

Comprehensive assessment of environmental fungus- reactive T cells response in hypersensitivity pneumonitis patients

Andreia Cristina Leite Coelho

Master's Degree in Biochemistry

Faculty of Sciences (FCUP), University of Porto

School of Medicine and Biomedical Sciences (ICBAS), University of Porto
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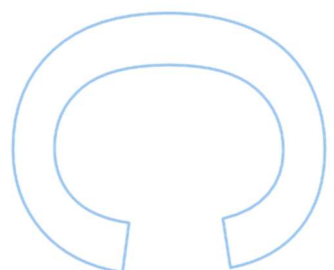
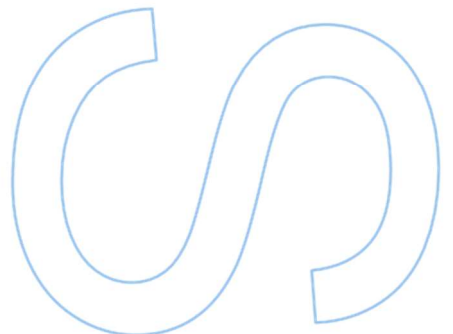
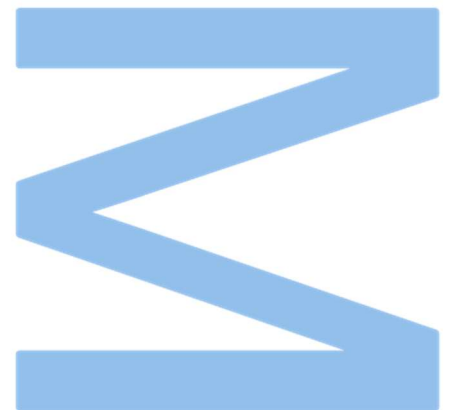
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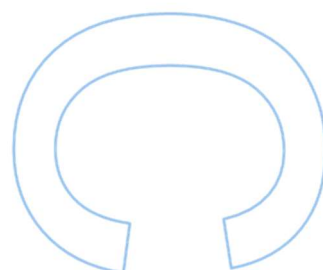
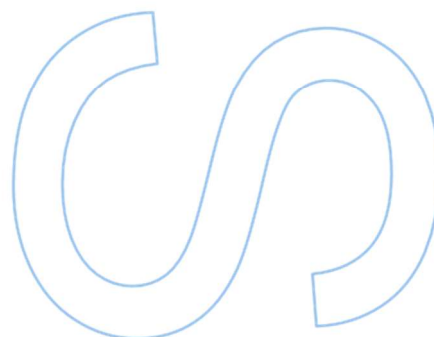
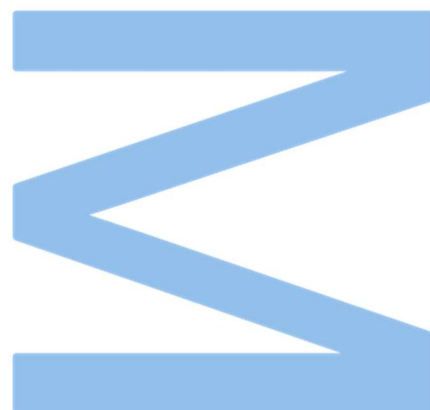
Ana Rita Faria dos Santos, Junior Researcher, Institute for Research and Innovation in Health (i3S), University of Porto

Co-supervisor

Elva Bonifácio Andrade, Junior Researcher, Institute for Research and Innovation in Health (i3S), University of Porto

Invited Assistant Professor, School of Medicine and Biomedical Sciences (ICBAS), University of Porto





*“Sou incapaz de responder às minhas próprias interrogações,
pois eu próprio sou um homem de interrogações”*

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Work Outcomes

During the time this work was developed, part of the results were presented in:

- I ImmunoHUB Annual Meeting: Infection and Immunity, held on the 17th of March 2023, at the Institute for Research and Innovation in Health (i3S), as a scientific poster (Annex I).
- XLVIII Annual Meeting of the Portuguese Society for Immunology, held between the 29th and 31st of March 2023, at the Aveiro Congress Center, as a scientific poster (Annex I).
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Resumo

A pneumonite de hipersensibilidade (PH) é uma doença pulmonar intersticial inflamatória, desencadeada pela inalação de partículas ambientais, com morbilidade e mortalidade significativas. A identificação do antigénio causador é crucial não só para um diagnóstico preciso, mas também dado que a evicção da exposição melhora a sintomatologia e consequentemente o prognóstico. No entanto, na maioria dos doentes, não é possível identificar a fonte da exposição.

A exposição prolongada a bolores é uma das principais fontes de antigénios desencadeantes e tem sido amplamente associada ao desenvolvimento de PH. Neste trabalho, pretende-se estabelecer uma correlação entre a elevada incidência de PH em Portugal e a exposição doméstica a bolores, através da estimulação de células T de doentes com pneumonite de hipersensibilidade com antigénios fúngicos.

Foram recolhidas amostras de ar de residências de doentes com pneumonite de hipersensibilidade com exposição relatada a bolores. De uma forma geral, *Cladosporium* spp. e *Penicillium* spp. foram os géneros mais prevalentes. No entanto, *Aspergillus fumigatus*, *Aspergillus niger* e *Rhizopus* spp. foram encontrados em maior quantidade no ar interior do que no exterior das casas destes doentes, podendo representar uma exposição relevante. Células mononucleares do sangue periférico de doentes com PH e de coabitantes sem doença respiratória foram estimuladas com cocktails fúngicos compostos por conídios dos fungos selecionados. Após a estimulação, observou-se uma forte resposta proliferativa das células T em relação a estes antigénios, bem como um aumento significativo da secreção de interferão gama nos doentes com PH em comparação com os coabitantes. Quando as células de doentes com suspeita de PH, ainda em fase de diagnóstico, foram estimuladas com os mesmos antigénios fúngicos, a resposta específica das células T observada, em alguns casos, foi semelhante à previamente observada para os doentes com diagnóstico definitivo.

Os resultados obtidos sugerem que doentes com PH desenvolvem uma resposta imune intensa e específica do tipo Th1 quando estimulados com um cocktail fúngico comum, evitando a necessidade de inspeções domiciliárias recorrentes. Avaliar a exposição específica a bolores permitirá satisfazer uma necessidade clínica em termos de identificação da exposição, contribuindo assim para um diagnóstico de HP de maior confiança.

Palavras-chave: pneumonite de hipersensibilidade, doença pulmonar intersticial, fungos ambientais, bolores, resposta células T, inflamação

Abstract

Hypersensitivity pneumonitis (HP) is an immune-mediated interstitial lung disease triggered by the inhalation of a variety of environmental particles, with significant morbidity and mortality. Identifying the inciting antigen is critical to obtain a confident diagnosis, and exposure avoidance ameliorates symptoms, and consequently clinical outcomes and prognosis. However, in most patients, the culprit exposure is not identified.

Moulds are considered a putative source of triggering antigens and have been extensively associated with the development of HP. The aim of this work is to establish an association between the high incidence of HP in Portugal and prolonged domestic exposure to moulds, through the assessment of specific fungi-reactive T cells in hypersensitivity pneumonitis patients.

Air samples were collected from patients' homes with reported mould exposure. *Cladosporium* spp. and *Penicillium* spp. were shown to be the most prevalent fungi, and *Aspergillus fumigatus*, *Aspergillus niger*, and *Rhizopus* spp. showed up in higher amounts indoors rather than in the outdoor environment. Peripheral blood mononuclear cells from HP patients and healthy co-inhabitants were stimulated with fungal cocktails comprised of conidia from the selected fungi. Upon stimulation, we observed a strong T cell proliferative response towards these antigens, as well as highly increased interferon-gamma secretion in HP patients in comparison with co-inhabitants. When cells from patients under diagnosis workup were stimulated with the same fungal antigens, the observed specific T cell response was similar to that of HP-diagnosed patients.

According to these results, HP patients develop a specific and intense Th1-like immune response towards a common fungal cocktail, avoiding the need for recurring house inspections. Assessing mould-specific response will fulfil a clinical need in exposure identification, therefore contributing to a higher confidence HP diagnosis.

Keywords: hypersensitivity pneumonitis, interstitial lung disease, environmental fungi, moulds, T cell response, inflammation

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Abbreviation List

AMB	AMPHOTERICIN B
APC	ANTIGEN-PRESENTING CELL
BAL	BRONCHOALVEOLAR LAVAGE
BCR	B CELL RECEPTOR
BFL	BIRD FANCIER'S LUNG
CFU	COLONY-FORMING UNIT
CTV	CELL TRACE VIOLET
DAMP	DAMAGE-ASSOCIATED MOLECULAR PATTERN
DC	DENDRITIC CELL
DLCO	DIFFUSING CAPACITY FOR CARBON MONOXIDE
FBS	FETAL BOVINE SERUM
FLD	FARMER'S LUNG DISEASE
FVC	FORCED VITAL CAPACITY
HP	HYPERSENSITIVITY PNEUMONITIS
IFN	INTERFERON
IG	IMMUNOGLOBULIN
IL	INTERLEUKIN
ILD	INTERSTITIAL LUNG DISEASE
IPF	IDIOPATHIC PULMONARY FIBROSIS
MEA	MALT EXTRACT AGAR
MHC	MAJOR HISTOCOMPATIBILITY COMPLEX
NK	NATURAL KILLER
PAMP	PATHOGEN-ASSOCIATED MOLECULAR PATTERN
PBMC	PERIPHERAL BLOOD MONONUCLEAR CELLS
PBS	PHOSPHATE BUFFER SALINE
RPMI	ROSWELL PARK MEMORIAL INSTITUTE
SDA	SABOURAUD DEXTROSE AGAR
SIC	SPECIFIC INHALATION CHALLENGE
TCR	T CELL RECEPTOR
TH	T HELPER
TNF	TUMOR NECROSIS FACTOR
UV	ULTRAVIOLET

1. Introduction

1.1. Immune System and Response

The immune system is a highly complex and interactive network of cells, tissues, and molecules, responsible for the host's defence against harmful organisms, such as bacteria or viruses. The immune system includes both cellular and humoral components that, together, work to identify and eliminate threats (1). The ability to recognize and respond to pathogens is complemented by the capacity to distinguish between self and non-self. Immune tolerance is a crucial mechanism, which allows the immune system to recognize and tolerate the host's cells and tissues, maintain integrity, and prevent unwanted reactions (2).

Classically, the immune response can be divided into two main branches: innate and adaptive immunity. Innate immunity is the first line of defence non-specific and provides immediate, but temporary, protection against several types of pathogens. The innate immune response is ensured both by cells of hematopoietic origin, such as macrophages, dendritic cells (DC), mast cells, neutrophils, eosinophils, and natural killer (NK) cells; but also of non-hematopoietic origin, such as epithelial cells of the mucosal membranes. In addition to these cellular defences, a humoral component is also present, comprised mainly of complement and C-reactive proteins, in circulation, which cannot only sense the pathogen but also aid infection clearance (3, 4).

Innate immune cells rely on germline-encoded receptors, the pattern recognition receptors (PRR). Despite the limited variety, these receptors can detect highly conserved and shared microbiological components: pathogen-associated molecular patterns (PAMP). Furthermore, innate cells have also evolved the capacity to detect damage-associated molecular patterns (DAMP), which are generally molecules expressed in the context of infection or inflammation, that may be released in the process of cell death, for example (5). The recognition of these molecules initiates a rapid and protective inflammatory response (6).

Adaptive immunity, on the other hand, is a specialized and targeted response, assured by T and B lymphocytes. It is characterized by life-lasting memory against a specific antigen, through the generation of antigen-specific memory cells (7, 8). On a first contact with the antigen, the adaptive immune response may take hours or days to fully develop: this comprises the primary response. However, due to the generation of memory cells,

further contacts with that same antigen will induce a quicker and more powerful immune response, so pathogen elimination is as effective as possible (9).

Nevertheless, the innate and adaptive immune systems are in constant communication to provide seamless defence against pathogens. Innate cells play a pivotal role in the subsequent activation of the adaptive immune response (**Figure 1**). Through their phagocytic capacity, innate antigen-presenting cells (APC), such as macrophages and DC, engulf the pathogen and process it. The smaller peptides derived from this process are then presented to T lymphocytes, major players of the adaptive immune response, through the interaction between major histocompatibility complex (MHC) molecules expressed at the surface of APCs, and the T cell receptor (TCR) (10). Contrary to innate immune cell receptors, adaptive immune cell receptors, such as the TCR, are the result of random gene rearrangement, which gives rise to an incredibly diverse collection of receptors. This confers the adaptive immune system the capacity to virtually recognize any antigen (11). Along with the TCR, T cells also express co-receptors, either CD4 or CD8, according to their effector function, which stabilize the MHC-TCR interaction (12).

During the T cell activation process, APCs and naïve T cells interact in secondary lymphoid organs, such as the lymph nodes, through antigen-loaded class II or class I MHC molecules, and the TCR. Naïve CD4-expressing (CD4+) T cells respond to processed antigens presented by class II MHC molecules, and become T helper (Th) cells, coordinators of the immune response; while naïve CD8-expressing (CD8+) T cells respond to antigens presented in class I MHC molecules, and develop into T cytotoxic (Tc) cells (13).

However, a second signal is necessary to fully activate naïve CD4+ T cells: interaction between co-stimulatory molecules present both in the APC and T cell surface, such as B7 molecules and CD28, but also CD40 and CD40L, respectively (14, 15). Without this signal, T cells become anergic and unresponsive (16).

Upon receiving both signals, naïve CD4+ T cells may differentiate into various Th cell subsets, depending on a combination of the perceived stimuli and the surrounding environment (17). The main subsets are Th1, Th2, or Th17, although others, such as Th22 and Th9, have been identified (18, 19). Each subset is characterized by the activation and expression of a specific transcription factor and set of cytokines: In Th1 cells, the master transcriptional regulator is T-bet, and these cells produce mainly interferon (IFN) gamma as an effector cytokine. In Th2 cells, the GATA-3 transcription factor is activated and these cells produce interleukin (IL) 4, 5, and 13, while in Th17

cells, the activated transcription factor is ROR γ t and they produce IL-17A, IL-17F, and IL-22 (17, 20). A successful activation process induces the expression of activation markers in these cells, such as CD69 (21) and CD25 (22), and culminates in the clonal expansion of the antigen-specific T cells, crucial for an effective immune response (23). Regulatory T cells (T regs) are also essential players not only in maintaining tolerance but also in dampening the strength of the immune response (24).

On the other hand, upon activation B cells secrete antibodies, or immunoglobulins (Ig), soluble versions of the B cell receptor (BCR), specific to the recognized antigen, that may bind and neutralize it. Along with cytokines, these proteins ensure the adaptive humoral response (25).

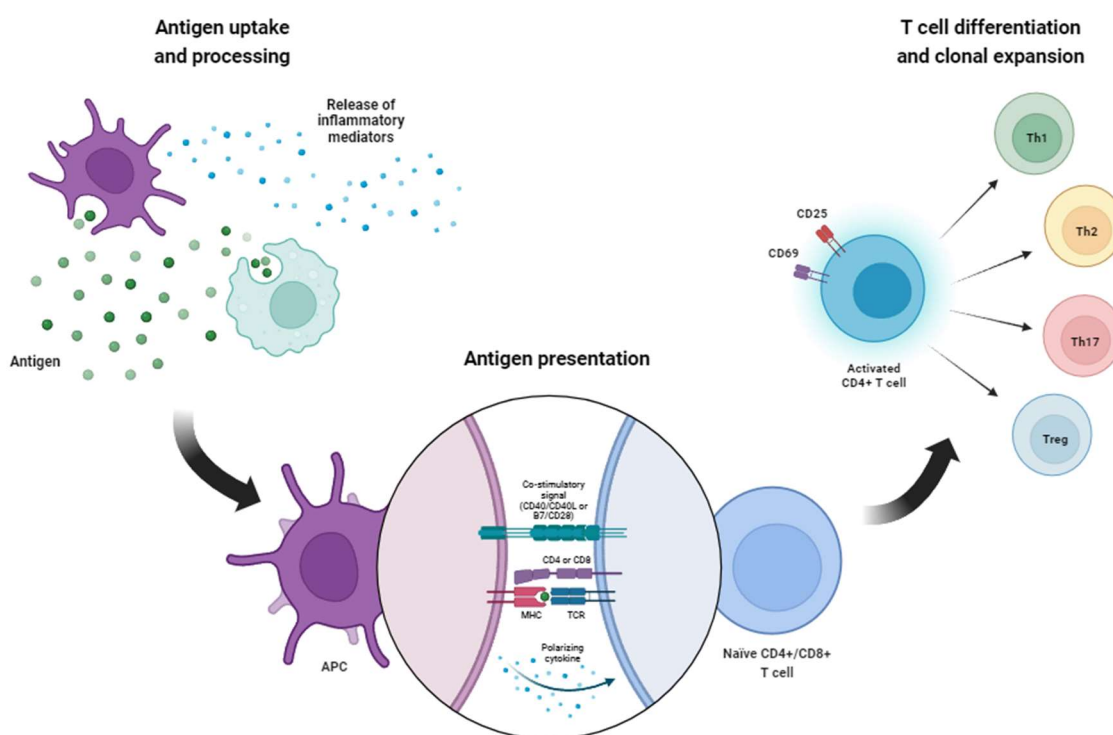


Figure 1. Initiation of the adaptive immune response and CD4+ T cell differentiation. Upon contact with a foreign antigen, an antigen-presenting cell (APC) engulf and process the antigen, which originates smaller peptides that will be presented to naïve T cells. When an antigen-specific T cell receptor (TCR) from a naïve T cell interacts with the antigen-loaded major histocompatibility complex (MHC) in the APC, the T cell becomes activated and initiates effector function. Activated T cells, either CD4+ or CD8+, express activation markers, such as CD69 and CD25. Additionally, depending on the perceived stimuli and surrounding environment, different metabolic changes occur within activated CD4+ T cells, causing them to differentiate into a specific T helper (Th) subset, such as Th1, Th2, Th17 or Treg, each characterized by the activation of a different transcription factor and set of cytokines, accompanied by the clonal expansion of the subset. Created with Biorender.com.

1.2. Hypersensitivity: dysregulation of the immune response

Despite its protective role, the immune system may malfunction. Hypersensitivity reactions are an extensively studied phenomenon, in which a disproportionate and excessive immunological response is initiated, damaging the host's tissues (26).

Hypersensitivity-related diseases show high prevalence and variability and include autoimmune diseases, which occur when the self-tolerance protective mechanism fails, and the body launches an immune response towards self-antigens, but also diseases that derive from a dysregulated and excessive immune response to foreign antigens (26, 27). In this case, the immune response is skewed towards a Th2-type response, mediated by a specific subset of T helper cells: Th2 lymphocytes (28). Cytokines secreted by this subset induce an antibody class switch, to produce antigen-specific IgE, and play a role in eosinophil and mast cell activation and function (29). Upon activation, these cells release potent inflammatory mediators stored in granules in their cytosol, such as histamine, tryptase, chymase, and proteoglycans, which further drive the immune response (30).

Furthermore, hypersensitivity reactions are generally classified as type I, II, III, or IV, depending on the mediator (31). Type I, II, and III hypersensitivity are considered immediate reactions, as symptoms develop within 24h, and are mediated mostly by immunoglobulins: IgE in the case of type I hypersensitivity, IgM and IgG in the case of type II hypersensitivity, and immunocomplexes (antigen-antibody complexes) in the case of type III hypersensitivity. Type IV hypersensitivity is T cell-mediated, and considered a delayed reaction (32, 33).

Foreign antigens can derive from highly variable sources. These can be pathogenic microorganisms, animal or plant proteins, and even pollen and chemicals, and are mainly ingested or inhaled by the host (34). After sensitization occurs, an aggressive immune response is triggered every time the same specific antigen is encountered. This causes significant morbidity, especially if the antigen is commonly found in the environment. Hypersensitivity-related diseases include asthma, rhinitis, food allergies, and atopic dermatitis, for example (35).

This work will focus on hypersensitivity pneumonitis (HP), a predominantly inflammatory interstitial lung disease (ILD). Initiated by an exaggerated immune response to the prolonged and continuous exposure to a certain antigen present in the environment (36), this disease is characterized by type III and type IV hypersensitivity reactions (37), which lead to damage to the lung tissue, with significant impact on the patient's quality of life and mortality.

1.3. Interstitial Lung Diseases

The interstitial lung disease group comprises a variety of complex pathologies characterized by inflammation or fibrosis of the pulmonary interstitium, associated with significant morbidity and mortality (38). This group comprises a broad spectrum of diseases, with sarcoidosis, connective tissue disease-associated interstitial lung disease (CTD-ILD), and idiopathic pulmonary fibrosis (IPF), among the most common (39), although IPF remains by far the most well-studied (40).

The causes of these pathologies are multiple and not yet fully understood. Some derive from an identifiable source, such as exposure to a certain etiologic agent (environmental exposure), tobacco smoke, or even some types of drugs; while genetic disorders and autoimmune disorders can be the root of other ILDs (41). Environmental exposure may occur in an occupational or recreational context. Silicosis, for example, a typical disease in mine, quarry, and construction workers, derives from the inhalation of silica particles (42), and farmer's lung disease (FLD), a form of HP directly related to farm work, is caused by the exposure to mould-contaminated hay or grain (43).

Symptom overlap between ILDs is common, due to the unspecificity of most symptoms. This can lead to misdiagnosis and, naturally, a delay in the diagnosis process, which is harmful to the patient. Therefore, clinicians rely on a combination of various types of medical examinations and complementary diagnostic tests, namely radiological, clinical, functional, and histopathological examinations. As such, a possible ILD patient should be evaluated by a multidisciplinary group of specialists (41) to obtain a faster and more accurate diagnosis and to define the best treatment strategy.

Treatment decisions require extensive multifactorial analysis, as evidence-based treatment is scarce and available only for some ILDs (38). This includes considering the suspected ILD classification, in terms of the main pathway driving disease progression. Most interstitial lung diseases possess an inflammatory component, which may be reversible, and may or not coexist with a fibrotic feature. The persistent activation of the immune system observed in the lung interstitium leads to a constant inflammatory state, damaging tissue, which leads to its substitution by non-functional connective tissue, or fibrosis (44). Thus, in some cases, immunosuppressive treatment may be advisable.

The most common immunosuppressants used in the management of inflammation-driven ILDs are corticosteroids, namely prednisone, as well as mycophenolate mofetil, azathioprine, and methotrexate (45).

Fibrosis-driven ILDs, on the other hand, are characterized by a predominantly fibrotic phenotype, so therapy is based on antifibrotic administration. Nintedanib and pirfenidone are two antifibrotic drugs approved for use in fibrosing ILD which have produced good results, with treated patients showing reduced annual forced vital capacity (FVC) decline, an important diagnostic tool used to assess lung capacity, in patients (46, 47).

1.4. Hypersensitivity Pneumonitis

1.4.1. Definition, Epidemiology and Clinical Presentation

Hypersensitivity pneumonitis is a common type of interstitial lung disease caused by an exaggerated immune response to an environmental antigen, with highly heterogeneous clinical manifestations. Incidence and prevalence are difficult to assess, as this disease often goes unrecognized, and is consequently underdiagnosed (36). Exposure also varies significantly between geographical regions, due to climate and occupational activities or working conditions (48). Epidemiological data on the incidence of HP in Europe show it ranges between 0.3 to 0.9 in 100,000 individuals (49-51). On the other hand, in India, for example, it is the most observed ILD, comprising almost 48% of cases (52). In a portuguese cohort, out of 634 ILD-diagnosed patients from the northern region, 123 (19.4%) were HP cases (53), highlighting a high HP incidence in Portugal.

Hypersensitivity pneumonitis' prevalence in terms of gender appears contradictory, with some studies showing this disease to be more common in female individuals (38), and others in males (50, 54). Nevertheless, most patients receive a diagnosis at 65 or older and are usually non-smokers (55).

Typical HP-related symptoms are usually non-specific, including dyspnoea and coughing, fatigue and inspiratory squeaks, weight loss, fever, chest tightness, and wheezing (53, 56). Due to its inflammatory origin, treatment often includes immunosuppressive drugs. However, antigen identification remains the ideal outcome for disease management, and its avoidance/removal is the forefront treatment with a great impact on disease prognosis (57).

Hypersensitivity pneumonitis has been classified as acute, sub-acute or chronic, depending on the duration of symptoms (48). However, this type of classification has become outdated and is no longer considered clinically significant, due to the heterogeneity of this disease's clinical presentation, which is highly dependent on many factors (55), such as the nature of the antigen, the intensity and duration of the exposure,

as well as host-related factors (58, 59). Since fibrosis is considered a highly important factor in disease prognosis (56, 60), HP classification has evolved to non-fibrotic, or inflammatory HP, and fibrotic HP. Non-fibrotic HP is generally associated with a better outcome and is putatively reversible. In its fibrotic form, this disease adopts an irreversible and progressive phenotype, with the possibility of stabilization when treated. It is generally more severe and associated with higher mortality (61).

In hypersensitivity pneumonitis, identification of the causative antigen is crucial, not only to obtain an accurate diagnosis but also because antigen avoidance is critical to ameliorate symptoms and stop progression (62). Though culprit exposure can derive from many sources, in about 30-60% of HP cases the causative antigen cannot be identified, despite the presence of typical symptoms and thorough evaluation. In this case, it is referred to as cryptogenic HP (51).

Thus, as mentioned above for ILD diagnosis, a multidimensional approach and a multidisciplinary team are of the utmost importance to obtain an accurate diagnosis, combining clinical, radiological, and, if necessary, histological findings (56) (**Figure 2**).

	HRCT					
	Typical for HP		Compatible with HP		Indeterminate for HP	
History of exposure and/or serum IgG testing	Exposure +	Exposure -	Exposure +	Exposure -	Exposure +	Exposure -
No BAL or BAL without lymphocytosis and either no histopathology or indeterminate histopathology	Moderate confidence	Low confidence	Low confidence	Not excluded	Not excluded	Not Excluded
BAL lymphocytosis without histopathology sampling	High confidence	Moderate confidence	Moderate confidence	Low confidence	Low confidence	Not excluded
BAL lymphocytosis with indeterminate histopathology	Definite	High confidence	Moderate confidence	Moderate confidence	Low confidence	Not excluded
Probable HP histopathology	Definite	High confidence	High confidence	Moderate confidence	Moderate confidence	Low confidence
Typical HP histopathology	Definite	Definite	Definite	Definite	Definite	High confidence*

Figure 2. Guidelines for the diagnosis of hypersensitivity pneumonitis. Diagnosis is based on exposure assessment, as well as radiological, clinical, and histopathological findings. Independently of how HP-compatible the radiological pattern is, the identification of a possible culprit exposure leads to a more confident diagnosis (green squares represent higher degree of confidence), avoiding the need for invasive diagnostic procedures. HRCT: high-resolution computed tomography; HP: hypersensitivity pneumonitis; BAL: bronchoalveolar lavage; Ig: immunoglobulin. From Raghu *et al* (2020) (56).

It is known that genetic factors predispose individuals to the development of HP, related mainly to the immune system, namely to the failure to control the immune response, for example (63). Other associated genetic variants have been reportedly associated with a

predisposition to develop HP, such as polymorphisms in the major histocompatibility complex proteins (64, 65), proteasome (66), and telomeres (67). A mucin 5B promoter single nucleotide polymorphism was reported to be more prevalent in fibrotic HP patients when compared to healthy controls and associated with a worse prognosis (68). The existence of a genetic susceptibility factor may explain why most individuals who have been exposed to environmental antigens do not respond at all, or develop only a mild reaction, which is ultimately resolved.

Curiously, hypersensitivity pneumonitis is less prevalent among smokers (69), with differences in specific antibody levels observed between smokers and non-smokers, with these levels being lower in the first group upon exposure to the same antigens (70). This seemingly impaired immune response may be protective in the case of HP. The processes involved in this protection are unclear, yet this effect is generally attributed to nicotine, which is known to impair lymphocyte and macrophage function, while also skewing the immune response towards a Th2-type response (71, 72). However, smokers who do develop HP generally show a more severe progressive fibrotic phenotype, associated with reduced survival (73).

1.4.2. Pathogenesis

Due to its gas exchange function, the lung is constantly exposed to several antigens. Additional barriers, such as the mucus produced by the lung epithelial cells, help deter the entrance of pathogens into the respiratory tract. Lung-resident innate immune cells, such as alveolar macrophages and dendritic cells, form the first cellular line of defence against inhalable pathogens (74). Once activated, these cells, along with lung epithelial cells, present the recognized antigens to T lymphocytes, inducing their activation (75).

In the case of HP, signalling through IFN- γ and IL-12, cytokines produced by activated innate cells, polarize the differentiation of naïve T cells into the Th1 phenotype, and drive the immune response, through a positive feedback mechanism (76). Additionally, the production of chemotactic factors induces the recruitment of T cells into the alveolar space, which consequently maintains an inflammatory environment and promotes granuloma formation (**Figure 3**). Due to this, the presence of lymphocytosis in the bronchoalveolar lavage (BAL) may be used as a distinguishing factor between non-fibrotic (inflammatory) HP and other ILDs (77).

Immunopathogenesis seems to be mediated by a combination of type III and type IV hypersensitivity reactions (78). In a first contact, as in inflammatory HP, the resulting immune response is humoral, mediated by immunocomplexes, comprising a type III

hypersensitivity reaction. However, in fibrotic HP, the pathogenesis process seems to be cell-mediated, comprising a more likely type-IV hypersensitivity reaction (59).

The development of fibrosis in HP is associated with a switch from a Th1 to a Th2-type response (79). The process is not clearly elucidated; however, it is believed that both the inhibition of regulatory T cell function (80), and Th2 cytokines, such as IL-4 and IL-13, are involved, through the promotion of fibroblast proliferation and production of extracellular matrix, which promotes fibrosis, and the development of the progressive fibrotic phenotype observed in fibrotic HP.

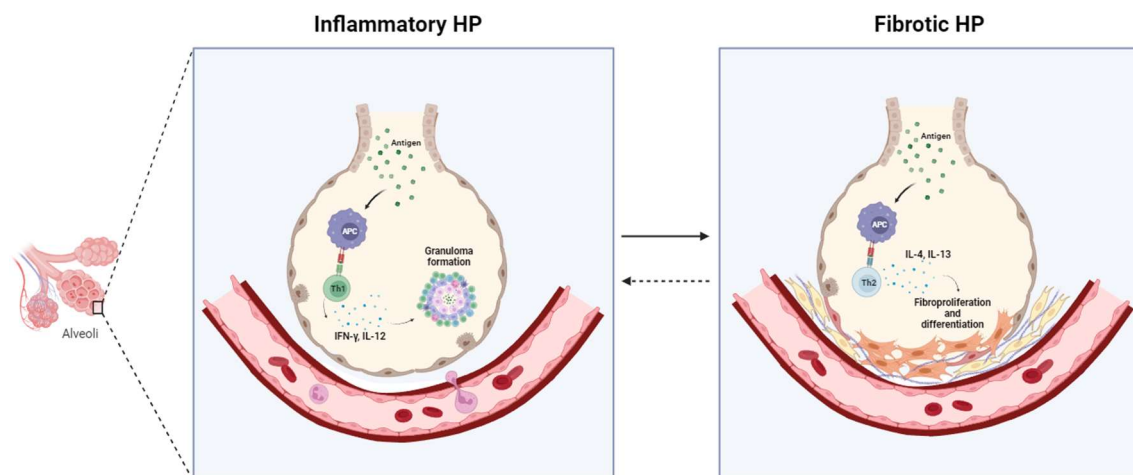


Figure 3. Pathogenesis of inflammatory and fibrotic hypersensitivity pneumonitis (HP). Inhaled antigens interact with antigen-presenting cells (APCs), such as macrophages and dendritic cells, via pattern recognition receptors. Neutrophils are present in early disease. Antigen-presenting cells stimulate a T helper (Th) 1 response, enhanced by cytokine production. Stimulated macrophages fuse into multinucleated giant cells, which in turn fuse with epithelioid cells, further enhancing granuloma formation, mediated by Th1 cytokine production. Inflammatory HP may progress to fibrotic HP, through what is believed to be a switch from Th1 to Th2-type response. Th2-associated cytokines, such as IL-4 and IL-13, are known to induce fibroblast proliferation and differentiation to myofibroblasts, as well as an increase in the production of extracellular matrix, resulting in lung fibrosis. IFN: interferon; IL: interleukin. Created with Biorender.com.

The immunopathological processes responsible for the development of HP seem to be shared by other ILDs. Lymphocytic alveolitis and granuloma formation, for example, are shared features between HP and sarcoidosis, with differences in exposure nature and duration, predisposing genetic factors and signalling pathways possibly holding responsibility for radiological and pathological differences between HP and other granulomatous diseases (81).

1.4.3. Exposure and Causative Antigens

More than 200 different inciting antigens have been identified and linked to the development of HP, with multiple sources, occupational or recreational (56). Most identified antigens derive from bacteria, fungi, animal proteins, and low-molecular-weight

chemical compounds (82). However, this list is chronically incomplete, as new sources and antigens are constantly described and associated with this disease. Even so, different inciting antigens have given rise to different disease forms.

Bird fancier's lung (BFL) is a form of HP that derives from the exposure to proteins present in bird feathers, faeces, or secretions via inhalation, first reported in 1965 (83). In most cases, BFL derives from exposure to pigeons or doves to birds from the *Psittaciformes* order, namely parakeets and parrots, but also to farm/poultry birds, such as chickens, geese, or turkeys (84). Inhalation of proteins from goose or duck feathers in duvets or pillows leads to the development of a sub-group of BFL, named feather-duvet lung (85).

Bird fancier's lung is one of the most common forms of HP, corresponding to 66-68% of all reported HP forms (84). The prevalence of bird fancier's lung is highly dependent on geographical location, due to climate conditions, economic and cultural status, and the duration and intensity of the exposure. In Portugal, a prevalence of 2-5% has been reported among pigeon breeders (86).

Less often, hypersensitivity pneumonitis may be caused by inorganic low-molecular-weight compounds, such as isocyanates and acid anhydrides, highly reactive compounds used widely across the paint and polymer industry (87). It is believed that these compounds can crosslink with human proteins, consequently impacting immune system function, and triggering the typical disproportionate immune response (88). Despite this, HP derived from exposure to these chemicals seems rather rare, with prevalence ranging from 1% to almost 5% in workers showing symptoms, as reported in two studies (89, 90).

Although rare, drugs may also cause HP in a non-inhalational manner. Numerous drugs have been associated with the development of HP, especially antineoplastic drugs (91), among others (92, 93). Therefore, the presence of this factor should be ruled out as early as possible, to prevent any further consequences for the patient.

Exposure to microbiological agents is also a frequent cause of hypersensitivity pneumonitis, with designations as diverse as the sources and microorganisms responsible. Farmer's lung disease, a type of HP caused by the inhalation of antigenic particles present in contaminated hay or grain, is considered the prototype of HP. Thermophilic actinomycetes, such as *Saccharopolyspora rectivirgula*, seem to be responsible for most FLD cases (94). Nevertheless, other microorganisms, namely moulds, from the *Alternaria*, *Aspergillus*, and *Botrytis* genera, for example, can also hold

responsibility for other FLD cases (95, 96). These organisms thrive in high temperatures and humidity levels, favoured by hay storage methods and climate conditions. Therefore, FLD prevalence heavily depends on geographical location and agricultural practices and is consequently more frequent in the wet season.

Gradually, other types of HP have been identified. Devices such as humidifiers or air conditioners can become contaminated with bacteria or moulds, and cause HP in sensitized individuals. Hot tubs have also been reported as a relevant source, when individuals are exposed to aerosolized water particles contaminated with *Mycobacterium avium* complex, which survive in the tubing due to their resistance to high temperatures (97). Metal worker's lung also derives from exposure to non-tuberculous mycobacteria-contaminated metal fluid, a form of HP especially relevant in the UK (98).

Fungi's relevance as inciting antigens continues to grow, with several sources already reported and mould-induced HP cases on the rise (99). Identifying a fungal causative antigen has proven difficult, due to the ubiquitous nature of these organisms, with numerous reported sources, both recreational and occupational. As such, it is worth highlighting that new antigen sources should be constantly researched, with special attention to the patient's home/work environment, proven to be more significant than previously thought.

1.5. Environmental Fungi Exposure in Hypersensitivity Pneumonitis

Among the possible environmental antigens, moulds are the most common, due to their ubiquitous distribution and adaptability. These are highly relevant antigen sources in high-humidity regions, such as the northern region of Portugal. Water damage in homes, derived from the existence of leaks or poor construction, may favour mould growth in homes. However, this type of exposure is very difficult to assess or even quantify, due to the lack of standardized methodologies and source diversity.

Occupational fungal exposure has been extensively described, as opposed to domestic exposure. Usually, patients report exposures of an occupational nature when asked, but often overlook possible domestic sources, so a thorough inspection of the patient's house, searching for signs of mould growth, such as mould stains on the walls or ceiling, is advisable to confirm a compatible exposure (100).

Mould spores are ubiquitous in outdoor environments and can present highly variable levels, generally lower in indoor environments unless conditions favour otherwise (101).

Most of the population can clear inhaled spores without developing any reaction, however, some develop allergic diseases, such as asthma (102). This is one of the most common non-communicable diseases, affecting at least 300 million people worldwide, with numbers expected to rise until 2025 (103).

Many fungi have been associated with the development of hypersensitivity-related diseases, such as *Aspergillus*, *Cladosporium*, *Penicillium*, and *Alternaria* genera (104). Several studies have established 1000 colony-forming units (CFU)/m³ as the upper limit for acceptable mould exposure (105). However, the prevalence and toxicogenic potential of the encountered species upon air sampling of the affected environment may affect these values.

Cladosporium and *Alternaria* genera are considered highly relevant outdoor airborne antigen sources. While these can also occur indoors, a significant correlation was observed between indoor and outdoor levels of these fungi, which suggests the outdoor environment as the spores' source (106). This correlation was not as marked in the case of *Penicillium* and *Aspergillus*, reported to be the most common airborne fungi indoors, associated with the development of asthma and hypersensitivity pneumonitis, among other respiratory diseases (106-110). Some *Aspergillus* species, such as *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus oryzae*, and *Aspergillus terreus*, are some of the most commonly associated with allergic respiratory diseases (111).

Several proteins from these fungi have been considered important allergens, mostly enzymes (111). In addition, and even though fungal cell wall composition can vary depending on environmental conditions and the growth stage of the fungi itself (112), its main components, β -glucans (mostly β -1,3-glucans), chitin, and mannans, are also highly relevant antigens, rapidly recognized by the immune system (113, 114).

However, assessing exposure to these microorganisms can be challenging. In patients with HP, a precipitin test is usually performed to assess antigen-specific IgG antibodies (115). Nevertheless, an elevation in the levels of these antibodies is evidence of sensitization, but not disease, further proven by positive results reported in exposed but healthy individuals, and with 10 to 15% of HP patients with no antigen-specific precipitins (36). This highlights the limitations of this method, which include cross-reactivity between antigens, and lack of standardization not only of the method itself, but also of the antigen preparations (116). Despite this, precipitins test is still encouraged to aid in the confirmation of a possible exposure in a suspicion of HP (56).

Occasionally, a specific inhalation challenge (SIC) may be performed, to complement other diagnostic tests and confirm a possible diagnosis (117). In a SIC, the patient is exposed to the suspected antigen through nebulization, or to the environment which is thought to be the source of the causative antigen (118). Alterations in FVC or diffusion capacity for carbon monoxide (DLCO), other clinical parameters, and the appearance or worsening of flu-like symptoms are seen as signs of a positive result (119). Due to the lack of validation and standardization, this test is not commonly performed and requires close medical supervision and specialized facilities, equipped to deal with possible side effects (120, 121).

To perform these tests, commercially available extracts of the suspected antigen, or recombinant versions, may be used (119). Yet, as mentioned before, it is important to consider the heterogeneity of exposure, as cases of multiple exposures are commonly seen in clinical practice, but also antigen cross-reactivity, especially in the case of moulds (117). Therefore, assessing the patient's environment and adapting the composition of the extract to match is of considerable importance. Thus, sampling of suspected antigen source environment, culture, and protein extraction of the microorganisms present in the sample (122), may be, in the case of moulds, an adequate measure to analyse and possibly obtain personalized extracts, to better assess patient exposure and increase the chance of identifying a culprit exposure, especially in the case of domestic HP (117).

1.6. Project Contextualization and Objective

Hypersensitivity pneumonitis is an immunologically mediated disease, caused by the inhalation of a wide variety of environmental antigens, leading to excessive lung parenchyma inflammation and damage, which results in severe morbidity. Diagnosing this disease requires multiple clinical, radiological, and pathological tests, due to the nature of the disease and phenotypical resemblances to other interstitial lung diseases. Early identification of a potential exposure can help prevent the need for invasive diagnostic procedures, such as lung biopsy. Symptom improvement in HP patients relies on antigen avoidance. However, the causative antigen remains unidentified in many of the cases.

A commonly overlooked, and especially relevant exposure in the northern region of Portugal is fungal exposure in a domestic setting, due to humidity conditions and poor construction, which favours mould growth. Thus, this work aims to establish a causal relationship between HP incidence in Portugal and mould exposure, through a

comprehensive assessment of environmental fungi-reactive T cells' response in HP patients, with the following objectives:

- Determine the most commonly found fungal antigens in the domestic environment, in order to develop a stimulation cocktail, powerful enough to induce T cell activation and cytokine secretion.
- Identify a signature response to the stimulation with fungal antigens.

Results obtained in this work will support clinicians in the confirmation of an HP diagnosis, by providing a way to confirm a possible environmental-fungi exposure as the culprit exposure in hypersensitivity pneumonitis patients, consequently impacting disease prognosis and patient's quality of life.

2. Methodology

2.1. Study design and participants

The present study included participants of the FIBRALUNG Portuguese cohort study (ClinicalTrials.gov Identifier: NCT05635032). Patients aged between 18-80 years, able to give informed consent, treatment-naïve for disease-modifying drugs, undergoing lung biopsy and/or BAL as part of their diagnostic workup, were enrolled in this prospective cohort study. Clinical information to be recorded includes comorbidities, smoking status, previous environmental exposures, clinical and serological autoimmunity findings, medication history, symptoms characterization, and physical examination.

The study protocol was reviewed and approved by the Ethics Committee of the Institution and performed in accordance with the Helsinki Declaration. Written informed consent was obtained from all participants.

2.2. House Air Sampling and Fungal Culture

Patients included in the FIBRALUNG cohort with a diagnosis of “cryptogenic” hypersensitivity pneumonitis and/or reported mould exposure, were selected for house inspection.

Sampling was performed using a single-stage microbiological air impactor (Air Ideal 3P®, bioMérieux®), according to previously validated methodology (123, 124), equipped with a sampling grid with 286 holes and culture plate support. Malt extract agar (MEA) culture plates supplemented with 1% chloramphenicol were used for fungi collection. Sequential duplicate air samples, each with a volume of 250 litres, were collected in the patient’s room, and one plate in the immediate exterior, if any windows or balconies were present. Additionally, one extra plate was collected if any other division showed evident signs of water damage or mould stains. Grid was cleaned with isopropyl alcohol between samples, and, as a control, a sampling blank was taken along with sampling plates, but not opened.

Fungal cultures were incubated at room temperature for a total of 7 days. At day 5, quantification of fungi levels was performed by naked eye count. The number of colonies recovered on the air sample plates was adjusted using a positive hole correction factor based on Feller’s law (125), and the results were expressed as the number of colony-forming units per cubic meter of air (CFU/m³). The quantification limit was established as

10 CFU per plate. Fungal identification was performed on day 7, either on the original MEA plates or after subculturing procedures, whenever colony isolation and growth observation were needed. Identification of fungal specimens was based on macroscopic and microscopic phenotypical characteristics, through the preparation of microscope slides and lactophenol cotton blue staining (126). Images were obtained using the Leica DM2000 LED brightfield microscope (Leica™). Conidia of individualized cultures were stored in glycerol at -80 °C.

2.3. Conidia Retrieval and Inactivation

When needed, fungi were cultured in Sabouraud dextrose agar (SDA) at room temperature for 7 days, to collect mature conidia, according to previously validated methodology (127). Colonies were scraped from the agar surface and resuspended in a sterile phosphate buffer saline (PBS) + 0.05% Tween20 solution. To remove debris, the suspension was filtered using a 40 µm cell strainer onto a 50-mL tube. Conidia were quantified and suspension was stored, for short periods, at 4 °C.

Inactivated conidia were obtained by resuspending 50×10^6 conidia in 12 mL of Roswell Park Memorial Institute (RPMI) 1640 cell culture medium (Gibco®), plated in an empty Petri dish, and ultraviolet (UV)-irradiated for 6h. Plates were then scraped using a cell scraper to ensure total retrieval of UV-inactivated conidia and suspension was collected onto a tube. After centrifugation at 4000 revolutions per minute for 10 minutes, conidia were resuspended to a final concentration of 100×10^6 conidia/mL, and aliquots were stored at 4 °C.

Dormant conidia were obtained by incubation with amphotericin B (AMB) (Gibco™) at a concentration of 5 µg/mL in the culture medium 24h prior to the addition of cells. Once cells were added, AMB concentration was adjusted to 2.5 µg/mL in the culture medium and was maintained throughout the stimulation assay.

2.4. Peripheral Blood Mononuclear Cell (PBMC) Isolation, Stimulation and Culture

Peripheral blood samples were collected from patients in ethylenediamine tetraacetic acid (EDTA)-treated tubes (BD Vacutainer®). PBMC fraction was isolated using Lympholyte®-H density gradient medium (CEDARLANE®) and 50-mL SepMate™

PBMC Isolation tubes (STEMCELL Technologies™). After isolation, cells were washed with PBS + 2% fetal bovine serum (FBS), counted, and stained with the proliferation dye CellTrace™ Violet (CTV) (ThermoFisher®). Cells were plated at 2.5×10^5 cells/well in RPMI 1640 cell culture medium supplemented with 1% penicillin-streptomycin, 1% pyruvate and 10% FBS (Gibco®), in a round-bottom tissue culture-treated 96-well plate (Corning Inc.®). To assess PBMC response to fungal exposure, cells were stimulated either with UV-inactivated or dormant conidia and incubated for 5 days at 37 °C and 5% CO₂ protected from light. Positive control (activation control) was obtained by stimulating cells with 1 µg/mL of anti-CD3 antibody (clone OKT3) and 3 µg/mL of anti-CD28 antibody (clone CD28.2) (BioLegend®) antibodies.

2.5. Flow Cytometry Analysis

T cell activation and proliferation were assessed using flow cytometry. Cells were washed and centrifuged at 300 x g for 3 minutes and stained with Zombie™ (BioLegend®) viability dye. Cell profile was determined using the following combination of antibodies: PE-Cy7 CD3 (clone UCHT1), BV650 CD4 (clone OKT4), BV785 CD8 (clone SK1), APC-Cy7 CD19 (clone HIB19), APC CD69 (clone FN50) and PE CD25 (clone BC96) (all from BioLegend®).

Immunophenotyping was performed at experimental day zero. Myeloid cell profile was determined using the following combination of antibodies: BV421 anti-human CD11c (clone 3.9), BV711 anti-human CD14 (clone M5E2), PE anti-human CD16 (clone 3G8), FITC anti-human CD45 (clone HI30), APC-Cy7 anti-human HLA-DR (clone L243) and PercP-Cy5.5 anti-human CD11b (clone M1/70). Cell profile of the lymphoid compartment was determined using the following combination of antibodies: APC anti-human CD45-RA (clone HI100), PercP-Cy5.5 anti-human TCR αβ (clone IP26), BV421 anti-human TCR γδ (clone B1), PE-Cy7 anti-human CD3 (clone UCHT1), BV650 anti-human CD4 (clone OKT4), BV785 anti-human CD8 (clone SK1), APC-Cy7 anti-human CD19 (clone HIB19), PE anti-human CD25 (clone BC96), FITC anti-human CD127 (clone A019D5) and BV711 anti-human CD62L (clone DREG-56) (all from BioLegend®).

Data was obtained using an LSRFortessa™ cell analyser (BD Biosciences®) and analyzed using FlowJo™ v10 software (BD Biosciences®).

2.6. Cytokine Measurement

Culture supernatants were harvested from 5 days-stimulated PBMC and analyzed using the LEGENDplex™ Human Th cytokine panel (12-plex), a bead-based multiplex immunoassay for the quantification of IFN- γ , TNF- α , IL-2, IL-4, IL-5, IL-13, IL-10, IL-22, IL-6, IL-9, IL-17A, and IL-17F (BioLegend®), according to the manufacturer's instructions and using BD Accuri™ C6 Plus for data acquisition (BD Biosciences®). Obtained data were analyzed using FlowJo™ v10 software (BD Biosciences®).

2.7. Statistical Analysis

All data were analyzed with GraphPad Prism™ v8 software (GraphPad Software®). Results are presented as means $\pm$ standard deviation (SD). Specific statistic tests are indicated in each figure's legend. Differences are considered statistically significant when $p < 0.05$.

3. Results and Discussion

In the FIBRALUNG cohort, which includes more than 600 patients from the north and central region of Portugal, almost 41% are cases of hypersensitivity pneumonitis, either fibrotic or non-fibrotic. However, in about 30-50% of these cases, a culprit exposure has not been identified - cryptogenic HP. Due to humidity conditions, associated with poor building ventilation, indoor mould growth is commonly reported, which could be associated with the high incidence of HP reported in Portugal.

3.1. Inspection to HP patient's homes reveals significant fungal presence and diversity

To validate this hypothesis, patients with a cryptogenic HP diagnosis, or a reported mould exposure, were selected for a house inspection, where indoor air samples were collected to assess the possible presence of mould spores and growth. Outdoor air samples were also collected to allow a comparison between both environments. Once collected fungi were quantified and identified, and conidia were obtained following the experimental setup highlighted in **Figure 4A**.

Collected plates showed significant mould growth and diversity on day 7 (**Figure 4B**). Total CFU quantification showed *Cladosporium* and *Penicillium* genera as the most prevalent, along with *Rhodotorula* spp., as seen in **Figure 4C**. Curiously, *Aspergillus fumigatus*, *Aspergillus niger*, and *Rhizopus* spp. showed up in higher amounts indoors rather than outdoors, which may suggest a potential indoor spore source, which in turn facilitates inhalation of the mentioned fungi. Importantly, due to its reported high seasonality, *Rhodotorula* spp. will not be used in this work, despite its relatively high prevalence.

Fungal identification, and consequent isolation, was based on morphological features, through microscope slide preparation and staining with lactophenol blue solution. Due to their prevalence and particular indoor counts, *Cladosporium* spp., *Penicillium* spp., *Rhizopus* spp., *Aspergillus fumigatus*, and *Aspergillus niger* were selected to be further studied in this work and are highlighted in **Figure 4D**. **Figure 4D₁** shows several conidiophores from *Aspergillus fumigatus*, whose structure is characteristic of this species, with conidia forming radiating chains, as can be observed. Hyphae and conidia from *Cladosporium* spp. are shown in **Figure 4D₂**. Extensive branching of hyphae, as well as the presence of shield-shaped cells, are characteristic of this genus. Contrary to

other moulds, fungi from the *Rhizopus* genus produce sporangia, instead of conidiophores, which hold sporangiospores. Additionally, this genus also produces rhizoids, which are specialized hyphae that aid the fungus with adherence to substrates. Both structures are visible in **Figure 4D₃**. **Figure 4D₄** depicts a long, symmetrical, and filamentous conidiophore, shaped like a brush, characteristic of the *Penicillium* genus. A dark-coloured radial conidiophore, characteristic of *Aspergillus niger*, is shown in **Figure 4D₅** (128). Microscopic observation of morphological characteristics limits identification range, as species from the same genus can be highly similar, and distinguishable only through molecular identification. Due to this, specific species of *Cladosporium*, *Rhizopus*, and *Penicillium* spp. could not be identified. As such, in this work, these fungi will be addressed by the genus name.

Filamentous fungi have the potential to infect human beings, mostly in an opportunistic manner (129). As such, indoor fungal count is regulated. Portugal's legislation relative to permitted indoor spore count is influenced by the toxicogenic or pathogenic potential of each fungus. Generally, if a mixture of several commonly found genera, such as *Cladosporium* spp. or *Penicillium* spp., is detected, the established limit is 500 CFU/m³. Toxicogenic fungi, such as *A. fumigatus*, should be quantified individually, with a limit of 12 CFU/m³. Additionally, indoor spore count should always be lower than outdoor spore counts (130).

The reported high prevalence of *Cladosporium* spp. is replicated in our house inspection results, highlighting its role as a major outdoor fungus, as mentioned previously. Allergies to this fungus are rare, and no significant correlation between exposure to this fungus and the development of hypersensitivity has been shown (131). As such, its prevalence in our sample does not seem to pose a significant threat to human health, however, frequent ventilation should be maintained.

On the other hand, the *Penicillium* genus, second most relevant in our results, and reportedly encountered in similar concentrations to *Cladosporium* spp., has been associated with higher rates of persistent coughs and wheezing (132), but also asthma severity (133). Major reported allergens for *Penicillium* spp. share a highly similar sequence to the homologous counterparts in *A. fumigatus*, a prominent fungus implicated in many respiratory diseases, highlighting the close taxonomical classification of both fungi (134) and reported similar association with the development of asthma (135). Additionally, both genera have been associated with the development of HP (109, 110). Some of the homes included in our sample exceeded the legislated indoor limit of

12 CFU/m³ for *A. fumigatus*, which could have worsened the respective patient's health status.

Similarly, *A. niger* and *Rhizopus* spp. were also detected in higher amounts indoors rather than outdoors in our sample, with *A. niger* showing the highest difference between both environments (**Figure 4C**). *A. niger* is a rather common fungus found in outdoor environments (136), however, it has been reported to cause allergic bronchopulmonary aspergillosis (137) and also reportedly correlates with increased asthma severity in children (138), similar to *A. fumigatus*. Moreover, *A. niger*-induced HP has also been reported (139, 140). Both fungi belong to the *Aspergillus* genus, a genus that presents a high frequency of pan-allergens that display cross-reactivity, which could explain the similarities in clinical relevance (106, 141).

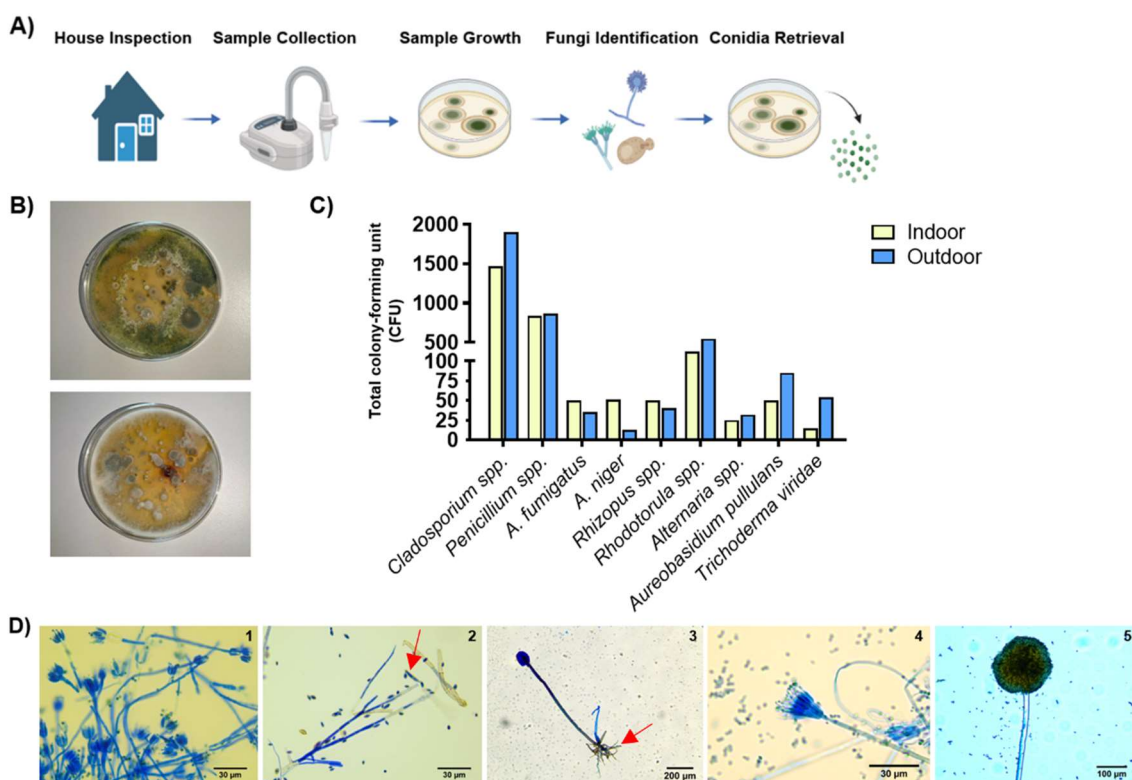


Figure 4. Air samples from hypersensitivity pneumonitis (HP) patients' homes reveal the presence of several fungi. Hypersensitivity pneumonitis patients with reported mould exposure were selected for a house inspection for the collection of air samples, to assess fungal presence and prevalence in the domestic environment. **(A)** Workflow of the house inspection and air sample collection process used to obtain conidia from pure mould cultures. **(B)** Representative malt extract agar (MEA) plates recovered from HP patients' homes after 7 days of growth at room temperature. **(C)** Number of colony-forming units (CFU) in the indoor and immediate outdoor environments of the patient's homes for the most prominent fungi. Values depicted represent the sum of CFU numbers in a total of 35 homes. **(D)** Microscopic images of the isolated fungi selected to be used in this work as stimulus after 7 days of growth and staining with lactophenol cotton blue. 1) Several *Aspergillus fumigatus* conidiophores and hyphae (400x). 2) Extensively branched hyphae, conidia and shield-cell (arrow) from *Cladosporium* spp. (400x). 3) Sporangiospore and rhizoids (arrow) from *Rhizopus* spp. (40x). 4) A single brush-like conidiophore from *Penicillium* spp., along with several released conidia (400x). 5) A radial, highly pigmented conidiophore, along with several released conidia, from *Aspergillus niger* (100x).

Although the difference between indoor and outdoor amounts of *Rhizopus* spp. is not particularly marked, similar to that of *A. fumigatus*, this fungus has also been reported to be capable of infecting the human respiratory tract (142) and causing HP in sensitized individuals (143).

Overall, the reported higher indoor prevalence of *A. fumigatus*, *A. niger*, and *Rhizopus* spp., the high general prevalence of *Penicillium* spp., as well as the fact that indoor spore count was close to or lightly exceeded the legislated limit of 500 CFU/m³ in some of the homes in this sample could have had a detrimental effect in patient's health in terms of disease development and progression and could constitute the culprit exposure for some of these individuals.

3.2. Optimization of cell seeding density for PBMC stimulation *in vitro*

To verify a direct link between exposure to these moulds and the development of HP in these patients, patient PBMCs will be stimulated with the isolated fungi. Cells from a healthy (no ILD/HP diagnosis) co-inhabitant will be used as comparison. Cells will then be incubated for 5 days in contact with different stimuli. As a readout of T cell-specific response, the expression of T cell activation markers, as well as proliferation, will be assessed using flow cytometry, as summarized in **Figure 5A**.

An optimization experiment was performed, using two different cell densities: 2.5×10^5 cells per well, and 5×10^5 cells per well, to determine the ideal seeding concentration. Healthy donor peripheral blood was collected and PBMCs were obtained through density gradient isolation. Upon 5 days in culture, cells were analyzed by flow cytometry. Cells of interest were isolated using size and complexity. From there, live T cells were isolated through Zombie staining and CD3 expression. CD4 and CD8 positive T cells were further selected. Expression of the activation markers CD69 and CD25, as well as proliferation, was assessed in each T cell subset (**Figure 5B**). As a positive control, T cell polyclonal activation was induced by anti-CD3 (1 µg/mL) and anti-CD28 (3 µg/mL) monoclonal antibodies (**Figure 5C**).

An increase in the percentage of CD3+CD25+ cells was observed, going from 10.5% in the unstimulated control (**Figure 5B**), to 75.7% in 2.5×10^5 cells/well, and 69.9% in 5×10^5 cells/well (**Figure 5C**) upon polyclonal stimulation. This shows that cell activation using the mentioned antibodies was successful in both conditions.

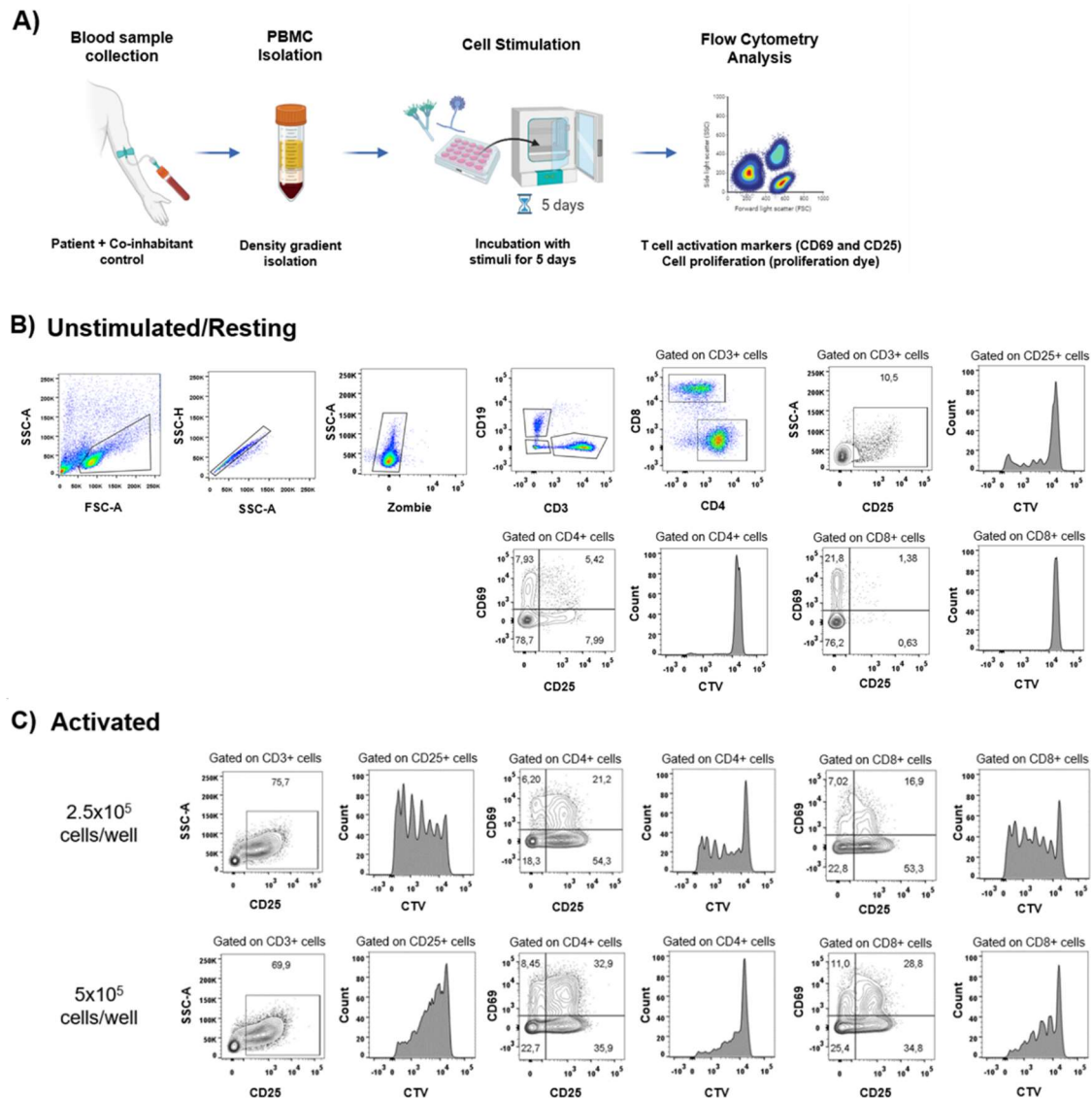


Figure 5. Cell seeding density affects activation profile upon polyclonal activation. (A) Workflow of peripheral blood mononuclear cells (PBMC) isolation and stimulation process. Upregulation of T cell activation markers and proliferation are the main readouts of this assay. Gating strategy used to isolate T cells is shown in **(B)**. PBMC from a healthy control were left unstimulated **(B)** or subjected to polyclonal activation in vitro with anti-CD3 (1 $\mu\text{g/mL}$) and anti-CD28 (3 $\mu\text{g/mL}$) monoclonal antibodies **(C)**, in two different seeding densities: 2.5×10^5 cells/well and 5×10^5 cells/well. Frequency of CD25+ cells in CD3+ cell population and histogram plots showing division peaks for resting/activated CD3+CD25+, CD3+CD4+ and CD3+CD8+ cells are shown for each condition. Histogram plots show CellTrace® Violet (CTV) proliferation dye fluorescence intensity on the X-axis and cell count on the Y-axis.

Proliferative profiles are shown in histograms, through CTV (proliferation dye) fluorescence values. This proliferation dye is incorporated in cells before cell stimulation and culture and becomes diluted each time cells undergo division. This way, CTV allows an assessment of the number of division cycles, as well as the number of cells undergoing division. Not only is the percentage of CD3+CD25+ cells higher when 2.5×10^5 cells are seeded per well, but there are also more cells undergoing division and

reaching a higher number of division cycles, as seen in the respective histograms (**Figure 5C, top**) when compared to 5×10^5 cells/well (**Figure 5C, bottom**).

When assessing CD69 and CD25 expression in CD4⁺ and CD8⁺ cells individually, double positive cells' (CD69⁺CD25⁺) percentage is higher in 5×10^5 cells/well, in both T cell subsets. It is known that CD69 expression precedes CD25 expression when T cell activation occurs (144). Nevertheless, the expression of CD69 decreases with activation time, whereas CD25 expression increases and peaks at later time points (145). Considering a 5-day stimulation, the frequency of CD25⁺ (single positive) cells, both CD4⁺ and CD8⁺, should comprise the highest percentage among the total T cell population, as CD69 expression is progressively lost, and double positive cells' percentage might decrease. Even though this seems to be the case for wells with 2.5×10^5 cells, this is not observed for wells with 5×10^5 cells: in this condition, percentages of double-positive cells are higher than observed in 2.5×10^5 cells/well, and rather similar to the respective percentage of CD25⁺ (single positive) cells. An impaired or delayed activation process in the 5×10^5 cells/well condition may be related to an insufficient activator antibody concentration for the number of seeded cells, which in turn negatively impacts T cell activation.

This is further supported by the lower number of cells dividing and reaching a lower number of division cycles in the wells with 5×10^5 cells when compared to wells with 2.5×10^5 cells/well, as seen for total T cells (**Figure 5C**).

Considering these results, CD25 expression and proliferation rates will be the primary outcomes of T cell activation in this work.

3.3. PBMC stimulation with fungal conidia retrieved from HP patient's home induces fungi-specific T cell activation and proliferation

Having defined the assay methodology, a proof-of-concept stimulation assay was set up. For this experiment, a treatment-naïve patient, with reported mould exposure and whose home was inspected, was selected along with the respective co-inhabitant.

In order to use the fungi as stimuli, fungal conidia must remain ungerminated, so as not to disturb cell culture. Inactivation of fungal conidia through exposure to ultraviolet radiation was used in previous publications (146), with the goal of maintaining the integrity of surface antigens, molecules that may otherwise be lost if other inactivation methods were employed. As such, UV inactivation seemed to best fit our needs, as UV-

inactivated conidia would not affect cell culture and antigen integrity would be maintained. However, *Penicillium* spp. and *A. niger* did not respond to this method. Resistance to UV radiation through the production of pigments, such as melanin, has been reported as the main resistance mechanism for both *Penicillium* spp. (147) and *A. niger* (148). This is an especially relevant mechanism in environmental fungi, contributing significantly to their survival in often unfavourable habitats. Moreover, fungal spores are generally hydrophobic and tend to cluster together, which may stunt UV inactivation (149).

Therefore, another method to prevent spore germination during cell culture was tested. In an effort to maintain antigen integrity, fungi were incubated with amphotericin B, a known antifungal agent, at a concentration of 5 µg/mL 24h prior to the addition of cells and maintained at the working concentration of 2.5 µg/mL throughout the 5 days of cell-fungi culture. When incubated with amphotericin B, the spores of *Cladosporium* spp., *A. fumigatus*, *Rhizopus* spp. and *Penicillium* spp. did not germinate, however, spores from *A. niger* germinated. Concentration of amphotericin B may have been too low to successfully prevent germination in this fungus, however, higher concentrations of amphotericin B in the cell culture medium have been shown to negatively affect cell viability (150), which could impact results. As *A. niger* did not respond to the inactivation methods tested at the time this work was performed, it could not be used as a stimulus.

In this patient's home inspection, *Cladosporium* spp. and *Penicillium* spp. were the most prevalent ones, although *Aspergillus fumigatus* was also detected. As such, cells were stimulated using conidia incubated with amphotericin B from *Cladosporium* spp., *Penicillium* spp., and *A. fumigatus*, in a ratio of 4:1 (conidia : cells) as suggested in a previous publication (127). A ratio of 8:1 was also tested, however, this amount of conidia seemed overpowering to cells, as not enough survived to obtain clear gates for activated cells or proliferative profiles (data not shown).

The expression of activation markers, as well as the proliferative profile of T cells, was assessed (**Figure 6**). The high number of cells in the proliferative profile of total T cells may hinder clear visualization of proliferating cells (**Figure 6A, middle histograms**), as not all cells will respond to this specific stimulus, and therefore will not proliferate. Thus, better visualization of cell division peaks can be obtained by further isolating activated cells, defined as CD25+, to assess specific-fungal stimulation response (**Figure 6A, right overlaid histograms**).

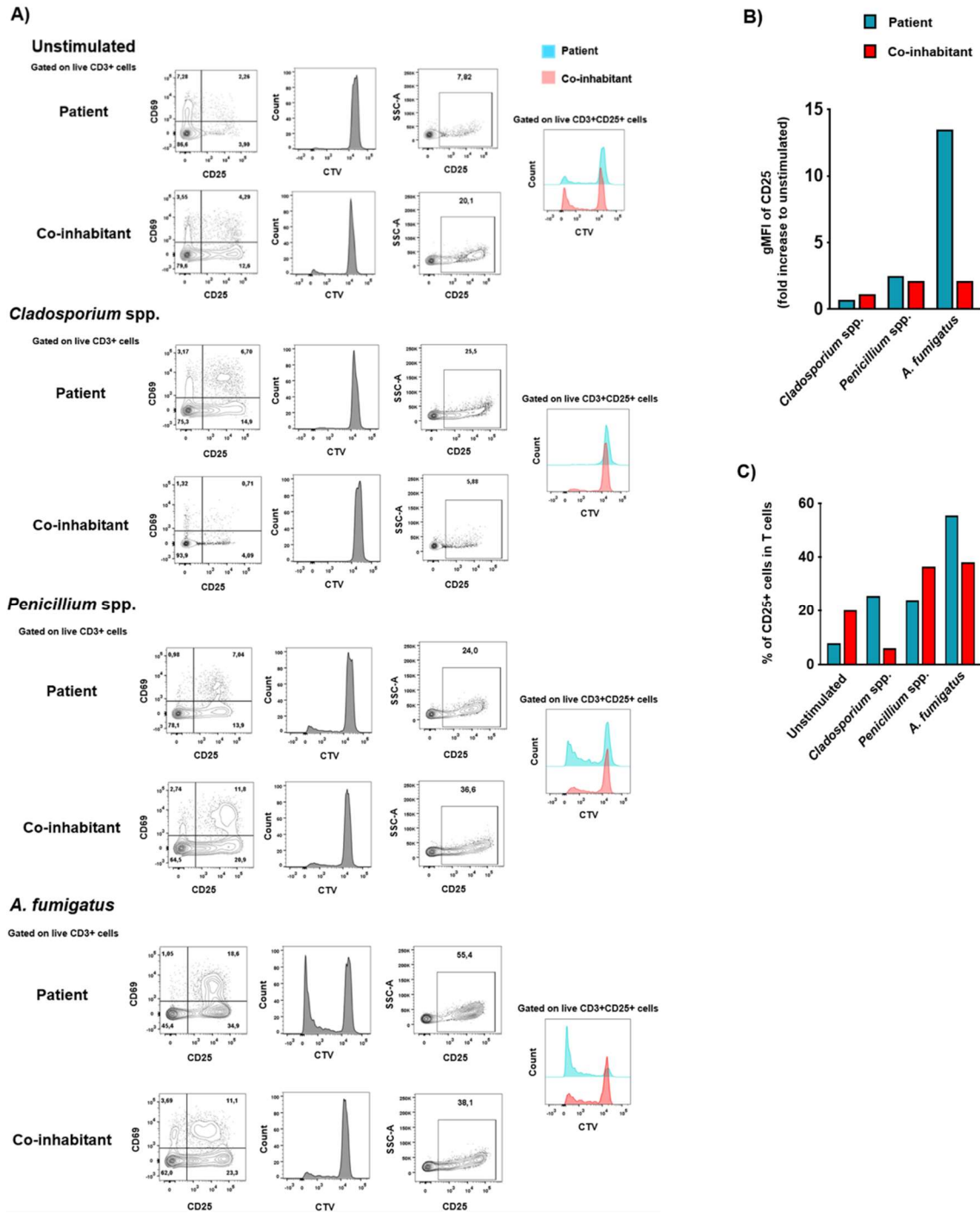


Figure 6. Stimulation with fungal conidia detected in patient's home induces T cell activation and proliferation. 2.5×10^5 peripheral blood mononuclear cells (PBMC) per well from a hypersensitivity pneumonitis (HP) patient and corresponding co-inhabitant were stimulated *in vitro* with conidia from the fungi detected in their home: *Cladosporium* spp., *Penicillium* spp. and *A. fumigatus*, at a ratio of 4:1 (conidia : cells). Conidia were incubated with amphotericin B at a concentration of 5 $\mu\text{g/mL}$ for 24 h prior to the start of the cell culture and maintained at 2.5 $\mu\text{g/mL}$ throughout cell culture duration. **(A)** Frequency of CD69+, CD25+ and double-positive cells, histogram plots showing division peaks and frequency of CD25+ cells in live CD3+ cell population for the unstimulated control and fungal stimuli. Overlaid histogram plots show division peaks in CD3+CD25+ cells for the patient (blue) and the co-inhabitant (red). Histogram plots show CellTrace® Violet (CTV) proliferation dye fluorescence intensity on the X-axis and cell count on the Y-axis. Geometric mean of fluorescence intensity values for T cell activation marker CD25 in the CD3+ cell population as a fold increase to the respective unstimulated control **(B)** and frequency of CD25+ cells in CD3+ cell population **(C)** for the patient (blue bars) and co-inhabitant (red bars) for every tested condition.

Patient's cells respond clearly upon stimulation with fungal conidia from *Penicillium* spp. and *A. fumigatus*, with no observable response towards *Cladosporium* spp. However, even though both seem to activate patient's cells, a much stronger response is observed upon stimulation with *A. fumigatus*, both in terms of proliferation (**Figure 6A, blue histograms**), CD25 expression (**Figure 6B, blue bars**), and CD25+ cells frequency (**Figure 6C, blue bars**). In contrast, the co-inhabitant seems to respond similarly to stimulation with all fungi, with comparable values of CD25 expression (**Figure 6B, red bars**), and frequencies of CD25+ cells (**Figure 6C, red bars**), although a weak proliferative response is visible upon stimulation with *A. fumigatus* (**Figure 6A, red histograms**).

It is not surprising to observe mild cell activation in healthy individuals upon stimulation with fungi, as fungal spores are naturally present in all environments, hence virtually all individuals will or have already encountered them. However, most individuals eliminate inhaled spores easily without developing any major inflammatory response, as previously mentioned (102). Further, the similarities between the stimulations with *Penicillium* spp. and *A. fumigatus* could derive from their taxonomical resemblance, hinting towards a non-specific response.

Curiously, when co-inhabitant's cells were stimulated with conidia from *Cladosporium* spp., the frequency of activated cells decreased (**Figure 6C, red bars**) and no proliferation was observed (**Figure 6A, red histograms**). This behaviour could derive from the fact that this fungus is commonly found in all environments, being the most prevalent in our sample (**Figure 4C**), and could simply not constitute a significant trigger to induce an immune response in healthy individuals. This, combined with possible toxicity due to the presence of fungal debris in the culture medium, may have caused a higher percentage of cell death upon stimulation with *Cladosporium* spp. when compared to the unstimulated control.

It is important to note that the frequency of CD25+ cells in the unstimulated control of the co-inhabitant is significantly higher than in the patient. *Ex vivo* steady-state cell profile is affected by any inflammation or prior activation suffered *in vivo*, which will affect resting behaviour, altering the percentage of activated/proliferating cells. Since this may affect data interpretation, immunophenotyping was performed in further experiments, for both patients and co-inhabitants, to assess baseline immune profile, and if in any patient/co-inhabitant cell frequencies were altered, cell stimulation was not performed.

All three fungi were detected in this patient's home, however, the mounted response against *A. fumigatus* seems to be specific. *A. fumigatus* is reportedly associated with the development of allergic respiratory diseases, as mentioned. Several species-specific antigens have been described for this fungus, and even though healthy individuals do not develop the disease, a rather diverse *A. fumigatus*-reacting T cell repertoire has been reported in humans, which could be related not only to their number but also to the high expression of the majority of these antigens *in vivo* (151), leading to enhanced recognition of these proteins by the host, and consequently amplified immune response, a phenomenon that could be further intensified in *A. fumigatus*-sensitized individuals, as observed in our results.

All in all, our observations seem to support the hypothesis that this patient may have become sensitized to fungal antigens, possibly to a species-specific antigen in *A. fumigatus*, prior to the stimulation assay, implying a possible culprit exposure for the development of HP in this patient.

3.4. Fungal cocktails composed of conidia from the fungi retrieved in the patient's home induce a strong T cell response

Taking these results into account, a fungal cocktail, containing conidia from multiple fungi, should be able to powerfully activate cells and could be used to assess possible mould exposure in putative HP patients, without the need for recurring house inspections.

We aim to develop a cocktail comprising the most prevalent fungi in our sample, *Cladosporium* spp., and *Penicillium* spp., as well as *A. fumigatus*, and *Rhizopus* spp., as these fungi showed up in higher amounts indoors rather than outdoors. The use of a cocktail may be able to induce a significant response in mould-sensitized cells, considering possible antigen cross-reactivity. Thus, managing the amount of conidia of each fungus included in the cocktail may help in achieving balance between response specificity and visibility.

Two different cocktails were used in further stimulation assays: a UV-inactivated and a dormant cocktail. The UV-inactivated cocktail contains UV-inactivated conidia from *Cladosporium* spp., *Rhizopus* spp., and *A. fumigatus*. To be able to include *Penicillium* spp., a relevant mould in terms of respiratory diseases, and to assure cell stimulation with fungal antigens that could be lost upon UV inactivation, a dormant cocktail is used,

containing conidia incubated with amphotericin B from *Cladosporium* spp., *Rhizopus* spp., *Penicillium* spp., and *A. fumigatus*. Different ratios were used in this work for both cocktails: 3:1 and 6:1 (conidia : cells) for the UV-inactivated cocktail, and 3:1, 6:1 and 10:1 for the dormant cocktail. These ratios were extrapolated from the results obtained on individual stimulations, based on the previously described methodology (127). A higher fungi-containing ratio, 10:1, was used for the dormant cocktail. Due to the higher number of fungi used in this cocktail, this ratio was used to ensure that enough conidia of each fungus are present in the culture to trigger cell activation, should these not share the same degree of immunogenicity.

Figure 7A shows the comparison between the cell proliferation profiles of the patient and respective co-inhabitant upon stimulation with each cocktail. When stimulated with the UV-inactivated cocktail, a proliferative response can be seen in the patient's cells in both ratios, although much stronger in the 3:1 ratio, with more proliferating cells. In the co-inhabitant, a low number of cells seems to be responding in the 3:1 ratio, however, in the 6:1 ratio, no cell response is visible.

Observations are similar when cells are stimulated with the dormant cocktail. In the patient, a strong response is elicited upon stimulation with a 3:1 ratio, with a high number of cells becoming activated and proliferating, a response that is almost completely lost in the other two ratios. Co-inhabitant's response to the 3:1 ratio of the dormant cocktail is similar to what is observed in the 3:1 ratio of the UV-inactivated cocktail. These observations may suggest that the highest fungi-containing ratios are extreme to the cells, with excessive conidia concentrations, leading to increased cell death, eventually related to cell toxicity and conidia impairing cell nutrient intake or metabolism. These observations are further supported by the data shown in **Figures 7B** and **7C**. As can be observed, overall differences in CD25 expression (**Figure 7B**) are higher in the patient (blue bars) when compared to the co-inhabitant (red bars) for the 3:1 ratio in both cocktails. This reflects a decrease in CD25 expression in the highest fungi-containing ratios of both cocktails and consequently, a decrease in cell activation, when compared to what is observed upon stimulation with a 3:1 ratio of both cocktails.

The percentage of CD25 positive cells (**Figure 7C**) in the co-inhabitant (red bars) appears slightly higher than in the patient (blue bars) for all cocktail ratios, both UV-inactivated and dormant. However, the frequency of CD25+ cells already present in the unstimulated control of this individual is much higher than in the patient. As such, differences between cocktail stimulations and baseline values are less significant than the differences observed in the patient in the same conditions. This highlights the

importance of normalizing data relative to baseline values, through an unstimulated control in this case.

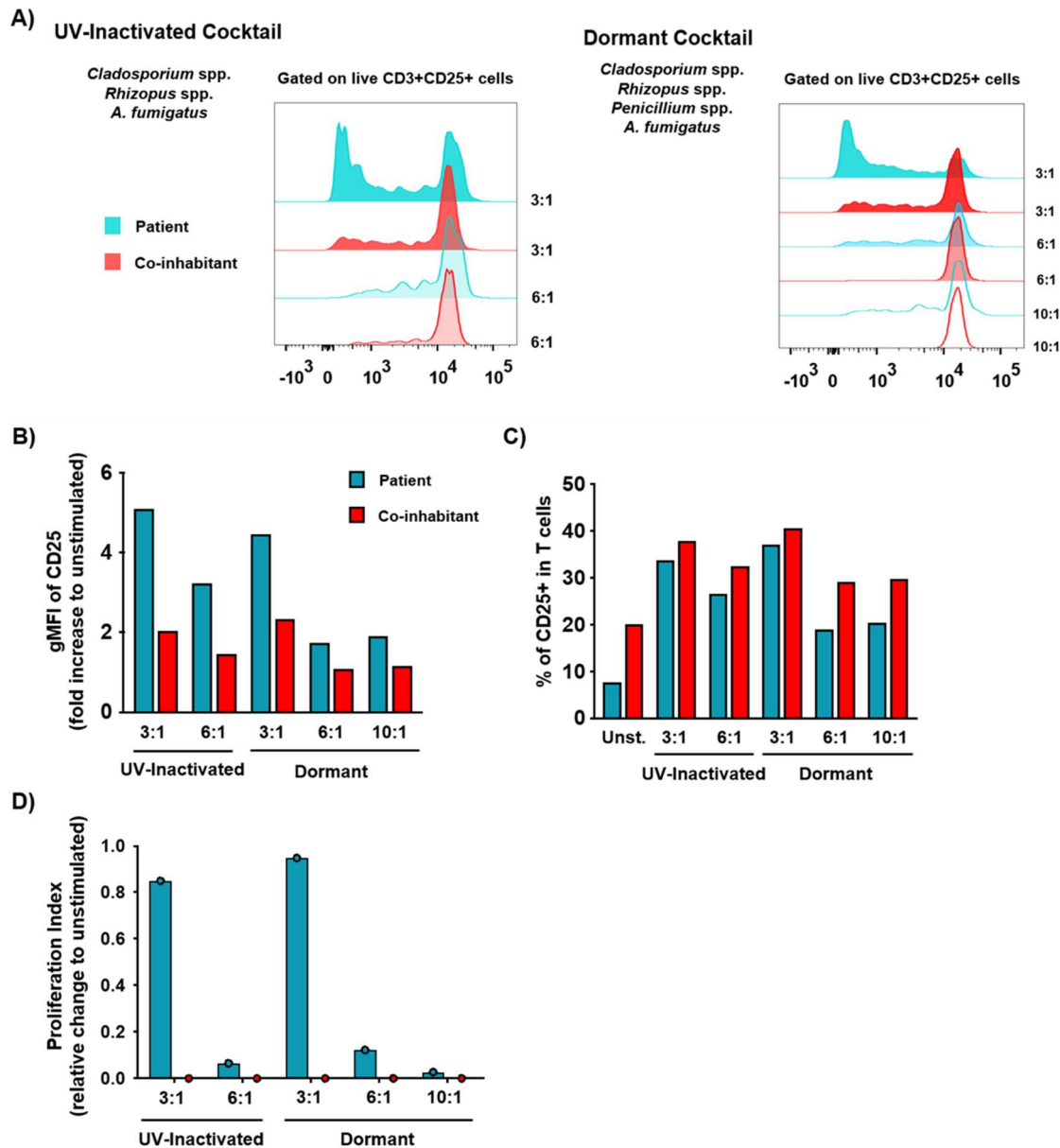


Figure 7. Fungal cocktails induce T cell activation and proliferation in a hypersensitivity pneumonitis (HP) patient, especially in a 3:1 ratio (conidia : cells). 2.5×10^5 peripheral blood mononuclear cells (PBMC) per well from an HP patient and corresponding co-inhabitant were stimulated *in vitro* with two fungal cocktails: one containing UV-inactivated conidia from *Cladosporium* spp., *Rhizopus* spp. and *A. fumigatus*, in two ratios, 3:1 and 6:1 (conidia : cells), and the other containing conidia incubated with amphotericin B (AMB) at a concentration of 5 $\mu\text{g/mL}$ for 24 h prior to the start of the cell culture and maintained at 2.5 $\mu\text{g/mL}$ throughout cell culture duration, from *Cladosporium* spp., *Rhizopus* spp., *Penicillium* spp., and *A. fumigatus*, in three ratios: 3:1, 6:1 and 10:1 (conidia : cells). **(A)** Overlaid histogram plots show division peaks in the CD3+CD25+ cell population for the patient (blue) and co-inhabitant (red) for all conditions. Histogram plots show CellTrace® Violet (CTV) proliferation dye fluorescence intensity on the X-axis and cell count on the Y-axis, in a modal scale. Geometric mean of fluorescence intensity values for T cell activation marker CD25 in the CD3+ cell population as a fold increase to the respective unstimulated control **(B)**, frequency of CD25+ cells in CD3+ cell population **(C)** and proliferation index as a relative change to the respective unstimulated control **(D)** for the patient (blue bars) and co-inhabitant (red bars) for all ratios of the UV-inactivated and dormant cocktails. Proliferation index values are obtained by subtracting the median proliferation dye fluorescence intensity in the CD3+CD25+ cell population to 1.

Considering what we observed in both **Figures 6** and **7**, where proliferation appears to be a more specific and robust readout, a proliferation index is shown (**Figure 7D**) for stimulations with fungal cocktails. Proliferation index is obtained through the median values of CTV fluorescence in T cells CD25+ cells as a fold change to the corresponding unstimulated control. Median values, rather than geometric mean values, of CTV fluorescence were used to obtain the proliferation index since observed cell proliferation does not follow a normal distribution. Indeed, differences in cell response can be clearly observed using this type of representation, mimicking the cell division peaks observed in histograms (**Figure 7A**). A much higher proliferation index is observed in the patient's cells upon stimulation with the 3:1 cocktails, however, as observed previously, this value decreases drastically in the other ratios of both cocktails. In the co-inhabitant, values for proliferation index are null in all cocktail ratios, showing no difference when compared to the control, which indicates no substantial number of cells is proliferating, and therefore not becoming specifically activated.

As such, using a fungal cocktail can be beneficial, as a specific response from a patient with possible mould-induced HP is distinguishable from a healthy (non-HP), but exposed, individual, as shown by our results, and could help avoid recurring house inspections.

3.5. Stimulation with fungal cocktails and individual fungi induces a Th1-type response in HP patients

As per results obtained regarding optimal stimulation ratios of both fungal cocktails, the 3:1 ratio of both the UV-inactivated and dormant cocktails was used in further experiments, along with *Rhizopus* spp., *Penicillium* spp., and *A. fumigatus* individually. Data from *Cladosporium* spp. individual stimulation is not included in the following results since it did not induce a response in any of the tested patients or co-inhabitant controls.

Some of the patients who agreed to a house inspection were tested in a stimulation assay. **Figure 8** depicts proliferative profiles (**Figure 8A**) proliferation index (**Figure 8B**) and IFN- γ concentration values in the culture supernatant (**Figure 8C**) for patients and co-inhabitants, in all conditions.

Comparing patient's proliferative profiles (**Figure 8A, blue histograms**) upon stimulation with either of the fungal cocktails, a higher number of cells seem to be proliferating in response to the dormant cocktail, specifically in patients FL146, FL52, and FL133, without any comparable response by the co-inhabitants. In contrast, only one of the

tested patients, FL133, seems to react to the UV-inactivated cocktail. We hypothesize that the enhanced response towards the dormant cocktail observed in the patients could imply the existence of a conidial factor exclusively present in this mixture, possibly lost when UV inactivation is employed.

Differences between proliferation index values for patients (**Figure 8B, blue bars**) and co-inhabitants (**Figure 8B, red bars**) seem to be the highest upon stimulation with both cocktails, seemingly more marked when the dormant cocktail is used as a stimulus, further supporting the fact that cells seem to respond more intensely to these conditions.

Regarding stimulations with individual fungi, a higher degree of activation is observed in the co-inhabitants, with the exception of the FL133 pair, whose patient response is always more intense than the respective co-inhabitants'. The co-inhabitant from patient FL52 (**Figure 8A, red histograms**) shows the highest response among the co-inhabitant group, with a high number of cells proliferating when stimulated with *Rhizopus* spp. and *A. fumigatus*, contrasting with observations for the respective patient (blue histograms). The intensity of the immune response observed for this individual suggests prior exposure to these specific fungi. This co-inhabitant control is not ideal, as they do not inhabit the same house as the patient permanently. As such sensitization may not be derived from a domestic exposure and could therefore be the result of a current occupational or recreational exposure. Although the domestic environment has become more and more relevant as a source of possible culprit exposures, workplace or school environments are highly common sources of culprit exposures in HP patients, as a significant part of the day is also spent in the workplace/school, and individuals could be recurrently exposed to a diverse array of organic or inorganic antigenic particles, that could influence or trigger the development of allergic disease (152, 153). Additionally, this co-inhabitant is much younger than the patient, and both are genetically related. Genetic factors are known to have an effect on HP development, therefore future development of respiratory disease cannot be ruled out in this individual.

Additionally, the co-inhabitant from patient FL174 and F133 (red histograms) also seems to have some cells proliferating upon stimulation with *Rhizopus* spp. and *Penicillium* spp., even though is not as marked as the one observed for the co-inhabitant of patient FL52, hinting towards a non-specific response, as proliferative responses to both mentioned fungi are similar, and no response is observed for neither of these patients when stimulated with the fungal cocktails.

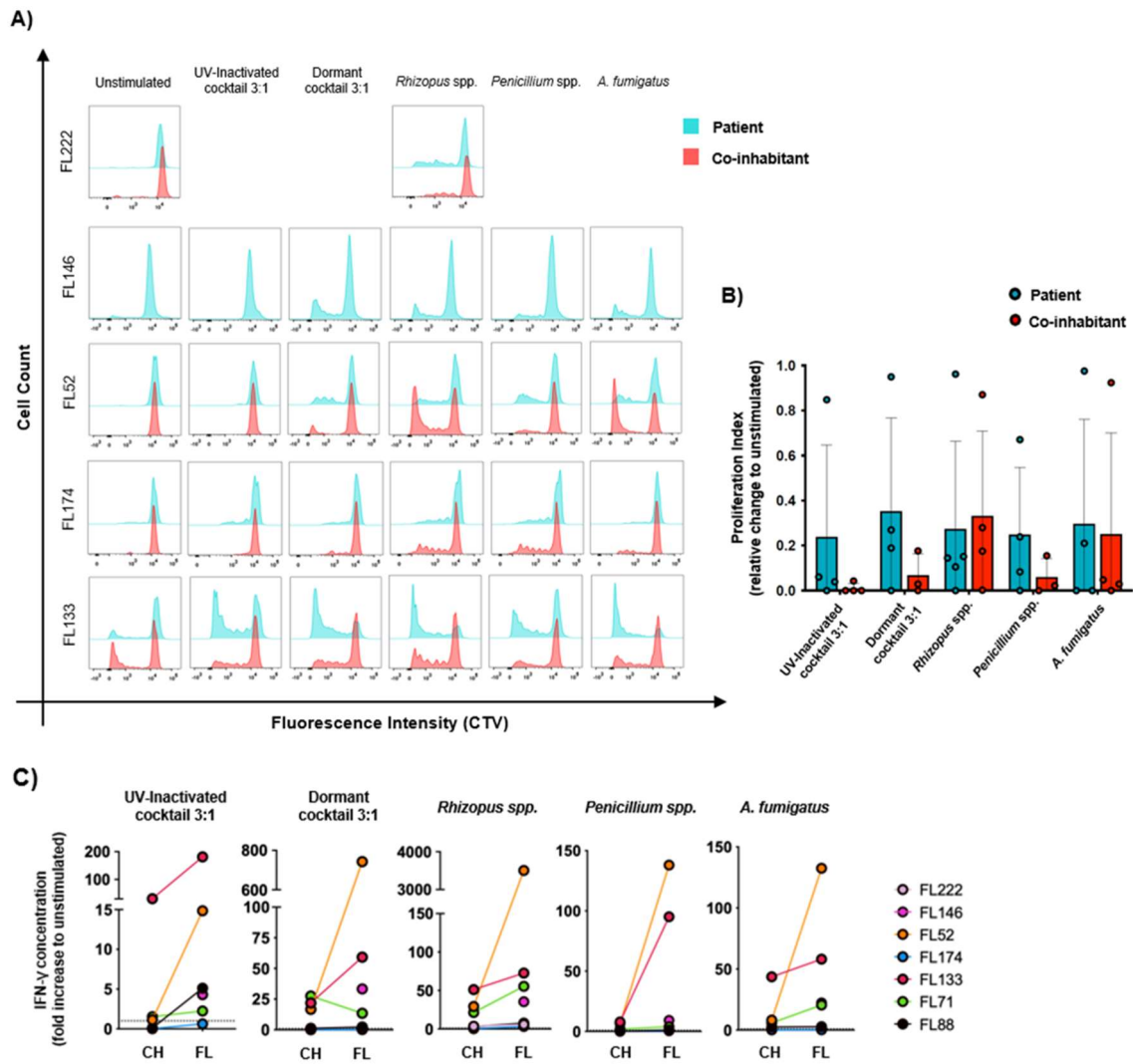


Figure 8. *In vitro* cell stimulation with fungal cocktails and individual fungi leads to a Th1-like inflammatory response in patients with a confirmed hypersensitivity pneumonitis (HP) diagnosis. 2.5×10^5 peripheral blood mononuclear cells (PBMC) per well from several patients with diagnosed HP and house inspection, and corresponding co-inhabitants were left unstimulated or stimulated *in vitro* with two fungal cocktails: one containing UV-inactivated conidia from *Cladosporium* spp., *Rhizopus* spp., and *A. fumigatus* and the other – dormant cocktail - containing conidia incubated with amphotericin B (AMB) at a concentration of 5 $\mu\text{g/mL}$ for 24 h prior to the start of the cell culture and maintained at 2.5 $\mu\text{g/mL}$ throughout cell culture duration, from *Cladosporium* spp., *Rhizopus* spp., *Penicillium* spp., and *A. fumigatus* both at a ratio of 3:1 (conidia : cells). Cells were also stimulated with AMB-incubated conidia from *Rhizopus* spp., *Penicillium* spp., and *A. fumigatus* individually, at a ratio of 4:1 (conidia : cells). **(A)** Overlaid histogram plots show division peaks in the CD3+CD25+ cell population for the patient (blue) and co-inhabitants (red) for all stimuli. Histogram plots show CellTrace® Violet (CTV) proliferation dye fluorescence intensity on the X-axis and cell count on the Y-axis, in a modal scale. **(B)** Proliferation index as a relative change to the respective unstimulated control for the patients (blue bars) and co-inhabitants (red bars), for all stimuli. Proliferation index values are obtained by subtracting the median of proliferation dye fluorescence intensity values in the CD3+CD25+ cell population to 1. Each dot represents one individual. Values are shown as mean ± SD. **(C)** Interferon-gamma (IFN-γ) concentration in the cell culture supernatant for each patient-co-inhabitant pair as a fold increase to the respective unstimulated control for all stimuli. Each dot represents one individual. Patient-co-inhabitant pairs are connected by a line.

Having validated a putative fungus-reactive T cell-specific proliferation, cell culture supernatants were also analyzed to assess cytokine production. The immunopathogenesis of HP is believed to be mainly characterized by a Th1-type

response, a highly inflammatory protective response, with IFN- γ as the main effector cytokine, and this cytokine has been shown to have an important role in HP pathogenesis (154). As such, interferon gamma production was analyzed and is shown in **Figure 8C**, as a fold increase to the unstimulated control, for each pair of patient-co-inhabitant.

Patients appear more responsive than the co-inhabitants in all conditions, however, differences to the corresponding unstimulated controls generally seem more significant when cocktails are used as stimuli. Additionally, patients FL52 (**Figure 8C, orange dot**) and FL133 (**Figure 8C, red dot**) show the strongest responses in terms of IFN- γ production in each condition. It is worth noting that the observed differences for patient FL52 in all conditions are the highest, especially when stimulated with *Rhizopus* spp. and the dormant cocktail, with a fold increase in IFN- γ concentration, to the respective unstimulated control, of nearly 3500 and 800, respectively. However, no proliferative response is observed in this patient. The fact that this patient was already under immunosuppressive treatment, with a full dose of mycophenolate mofetil, may help explain this behaviour, as this drug negatively impacts the proliferation capacity of T and B lymphocytes specifically (155, 156), preventing the visualization of a proliferative response, which could accompany the marked differences in IFN- γ concentration observed for this patient.

All in all, stimulation with the dormant cocktail generally seems to induce a more intense inflammatory response in patients than in co-inhabitants, hinting at a possible prior sensitization to mould antigens. However, some individual stimulations also seem to induce significant responses in patients. Factors such as natural inter variability in cytokine production or immunosuppressive treatment can make it difficult to take definitive conclusions from cytokine data only. As this evaluation would be performed as an aid to the diagnosis process, before treatment decisions, the effect of immunosuppressive treatment would not be a confounding factor.

To validate this hypothesis, 6 patients with suspected HP (characteristic radiological pattern and symptomatology) and reported mould exposure, still in the diagnosis process and without house inspection, were selected for a stimulation assay, using both cocktails and individual fungi.

Overall, the patient's proliferative response (**Figure 9A, blue histograms**) appears stronger upon stimulation with fungal conidia, when compared to the respective co-inhabitants (**Figure 9A, red histograms**), with no significant visible proliferation. Not all patients develop a response to the fungal conidia, however, cells from patients FL534,

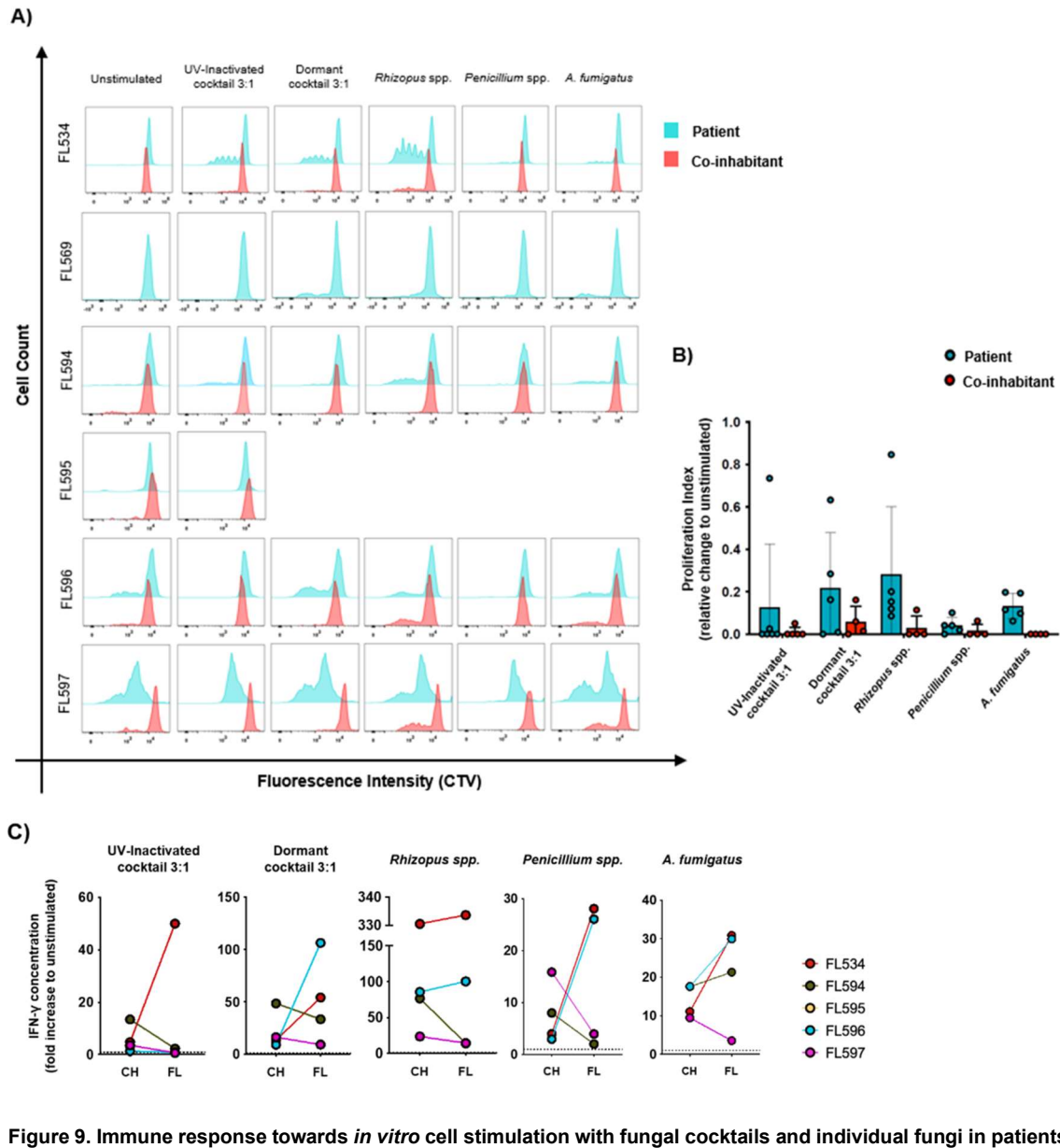


Figure 9. Immune response towards *in vitro* cell stimulation with fungal cocktails and individual fungi in patients with suspected hypersensitivity pneumonitis (HP) may help to achieve a final and highly confident diagnosis. 2.5×10^5 peripheral blood mononuclear cells (PBMC) per well from several patients with suspected HP and corresponding co-inhabitants were stimulated *in vitro* with two fungal cocktails: one containing UV-inactivated conidia from *Cladosporium* spp., *Rhizopus* spp., and *A. fumigatus* and the other – dormant cocktail - containing conidia incubated with amphotericin B (AMB) at a concentration of $5 \mu\text{g/mL}$ for 24 h prior to the start of the cell culture and maintained at $2.5 \mu\text{g/mL}$ throughout cell culture duration, from *Cladosporium* spp., *Rhizopus* spp., *Penicillium* spp., and *A. fumigatus* both at a ratio of 3:1 (conidia : cells). Cells were also stimulated with dormant conidia from *Rhizopus* spp., *Penicillium* spp., and *A. fumigatus* individually, at a ratio of 4:1 (conidia : cells). **(A)** Overlaid histogram plots show division peaks in the CD3+CD25+ cell population for the patient (blue) and co-inhabitants (red) for all stimuli. Histogram plots show CellTrace® Violet (CTV) proliferation dye fluorescence intensity on the X-axis and cell count on the Y-axis, in a modal scale. **(B)** Proliferation index as a relative change to the respective unstimulated control for the patients (blue bars) and co-inhabitants (red bars), for all stimuli. Proliferation index values are obtained by subtracting the median of proliferation dye fluorescence intensity values in the CD3+CD25+ cell population to 1. Each dot represents one individual. Values are shown as mean $\pm$ SD. **(C)** Interferon-gamma (IFN- γ) concentration in the cell culture supernatant for each patient-co-inhabitant pair as a fold increase to the respective unstimulated control for all stimuli. Each dot represents one individual. Patient-co-inhabitant pairs are connected by a line.

FL596, and FL597 seem to respond when cocktails are used as a stimulus, especially the dormant cocktail.

Additionally, some patients develop a response when fungi are used individually. Patient FL534 (blue histograms) displays an intense proliferative response when stimulated with *Rhizopus* spp., an intensity that is not observed in the respective co-inhabitant (red histograms). Additionally, the response that is observed in the cocktails for this patient could be related to the presence of *Rhizopus* spp. in the mixtures, as it is of lower magnitude than the one observed for the fungus individually. Even though no house inspection was performed, we hypothesize that *Rhizopus* spp. may be present in this patient's home, as the observed response seems to be specific for this fungus, hinting towards the possibility that this could be the culprit exposure in this case.

Few cells from patients FL594 and FL596 also appear to respond to this fungus, albeit much less intensely, with a comparable response in the co-inhabitant of patient FL596 in the same conditions.

Curiously, proliferation peaks for patient FL597 (blue histograms) are unclear. As the larger peak, with higher CTV fluorescence corresponding to non-proliferating cells, also seems affected, the lack of clarity could be related to a possible issue with CTV incorporation by the cells. Nevertheless, cells seem to respond when stimulated with the dormant cocktail, *Rhizopus* spp. and *A. fumigatus*. Few cells from the respective co-inhabitant (red histograms) seem to become activated and proliferate, although this only happens upon stimulation with the individual fungi, not with the dormant cocktail, as happens with the patient.

Differences in proliferation index values between patients (**Figure 9B, blue bars**) and co-inhabitants (**Figure 9B, red bars**) seem markedly higher for the patients when cells are stimulated with the cocktails, especially the dormant cocktail, further supporting proliferation observations (**Figure 8A**). Additionally, differences between both groups when stimulated with *Rhizopus* spp. or *A. fumigatus* are also markedly higher in the patients when compared to the co-inhabitants.

Concentration of IFN- γ was also assessed in the cell culture supernatant (**Figure 9C**). Patient FL534 (red dot) is one of the strongest responders in terms of IFN- γ production when stimulated with *Rhizopus* spp. and the cocktails, which correlates with respective proliferation results. Additionally, patient FL596 (blue dot) also displays a strong inflammatory response, especially in the dormant cocktail, and a comparable response is observed in the respective co-inhabitant upon stimulation with *Rhizopus* spp., similar

to the profile of patient FL534 in this same condition. Interestingly, the concentration of IFN- γ in patients FL594 (green dot) and FL597 (pink dot) is lower than in the respective co-inhabitants, in all conditions.

Generally, these results seem to support our previous observations. When the cocktails, especially the dormant cocktail, are used as stimuli, production of IFN- γ seems higher in the patients than in the co-inhabitants. Patients FL534 and FL596 are the highest responders in terms of cytokine response when stimulated with the UV-inactivated cocktail and the dormant cocktail, respectively. Evidently, stimulation with the cocktails seems to provide a way to distinguish possible mould-induced HP, as IFN- γ production is markedly increased in patients whose cells have proliferated in contact with this mixture.

3.6. Patients diagnosed with HP tend to develop a stronger immune response towards fungal conidia, as opposed to other ILD diagnosis

After the diagnosis process of these patients was completed, four of these patients were diagnosed with HP, one with IPF, and one with smoke-related ILD, caused by continued cigarette smoking. **Figure 10** shows both the proliferative index (**Figure 10A**) and production of IFN- γ as a fold increase to unstimulated control (**Figure 10B**) for all tested patients, stratified according to the diagnosis.

Proliferation index generally appears to be higher in patients with an HP diagnosis (blue bars) when compared to non-HP patients (orange and pink bars) in all conditions. When the dormant cocktail is used, there is a tendency for values to be higher in HP patients, however, proliferation index values for HP and IPF patients are not as markedly different as expected, when considering previous results. It is known that IPF shares similarities with HP in terms of phenotype, especially fibrotic HP, sometimes misdiagnosed as IPF (157). Nonetheless, the specific cause of IPF has not been fully elucidated, despite several risk factors for the development of IPF having been described. Even though the pathogenesis of IPF does not seem to comprise a significant immune component, it is known that certain environmental exposures may be responsible for the worsening of symptomatology and clinical outcome, as well as a higher risk of developing this disease (158, 159). Thus, the similarity between HP and IPF in terms of proliferation index values may derive from the fact that mould exposure could have been an inciting factor for the development of IPF in this patient, who could have become sensitized, showing a stronger proliferative response when stimulated with the dormant cocktail, similarly to HP

patients. However, in individual stimulations, the proliferation index for this patient is markedly lower when compared to HP patients. As such, a higher number of samples could help to better visualize differences between the groups and distinguish possible HP from other diagnoses.

In terms of IFN- γ production, differences seem markedly augmented in HP patients when compared to non-HP patients, which is in line with observations for the proliferation index values. Similarly, there is a tendency for the production of IFN- γ to be higher for HP patients upon stimulation with the dormant cocktail, with even higher values when cells from these same patients are stimulated with *Rhizopus* spp., whereas values for the IPF patient are comparable between these two conditions, and between *Penicillium* spp. and *A. fumigatus*, despite lower values in these last two conditions. Additionally, the smoke-related ILD patient does not seem to develop an immune response towards any of the stimuli, showing comparable low responses throughout.

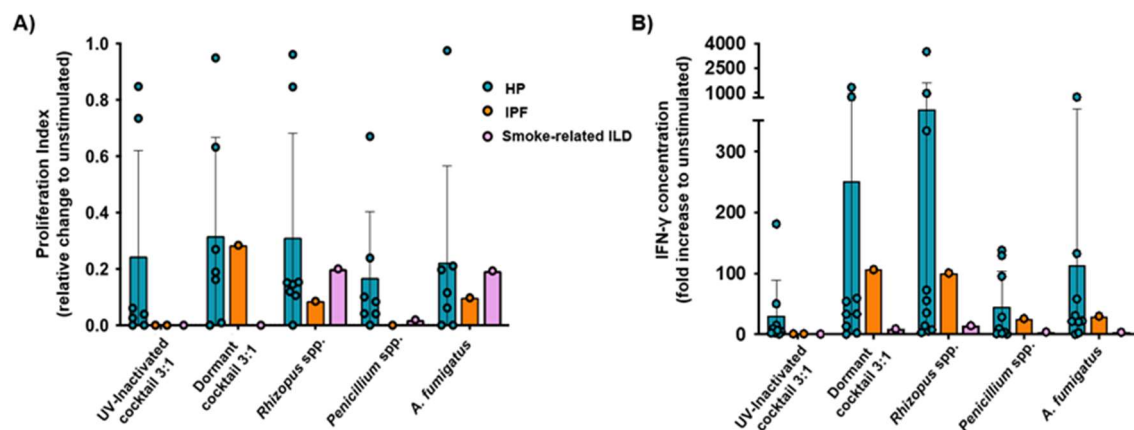


Figure 10. A stronger immune response towards fungal antigens associates with an HP diagnosis. Proliferation index as a relative change to the respective unstimulated control (A) and interferon-gamma (IFN- γ) concentration in the cell culture supernatant as a fold increase to the respective unstimulated control (B) for all tested patients (with house inspection and without house inspection) for all stimuli according to final diagnosis: HP (blue bar), idiopathic pulmonary fibrosis (IPF) (orange bar) and smoke-related interstitial lung disease (ILD) (pink bar). Proliferation index values are obtained by subtracting the median of proliferation dye fluorescence intensity values in the CD3+CD25+ cell population to 1. Each dot represents one individual. Values are shown as mean $\pm$ SD.

Altogether, there seems to be a tendency for HP patients to respond more intensely to stimulation with fungal conidia, a response which curiously appears more powerful when the dormant fungal cocktail is used to stimulate cells, as opposed to the UV-inactivated cocktail. Even though the UV-inactivated cocktail does not contain conidia from *Penicillium* spp., we believe the intensity of the response observed in the dormant cocktail is not related to the presence of this fungus in the mixture, as no marked proliferative or cytokine response is observed when it is used in cell stimulations

individually, cueing towards the existence of a factor exclusive to the dormant cocktail, which may be inducing a more intense immune response, as previously mentioned.

Furthermore, we believe that the highest differences in the production of IFN- γ upon stimulation with the dormant cocktail and *Rhizopus* spp., mostly, could be the result of an immune response mounted by HP patients whose culprit exposure comprises mould particles, either in a domestic, occupational, or recreational setting. In fact, the highest values of the production of IFN- γ among HP patients in both conditions reach nearly 1000 times higher values of IFN- γ when compared to the respective unstimulated control and range from 350 to nearly 4000 times higher values with *Rhizopus* spp., markedly different from what is observed for the other ILDs. As such, the use of a fungal cocktail for cell stimulations appears to allow for the distinction of HP patients in a diagnosis scenario, with a seemingly more intense response in patients whose disease development may be derived from the inhalation of mould particles.

4. Conclusion and Future Perspectives

In this work, we aimed to establish a correlation between the high incidence of HP in the northern region of Portugal and domestic exposure to moulds, by establishing the most common fungal antigens in the domestic environment and developing a fungal cocktail capable of inducing T cell response and cytokine secretion that would allow the identification of a signature response in patients with mould-induced HP.

Air samples were collected in patient's homes. Several different fungi were detected, however *Cladosporium* spp. and *Penicillium* spp. were shown to be the most prevalent. Indoor spore count was also shown to be higher than outdoor spore count for some of the fungi, namely *Aspergillus fumigatus*, *Aspergillus niger*, and *Rhizopus* spp., which could indicate a detrimental role for these fungi in the health of these patients.

We show that cells from some of the tested patients become activated and proliferate extensively when stimulated with fungal conidia, whereas the same profile is not observed in co-inhabitants. Even though some of these healthy but exposed individuals also appear to develop a mild response, the intensity is always lower than what is observed in the patient, cueing towards an unspecific immune response. An upregulation of the production of interferon-gamma, a highly inflammatory cytokine and main effector of the Th1 response characteristic of HP pathogenesis, is also observed when patient's cells are stimulated with fungal conidia, when compared to the respective co-inhabitant controls. Not all tested HP patients responded to the fungal stimulation. With almost 200 different exposures reported to potentiate the development of HP in sensitized individuals, mould may simply not be the inciting antigen in these cases, despite reported compatible exposure.

When patients with suspected HP, who also reported mould exposure, were tested, a similar profile was observed: cells from some of the patients also proliferated and produced higher amounts of IFN- γ when compared to the co-inhabitant controls. Similar to what was observed in diagnosed patients, not all individuals developed a response. Additionally, an HP diagnosis was confirmed for most of the patients in this group, including the patient with the strongest observed response both in terms of proliferation and IFN- γ production. Despite no house inspection being performed for this patient, the observed response seems to hint towards moulds as culprit exposure.

Importantly, a more intense response was observed when fungal cocktails, a mixture of conidia from the selected fungi, were used in both groups, as opposed to stimulations

with individual fungi. This may derive from antigen cross-reactivity, which may have exacerbated the immune response to the organisms in sensitized patients, an aspect that further supports the use of a fungal cocktail to potentially activate patient's cells and identify a signature response. Curiously, the same response to the cocktails is not observed for the co-inhabitants. Further, the dormant cocktail seems to induce a stronger response than the UV-inactivated one, possibly due to the presence of other antigens, which may be lost upon UV inactivation of the conidia. One of the fungi we considered relevant for this work, *Aspergillus niger*, could not be added to the cocktails as it did not respond to the inactivation methods. As such, we plan to produce whole protein extracts of the fungi used in this work, and use these extracts in cell stimulation assays, to maintain as many fungal antigens as possible in contact with the cells when in culture and determine their immunogenic potential.

All in all, we believe the combination of T cell proliferation and elevated secretion of IFN- γ induced in HP patients by the stimulation with the dormant cocktail developed in this work, comprises a specific response towards moulds, and could help in the identification of environmental fungi as a culprit exposure for HP patients with reported mould exposure, further facilitating a diagnosis. Nevertheless, a larger sample will be useful in establishing stronger correlations going further.

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Annexes

Annex I: Scientific poster presented in the I ImmunoHUB Annual Meeting – Infection and Immunity and the XLVIII Annual Meeting of the Portuguese Society for Immunology.

Comprehensive assessment of environmental fungus-reactive T cells in hypersensitivity pneumonitis patients

Rita F. Santos^{1,2}, Andreia L. Coelho^{1,3,4}, João Cavaleiro Rufo^{5,6}, David Coelho^{7,8}, Melany Gonçalves^{1,2}, Samuel M. Gonçalves⁹, Cristina Cunha⁹, Agostinho Carvalho⁹, Luis Delgado^{7,8,10}, António Morais^{7,8}, Margarida Saraiva¹ & Helder Novais-Bastos^{1,7,8}

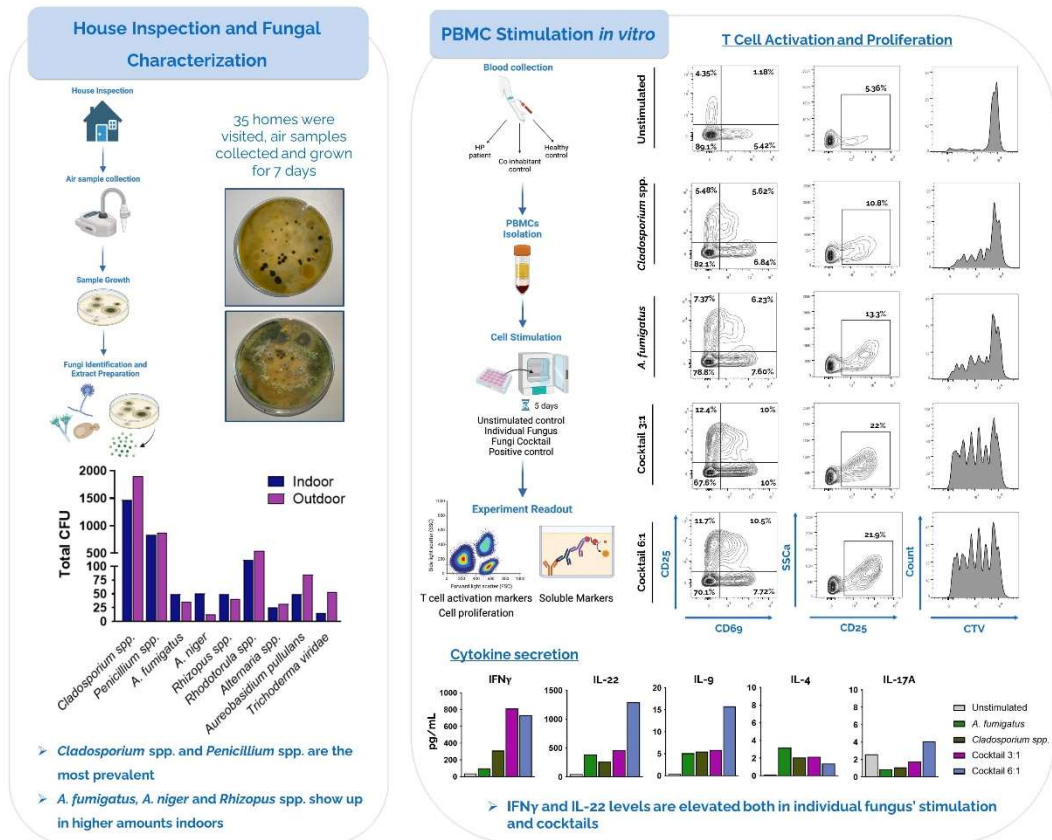
¹Instituto de Investigação e Inovação em Saúde (i3S), Universidade do Porto; ²Escola Superior de Saúde, Instituto Politécnico do Porto; ³Faculdade de Ciências da Universidade do Porto (FCUP); ⁴Instituto Ciências Biomédicas Abel Salazar (ICBAS); ⁵EPIUnit – Instituto de Saúde Pública, Universidade do Porto; ⁶Laboratório para a Investigação Integrativa e Translacional em Saúde Populacional (ITR), Universidade do Porto; ⁷Faculdade de Medicina da Universidade do Porto (FMUP); ⁸Centro Hospitalar Universitário de São João, EPE; ⁹Instituto de Investigação em Ciências da Vida e Saúde (ICVS); ¹⁰Basic/Clinical Immunology Unit, Department of Pathology, Faculty of Medicine, University of Porto;



Introduction

- Hypersensitivity pneumonitis (HP) is an interstitial lung disease that results in parenchymal and small airways inflammation, initiated by an exaggerated immune response triggered by the inhalation of a wide variety of environmental antigens.
- Exposure identification is the diagnosis' cornerstone. However, the inciting antigen is not identified in a large proportion of patients. A difficult to identify, but common inciting antigen, is **exposure to household fungi**.

Based on this, we aim to develop a novel diagnostic method for HP, through the analysis of mold-reactive T cells response



Future Perspectives

- Define a fungi stimulating cocktail to avoid recurring house inspection
- Establish response threshold in comparison to co-inhabitant controls
- Assess T cell response upon fungal stimulation in patients under immunosuppressive treatment

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INSTITUTO DE INVESTIGAÇÃO E INOVAÇÃO EM SAÚDE
UNIVERSIDADE DO PORTO
Rua Alfredo Allen, 208
4200-135 Porto
Portugal
+351 220 408 900
www.i3s.up.pt