and CF patients with or without sensitization to *A. fumigatus* to three additional *A. fumigatus* proteins was analyzed. PBMCs were stimulated with the whole *A. fumigatus* lysate and the proteins CsnB, Aspf4 and ScwA. CsnB is a chitosanase, an enzyme that utilizes chitosan, a derivate of chitin which is described to be an important part of the fungal cell wall (Beck et al. 2014; Baker et al. 2007). Aspf4 is described as an *A. fumigatus* allergen which has been suggested to elicit increased IgE-reactivity in ABPA patients (Kurup et al. 2006). ScwA (small cell wall peptide A) is reported to be a highly abundant small *A. fumigatus* surface protein which is involved in fungal stress regulation (Blango et al. 2020). After a stimulation period of 7 hours, the cells were processed according to the ARTE procedure and afterwards analyzed for phenotype and effector function. Figure 9A shows exemplary dot plots of a Th cell response from an allergic CF patient with the unstimulated condition as negative and the *A. fumigatus* condition as positive control. The strongest reaction can clearly be seen for the protein CsnB, whereas only few (Aspf4) to no (ScwA) CD154 positive cells was detected after stimulation with the other proteins. This can also be seen in the summarized data for frequencies of CD154+ and memory Th cells among CD4+ cells (Fig. 9B). For the calculation of the frequency of CD154+ among CD4+ cells, the background activation was subtracted. ScwA induced the lowest overall frequencies, but especially for the allergic CF patients. Interestingly, in those donors with a detectable Th response after subtraction of the background, the enrichment factor indicates that all three proteins are strong memory targets. In the allergic cohort, Ki-67 production can be noted for all three proteins as a marker of activation. For further characterization of the Th cell response, cytokine expression of the memory Th cells was analyzed (Fig. 10). As expected, the overall cytokine expression for ScwA was very low. For Aspf4, the levels of GM-CSF, IFNγ and IL-17A are slightly increased in all three cohorts. Some of the allergic patients also react with Th2 cytokine production against Aspf4, but the majority does not react. The most interesting protein is CsnB as it provokes Th cell responses which are mimicking the reaction to the whole *A. fumigatus* lysate. Healthy donors and not allergic CF patients are responding with IFNγ and IL-17A production against CsnB while there is a clear increase of Th2 cytokines in the allergic cohort.

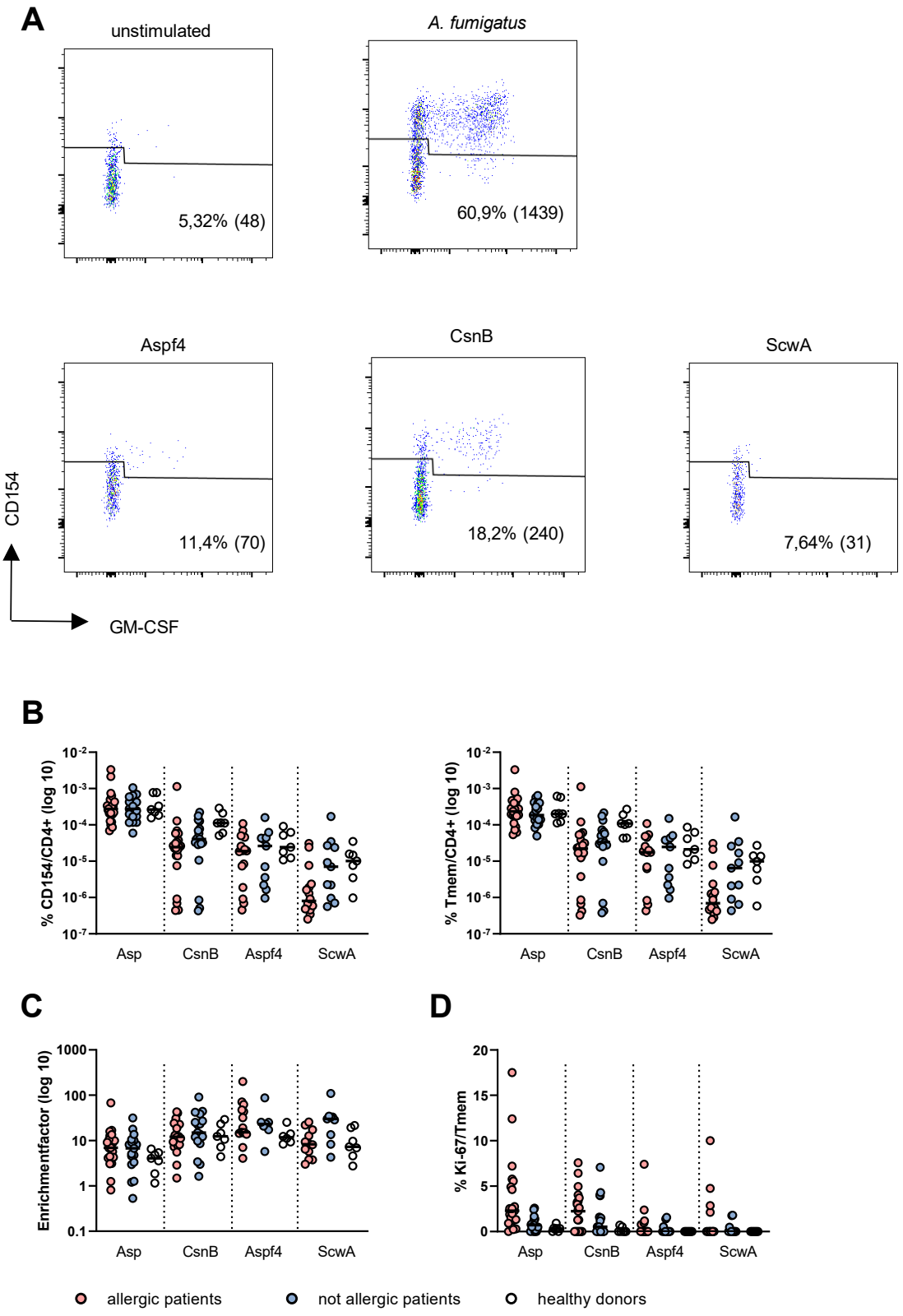

Figure 9: **CD4+ T cell responses of CF patients and healthy controls towards the whole *A. fumigatus* lysate and three *A. fumigatus* proteins (CsnB, Aspf4 and ScwA).** Patients are grouped into “allergic” and “not allergic” depending on their *A. fumigatus* specific IgE levels (cut-off 0,35 kU/l). **A**: exemplary dot plots from an allergic CF patient. Percentages of parental gate and cell number (in brackets) are written in the plots. **B**: frequencies of CD154+ and memory T cells among CD4+ T cells on logarithmic scale **C**: enrichment factor on logarithmic scale **D**: Ki-67 frequency among memory cells

Similar to the “Th2 target” protein pool, some allergic patients are only reacting to the protein, but not the lysate, underlining the importance of knowing the Th2 targets for more specific diagnosis. These results indicate that CsnB can be a target for different Th subsets as it can induce a Th1/Th17 response and a Th2 reaction in predisposed patients.

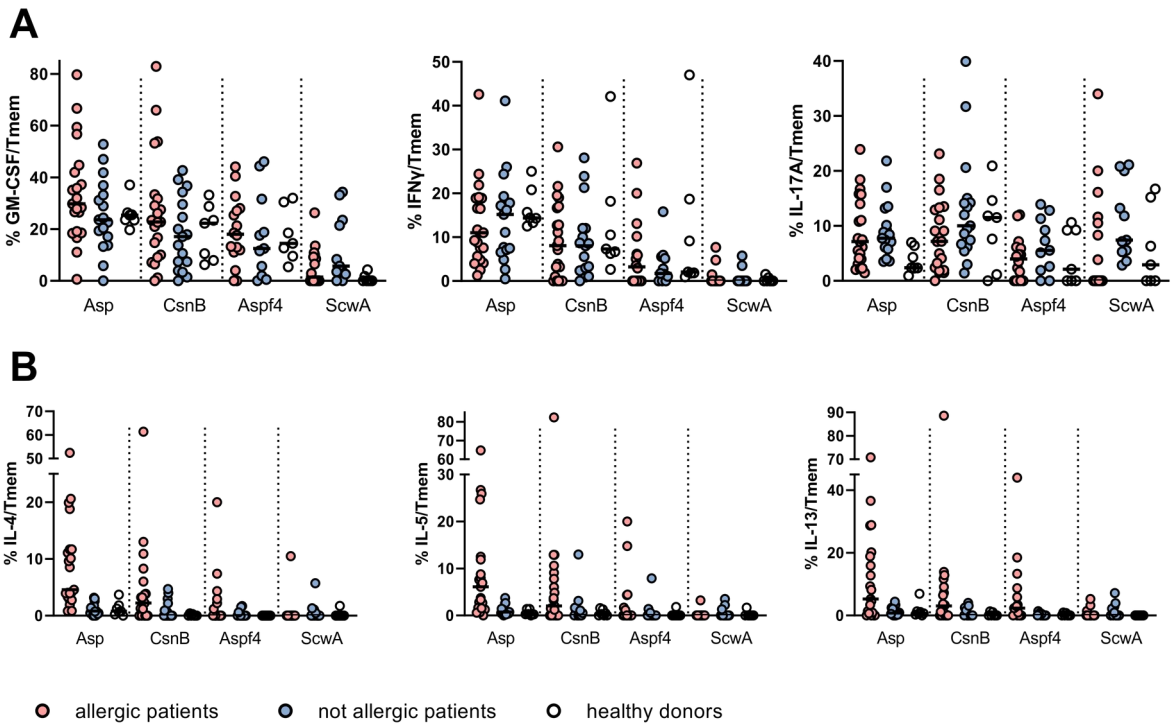

Figure 10: **Cytokine release of CD4+ T cells of CF patients and healthy controls in response to the whole *A. fumigatus* lysate and three *A. fumigatus* proteins (CsnB, Aspf4 and ScwA).** Patients are grouped into “allergic” and “not allergic” depending on their *A. fumigatus* specific IgE levels (cut-off 0,35 kU/l). **A**: GM-CSF, IFNγ and IL-17A frequencies among memory T cells **B**: Th2 cytokines among memory T cells

3.5 Results of T cell expansion

To further characterize the Th cell response to CsnB, protein-specific and *A. fumigatus* specific memory Th cells were expanded and re-stimulated after 3 to 4 weeks with PMA/ionomycin as a positive control*, C. albicans*, *A. fumigatus* and CsnB. Additionally, a titration of the protein and the whole fungal lysate was performed to analyze the functional affinity of the cells. Expansion and re-stimulation were done with cells of healthy donors and CF patients to see potential differences in the reactions. The reactivity upon re-stimulation is indicated by the frequency of CD154+/TNFα+ cells among CD4+ cells. The *A. fumigatus* specific cells of the healthy donors and the CF patients show similar frequencies after re-stimulation with PMA/ionomycin, *A. fumigatus* and CsnB. For *C. albicans*, there is a higher cross-reactivity within the CF patients (Fig. 11B). *C. albicans* is in contrast to *A. fumigatus* a strong Th17 inducer (Bacher et al. 2019) and there is an increase for IL-17A towards *A. fumigatus* in the CF patients (ARTE results 4.1.2), indicating a preferential expansion of *A. fumigatus*-*C. albicans* cross-reactive T cells in CF patients (Bacher et al. 2019). Upon re-stimulation, the cells were also evaluated for cytokine production. For both groups, the cells show pre-dominantly IFNγ production and IL-17A production to a lesser extent. Surprisingly, the IL-4 frequencies are low for all analyzed individuals (Fig. 11D and E) although 2 of the CF patients presented with a Th2 phenotype towards the *A. fumigatus* lysate in the ARTE experiments. The results for the CsnB specific cells are similar. Although, the expanded cells re-reacted against CsnB, the reactivity to the original concentration of the protein is rather low in both groups (Fig. 11C). The CsnB specific cells of the healthy donors show IFNγ and IL17A production after re-stimulation with CsnB, indicating that the cells conserve the original phenotype. In one of the CF patients, the re-stimulation of the protein-specific cells with CsnB elicits an IFNγ response without expression of IL-17A whereas the cells of the other two patients are not reacting with cytokine release (Fig.11E).

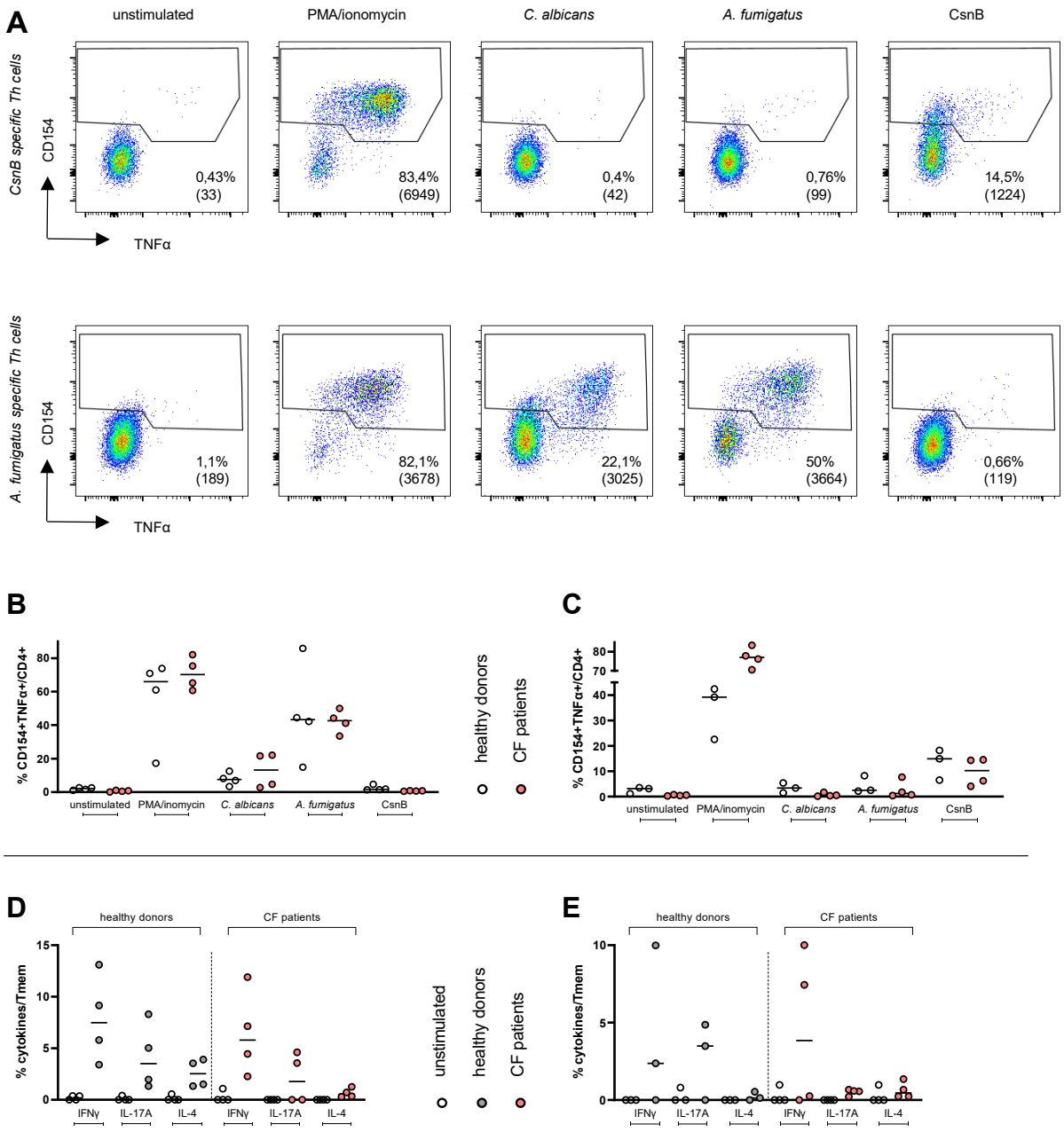

Figure 11: **Reactions of antigen specific Th cells after re-stimulation. A**: exemplary dot plots showing reactions of CsnB specific and *A. fumigatus* specific memory Th cells after re-stimulation with PMA/ionomycin, *A. fumigatus*, *C. albicans* and CsnB and an unstimulated control. Plotted are CD154+ cells against TNFα+ cells. Percentages and cell numbers (in brackets) are written in the plots. **B**: frequencies of CD154+/TNFα+ *A. fumigatus* specific cells among CD4+ T cells for CF patients and healthy donors after re-stimulation with PMA/ionomycin, *C. albicans*, *A. fumigatus*, CsnB and an unstimulated control. **C**: frequencies of CD154+/TNFα+ CsnB specific cells among CD4+ T cells for CF patients and healthy donors after re-stimulation with PMA/ionomycin, *C. albicans*, *A. fumigatus*, CsnB and an unstimulated control. **D** and **E**: frequencies of IFNγ, IL-17A and IL-4 of *A. fumigatus* (D) and CsnB (E) specific memory Th cells after re-stimulation with the specific antigen and an unstimulated control.

The titration experiments also show similar results for the healthy donors and the CF patients with a strong decrease of reactivity for the lower antigen concentrations for CsnB- and *A. fumigatus*- specific Th cells. These findings indicate rather low affinity

which is also shown by comparable levels of the EC50 values (Fig. 12C and 13C). The EC50 describes the amount of antigen which is necessary to provoke a response in 50% of the expanded cells, thereby low values indicate high affinity. To increase validity, titrations would need be performed with more samples of both cohorts as the results are spreading rather widely.

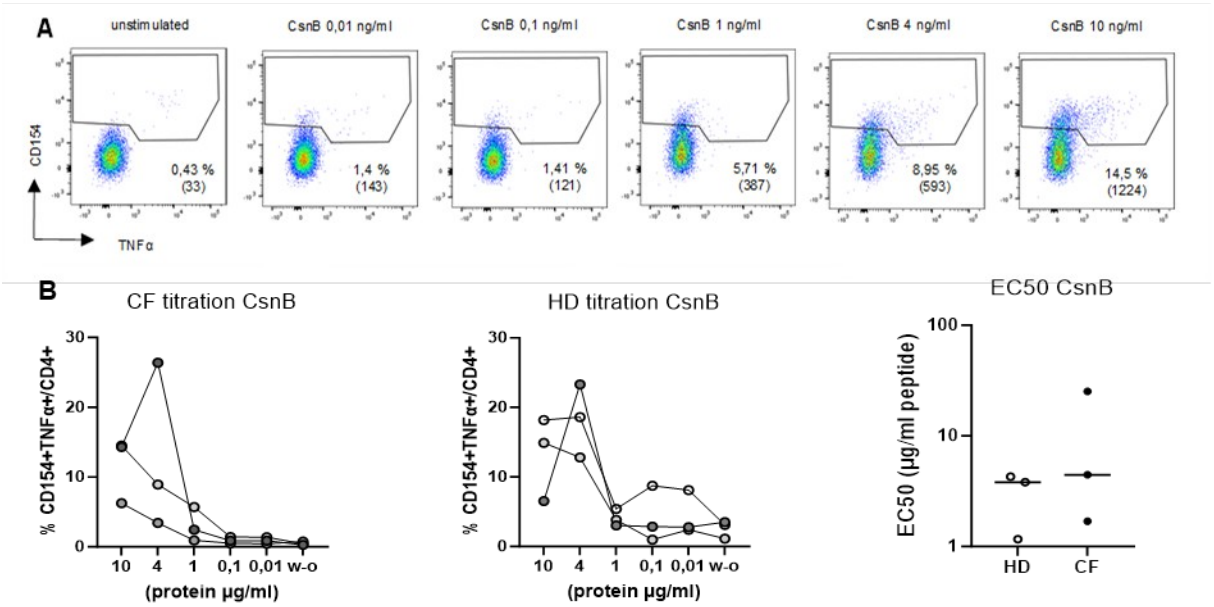

Figure 12 **Reaction of CsnB specific cells to decreasing protein concentrations. A**: exemplary dot plots of a CF patient. Plotted are CD154+ cells against TNFα+ cells. Percentages and cell numbers (in brackets) are written in the plots. **B**: frequencies of CD154+TNFα+ Th cells in response to CsnB titration for 3 healthy donors and 3 CF patients. **C**: EC50 values of healthy donors and CF patients.

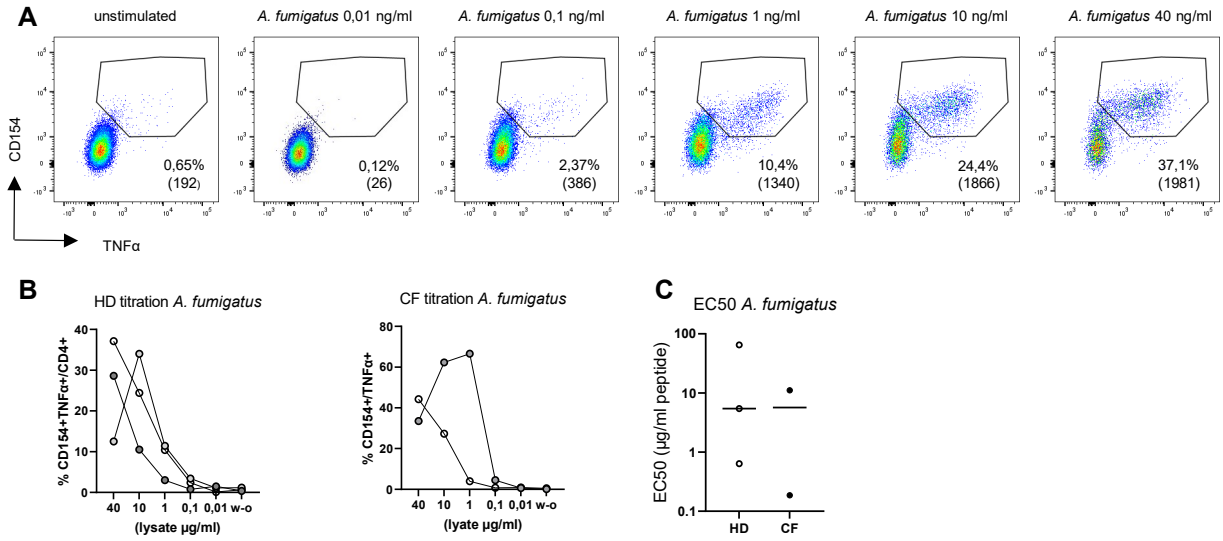

Figure 13: **Reaction of *A. fumigatus* specific CD4+ cells to decreasing concentrations of the lysate.** **A**: exemplary dot plots of a healthy donor. Plotted are CD154+ cells against TNFα+ cells. Percentages and cell numbers (in brackets) are written in the plots. **B**: frequencies of CD154+TNFα+ Th cells in response to *A. fumigatus* titration for 3 healthy donors and 2 CF patients. **C**: EC50 values of healthy donors and CF patients

4 Discussion

4.1 GM-CSF and the pathological Th2 response

CF patients are frequently sensitized to *A. fumigatus*. Allergic reactions to *A. fumigatus* form a spectrum of different diseases ranging from IgE-mediated sensitization to the severe allergic complication ABPA. The spectrum of clinical manifestations caused by *A. fumigatus* suggests different fungal-host-interactions, but the types of anti-*A. fumigatus* immune reactions and the extent to which they influence CF pathology are poorly understood. In this thesis, the specific Th cell response against *A. fumigatus* was analyzed, which enabled to sub-classify patients according to different reactivity patterns. *A. fumigatus* sensitized patients could clearly be detected based on the increase of the Th2 cytokines IL-4, IL-5 and IL-13 within *A. fumigatus* specific CD4+ T cells. Patients with acute/subacute ABPA show a trend for higher frequencies of Th2 cytokines than sensitized patients without ABPA. Strikingly, strong Th2 reactions are associated with high GM-CSF secretion. Further analysis of GM-CSF secreting cells allowed the classification of two subgroups of patients with a Th1 or Th2 cytokine profile, respectively. All but one patient diagnosed with ABPA were contained within the GM-CSF+Th2 subgroup. These results suggest that GM-CSF is involved in a more pathological Th2 response towards *A. fumigatus* which might contribute to the development of ABPA. Interestingly, the pathogenic potential of GM-CSF is already described for other Th cell subsets like Th1 and Th17 cells (McGeachy 2011; Yamada et al. 2017; Codarri et al. 2011). In general, Th cells are described as the main producers of the inflammatory cytokine GM-CSF which serves as an important mediator between lymphocytes and myeloid cells (Becher et al. 2016; Croxford et al. 2015). GM-CSF has been shown to be important for the development of alveolar macrophages in the lung (Guilliams et al. 2013). However, it is found in increased concentrations in various sites of infection (Kay et al. 1991; Carrieri et al. 1998). Especially in chronic inflammation, the repetitive release of GM-CSF might be detrimental for the organism as it potentially leads to continuous activation of phagocytes and granulocytes as “executive” cells which cause tissue damage (Becher et al. 2016). Several studies suggest that GM-CSF is crucial in pulmonary allergic disorders (Nobs et al. 2021; Llop-Guevara et al. 2014). Llop-Guevera et al. could show that in the presence of GM-CSF lower concentrations of house dust mite (HDM) are

sufficient for sensitization leading to long-lasting memory reactions (Llop-Guevara et al. 2014). Another suggested role for GM-CSF is the mediation of eosinophilic inflammation in allergic airway inflammation (Nobs et al. 2019). Thus, these reports support a role for GM-CSF in priming and maintaining the allergic immune response in the lung. Other groups described IL-5 as the main driver of pathogenic Th2 reaction in allergic diseases, thereby maintaining eosinophilic inflammation and structural re-organization (Upadhyaya et al. 2011; Nakayama et al. 2017; Hirahara et al. 2021). Interestingly, in the *A. fumigatus* specific GM-CSF secreting cells, IL-5 was in general expressed in higher frequencies than IL-4 or IL-13. This suggests a pathogenic Th2 phenotype, characterized by high levels of GM-CSF and IL-5, in addition to IL-4 and IL-13 secretion.

Previous reports show that both, GM-CSF and IL-5, are increased in the presence of IL-33 (Llop-Guevara et al. 2014; Endo et al. 2015). IL-33 is considered as a member of the “alarmins” which are produced by non-lymphoid cells like epithelial and endothelial cells as well as smooth muscle cells in the bronchial system in reaction to stress and non-apoptotic cell death (Préfontaine et al. 2009; Préfontaine et al. 2010). The membrane bound receptor for IL-33, ST2, is expressed by a variety of cells of the innate and the adaptive immune system (Schmitz et al. 2005; Griesenauer and Paczesny 2017). ST2 expressing dendritic cells (DCs) and innate lymphoid cells 2 (ILC2) are described to generate a milieu that promotes Th2 differentiation in naïve CD4+ T cells in presence of IL-33 (Rank et al. 2009; Petrova et al. 2020; Chu et al. 2013). Additionally, memory Th2 cells express ST2 and can directly react to IL-33 which is serving as a chemoattractant on the one hand and also influences the cytokine release of the Th2 cells in a pro-allergic/pro-inflammatory manner (Louten et al. 2011; Kearley et al. 2009; Endo et al. 2015). Many reports show a correlation between enhanced IL-33 levels and allergic inflammation, especially in asthma (Lloyd 2010; Cayrol and Girard 2014; Préfontaine et al. 2010), but also for CF (Roussel et al. 2013; Tiringer et al. 2014). Eosinophils express receptors for GM-CSF, IL-5 and IL-33 and are important at different stages of eosinophilic maturation and activation (Rosenberg et al. 2013), thereby contributing to the development and maintenance of allergies and asthma (Asquith et al. 2008). Anti-IL-33 and anti-ST2 antibodies are even evaluated as a novel treatment option for asthma patients (Wechsler et al. 2021; Kelsen et al. 2021). In this thesis, a Th2+GM-CSF+ phenotype of *A. fumigatus*-specific T cells was

identified in CF patients with acute or subacute ABPA. As IL-33 can be connected to both cytokines, it might be involved in the pathogenic pathway of ABPA development. To address this question, the analysis of IL-33 and ST2 expression in lung tissue from *A. fumigatus*-sensitized patients could be performed, as it has been done in asthma animal model (Louten et al. 2011). Further, human Th2 cells could be analyzed with ARTE for expression of ST2 to see whether an increase of the receptor can be correlated to *A. fumigatus* sensitization level and ABPA as the upregulation of ST2 by activated Th2 cells could be shown (Peine et al. 2016; Löhning et al. 1999). A better understanding of these pathways could probably help to illuminate why some CF patients develop ABPA whereas most sensitized patients do not. Another interesting question concerns the patients without ABPA which present with the Th2+GM-CSF+ phenotype. A follow up on these patients would be interesting to see if they are at risk to develop ABPA and to see if the Th cell phenotype is stable. In addition to the pathophysiology, the characterization of pathogenic Th cells could also be interesting regarding therapeutical options as it could already be shown that allergen-specific Th cells decrease in patients that received desensitization treatment (Wambre et al. 2017).

4.2 Influences of soluble and non-soluble proteins on the Th cell fate

The CF lung is confronted with many pathogens on a daily basis. The changed physiology of the lung environment caused by thick mucus production and chronic inflammation allows pathogens like *A. fumigatus* to survive longer after the encounter with the host, leading to growth and maturation. Importantly, further developed fungal states such as germinating conidia or hyphae contain more Th cell epitopes than the resting conidia (Bacher et al. 2014b). However, the precise antigenic targets of *A. fumigatus*-specific T cells remain largely undefined. CF patients with prior sensitization to *A. fumigatus* (indicated by specific IgE levels >0,35kU/l) show a clear Th2 phenotype to a distinct *A. fumigatus* protein pool (CatB, Asp2, Aspf3, CpcB, FgGAP). These proteins are labelled as “Th2 targets” due to previous results showing that these soluble proteins are Tcon inducers with the potential of provoking Th2 responses in allergic individuals. The same study could show that on the other hand particle-associated proteins (CRF1, SHMT, SOD3, Aspf22, Scw4, TpiA, GliT, Pst, Gel1, CcpA,) are Treg targets which successfully suppress Tcon activation (Bacher et al. 2016). Appropriately, these proteins, classified as “non Th2 targets”, do not induce

strong Th2 reactions in the CF cohort. Thus, the development of allergy is not due to an impaired Treg function but caused by different antigen specificity of Th2 and Treg cells. Interestingly, non-allergic and allergic CF patients show similar memory Th cell frequencies in response to the “Th2 target” protein pool, indicating that there has been prior contact *in vivo* in both patient groups, raising the question why some patients develop a Th2 response against these proteins, but others do not. Bacher et al. propose a two-checkpoint model for the development of tolerance vs. allergy. They describe the induction of Tregs for particle-associated proteins after presentation via APCs whereas soluble proteins are not taken up and processed by APCs probably due to low doses, leading to a metastable state of immune ignorance without Treg or Tcon induction. This ignorance state can turn into Th2 activation and allergy development under special genetic and/or environmental circumstances (Bacher and Scheffold 2018). The non-allergic CF patients present a Th cell response towards the “Th2 target pool” with a Th1/Th17 phenotype, indicating that their immune system is no longer in the “ignorance” state and thus protected from Th2 differentiation. This underlines the importance to understand the processes which drive the different Th cell differentiation pathways against individual antigenic proteins. Bronchial epithelial cells (BECs) are the first cells that encounter the potential allergen and additionally soluble proteins might be more stressful for the epithelial barrier than particle-associated proteins. Furthermore, there are many reports describing intense collaboration between epithelial cells and cells of the innate and adaptive immune system in the differentiation of Th2 reactions and the development of allergy. BECs are able to recognize allergens via PRRs leading to the release of alarmins, including IL-33, IL-25 and thymic stromal lymphopoietin, as well as GM-CSF which are described to activate DCs in a “Th2-biased” way (Besnard et al. 2011; Hammad et al. 2009; Sheih et al. 2017; Willart et al. 2012). Another part of the innate immunity that seems to be influenced by epithelial cells after allergen recognition consists of type 2 innate lymphoid cells (ILC2s) which additionally promote Th2 responses (Lambrecht and Hammad 2014). Furthermore, the release of alarmins might directly influence memory Th2 cells as discussed above. Strikingly, the exposure to certain environmental factors like cigarette smoke and viral infections increases the expression of certain allergen recognizing PRRs on epithelial cells, thereby induces the release of cytokines and alarmins directly (Pace et al. 2008; Monick et al. 2003). Thus, environmental factors are lowering the allergen threshold and are contributing to the “allergic cascade” in a supplemental or cumulative pathway.

A rather passive mechanism which could contribute to a higher concentration of allergens is described by Leino et al. as they show an increased permeability of cultured bronchial epithelial cells in asthmatic patients compared to healthy donors after stimulation with *Alternaria species* (Leino et al. 2013). It would be interesting to see, if these results can be extended to *A. fumigatus* sensitization in CF patients. Thus, dysregulated BECs could passively support the encounter of higher allergen doses and can be more sensitive due to prior exposure of certain environmental factors which could influence the “ignorance state” of an individual. Furthermore, the changed physiology in the CF lung favors the longer persistence and maturation of *A. fumigatus*, thereby leading to the exposure of higher fungal concentrations in general and the encounter with more potential allergens which are only expressed by more developed stages of *A. fumigatus*.

In summary we could show that *A. fumigatus* sensitized CF patients show a clear Th2 phenotype towards a pool of secreted *A. fumigatus* proteins which is in line with previous results and supporting the hypothesis that especially soluble particles can serve as allergen inducing Th2 targets. Interestingly, not all patients who reacted with a Th2 response against the analyzed “Th2 target” proteins, show a Th2 response against the whole lysate. An explanation could be that the recognized allergens are present in too low concentrations in the total lysate or that the Th2 response is masked by a higher reactivity against other proteins contained in the total lysate. In any case, this underlines the importance of defining Th2 target proteins. As not all targets are identified yet, further screening for Th2 targets is necessary.

4.3 CsnB – a new *A. fumigatus* Th2 target protein in CF patients

In this study the *A. fumigatus* chitosanase CsnB was identified as a new Th2 target in CF patients. Chitosanases are produced by a variety of bacteria and fungi, but the physiological function in fungi is still unclear. Cell wall recognition, autolysis, cell division and antagonistic interactions between microbes are proposed roles (Beck et al. 2014). CsnB utilizes chitosan, a derivate of chitin which is described to be an important part of the fungal cell wall (Baker et al. 2007; Beck et al. 2014). Contradictory reports describe inflammatory responses towards chitosan whereas others claim chitosan is a molecule which is used as a shield to prohibit the exposure of cell wall structures that would elicit immune responses (Gorzelanny et al. 2010; El Gueddari et al. 2002). Beck et al. identified the encoding gene (AFUA_G01290) and could describe

specific IgG and IgM antibodies in humans as well. As the antibody titers did not differ between healthy controls and patients with invasive aspergillosis, they cannot be used as predictors in invasive *A. fumigatus* related disease (Beck et al. 2014). Although CsnB was already mentioned as a potential new allergen based on an immunoproteomic approach combined with mass spectrometric analysis from *A. fumigatus*-sensitized patients (Gautam et al. 2007) there is so far no description of CsnB specific IgE levels. Our results confirm CsnB to be an *A. fumigatus* allergen as CsnB-specific Th cells produce high levels of IL-4, IL-5 and IL-13 in the allergic CF patients. CsnB is described as one of the major secreted antigens of *A. fumigatus* by immunoblot analysis of sera from patients with invasive aspergillosis or aspergilloma (Schwienbacher et al. 2005) and thereby supporting the hypothesis of soluble proteins as main Th2 targets. We could show strong Th2 responses to CsnB in the allergic CF patients (indicated by *A. fumigatus* specific IgE >0,35 kU/l). Interestingly, non-allergic CF patients as well as healthy donors, showed Th cell responses to CsnB. In accordance with the results for the protein pools discussed above, the not allergic CF group presents a memory Th cell phenotype, accompanied by a predominant Th1/Th17 phenotype. Additionally, the healthy donors show rather strong CsnB-specific responses. However, in difference to the patients, the healthy donors display a pre-dominant IFNγ and/or IL-17A response. *A. fumigatus*-reactive Th17 cell have been shown to by induced by cross-reactivity to *C. albicans* (Bacher et al. 2019). However, in the re-stimulation data of expanded CsnB-specific Th cell cultures, cross-reactivity to *C. albicans* lysate could not be confirmed. This might be due to the low overall reactivity of the expanded cells or the lack of potential CsnB-homologs in the Candida lysate. In any case, it would be interesting to analyze whether a homologous protein exists in other fungal species. In summary, the chitosanase CsnB, one of the major secreted antigens of *A. fumigatus*, is a newly identified Th cell target with the capability to induce Th2 responses in susceptible individuals, but also Th1/Th17 reactions in non-allergic donors.

5 Summary

Patients with cystic fibrosis suffer from a variety of pathogen-related complications due to changed physiology of the patient's lung. One of these problematic pathogens is *Aspergillus fumigatus*, a ubiquitous airborne fungus, that is one of the major allergens in cystic fibrosis patients. Although *Aspergillus fumigatus* associated allergic diseases

are rarely found in healthy individuals, almost 40% of patients with cystic fibrosis develop *Aspergillus fumigatus* sensitization which can lead to a severe complication: acute bronchopulmonary aspergilloses which is challenging to diagnose as it lacks specific diagnostical markers and clinical symptoms are overlapping with general conditions of cystic fibrosis. Importantly, acute bronchopulmonary aspergillosis is described to be a Th2 driven disease and CD4+ T cells are known to be important mediators of the anti-fungal immune response in general, making them a promising diagnostic target. So far, the analysis of antigen-specific Th cells was limited due to their rare frequencies in peripheral blood. A new method, the antigen-reactive T cell enrichment, allows the direct *ex vivo* characterization of activated conventional Th cells by indirect magnetic labelling of the activation marker CD154. This enrichment allows the further in-depth analysis of the phenotype and effector function of the Th cells which are specific for the antigen of interest.

The aim of this thesis was to analyze the CD4+ T cell response to *Aspergillus fumigatus* from cystic fibrosis patients with different history of fungal exposure to identify potential patterns of Th cell reactions and to correlate these results with clinical data. To identify T cell target proteins of *Aspergillus fumigatus*, the antigen-reactive Th cell enrichment was performed with different *Aspergillus fumigatus* single proteins and protein pools, with a special interest on Th2 targets. Cystic fibrosis patients with sensitization to *Aspergillus fumigatus* and/or acute bronchopulmonary aspergillosis could clearly be identified by the expression of a Th2 cell profile, indicated by increased frequencies of IL-4, IL-5 and IL-13. Interestingly, Th2 patients could further be divided by the intensity of this response with strong Th2 reactions being accompanied by increased levels of the pro-inflammatory cytokine GM-CSF. All but one patient with acute or subacute forms of acute bronchopulmonary aspergillosis were contained in the Th2+GM-CSF+ group. Thus, this Th cell phenotype marks an interesting potential marker for the diagnosis of this hypersensitivity disorder. Additionally, distinct patterns in the Th cell responses could be detected for protein pools, containing “Th2 targets” and “non Th2 targets”. Finally, the chitosanase CsnB was identified as a new Th2 cell target, generating Th2 responses in the majority of *Aspergillus fumigatus* sensitized cystic fibrosis patients.

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7 Appendix

7.1 Supplementary data

7.1.1 Supplementary 1: data for dot plots

Figure	Pseudonym	Date of processing
Figure 1	NA10	16.11.2018
Figure 2	JDAVDHD9	11.04.2019
Figure 3	HD10 *C. albicans* - *C. albicans*	19.02.21
Figure 6	Y85VPNG6	18.01.19
Figure 9	JDAVDHD9	11.04.2019
Figure 11	*A. fumigatus* specific cells NA8,	28.05.21
Figure 12	P48K5MPG	28.05.21
Figure 13	HD14	25.11.21

7.1.2 Supplementary 2: data table all samples

CF patients (n=98) and healthy donors (n=12) who were analyzed for the characterization of the T cell response towards *A. fumigatus* with the ARTE screening and the CF patients (n=5) and healthy donors (n=6) that were recruited to perform the expansion of antigen specific T cells

pseudonym	date of processing	gender	cohort	experiment
KVJX8240	11/10/2018	M	CF patient	ARTE
0U74F2QQ	11/10/2018	W	CF patient	ARTE
RK2HQ7RQ	01/11/2018	M	CF patient	ARTE
C6UK307V	01/11/2018	M	CF patient	ARTE
FR8NA44A	08/11/2018	W	CF patient	ARTE
UNU01ZWN	08/11/2018	M	CF patient	ARTE
E01NK3C1	08/11/2018	M	CF patient	ARTE
NA10	15/11/2018	W	CF patient	ARTE
NA33	06/12/2018	W	CF patient	ARTE
VR17SSO	06/12/2018	W	CF patient	ARTE
5XJ3RDRR	06/12/2018	M	CF patient	ARTE
LQN71V33	06/12/2018	M	CF patient	ARTE
9JHUMLN4	20/12/2018	M	CF patient	ARTE
SVJZ4T69	20/12/2018	W	CF patient	ARTE
X328X5PH	10/01/2019	W	CF patient	ARTE
LQ290C57	10/01/2019	W	CF patient	ARTE
JXREAP3W	10/01/2019	W	CF patient	ARTE
QMTEKRKN	10/01/2019	W	CF patient	ARTE
Y85VPNG6	17/01/2019	M	CF patient	ARTE
QAYNP0YL	17/01/2019	W	CF patient	ARTE
VKPV9KJQ	17/01/2019	M	CF patient	ARTE
NA9	17/01/2019	W	CF patient	ARTE
NA52	17/01/2019	W	CF patient	ARTE

NA16a	17/01/2019	W	CF patient	ARTE
NA49	24/01/2019	M	CF patient	ARTE
GEER8M0T	24/01/2019	W	CF patient	ARTE
NA8	31/01/2019	W	CF patient	ARTE
DYTER3YA	31/01/2019	M	CF patient	ARTE
GEJH9TQ5	31/01/2019	W	CF patient	ARTE
F9N39KL1	07/02/2019	W	CF patient	ARTE
TM29KG0R	31/01/2019	M	CF patient	ARTE
T9NXWHYR	14/02/2019	W	CF patient	ARTE
5T63FJ9L	07/02/2019	W	CF patient	ARTE
QV7N87W8	14/02/2019	M	CF patient	ARTE
MT40RRZV	21/02/2019	W	CF patient	ARTE
Q30L7W2V	21/02/2019	M	CF patient	ARTE
NA25	21/02/2019	W	CF patient	ARTE
H18XRXL3	28/02/2019	W	CF patient	ARTE
NA53	28/02/2019	W	CF patient	ARTE
NA28	14/03/2019	M	CF patient	ARTE
LATHJG34	14/03/2019	M	CF patient	ARTE
NA78	14/03/2019	M	CF patient	ARTE
RA1UH388	14/03/2019	W	CF patient	ARTE
NA22	21/03/2019	M	CF patient	ARTE
NA54	21/03/2019	W	CF patient	ARTE
P48K5MPG	21/03/2019	M	CF patient	ARTE
8NDG9VQZ	28/03/2019	M	CF patient	ARTE
YH5ZN0LH	28/03/2019	M	CF patient	ARTE
2FK4AMDU	28/03/2019	M	CF patient	ARTE
79HR4PYM	28/03/2019	W	CF patient	ARTE
L8YMNJU8	28/05/2019	M	CF patient	ARTE
3ARWDZ7Q	28/03/2019	W	CF patient	ARTE
NA65	28/03/2019	M	CF patient	ARTE
NA44	04/04/2019	W	CF patient	ARTE
G0U75840	04/04/2019	M	CF patient	ARTE
NA21	04/04/2019	W	CF patient	ARTE
Z01QN9U2	11/04/2019	W	CF patient	ARTE
JDAVDHD9	11/04/2019	M	CF patient	ARTE
WLUVNCP9	11/04/2019	W	CF patient	ARTE
H7D7NGCP	11/04/2019	M	CF patient	ARTE
Q5YCG4FQ	25/04/2019	M	CF patient	ARTE
AL1GER6T	25/04/2019	M	CF patient	ARTE
NA66	25/04/2019	W	CF patient	ARTE
3NZ8ED45	25/04/2019	W	CF patient	ARTE
FKZFKZFH	09/05/2019	M	CF patient	ARTE
NA48	09/05/2019	W	CF patient	ARTE

NA19	09/05/2019	W	CF patient	ARTE
59MZ8Z9M	16/05/2019	W	CF patient	ARTE
00VCUM65	16/05/2019	M	CF patient	ARTE
RVZ72AFT	16/05/2019	W	CF patient	ARTE
F5PCFCZ7	16/05/2019	W	CF patient	ARTE
TMZ74NLM	23/05/2019	M	CF patient	ARTE
0XJLNKJ4	23/05/2019	W	CF patient	ARTE
WZP6VYA8	26/05/2019	W	CF patient	ARTE
CAG4TQP4	23/05/2019	W	CF patient	ARTE
98Z5H1Y2	04/06/2019	W	CF patient	ARTE
97M7JVAY	18/07/2019	W	CF patient	ARTE
QXRJ2M5W	18/07/2019	M	CF patient	ARTE
9Z6QWC1M	01/10/2019	M	CF patient	ARTE
0DCEA599	10/10/2019	W	CF patient	ARTE
NA70	17/12/2019	W	CF patient	ARTE
KN31T1GR	17/12/2019	W	CF patient	ARTE
G0DG3WL0	17/12/2019	M	CF patient	ARTE
LJQEYNN2	17/12/2019	W	CF patient	ARTE
NA18	17/12/2019	M	CF patient	ARTE
J39Q5YRT	17/12/2019	W	CF patient	ARTE
KMR86TFA	13/02/2020	M	CF patient	ARTE
P90RJX24	13/02/2020	M	CF patient	ARTE
NA20	13/02/2020	M	CF patient	ARTE
FTTJYUA6	10/03/2020	M	CF patient	ARTE
PJT7LE6G	10/03/2020	W	CF patient	ARTE
CCP54N6U	05/05/2020	M	CF patient	ARTE
AZN2NEE9	19/05/2020	W	CF patient	ARTE
CWL04LYK	19/05/2020	W	CF patient	ARTE
H3U9P07Y	14/07/2020	W	CF patient	ARTE
HUR31K9W	21/07/2020	W	CF patient	ARTE
4XJWMEGG	17/08/2020	W	CF patient	ARTE
8K1XW9AD	17/08/2020	W	CF patient	ARTE
HD1	02/04/2019	W	Healthy donor	ARTE
HD2	02/04/2019	M	Healthy donor	ARTE
HD3	02/04/2019	M	Healthy donor	ARTE
HD4	17/04/2019	M	Healthy donor	ARTE
HD5	17/04/2019	W	Healthy donor	ARTE
HD6	17/04/2019	W	Healthy donor	ARTE
HD7	17/04/2019	W	Healthy donor	ARTE

HD8	02/05/2019	W	Healthy donor	ARTE
HD9	20/06/2019	M	Healthy donor	ARTE
HD10	20/06/2019	W	Healthy donor	ARTE
HD11	20/06/2019	W	Healthy donor	ARTE
HD12	20/06/2019	M	Healthy donor	ARTE
QV7N87W8	30/04/2021	M	CF patient	Expansion antigen specific T cells
P48K5MPG	05/05/2021	M	CF patient	Expansion antigen specific T cells
NA8	05/05/2021	W	CF patient	Expansion antigen specific T cells
Q5YCG4FQ	05/10/2021	M	CF patient	Expansion antigen specific T cells
FTTJYUA6	05/10/2021	M	CF patient	Expansion antigen specific T cells
HD10	26/01/2021	W	Healthy donor	Expansion antigen specific T cells
HD13	08/02/2021	W	Healthy donor	Expansion antigen specific T cells
HD14	14/02/2021	W	Healthy donor	Expansion antigen specific T cells
HD15	14/02/2021	M	Healthy donor	Expansion antigen specific T cells
HD11	28/03/2021	W	Healthy donor	Expansion antigen specific T cells
HD16	28/03/2021	W	Healthy donor	Expansion antigen specific T cells

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9 Publications

03/2023: co-authorship of the paper „Antigen specificity and cross-reactivity drive functionally diverse anti-Aspergillus fumigatus T cell responses in cystic fibrosis“ (Schwarz, C., Eschenhagen, P., **Schmidt, H**., Hohnstein, T., Iwert, C., Grehn, C., Roehmel, J., Steinke, E., Stahl, M., Lozza, L., Tikhonova, E., Rosati, E., Stervbo, U., Babel, N., Mainz, J., Wisplinghoff, H., Ebel, F., Jia, L.-J., Blango, M., Hortschansky, P., Brunke, S., Hube, B., Brakhage, A., Kniemeyer, O., Scheffold, A. and Bacher, P. *J Clin Invest.* 2023;133(5):e161593. https://doi.org/10.1172/JCI161593)

03/2022: poster presentation at the European CF Young Investigators Meeting