Universidade de Aveiro Departamento de Ciências Médicas
2018

ANA FILIPA NINA E CUNHA

DIAGNOSTIC METHODS FOR IMMUNE-RELATED PULMONARY DISEASES

MÉTODOS DE DIAGNÓSTICO PARA DOENÇAS PULMONARES COM UMA COMPONENTE IMUNITÁRIA

Universidade de Aveiro Departamento de Ciências Médicas
2018

ANA FILIPA NINA E CUNHA

DIAGNOSTIC METHODS FOR IMMUNE-RELATED PULMONARY DISEASES

MÉTODOS DE DIAGNÓSTICO PARA DOENÇAS PULMONARES COM UMA COMPONENTE IMUNITÁRIA

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Doutora Catarina Rodrigues de Almeida, Professora Auxiliar do Departamento de Ciências Médicas da Universidade de Aveiro

Este projeto foi financiado pela Fundação para a Ciência e Tecnologia I.P. (Orçamento de Estado) e COMPETE 2020 – Programa Operacional Competitividade e Internacionalização, (FEDER) – referências UID/BIM/04501/2013 e POCI-01-0145-FEDER-007628

Cofinanciado por:

Dedico este trabalho à minha família e aos meus amigos.

o júri

presidente

Prof. Doutora Ana Margarida Domingos Tavares de Sousa
professora auxiliar convidada da Universidade de Aveiro

Prof. Doutora Rita Maria Pinho Ferreira
professora auxiliar da Universidade de Aveiro

Prof. Doutora Catarina Rodrigues de Almeida
professor auxiliar da Universidade de Aveiro

agradecimentos

Antes de mais queria agradecer à Professora Catarina Almeida por me ter aceite no seu laboratório e pelo apoio incansável e essencial para todas as etapas desta tese e sem o qual não teria a oportunidade de trabalhar na área de diagnóstico em imunologia que me fascina. Seguidamente, agradeço a todos os elementos do grupo Immune Cell Biology, em especial à Carolina Silva por toda a paciência e persistência para me ensinar todas as técnicas, como trabalhar em laboratório, estimulando um espírito crítico; e ao Paulo Antas, pela ajuda dentro e fora do laboratório, pela disponibilidade total para me motivar nas idas ao citómetro.

Agradeço também o interesse do Dr. Pedro Ferreira em estudar e desenvolver ensaios em imunologia de doenças pulmonares, fornecendo amostras e novas perspetivas, e sem o qual este projeto não teria sido possível. Ao Edgar Lopes, que foi uma ajuda imprescindível na área da microbiologia, na qual tenho muito pouco conhecimento, na disponibilização dos reagentes, protocolos e sobretudo tempo e ideias que permitiram obter resultados interessantes neste trabalho.

Não poderia deixar de mencionar o apoio incondicional dos meus pais ao longo de todo o mestrado em Aveiro e da minha vida académica e pessoal, ao Diogo e à Ana que me impulsionaram a aceitar desafios literalmente fora da minha zona de conforto, e pelos debates científicos. Tenho de agradecer ainda aos meus amigos fora e à Marta, aos meus amigos de mestrado ‘colegas’ nesta jornada em especial ao Rui.

Á Avelab, e à Dr. Ester em particular, pela colheita de amostras e fornecimento de material essencial para terminar esta tese.

palavras-chave

imunologia, diagnóstico, doenças pulmonares, proteinose alveolar pulmonar pneumonite de hipersensibilidade, GM-CSF, autoanticorpos, ELISA, CFSE, proliferação, linfócitos T, citometria de fluxo.

resumo

O desenvolvimento de ensaios para clínica laboratorial que auxiliem no diagnóstico precoce de doenças raras, em conjunto com os exames de diagnóstico médico, é essencial para a sua gestão e tratamento. Neste projeto foram estudadas duas doenças: a proteinose alveolar pulmonar autoimune (aPAP) e a pneumonite de hipersensibilidade crónica (HP).

O autoanticorpo contra o fator estimulador de colónias de granulócitos e macrófagos (GM-CSF) é um biomarcador da proteinose alveolar pulmonar autoimune, que quando presente em níveis elevados compromete a sinalização GM-CSF, levando à acumulação de surfactantes nos alvéolos e consequentemente a disfunção pulmonar. Esta proteína pode ser facilmente detetada em amostras de plasma através de um ensaio de imunoabsorção enzimática (ELISA). Comparando os níveis de autoanticorpos contra GM-CSF presentes tanto em amostras de plasma de indivíduos saudáveis como pacientes com suspeita de aPAP ou diagnosticados com a doença, torna-se útil para o diagnóstico clínico da forma autoimune da doença.

A pneumonite de hipersensibilidade crónica desenvolve-se após exposição a certos agentes indutores (microorganismos, proteínas aviárias, metais, químicos…). A característica diagnosticante desta doença é a migração e proliferação excessiva para o parênquima alveolar por parte de linfócitos T prévia e cronicamente sensitizados para um determinado agente(s) (antigénico/alergénico). Foi desenvolvido um ensaio de linfoproliferação *in vitro* para avaliar hipersensibilidade mediada por células em resposta a diferentes estímulos. Através de citometria de fluxo e com a marcação prévia com éster sucidinil de carbofluoresceína (CFSE), pode ser medida esta resposta proliferativa dos linfócitos T sensitizados devido à estimulação com mitogénio ou antigénio/alergénio. Utilizando células mononucleares do sangue periférico (PBMCs) isoladas de dadores saudáveis, foi possível detetar a proliferação induzida por fitohemaglutinina (PHA) e leveduras *C. albicans* inactivadas (por temperatura ou quimicamente). A optimização deste ensaio para pacientes com suspeita de pneumonite de hipersensibilidade para identificar o agente indutor da resposta inflamatória permitirá o seu tratamento, evitando a exposição ao agente que está a causar ou agravar a doença. A implementação destes ensaios pode ser fundamental na deteção precoce de doenças pulmonares com uma componente imune.

keywords

immunology, diagnostic, pulmonary diseases, pulmonary alveolar proteinosis, hipersensitivity pneumonitis, GM-CSF, autoantibodies, ELISA, CFSE, proliferation, T lymphocytes, flow cytometry.

abstract

The development of clinical laboratory assays to help interpret medical examination is essential for the early diagnosis of rare diseases, facilitating their management and treatment. Two diseases were studied in this project: autoimmune pulmonary alveolar proteinosis (aPAP) and chronic hypersensitivity pneumonitis (HP).

Granulocyte-macrophage colony-stimulating factor (GM-CSF) autoantibody is a biomarker of autoimmune pulmonary alveolar disease, with elevated levels resulting in impaired GM-CSF signalling, and on the surfactant alveolar accumulation and consequently lung dysfunction. This protein can be easily detected in plasma samples by enzyme-linked immunosorbent assay (ELISA). Comparing the levels of GM-CSF autoantibody present in plasma samples from healthy individuals along with patients with suspicion of aPAP and individuals diagnosed with aPAP is helpful in the clinical diagnosis of the autoimmune form of the disease.

Chronic hypersensitivity pneumonitis develops following exposure to certain inciting agents (microoganisms, avian proteins, metals, chemicals…). The key feature of this disease is an excessive migration and proliferation of primed T lymphocytes into the alveolar parenchyma as a consequence of chronic pre-sensitization to the agent (antigen/allergen). An *in vitro* lymphoproliferation assay was developed to assess delayed-type hypersensitivity in response to different stimulus. The measurement of the proliferative response of primed T-lymphocytes stimulated with mitogen or antigen/allergen, by flow cytometry is possible with carboxyfluorescein succinimidyl ester (CFSE) dilution. Using peripheral blood mononuclear cells (PBMCs) isolated from healthy donors, proliferation was detected following challenge with phytohaemagglutinin (PHA) or inactivated (heat and chemically) *C. albicans* yeast cells. Optimization of this assay for patients with suspicion of hypersensitivity pneumonitis will contribute for the identification of the inducing causal agent of the inflammatory response, enabling the treatment and avoidance of the exposure that could be causing or aggravating the disease.

Implementation of these assays will be valuable for the early detection of rare pulmonary diseases

Contents

Figure index

Table index

Abbreviations

APC	Allophycocyanin; Antigen Presenting Cells
BALF	Bronchoalveolar Lavage Fluid
BSA	Bovine Serum Albumin
CFSE	Carboxyfluorescein Succinimidyl Ester
CSF2RA	Granulocyte-Macrophage Colony-Stimulating Factor Receptor Subunit Alpha
DSP	Disorders of Surfactant Production
EAA	Extrinsic Allergic Alveolitis
EAE	Experimental Autoimmune Encephalomyelitis
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
G-CSF	Granulocyte Colony-Stimulating Factor
GMAb	Granulocyte-Macrophage Colony-Stimulating Factor Autoantibody
HLA	Human Leukocyte Antigen
HP	Hypersensitivity Pneumonitis
HRP	Horseradish Peroxidase
ILD	Interstitial Lung Disease
iNKT	Invariant Natural Killer T
M-CSF	Macrophage Colony-Stimulating Factor
MHC	Major Histocompatibility Complex
NEAA	Non-Essential Amino Acids
PAP	Pulmonary Alveolar Proteinosis
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
PHA	Phytohemagglutinin

rhGM-CSF	Recombinant Human Granulocyte Macrophage Colony Stimulating Factor
SDS	Sodium Dodecyl Sulfate
Th	T Helper cells
TMB	3,3',5,5'-Tetramethylbenzidine
Treg	T regulatory cells
YPD/YEPD	Yeast Extract Peptone Dextrose

1. Introduction

1 Introduction

1.1 Immunity of pulmonary diseases

Autoimmune diseases are chronic, progressive and relatively rare affecting only 2 to 5% of individuals in the developed countries, however their incidence is increasing [1,2]. Autoimmune diseases develop when there is a shift in the natural tolerance to self-molecules or antigens, leading to an immune system's response to the molecules produced by our own organism. When the mechanisms that regulate the destruction of foreign antigens and the tolerance to self breaks, there is production of autoantibodies [1,3,4]. This is particularly important in the pathogenesis of Pulmonary Alveolar Proteinosis (PAP), that will be described further on.

People are exposed every day to pathogens, developing host responses where adaptive immune responses are essential [1,3]. In some situations these responses are deregulated, leading to inflammation, tissue damage and disease, or causing hypersensitivity disorders [1,3]. Hypersensitivity reactions involve both humoral and cellular immunity and may be divided in 4 subtypes [1,3,5]. Immediate hypersensitivity (type I) results from the formation of IgE antibodies after sensitization by an environmental antigen [1]. The antigen re-exposure leads to an anaphylactic reaction of the host [1,5]. Type II hypersensitivity can occur via two pathways: complement-mediated tissue destruction (by IgG and IgM antibodies that bind to their antigens in the target cell leading to cytolysis) or antibody dependent cell cytotoxicity resulting in targeted cell death effected by NK cells [1,5]. Immune complex–mediated (type III), where deposition of immune complexes of circulating antigens and IgM or IgG antibodies occurs after exposure to an antigen, leading to the recruitment and activation of leukocytes [1]. Delayed-type reactions (type IV) are mediated by T cells and antigen presenting cells (APCs) as a response to an antigen stimulus [1]. For this response to occur it is required firstly antigen incorporation, processing and presentation by antigen presenting cells (macrophages or dendritic cells). Next, antigen primed T helper 1 (Th1) cells travel to the site where the antigen is present and become activated. This whole process between antigen exposure and the development of a visible inflammatory

response could take 48 hours [1,4]. Types III and IV hypersensitivity are thought to be responsible for the acute and chronic inflammatory reactions present in Hypersensitivity Pneumonitis (HP) [6–8].

1.2 Pulmonary Alveolar Proteinosis

1.2.1 Pathogenesis

PAP is a syndrome that comprises a group of heterogeneous phenotypes being divided in a primary and a secondary form of the disease [9–11]. It is a rare lung disease thought to affect less than 5 individuals in 100 000, based on a Japanese study with a large cohort [12]. Mainly, this disease is a consequence of the accumulation of surfactants in the alveoli due to macrophage dysfunction, resulting in a diffuse lung parenchyma and eventually reduced pulmonary function [9–11,13–18]. PAP's pathogenesis was unknown until several studies with mice GM-CSF knock-outs, intended to understand the physiological role of this cytokine, revealed that GM-CSF was vital for the normal development of the lungs [19–21]. These studies led to the conclusion that this cytokine is not essential in haematopoiesis. However, these mice exhibited a phenotype similar to human PAP alveoli, implicating a pathogenic role for this cytokine [19–21]. The primary form of PAP is directly associated with the disruption of GM-CSF signalling either by the presence of neutralizing autoantibodies against GM-CSF (autoimmune or idiopathic PAP) or mutations in the α or β-chains of the GM-CSF receptor (hereditary PAP) [9,11,22]. On the other hand, a variety of exogenous factors and certain diseases leads to a decreased macrophage number and function culminating in the secondary form of the disease [9,11,13]. Its pathogenesis is still quite unknown and will be discussed further on.

1.2.1.1 Hereditary PAP

This form of the disease affects mainly neonates and children and accounts only less then 1% of the cases of PAP [9,18]. It has been associated with genetic abnormalities in the receptor for GM-CSF on either its α or β-chains in an autosomal recessive mode of inheritance [10,14,23]. *CSF2RA* (Granulocyte-Macrophage Colony-Stimulating Factor Receptor Subunit Alpha) mutations may be missense, nonsense, duplication, deletion, and

frameshift mutations [10]. As a result, a different GM-CSF receptor protein is formed which may be dysfunctional at recognizing GM-CSF, resulting in a faulty GM-CSF signal transduction and consequently, impaired stimulation and activation of neutrophils and macrophages [13,23].

1.2.1.2 Autoimmune PAP (aPAP)

Autoimmune PAP is a far more prevalent clinical form of the disease, accounting for 90-95% of the cases disregarding the age onset [10,14,18]. It is characterized by the presence of autoantibodies against GM-CSF (GMAb) with the ability to bind and neutralize GM-CSF in the alveoli [23]. Since the remaining GM-CSF is very scarce, it is no longer capable of activating and inducing maturation and differentiation of alveolar macrophages, and therefore surfactants accumulate in the lung [9,11,18,24–26]. For a long time, it was denominated idiopathic because its pathophysiology was unknown [18]. After Nakata and colleagues detected these autoantibodies in the patients serum and bronchoalveolar fluid, the pathogenic mechanism became clearer raising the possibility of these proteins becoming biomarkers of aPAP disease [9,11,13,23,27–33]. In healthy people, GM-CSF autoantibodies are present but in low concentration levels, possibly neutralizing some of the GM-CSF available, working as negative regulators of GM-CSF signalling in health and disease states [13,23,25,29]. The (dis)equilibrium between GM-CSF and its autoantibodies determines the emergence of this autoimmune disease (Figure 1) [29].

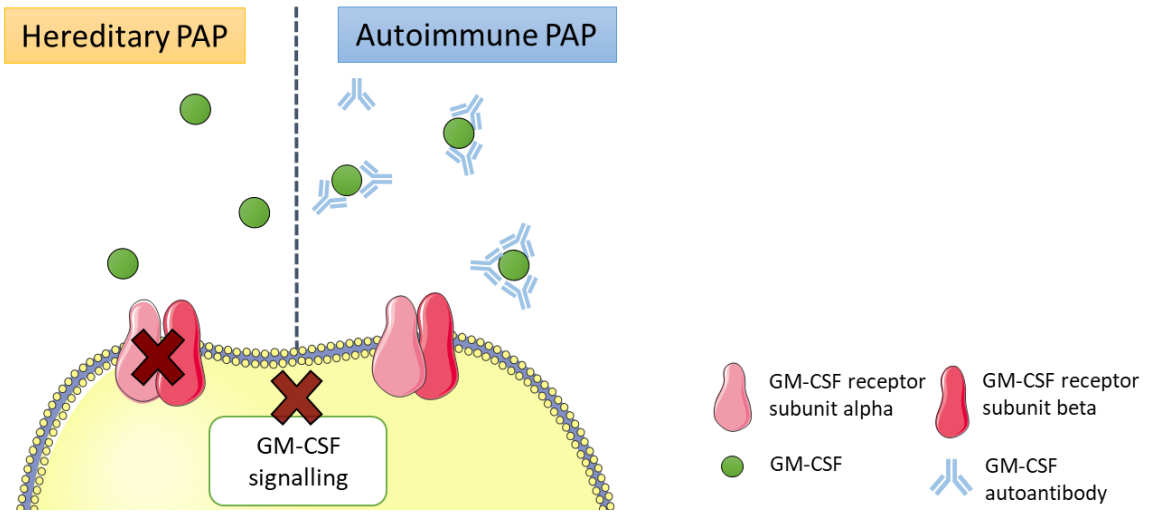


Figure 1 - Signalling triggered by GM-CSF in macrophages is impaired through different mechanisms in hereditary and autoimmune PAP.
This illustration was made with the help of Servier Medical Art (smart) by Servier

1.2.1.3 Secondary PAP

This type of pulmonary alveolar proteinosis affects mostly adults, corresponding to 5-10% of PAP cases [18]. It is characterized by a reduction in the number of alveolar macrophages or functional ones resulting in a disfunction of the surfactant's clearance [9,11,16]. It has been proposed that an acquired loss of GM-CSF signalling is responsible for secondary PAP [18]. Overall, the causes of the disease are still not well understood but it is most commonly related with comorbidities such as haematological disorders (myeloid leukaemia or lymphoma), immunological disorders (severe combined immunodeficiency or lung transplantation), due to exposure and inhalation of dust, lysinuric protein intolerance, drug-induced or iatrogenic, viral and bacterial infections [9,18]. Since this form of the disease is usually associated with comorbidities, the prognosis is worse than the autoimmune PAP and could be fatal [9,18]. Occupational exposure to silica, aluminium, titanium dust have also been associated with the secondary type of PAP disease [18].

1.2.1.4 GM-CSF

Neutralization or impairment of GM-CSF signalling leads to PAP

On autoimmunity and in inflammation responses, GM-CSF and M-CSF are expressed at high concentrations [34]. Recent studies have unveiled a pathogenic role of GM-CSF in several autoimmune diseases [35,36]. In experimental autoimmune encephalomyelitis (EAE),

arthritis, arthritis-related interstitial lung disease, nephritis, or psoriasis disease models, GM-CSF neutralization or depletion has been proven helpful [35]. In rheumatoid arthritis, GM-CSF and its receptor are being targeted for therapy in ongoing clinical trials [36].

Colony Stimulating Factors (CSF) are cytokines that have a proinflammatory role, mobilizing myeloid cell populations from the bone marrow[37]. First described as hematopoietic growth factors, these proteins also act as pro-survival, activation or differentiation factors on myeloid cells [34,37]. This group of proteins include macrophage-CSF (M-CSF or CSF-1), granulocyte macrophage-CSF (GM-CSF or CSF-2) and granulocyte-CSF (G-CSF or CSF-3) [34]. M-CSF is constitutively expressed and is responsible for the activation, survival, proliferation, differentiation of macrophage lineage cells while G-CSF has the same function but in granulocyte lineage populations [34,37]. On the other hand, besides activation (priming) of macrophages, GM-CSF can promote the survival and activation of neutrophils and eosinophils and also dendritic cell (DC) maturation (Figure 2) [34,38]. T lymphocytes, macrophages, endothelial cells and fibroblasts are the main sources of GM-CSF [39].

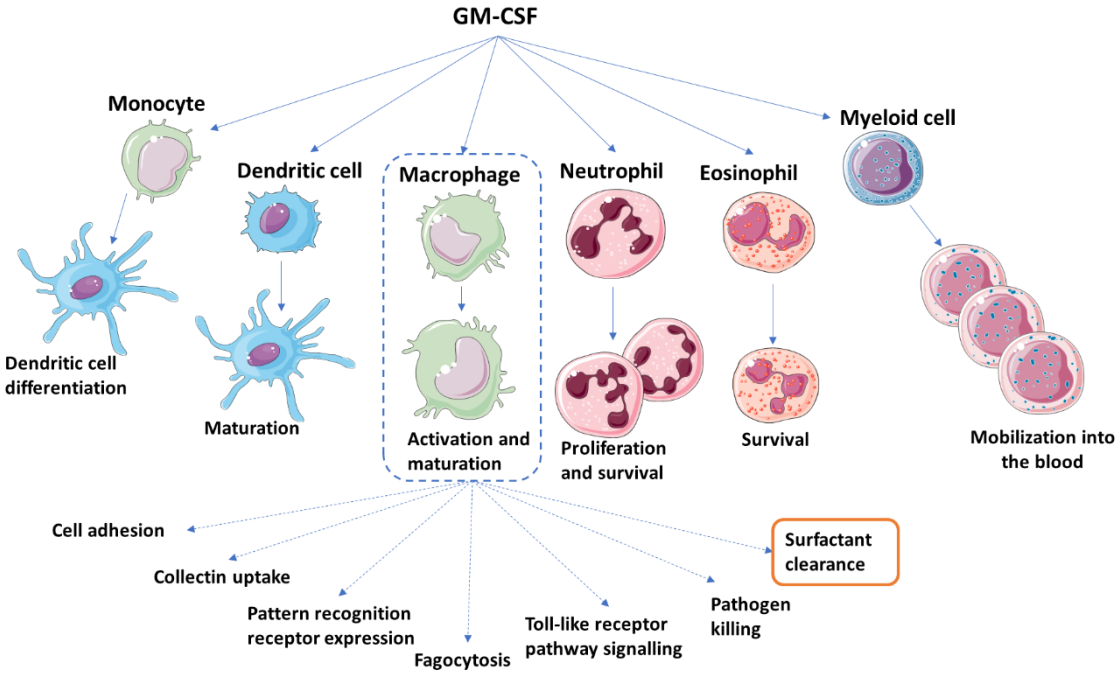


Figure 2 - Effects of GM-CSF on cells of the immune system.
Based on Kaufman et. al, 2016 and Shibata et. al, 2001. This illustration was made with the help of Servier Medical Art (smart) by Servier

GM-CSF has a critical role in pulmonary host response, macrophage function and surfactants clearance [9,11,13]. In the lungs, GM-CSF stimulates expression of PU.1, a myeloid

master transcription factor correlated with differentiation of alveolar macrophages, essential for their regular immune function [11,13,26]. In steady state, GM-CSF is essential for maturation of alveolar macrophages and invariant natural killer T (iNKT) [34]. While high concentrations of GM-CSF stimulate alveolar macrophage activation, survival and proliferation, low concentrations of this cytokine only promote cell survival [13].

1.2.1.5 Macrophage dysfunction

Macrophages are immune cells participating in the innate and adaptive response [1]. Their several functions include: host defence by ingesting and killing pathogens, phagocytosis of apoptotic cells, antigen presentation (antigen display to T-cells and their activation - APCs), inflammation and tissue regeneration. [1,4] Macrophages secrete cytokines that will recruit monocytes and other leukocytes to the site of injury, amplifying the immune response and also promoting angiogenesis and fibrosis, essential processes in the repair of injured tissue [1,4]. These cells differentiate from monocytes, circulating cells that acquire different functions according to the tissue or organ where they will reside and mature into tissue resident macrophages [1]. For instance, macrophages in the brain become microglial cells, Kupffer cells in the liver and sinusoidal macrophages in the spleen. In the lungs, resident alveolar macrophages are responsible for clearing inhaled microbial pathogens, toxins and debris, apoptotic cells, inflammatory response and lung remodelling and specially clearance of alveolar surfactants [22].

1.2.1.6 Surfactants and clearance

Surfactants are phospholipids and proteins such as SP-A, -B, -C and -D that accumulate in the terminal bronchioli and alveoli [24]. These proteins, produced by alveolar epithelial cells type II, are important for the lung structure, reducing the surface tension between the air-liquid-tissue interface and preventing collapse of the alveoli [11,26]. Host defense is another of its main functions as surfactants - belonging to the collectin protein family - bind, opsonize and directly kill pathogens in the alveoli [11,40,41]. The maintenance of pulmonary surfactant homeostasis is indirectly regulated by GM-CSF signalling in alveolar macrophages being essential for regular lung function [11,15,22]. Since alveolar macrophages catabolize surfactants, a lack of GM-CSF leads to a disfunction of these immune cells,

causing an excessive accumulation of surfactants and respiratory failure as a consequence (Figure 3) [11,13,15,16,42]. Excess of surfactants is thus associated with several other lung disorders like asthma and COPD, implying that GM-CSF might be also implicated in these pathologies [34]. Also, unstimulated immature macrophages are not able to phagocyte and kill microbes, express pathogen recognition receptors, generate reactive oxygen species, decreased adhesion and secretion of proinflammatory chemokines [11]. There is also a group of diseases associated with disorders of surfactant production (DSP). However it is unrelated to and distinguishable from PAP, since in PAP, there is only surfactant accumulation not a deficiency in production, uptake, function or lung parenchymal architecture [11].

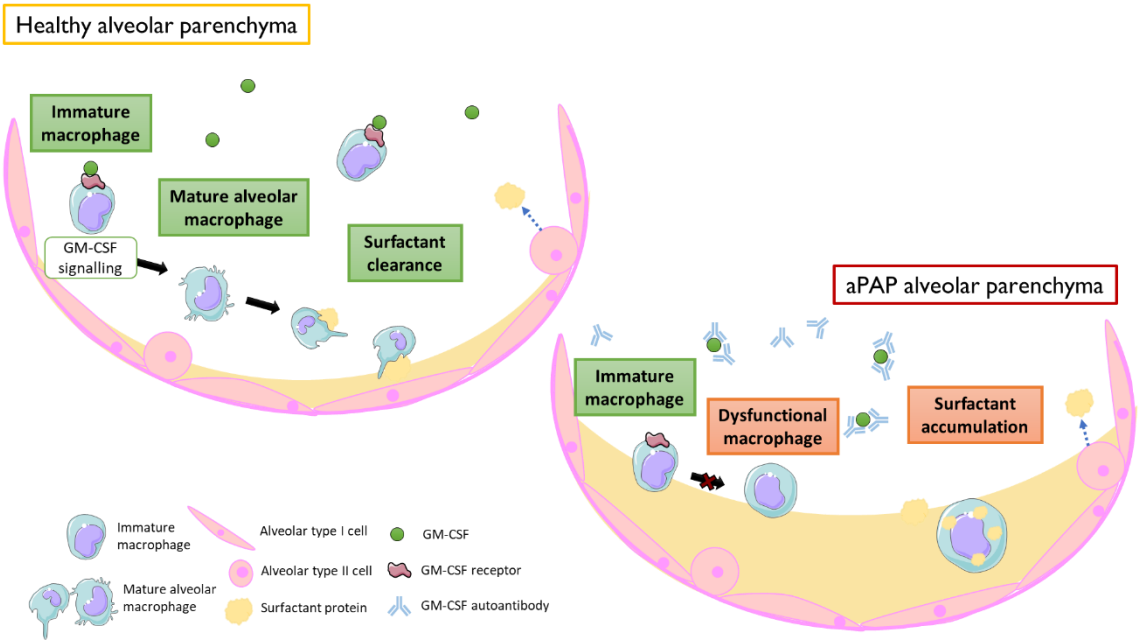


Figure 3 – Pathogenesis of autoimmune pulmonary alveolar proteinosis.
In healthy alveolar parenchyma, alveolar macrophages are responsible for surfactant clearance. In aPAP, macrophages remain immature, leading to accumulation of surfactant. This illustration was made with the help of Servier Medical Art (smart) by Servier

1.2.2 Current diagnostic approaches

Currently, the clinical diagnosis of this rare disease is based on symptoms and evaluation of physical signs, elevated lactate dehydrogenase (LDH), high resolution computed tomography chest (HRCT), pulmonary function tests (e.g. forced expiratory volume in one second - FEV_1) and pulmonary biopsy [9,14]. Also, cytologic analysis with

periodic acid-Schiff (PAS) are used to detect eosinophilic granular material in the bronchoalveolar lavage [9].

Genetic biomarkers such as SFTPB, SFTPC, ABCA3, TTF1, CFS2RA and CSF2RB are recommended for the diagnosis of hereditary PAP [14]. Serum GM-CSF can also be detected by enzyme-linked immunosorbent assay (ELISA) and higher concentrations of this protein can indicate a dysfunction of GM-CSF receptor and signalling, resulting in the development of PAP [10]. Increased levels of LDH, CEA, CYFRA 21-1, SP-A, SP-D or KL-6 serum biomarkers are related to the severity of the disease and could help for diagnostic purposes, although not specific of this disease and only used in certain countries [9,18].

However, these exams and tests are not enough for a conclusive clinical diagnosis of autoimmune PAP [43]. Since the treatment differs according to the form of the disease, it is necessary a better, non-invasive, sensitive and specific test, that along with the other exams, could help in identifying the most common type of this rare lung disease [43].

1.2.3 GM-CSF autoantibodies assay

Since the aPAP disorder is characterized by the presence of autoantibodies against GM-CSF in circulation, the detection of this protein is a desirable choice as an aPAP biomarker [9,14]. It can be detected in serum, plasma and bronchoalveolar lavage fluid (BALF) samples of patients through techniques such as Western Blot or ELISA [27,28,43]. Detection of GMAb helps in the distinction between hereditary and autoimmune forms of the pathology. As a serum biomarker, GM-CSF autoantibody has a high sensitivity and specificity, is minimally invasive and usefull tool for aPAP diagnosis [9,14]. Several articles describe the importance of the use of this autoantibody as a serological diagnosis marker for autoimmune PAP [9,11,13,23,27–33]. One article even described a case of a patient with elevated GM-CSF antibody levels before the onset of the disease, demonstrating the importance of this marker for monitoring the disease [29]. GMAb may also be present In healthy individuals, but in a very low concentration when compared to aPAP patients and studies have defined a critical threshold for the serum detection of this biomarker [14,23,33,43]. Accordingly, a patient might present aPAP if the concentration of GMAb is higher than approximately 5 µg/ml [23,25,33]. In other countries, GM-CSF autoantibody testing is already

being used to help diagnose aPAP, but it is only available in specific clinical research centers [18]. For instance, PAP testing is available at the Diagnostic Immunology Laboratory in Cincinnati Children's Hospital Medical Center (USA), as part of an ongoing PAP clinical research. Also, in Munich, there is a laboratory running PAP tests for the determination of GM-CSF autoantibodies at the Dr von Hauner Children's Hospital [14]. Even though PAP is a rare disease, the autoimmune form is responsible for the vast majority of the cases, raising the importance of the widespread use of GMAb test. If this test was available on a regular basis, it would help in identification of the type of disease: either detecting autoantibodies against GM-CSF or ruling out the autoimmune disorder, focusing on the secondary or the hereditary form of PAP [18].

1.3 Hypersensitivity Pneumonitis

1.3.1 Pathogenesis

Also known as extrinsic allergic alveolitis, this complex disease belonging to the group of interstitial lung diseases (ILD), presents a variety of clinical phenotypes being difficult to differentiate from idiopathic pulmonary fibrosis (IPF) [44–46]. Some authors categorise it in two different clinical forms - acute and chronic - while others consider a subacute phase [44–46]. This disease is characterized by an exaggerated humoral and cellular immune response resulting in a granulomatous inflammation in the pulmonary parenchyma (alveoli, terminal bronchioli and alveolar interstitium) and respiratory failure [8,46]. This disease is triggered by inhalation of an antigen/allergen (or a group of antigens) to which the individual was previously exposed and sensitized, resulting in a T lymphocyte proliferative response [7,44]. Following the exposure to an antigen, several responses can occur: one can either be sensitized and not develop any disease, other individuals may not ever become sensitized, and only a minority of the exposed individuals develop hyperresponsiveness to the antigen resulting in hypersensitivity pneumonitis [44]. However, the exposure to certain stimuli is not enough to develop the disease, which explains the low prevalence of HP [44,46]. The most accepted pathogenesis of HP is a two-hit model in which the development of the disease arises from antigen inhalation allied with genetic or environmental factors, the 'triggers' of the disease [44,46] (Figure 4). Currently, an IgG immune complex mediated response is the putative immunopathogenic mechanism for acute HP resulting in an increase of lung neutrophils, while in subacute/chronic HP it is a T-cell delayed mediated response mechanism [26,29].

The disease's phenotype and severity are highly variable among patients being mediated by the type of antigen, the duration, amount and frequency of the exposure to the antigen, host immune and genetic co-factors, seasonal and geographic variations [8,44,46]. The time between the exposure and the onset of the disease (latency) varies from weeks or months to years making it difficult to identify the causative antigen [45].

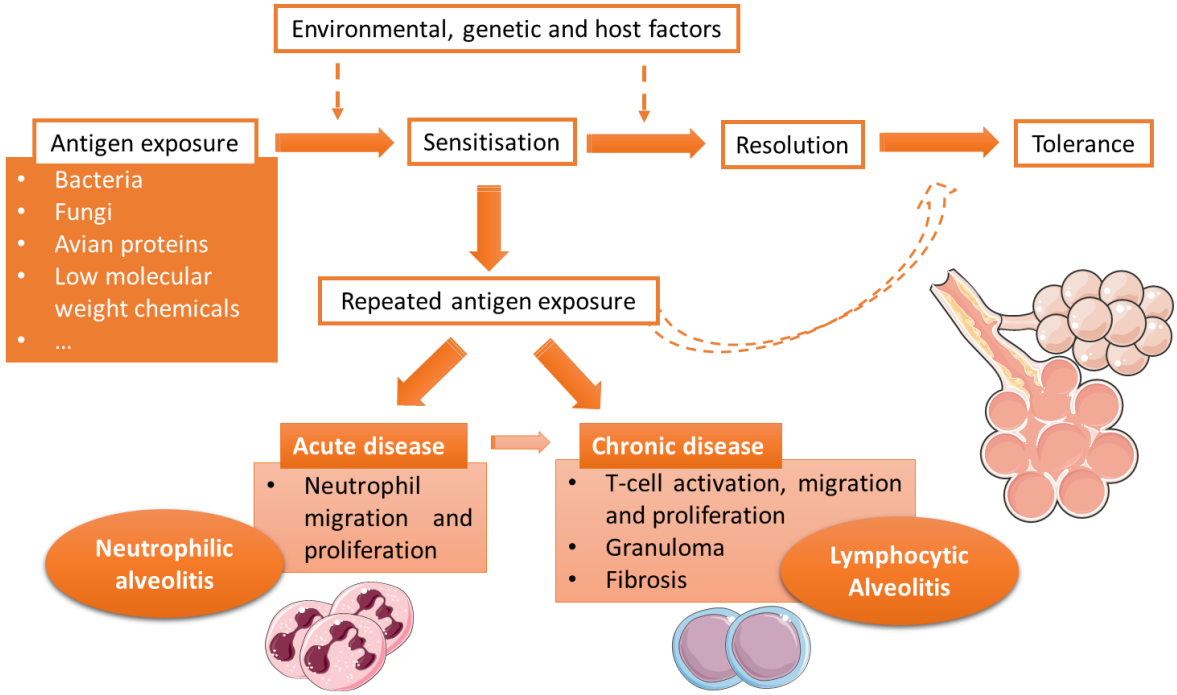


Figure 4 - Pathogenic mechanisms in hypersensitivity pneumonitis
(Based on Fig. 1, Agache & Rogozea, 2013). This illustration was made with the help of Servier Medical Art (smart) by Servier.

1.3.1.1 Acute HP/inflammatory phase

This non-progressive form of the disease is similar to a flu or viral infection's symptoms (headache, chills, sweating, fever and less frequently dyspnea) occurring hours after an intermittent and intense exposure to the inducing antigen and lasting hours to days [8,44,46]. Patients improve spontaneously after antigen avoidance. However, symptoms recur after re-contact with the pathogen [46]. Episodes of the acute phase are often misinterpreted as a common bacteria or viral infection making it problematic to diagnose [8]. It is particularly concerning in individuals working in farms in contact with livestock because they are at risk of developing Farmer's lung disease (FLD), a type of acute HP that may evolve to more severe states of the disease [7,8,44,45,47].

The acute phase is characterized by the presence of antigen-specific IgG in circulation and neutrophilic alveolitis caused by an increase in the number of neutrophils present in the alveoli, which could release reactive oxygen species (respiratory burst) [44,46]. Then, macrophages and neutrophils are activated by Fcγ receptors evolving to a subacute and chronic phase of the disease [7].

1.3.1.2 Chronic HP/fibrotic phase

The continuous antigen exposure and untreated acute reaction (for months or even years) results in a chronic progressive respiratory disease with a very indistinguishable phenotype from idiopathic pulmonary fibrosis (IPF) and usual interstitial pneumonia (UIP) [8,46]. Dyspnea, cough, fatigue and weight loss are common symptoms of this phase [46]. The main histopathological features of HP are interstitial cellular infiltrates, cellular bronchiolitis and poorly formed granulomas [8,48]. In this disease, granulomas are frequently small non-caseating/necrotising in the peribronchiolar location and composed mainly by lymphocytes, epithelioid and multinucleated giant cells [8,48]. Interstitial infiltrates of lymphocytes, monocytes, macrophages and plasma cells are present [49].

A type IV reaction occurs leading to T lymphocytic migration and local proliferation (lymphocytic alveolitis) and decreased apoptosis [8].

Granulomas start to form mediated by Th1 immune response under the regulation of T-Bet transcription factor [7,8]. IFNγ and IL-12 also help Th1 differentiation, playing a part in granuloma formation [7,8]. Furthermore, expression of CD34 and toll-like receptor 9 (TLR9) in dendritic cells are thought to be necessary for the migration of DC (antigen presenting cells) to the lymph nodes, leading to activation and influx of T lymphocytes to the lungs [7]. On the other hand, Th17 has also been implicated in the granuloma inflammation in HP since IL-17 is associated with disease severity [7,8]. Overall, Th1/Th17 cell responses are thought to be responsible for hypersensitivity pneumonitis inflammation [7]. Over time, the production of angiogenic chemokines leads to fibrosis, another characteristic of this phase and eventually destruction of lung architecture [50].

Pigeon breeder's lung or bird fancier's lung are well studied forms of hypersensitivity pneumonitis, considered the prototypes of subacute and chronic HP, developed after contact with avian antigens such as pigeon dropping extracts or feathers [49,51].

1.3.1.3 Inducing factor (antigen exposure)

A wide array of antigens may trigger the disease in the different environments to which individuals are in contact with daily [44]. The common classes of antigens causing a variety of HP forms are bacteria, fungi, yeast, viruses, (in)organic chemicals, metals, animal and plant proteins (Table 1) [44,52].

Table 1 - Common types of Hypersensitivity Pneumonitis according to major classes of antigens (Based on Table 1, Spagnolo et. al, 2015).

Class of Antigens	**Specific Antigens**	**Sources**	**Type of Disease**
Bacteria	*Saccharopolyspora rectivirgula, Thermoactinomyces vulgaris*	Moldy hay, grain	Farmer's lung
Fungi, yeasts	*Aspergillus* species	Moldy hay, grain	Farmer's lung
	Aspergillus species	Moldy compost and mushrooms	Mushrooms worker's lung
	Trichosporon cutaneum	Contaminated houses	Japanese summer-type HP
	Penicillium species	Moldy cork	Suberosis
	Penicillium casei	Moldy cheese or cheese casings	Cheese washer's lung
	Alternaria species	Contaminated wood pulp or dust	Woodworker's lung
Mycobacteria	*Mycobacterium avium-intracellulare*	Mold on ceiling, tub water	Hot tub lung
	Mycobacterium avium - intracellulare	Mist from pool water, sprays and fountains	Swimming pool lung
Animal proteins	Proteins in avian droppings and serum and on feathers	Parakeets, budgerigars, pigeons, parrots, cockatiels, ducks	Pigeon breeder's lung, bird fancier's lung
	Avian proteins	Feather beds, pillow, duvets	Feather duvet lung
	Silkworm proteins	Dust from silkworm larvae and cocoons	Silk production HP
Chemicals	Diisocyanates, trimellitic anhydride	Polyurethane foams, spray paints, dyes, glues	Chemical worker's lung

1.3.1.4 Causative agents (genetic, environmental and host factors)

1.3.1.4.1 Genetic predisposition

Few studies focused in genetic susceptibility as an additional risk factor for HP [46]. Polymorphisms and heterozygosity in the major histocompatibility complex (MHC) genomic region (which is a known immune response regulator), and associated with human leukocyte antigen (HLA)-DR e DQ have been associated with increased risk for developing HP in different populations [53]. Moreover, genetic variants in the immunoproteasome catalytic subunit b type 8 (PSMB8) and transporters associated with antigen processing (TAP) genes have been implicated with high HP susceptibility, while a protective effect might be due to promotor variants in tissue inhibitor of metalloproteinase-3 (TIMP-3) [54–56].

1.3.1.4.2 Environmental factors

Interestingly, cigarette smoking has been implied as a protective factor for HP, through an unknown mechanism [57,58]. Lower prevalence of HP and lower quantity of antibodies specific to the causing antigen was observed in smoker individuals [57,58]. It appears that nicotine decreases lymphocyte proliferation and function, and affects activation of macrophages [44,46]. Although, less frequent in smokers when the disease is present it is usually chronic, resulting in lower survival rate [59].

1.3.1.4.3 Immune tolerance

The mechanism by which individuals exposed to the antigen gain tolerance and do not develop the disease is thought to be mediated by regulatory T cells (T_{reg}), $CD4^+$ cells involved in supressing Th1 and Th2 immune responses [46]. T_{reg} demonstrated an anti-inflammatory protective effect in an experimental model of HP [60]. T_{reg} isolated from blood and BAL samples from HP patients were incapable to supress lymphocyte proliferation, unlike asymptomatic exposed individuals [61].

1.3.2 Current diagnostic approaches

Hypersensitivity pneumonitis is often a misdiagnosed disease, which is one of the reasons why standardized diagnostic criteria and a set of complementary tests are essential for a more precise and conclusive diagnosis [7,8,44,52]. Personal exposure at work or at home

might be important to be evaluated for identifying the antigen's type and source, essential for doctors to correctly diagnose and treat the patient and particularly to raise awareness to avoid re-contact or exposure to that antigen source [7,52].

Chest radiography is usually evaluated first after HP suspicion [44]. In abnormal cases, ground glass opacities indicated the presence of the disease [45]. Lung function evaluation and gas transfer (DLCO levels) are also important for a complementation test [7,8]. Another currently used approach is to measure specific precipitating IgG antibodies in the patient's serum to detect an antigen exposure, through ELISA, immunodiffusion or ImmunoCAP techniques [52,62]. It can specifically detect a variety of antibodies against avian antigens, for example. However, one downside of these HP diagnosis techniques is the fact that the detection of an antigen in circulation does not necessarily imply that it is the causative antigen [45].

BAL fluid cellular analysis is also helpful in HP diagnosis since it allows counting lymphocytes, CD4+/CD8+ proportion and neutrophil count [45].

Certain inflammatory biomarkers in the peripheral blood, BALF, and lung tissue such as [KL]-6, SP-D, and chitinase 3–like protein 1 [YKL-40] and chemokines (CCL17, CCL18, IL-8, vascular endothelial growth factor (VEGF), epithelial neutrophil-activating peptide 78 [ENA-78] and chatepsin-K might be helpful, although there is a need for further investigation concerning diagnosis and prognosis use [45].

The detection of fungal DNA in serum and BALF might prove to be a useful diagnostic tool [49,63]. In certain cases, a lung biopsy might also be required to detect granulomas followed by immunohistochemistry of the causal antigen, to help in HP diagnosis, but it is an invasive technique [7,44,45].

An alternative test is the measurement of antigen-induced lymphocyte proliferation in peripheral blood or BALF cells to assess T-cell function, which could help differentiate between asymptomatic patients and identify the causal agent of the hypersensitivity reaction [7]. Also, environmental or laboratory-controlled inhalation challenge could be a good tool to detect HP [7].

1.3.3 Lymphocyte Proliferation Assay

One of the key features in chronic hypersensitivity pneumonitis is the excessive antigen specific lymphocyte proliferation in the alveoli, giving importance to lymphoproliferation assays as an approach for the disease detection [7]. Cell cultures of PBMCs, which contain lymphocytes (T, B and NK cells) and monocytes and BAL fluid cells from patient samples can be used to assess proliferation of T cells [51]. Lymphocyte proliferation assays are used to assess T-cell function in patients with severe immunodeficiency as a response to antigenic specific stimuli [64,65].

Several techniques allow measuring cell division, such as incorporation of BrdU or Violet Cell Tracers dyes. Some studies suggest radioactivity with incorporation of tritiated thymidine (3H-thymidine) for proliferation measurement [51,66]. However, vital dyes are a safer alternative. Carboxyfluorescein succinimidyl ester (CFSE) dye is an intracellular marker that can be used together with a variety of monoclonal antibodies [67–69]. It covalently binds to certain cytoplasmic components [67–69]. It allows visualization of proliferating cells in a flow cytometer, as in each cell division, the dye divides equally through the daughter-cells, until the fluorescein molecule loses its ability to fluoresce, returning to the basal background level of autofluorescence in unlabelled cells, which usually happens after 8 to 10 cycles [67,68]. A rapid cell fluorescence loss is related to more cycles indicating a higher cell proliferation rate. Very few studies mention the use of CFSE proliferation assays in pulmonary inflammatory diseases like hypersensitivity pneumonitis [70,71]. Labelling PBMCs with CFSE prior to cell culture with the cell stimulus (allergen or mitogen) allows the detection of specific lymphocyte proliferative response.

1.3.3.1 Allergen/antigen/mitogen stimulation in hypersensitivity pneumonitis

Some studies have used the mitogens phytohemagglutinin (PHA) or concanavalin A (Con A) and antigens such as tetanus toxin, HIV, or yeast like *C. albicans* to stimulate T-cells for proliferation using PBMC or cells from BALF [65,67,71–73]. Phytohemagglutinin is a known T lymphocyte mitogen inducer used in lymphocyte proliferation assays. *Candida* is a genus of very prevalent fungi present in the human microbiota [74,75]. It can be found in the skin and oral cavity mucosa, gastrointestinal, respiratory and vaginal tracts as a commensal

organism [74–78]. However, in immunocompromised or critically ill individuals, for example, this organism is capable of causing infections becoming an opportunistic pathogen [75,76,79].

Very few cases of hypersensitivity pneumonitis have been associated to *Candida* as its causal agent [77,80]. Nonetheless, considering the fact that almost every individual has been exposed to this organism, it is likely that this agent could induce a proliferative lymphocytic response in healthy donors after *Candida* stimulation. Using multiple allergens as cell stimulators, it should be possible to identify the agent(s) causing the hypersensitivity response, which is essential in disease management, treatment and avoidance of the organism.

2. Aims

2 Aims

This work aimed to develop and optimize diagnostic assays for two specific pulmonary diseases derived from de-regulated immune responses. More specifically, it aimed to:

- detect the presence of anti-GM-CSF autoantibodies, for Autoimmune Pulmonary Alveolar Proteinosis diagnosis;
- quantify antigen specific T lymphocyte proliferation for diagnosis of Hypersensitivity Pneumonitis.

3. Materials and methods

3 Materials and Methods

3.1 Detection of GM-CSF antibodies by ELISA

Autoimmune pulmonary alveolar proteinosis is characterized by the presence of the autoantibody against GM-CSF, a biomarker of this form of the disease. Based on a protocol described by Uchida and colleagues in 2014, an ELISA was developed to detect and quantify GM-CSF autoantibody in blood samples of individuals [23]. In our assay, a recombinant human GM-CSF protein is added to the plate to capture the antibody against GM-CSF present in plasma samples or the commercial standard antibody (Figure 5). After incubating with the secondary antibody conjugated with horseradish peroxidase, TMB substrate solution was added and converted, developing color, whose intensity is proportional to the concentration of the antibody. Using the equation of the GM-CSF commercial antibody standard curve, it is possible to estimate the concentration of the autoantibody in circulation.

3.1.1 Experimental design

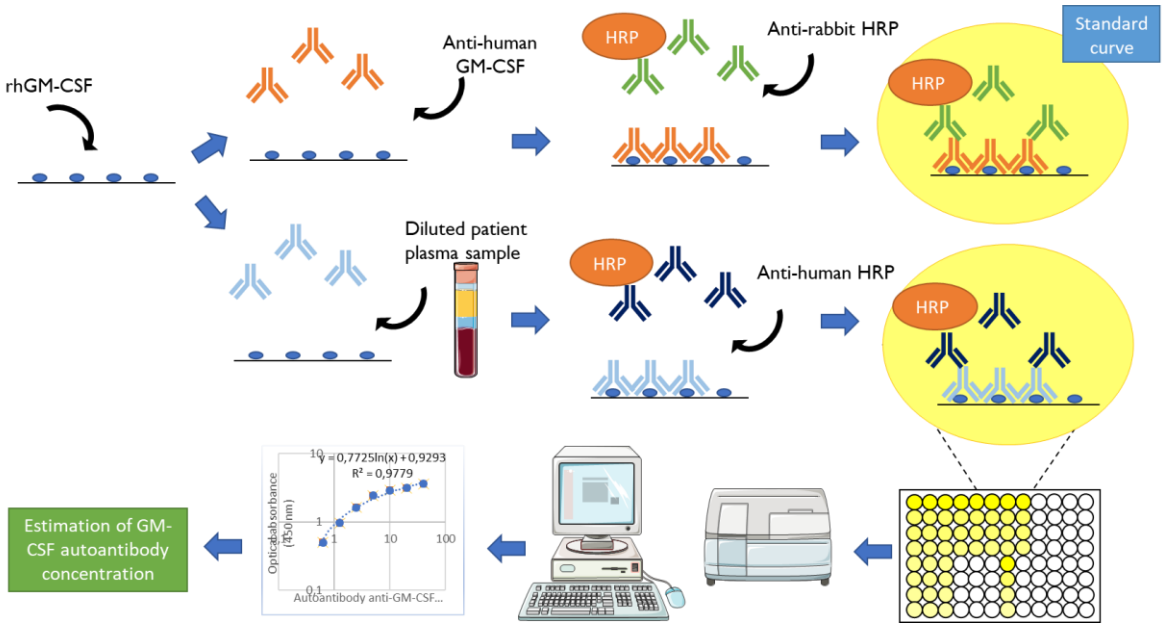


Figure 5 - ELISA experimental design.
This illustration was made with the help of Servier Medical Art (smart) by Servier.

3.1.2 Sample collection

The subjects in this study were healthy donors, patients with aPAP and patients with suspected aPAP from Centro Hospitalar do Baixo Vouga (CHBV) - Hospital Infante D. Pedro.

3.1.3 Sample processing

Blood samples were collected in tubes containing EDTA. Samples were transferred to a 15ml tube and centrifuged at 2000g for 10 min, at 4^{o}C without break. Then, plasma was carefully collected, aliquoted and stored at -80^{o}C until use.

3.1.4 ELISA protocol

The microplate was sensitized with 50 µl of capture antigen solution (1 µg/ml rhGM-CSF (GF004, Millipore) diluted in PBS) and incubated overnight at 4^{o}C, shaking (Figure 6). To remove the recombinant protein that did not bind, 300 µl of washing solution (PBS + 0.1% Tween 20) was added four times. An incubation with 100 µl blocking solution (PBS, 1% BSA, TMB ELISA Buffer Kit, 900-T00, PeproTech) for 1h was performed at room temperature, with shaking. Meanwhile, plasma samples were prepared by diluting with PBS, 1% BSA, 0.1% Tween 20 in serial dilutions (1:3000, 1:6000, 1:9000, 1:12000, 1:15000). Also, the antibody solution (anti-human GM-CSF) was prepared in the concentration of 20, 10, 5, 2.5, 1.25, 0.63, 0.315, 0 ng/ml with diluent (PBS, 1% BSA, 0.1% Tween 20). Then, aliquots of 50 µl of diluted plasma (patient, positive and negative control) or the anti-human GM-CSF antibody (500-P33, PeproTech) for the calibration curve were pipetted and the plate was incubated for 40 min at room temperature while shaking, to allow the antibody to bind to the recombinant protein (capture antigen). Triplicates of every sample and standard were performed to evaluate well-to-well variability, reducing the variability of the calculated concentrations. Then, the unbound antibody was removed with 300 µl of washing buffer (PBS, 0.1% Tween 20) four times. Next, 50 µl of detection antibody was pipetted to each well (anti-human IgG conjugated HRP, 109-035-003, Jackson Immuno Research) or the anti-rabbit HRP antibody (7074S, Cell Signalling) for the calibration curve, prepared by diluting 1:5000 and 1:1000, respectively (anti-human IgG HRP conjugate for

the plasma sample wells, anti-rabbit HRP for the wells containing anti-human GM-CSF). After 40 min incubation at room temperature, the plate was washed again four times with buffer (PBS, 0.1% Tween 20). 50 µl of TMB were added to each well at room temperature to allow color development (protecting from light) and five minutes later, 50 µl of Stop solution was added.

3.1.5 Estimation of the GM-CSF autoantibody

The optical absorbance was measured in the 96 well microplate reader (Infinite M200 – TECAN) at 450 nm, calculating the difference from the reference 650 nm. The calibration curve was analysed with Microsoft Excel software, plotting the average subtracted optical density of anti-GM-CSF triplicates with the antibody concentrations ranging from 0 to 20 ng/ml. The power curve was chosen for the trendline, using a log-log transformation. Then, the average GM-CSF autoantibody concentration (µg/ml) of the triplicates of the diluted plasma samples was estimated.

To analyse the statistical significance of the estimated antibody concentration data, a non-parametric test was applied on GrahPad Prism 7, Wilcoxon matched-pairs signed rank test was performed to compare the α-GM-CSF average concentration of the triplicates of patient's diluted plasma samples with the negative control. No statistical significance was detected due to the low sampling number (n=3) and high variability in the calculated concentration of the autoantibody between assays (p-value = 0,25 > 0,05).

Figure 6 - ELISA plate layout.

3.2 *Candida albicans* SN152 inactivation treatments

20 µl of *Candida* inoculum (4.5 x 10^6 cells/mL) was pipetted in 6 1.5 ml tubes and subjected to the following treatments:

- Addition of 0.04% SDS and overnight incubation
- Addition of 1% PFA and overnight incubation
- Addition of 7% hydrogen peroxide and overnight incubation
- Autoclaving the tube inside a glass container
- Heating at 100^{o}C for 20 min
- Control without treatment

After all treatments, cells were washed with 1 ml of sterile PBS, centrifuged at 13000 rpm for 5 min, resuspended in 40 µl of PBS and left at 4^{o}C (at this temperature, growth is inhibited). In a 12 well microplate, 2 ml of medium (RPMI-1640, 10% FBS, 1% penstrep, 1% Hepes, 1% Sodium citrate, 1% non-essential aminoacids (NEAA)) was placed in each well and 20 µl of each condition were added respectively. This incubation was made to observe cell morphology and count the cell density. The microplate was incubated at 37^{o}C, 5% CO_2 for 3 and 7 days. Yeast cells were counted with trypan blue in a hemocytometer before and after treatment. Depending on the efficiency of each treatment, the effectively inativated inoculum could be used as an antigen stimuli for the lymphocyte proliferation assay.

3.3 Lymphocyte antigen-specific proliferation assay

The CFSE lymphocyte proliferation assay is based on the incorporation of a fluorescent dye (CFSE) that binds to intracellular components of the cytoplasm and divides equally in daughter cells, allowing the detection of up to 8 cellular divisions (Figure 7). The more the fluoresce peaks, the more cells proliferated.

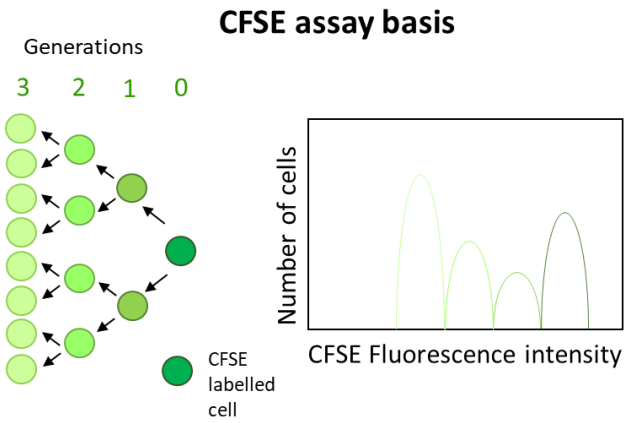


Figure 7 - CFSE assay basis.
Each cell division will correspond to a CFSE peak of fluorescence intensity.

Incubating PBMCs isolated from blood samples of donors, with a mitogen or an antigen will induce T cell proliferation that can be detected by flow cytometry (Figure 8). If T-cell priming to a specific antigen has previously occurred, lymphoproliferation will occur after stimulation with that antigen. The yeast *C. albicans* is a commensal organism to which sensitization has probably occurred. Using several inactivating methods for *C. albicans*, the treated yeast cells were used to stimulate T-lymphocytes antigen specific response.

3.3.1 Experimental design

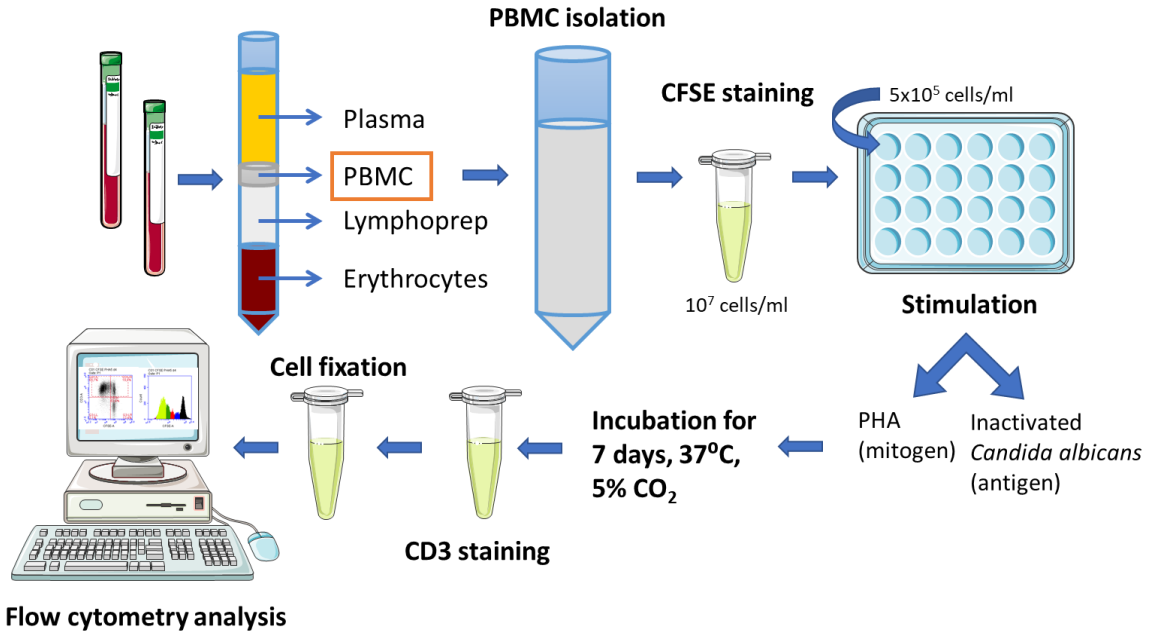


Figure 8 – Proliferation assay experimental design.
This illustration was made with the help of Servier Medical Art (smart) by Servier

3.3.2 Sample collection

Samples were collected from healthy individuals from Escola Superior de Enfermagem da Universidade de Aveiro (ESSUA) and Centro Hospitalar do Baixo Vouga (CHBV) - Hospital Infante D. Pedro. The protocol for sample collection was submitted to the Ethics Commission from CHBV. Collection tubes containing the anticoagulant sodium citrate were used and the blood was processed immediately.

3.3.3 Sample processing and PBMC isolation

Blood samples were collected in tubes with sodium citrate. Blood was diluted in sterile filtered PBS in 1:1 ratio (5 ml blood + 5 ml PBS), then carefully overlaid in Lymphoprep (12IHS13, Axis-Shield) on a 2:1 ratio (10 ml blood diluted in PBS + 5 ml Lymphoprep), to avoid mixture with Lymphoprep. The mixture was centrifuged at 800g for 30 min, at room temperature without break and low acceleration. After the density centrifugation, peripheral blood mononuclear cell (PBMC) layer forms a ring, which was

carefully removed to a tube with a Pasteur pipette. Cells were washed with 40 ml of cold PBS and centrifuged at 300g for 10 min in the maximum (des)acceleration. The supernatant was removed and this wash repeated 4x. The pellet was ressuspended in 1 ml of PBS.

3.3.4 CFSE staining

The cell number was counted in a hemocytometer upon trypan blue exclusion. One million cells were saved for the unstained and CD3 compensation conditions for flow cytometry analysis. 10^7 cells/mL in PBS were incubated with 0.5 µM CFSE (423801, Biolegend) for 15 min at 37^{o}C, protecting from light. The cell solution was then washed with PBS+20% FBS and centrifuged for 10 min at 300g. Cells were resuspended in 1 ml of culture medium (RPMI, 10% FBS, 1% penicillin-streptomycin, 1% Heppes, 1% sodium pyruvate, 1% NEAA) and counted again. In a 24 well plate, 1 ml of medium was added to each well and the volume corresponding to a cell density of $5X10^5$ cells per ml was plated. Also, the remaining CFSE stained cells were saved for cytometry analysis (CFSE only) and CD3 staining (CFSE+CD3). Cells were incubated in a final volume of 9 ml of culture medium.

3.3.5 Cell stimulation

5 or 20 µg/mL of phytohemagglutinin (PHA-P, L8754, Sigma-Aldrich) was added to the corresponding wells for the positive control condition (Figure 9). Treated inactivated inoculum of *Candida* cells were added to other wells with different concentrations (0.5 or $1x10^6$ yeast cells). Cells were incubated in a controlled humidifier at 37^{o}C, 5% CO_2 for 7 days until analysis by flow cytometry.

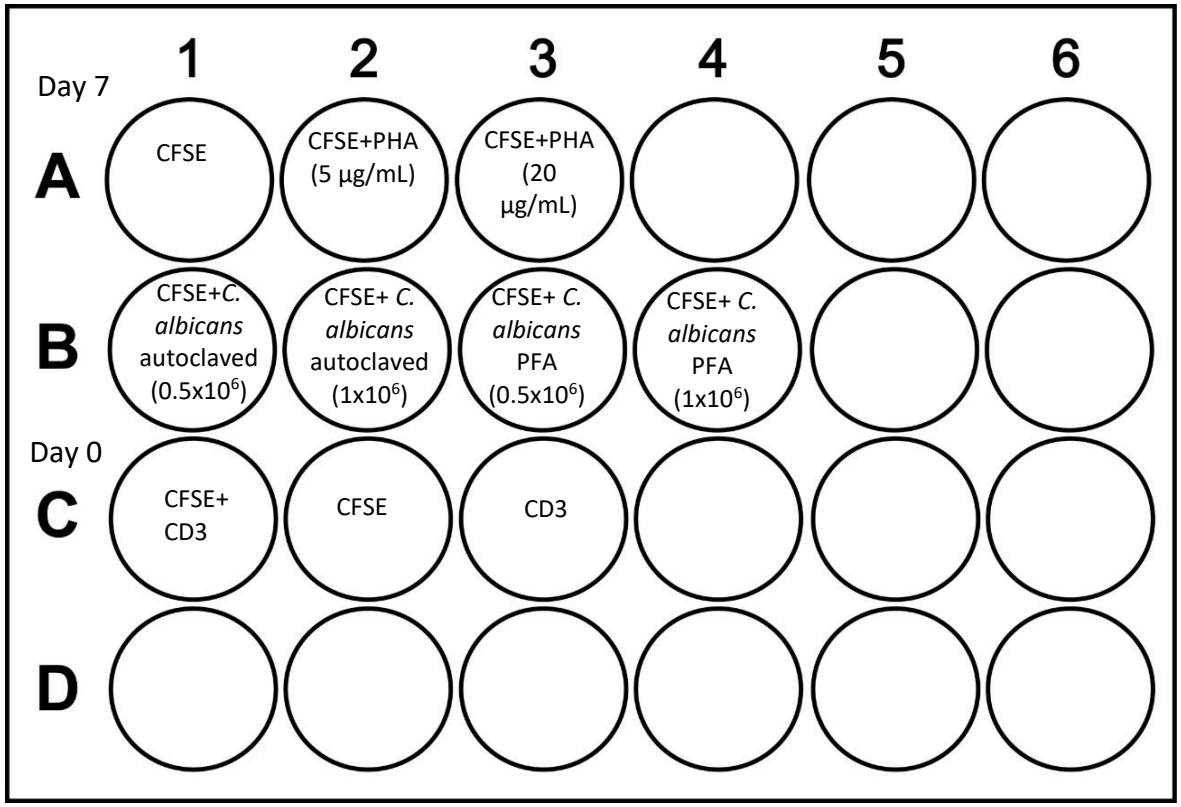


Figure 9 - Cell culture plate layout.

3.3.6 CD3 staining

Cells were resuspended in each well, transferred to a microtube and resuspended with 400 µl of PBS to take the maximum cells possible from the well. After 6 min centrifugation at 400 g at 4^{o}C, the supernatant was discarded. The pellet was washed with 1 ml FACS buffer (0.5% BSA + 0.01% sodium azide + PBS) by centrifuging again. On the last centrifugation, the supernatant was carefully removed. FACS solution with CD3-APC antibody (21620036, Immunotools) (45 µl of FACS buffer + 5 µl CD3-APC antibody) was prepared and 50 µl of CD3-APC antibody solution was resuspended in each sample. After 30 min incubation on ice protected from light, the cell suspension was washed twice with 1 ml of FACS buffer and 1x with cold PBS centrifuging 6 min at 400 g at 4^{o}C. In the last wash, the supernatant was removed and the pellet fixed in 150 µl of PFA 4% for 15 min. After washing with 1 ml of PBS and centrifuged, the pellet was resuspended in 150 µl of PBS. The samples were kept at 4^{o}C protected from light until analysis.

3.3.7 Flow cytometry analysis

The cell suspension was filtered using a 40 µm cell strainer and analysed in the flow cytometer BD Accuri C6. The analysis was made in the FL1 region (corresponding to CFSE fluorescence intensity) and FL4 (to view CD3 APC fluorescence intensity). Control conditions were run in the cytometer to determine the regions of autofluorescence corresponding to unstained cells, single stained cells for CFSE and CD3 only, double stained CFSE+CD3 cells. Lymphocytes were gated in a forward x side scatter dot plot (P1) and in this gate, dotplots with CSFE (x axis) vs CD3 (y axis) fluorescence were drawn (Figure 10).

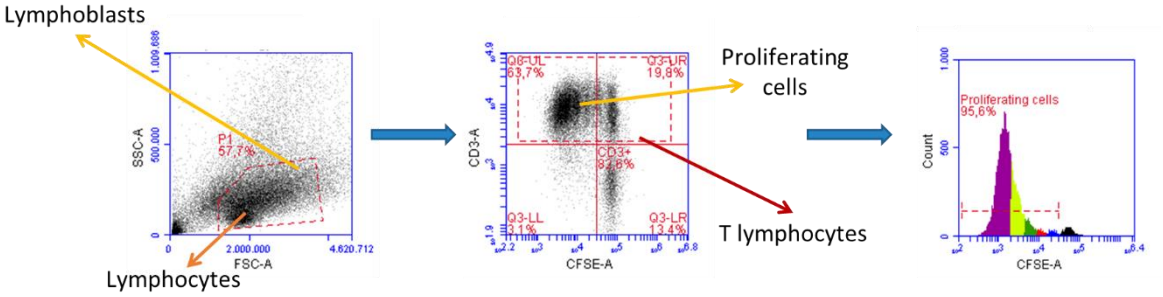


Figure 10 – Gating strategy.
Gating lymphocytes (P1) in the forward/side scatter plot (A) and CD3+ cells double stained for CFSE and CD3 (B) allows the assessment of T-cell proliferation due to a specific stimulus.

After gating of CD3+ cells in the dotplots, an histogram of CSFE fluorescence intensity of the gated T-lymphocytes allows the visualization of CFSE fluorescence decay corresponding to cell divisions. Each peak of CFSE fluorescence intensity correspond to different cell generations. Six to eight peaks of divisions can be detected in the CFSE fluorescence histogram. 15000 events were acquired in the CD3+ gate within P1 for every condition, whenever possible. The gates drawn were the same for all conditions in each day, however different between day 4 and 7.

The fluorescence peak corresponding to the CSFE intensity in day 0 is the initial generation of cells. Establishing a gate englobing all the events below that level of initial fluorescence intensity (to exclude the initial cell generation), gives the percentage of CFSE proliferating T-cells. Comparing with the unstimulated and stimulated cell conditions from the same day, a baseline lymphoproliferation can be taken into consideration when evaluating lymphocyte specific proliferation induced by each stimulus.

To test statistical differences between the stimulation conditions and the control (unstimulated), Friedman's non-parametric test was applied and detected that the groups (conditions of stimulation) and the control column were statistically different (**p value = 0.004 < 0.05). Then, a multiple comparison with Dunn's correction was performed to detect the significant stimulation condition, showing that only PHA (20 µg/ml) exhibited a statistically significant difference from the unstimulated condition (*p = 0.018 < 0.05).

4. Results and Discussion

4 Results and discussion

Certain pulmonary diseases are relatively rare diseases that are under-diagnosed because current detection methods are based mainly in the performance of clinical examination. Investigation in the area of clinical immunology will likely contribute for a more precise disease diagnosis, when evaluated alongside with clinical tests, relying in a collaboration between hospital staff, investigators, technicians and patients.

4.1 Detection of GM-CSF autoantibodies

The detection of the autoantibody GM-CSF in high concentrations in plasma samples of patients indicates that GM-CSF signalling is defective, directly reflecting the presence of autoimmune pulmonary alveolar proteinosis (aPAP) disease. Here, we optimized an ELISA method for detection of these antibodies, based on a previous study by Uchida and colleagues [23].

4.1.1 Protocol optimization

Firstly, an ELISA assay to detect anti-GM-CSF antibodies was optimized. The effect of the incubation time of a primary (rabbit anti-human GM-CSF standard) and secondary (goat anti-rabbit IgG HRP conjugated) antibodies was evaluated. Each antibody was either incubated for 40 min or 1 hour in two microplates. The range of concentration of the antibody for the standard curve was also assessed ranging from 20 to 0.315 ng/ml, with 0.315 ng/ml of anti-GM-CSF being the lower limit of detection. Incubations of 40 minutes already allowed the detection of the anti-GM-CSF antibody and also corresponded to the best fitted curve ($y = 0.7725\ln(x) + 0.9293$; $R^2 = 0.9779$) (Figure 11).

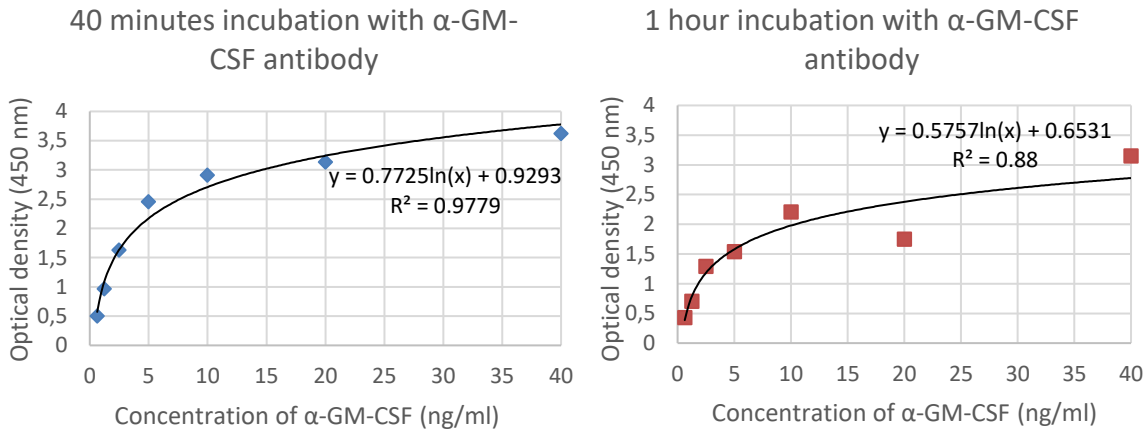


Figure 11 - Effect of primary and secondary antibody incubation time (40 min or 1 hour) in the detection and quantification of α-GM-CSF antibody concentration by ELISA.

A previously published study describes a standardized test for the diagnosis of aPAP using serum [23]. Here, we analysed if the results of anti-GM-CSF detection and concentration differed between human plasma and serum samples (Figure 12). Samples were diluted in different concentrations, to ensure that the obtained values of optical absorbance are within the calibration curve.

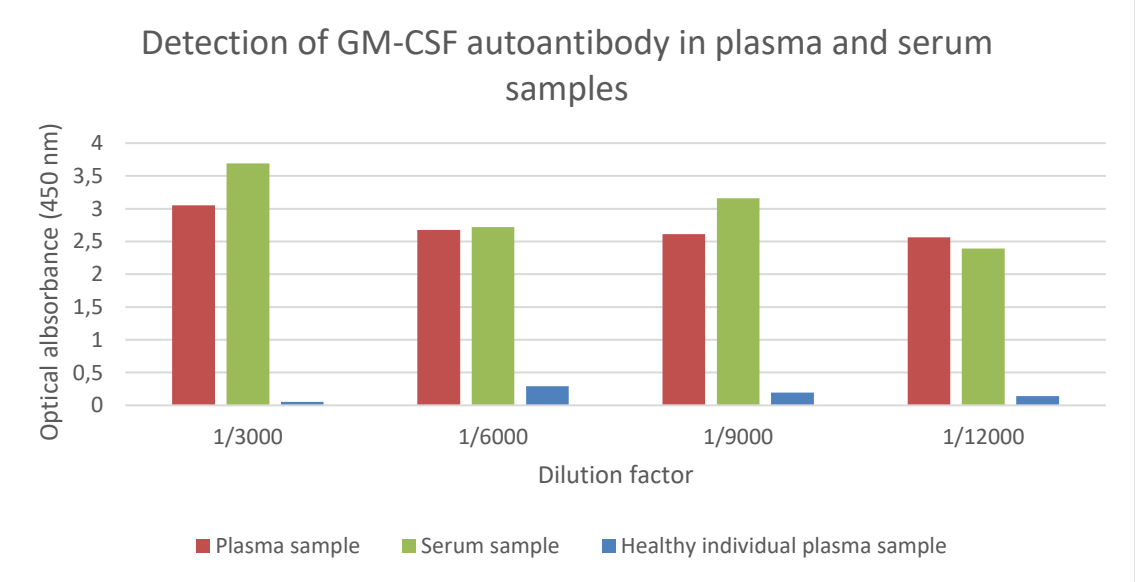


Figure 12 - Optical absorbances indicating the presence of anti-GM-CSF autoantibodies in plasma and serum samples of an individual with suspected aPAP and plasma samples from a healthy subject.

No substantial difference was observed in detection of the antibodies in serum and plasma samples which means that both samples may be used for the detection of anti-GM-

CSF in this diagnosis assay (Figure 12). In this particular assay, none of the dilutions from the tested sample provided absorbance values in the calibration curve, so 1/15000 dilution was also tested in the consequent assays.

The previously mentioned paper (Uchida *et al.*, 2014) advised 15 minutes of incubation with TMB substrate solution. With our reagents, 5 minutes were sufficient for color development, and more time with TMB resulted in oversaturation of the plate to an unreadable absorbance value. The reagents used in our assay are from a different brand, so the TMB substrate used could be more sensitive than the one advised in the article [23]. Besides, two different secondary antibodies conjugated to HRP were used: an anti-rabbit for the standard curve and an anti-human IgG for the plasma samples.

4.1.2 Detection of human anti-GM-CSF autoantibodies in plasma samples

Using the optimized ELISA, we estimated the average concentration of autoantibody anti-GM-CSF (µg/ml) present in human plasma samples. Plasma samples from a healthy individual presented a low concentration of circulating autoantibody, corresponding to the negative control for these assays (2.75 ± 0.36 µg/ml). It is important to clarify that these antibodies may be present in healthy individuals, although in small concentrations. In the study by Uchida *et. al*, 2014, a patient's plasma concentration of anti-GM-CSF higher than 5 µg/ml indicated that the individual was suffering from autoimmune pulmonary alveolar proteinosis disease. Furthermore, low fluctuations of plasma anti-GM-CSF could be detected in inflammatory and autoimmune conditions such as Crohn's disease, which is why a radiological examination, for instance, would help clearing aPAP suspicion [81]. As a positive control, we analysed plasma samples from an individual clinically diagnosed with aPAP. These samples showed high levels of anti-GM-CSF (170.518 ± 2.95 µg/ml) in circulation, confirming the reliability of the assay to detect this biomarker in blood samples (Figure 13).

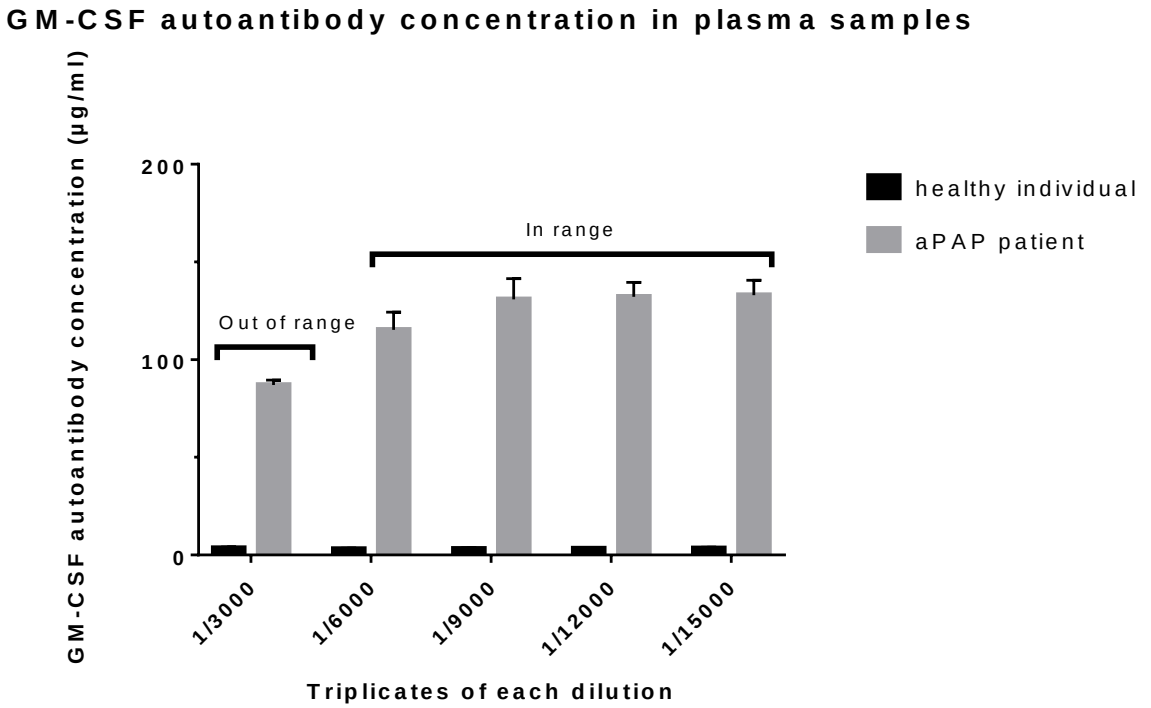


Figure 13 - Estimation of GM-CSF autoantibody concentration in plasma samples from a healthy individual and a patient diagnosed with aPAP.
Five dilution factors were used in triplicates and the corresponding average and standard deviaton are shown. The optical absorbances of the 1/3000 diluted aPAP individual's plasma was out of the calibration curve range.

We then analysed blood samples from an individual with suspicion of aPAP (Table 2). These samples had, on average 111.58 ± 109.42 µg/ml of anti-GM-CSF antibodies. This result was obtained both in plasma and serum samples of the individual tested. Values were consistently superior to the ones found in healthy individual samples, although no statistical significance was found (p-value = 0.25), due to the high variability between assays.

The fold increase in the concentration of GMAb of the patient tested versus the healthy individual sample was higher than fiftyfold in two of the assays. In one of the assays, the values of GM-CSF autoantibody concentration were relatively low in the patient, but still higher than in the healthy individual. However, this assay may have had technical failures to be accounted such as freeze/thawing cycles, excessive time in the preparation and addition of the plasma sample dilutions, cross-well contamination [82]. Besides, the sample of the healthy individual was a plasma sample from a donor whose clinical history is unknown; this individual could have had comorbidities that could influence the detection

of the anti-GM-CSF antibody. Another aspect to ponder is the fact that in the 10th assay, a newly reconstituted recombinant human GM-CSF protein (capture antigen) was used, giving high levels of anti-GM-CSF concentration comparing with other assays in which the recombinant protein had been stored for a long period of time, so the primary antibodies could not be so avid to bind this less efficient capture protein.

Table 2 - Concentration of GM-CSF antibodies detected in plasma samples from a healthy individual and the individual tested.
The fold increase was calculated for the antibody concentration detected in the individual tested sample relative to the healthy subject

Sample	Healthy individual (Negative control)	Individual tested	Fold change
6th assay	11.00	18.59	1.69
8th assay	1.53	83.99	54.74
10th assay	2.75	232.16	84.53
Average	5.09	111.58	46.99
Standard deviation	5.15	109.42	41.96

An aspect to be taken into account is the fact that the secondary antibody used is different for the standard curve (anti-rabbit) and the plasma samples (anti-human) because the standard primary antibody against GM-CSF is produced in rabbit, and not in human as would be preferable. If the secondary antibodies were the same, a more precise calculation of the GM-CSF autoantibodies present in the plasma samples would be possible. Even so, the detection of this biomarker in plasma samples of individuals in substantially higher levels than in healthy controls is helpful for the early diagnosis of aPAP.

Overall, the results above indicate that the individual tested had a high concentration of GMAb in circulation, around 47 times higher than the healthy subject, so it is highly probable that this individual was indeed suffering from aPAP.

4.1.3 Importance of an assay detecting anti-GM-CSF antibodies for aPAP diagnosis

The anti-GM-CSF ELISA assay is a relatively sensitive, specific and rapid experimental protocol, giving an easy interpretation of results. It is helpful in the diagnosis of aPAP since it directly detects the biological cause of the disease, making anti-GM-CSF antibody a good biomarker of aPAP. Also, each assay requires a small volume of the patient's sample besides giving the possibility to evaluate several patient samples at the same time.

On the other hand, this assay is not sufficient to allow an exact diagnosis. Therefore, analysing the ELISA results along with clinical history, radiological exams, biomarkers evaluation and pulmonary function tests is essential for a more assertive detection of aPAP pathology. Furthermore, if the physician suspects the patient has PAP but is unsure of the cause of the disease, a negative result in the ELISA GMAb assay could exclude the autoimmune form of the disease, allowing the diagnosis of the other forms of PAP.

In order to implement our ELISA GM-CSF autoantibodies detection protocol as diagnostic tool for autoimmune pulmonary alveolar proteinosis, a Standard Operation Procedure (SOP) was created (Appendix). A SOP is a document with precise detailed written instructions for a routine activity or protocol performed by an organization [83,84]. A successful quality system relies in SOP's creation and usage for the fact that it facilitates job performance, improving quality, safety and consistency of the product and ultimately of the laboratory results [83,84]. These procedures are extremely valuable and demanded for study validation in clinical laboratory diagnostic [84]. The fact that a similar protocol is already standardized as a diagnostic assay in other countries could ease the implementation of the anti-GM-CSF ELISA assay in hospital laboratories or clinical diagnostic institutes in Portugal. A limitation of the assay is that it is relatively expensive.

The early diagnosis of aPAP eases disease management, allowing a treatment that is mainly whole-lung lavage. Another option is treatment with GM-CSF, which is available via inhalation or a subcutaneous injection. It has also been reported that plasmapheresis could reduce GM-CSF autoantibodies in circulation or antibody treatment with rituximab to decrease B-cells, in order to diminish the formation of anti-GM-CSF.

4.2 Quantification of T lymphocyte proliferation by flow cytometry

An important aspect for diagnosis of hypersensitivity pneumonitis is the ability to determine which antigens stimulate T cell proliferation. Here, several stimuli were used and their concentration was optimized to challenge PBMCs isolated from healthy donors labelled with CFSE proliferation dye. Proliferation was induced with a mitogen stimulator of T-cells (PHA) or an antigen extract (heat or chemically inactivated *C. albicans*) at several concentrations.

4.2.1 T-lymphocyte proliferation can be induced *in vitro* by a mitogen (PHA)

As a positive control of the experiment, we analysed proliferation of T lymphocytes upon stimulation of freshly isolated PBMC with phytohemagglutinin (PHA), a known T-cell mitogenesis inducer. T cell proliferation is inversely correlated with CFSE fluorescence intensity, which is visible in the histogram of CFSE fluorescence intensity.

Observing the histograms of Figure 14, each color corresponds to one generation of cells, considering that the initial generation of cells is the peak colored in black. For estimation of lymphoproliferative responses to each condition of stimulation, the condition CFSE+CD3 (unstimulated) measured at the 7^{th} day is the negative control of the experiment being considered the baseline level of cell proliferation. The induction of proliferation with the mitogen condition is evident after 7 days of stimulation with PHA (Figure 14). As expected, phytohemagglutinin stimulation resulted in a high lymphoproliferative response, and incubation during 7 days with 20 µg/ml of PHA promoted a higher proliferative response (90.6 ± 7.047 = average percentage of proliferating cells) than with 5 µg/ml (71.81% ± 21.79 of proliferating cells) in all the assays. As seen in the assay of Figure 14, the percentage of proliferating cells stimulated with both concentrations of PHA (5 and 20 µg/ml) was higher than 90%, with the majority of cells in the CFSE fluorescence peak with the lower intensity, suggesting that almost all the cells divided until they reached a limit of proliferation and lost CFSE fluorescence.

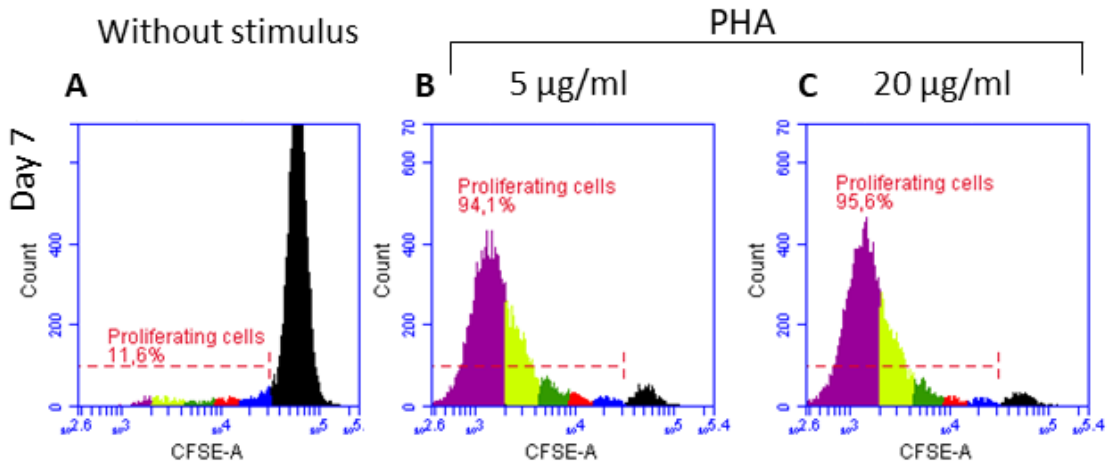


Figure 14 - Flow cytometry analysis of lymphocyte proliferation upon PHA stimulation after 7 days.
These plots are data from a representative assay (n=3).

In two assays, 2 timepoints were evaluated corresponding to day 4 and 7 of incubation. It was observed that, after 4 days of culture, lymphocyte proliferation could already be detected in the condition of mitogen (PHA) stimulation (with 5 and 20 µg/ml), reaching slightly higher levels after 7 days (Figure 15).

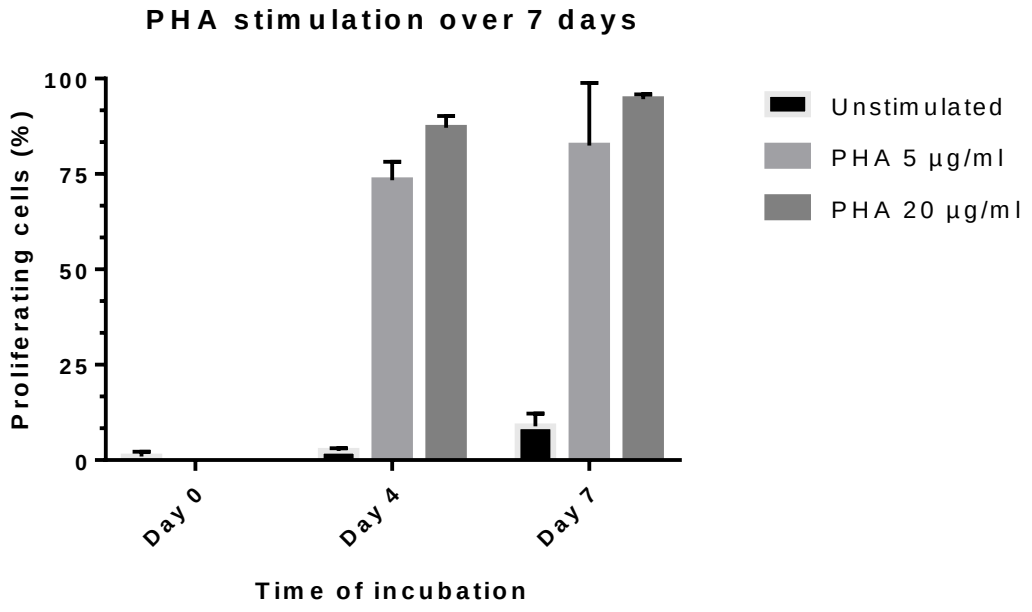


Figure 15 - Assessment of the percentage of proliferating T-cells by flow cytometry.
Three timepoints (day 0, 4 and 7) were analysed following PHA stimulation (5 or 20 µg/ml) or not (n=2). Graph shows average and standard deviation.

4.2.2 Inactivation of *Candida albicans*

The organism *C. albicans* was used to stimulate lymphocyte proliferation since most donors have probably been in contact with this organism, being sensitized to it and developing a delayed-type response when re-exposed to it. Since this organism has the ability to disseminate through hyphal growth depending on temperature and pH levels [75,79], several methods of inactivation were used to kill this organism, to assure the maintenance of its innocuous unicellular form, avoiding hyphae formation and ultimately cell culture contamination.

The strain SN152 of *Candida albicans* was chosen for the inactivation treatments and later antigen challenge, since this strain is known to have an attenuated virulence compared to other strains due to its triple deletion of virulence genes. Firstly, 100 µl of a saturated solution with *C. albicans* SN152 strain was added to 10 ml of cell culture medium to verify if the yeast grew in this medium. As a positive control, 100 µl of *C. albicans* inoculum was added to 10 ml of Yeast Peptone Dextrose (YPD) medium, the optimal growth medium. The two petri dishes were incubated one day at 37^{o}C, 5% CO_2. Comparing the two plates, it was verified that hyphae began to form, which indicated that the yeasts indeed grow in the cell culture medium used for the lymphocyte proliferation. Thus, multiple methods such as heat and chemical inactivation were evaluated. Yeast cells were either heated to 100°C for 20 min or autoclaved. The chemical inactivation consisted in treatments with 0.04% SDS, 1% PFA and 7% hydrogen peroxide (H_2O_2) overnight. Treated cells were then incubated for 3 days in cell culture medium and observed by microscopy and counted.

Microscopic observation of the inactivated yeast cells after 3 days of culture are shown in Figure 16. *C. albicans* adopted several morphologies depending on the treatment chosen: with PFA, H_2O_2, 100^{o}C and autoclave inactivation, the yeast form is visible. The maintenance of yeast unicellular form after 3 days of culture in a medium with serum indicates a successful inactivation of these cells.

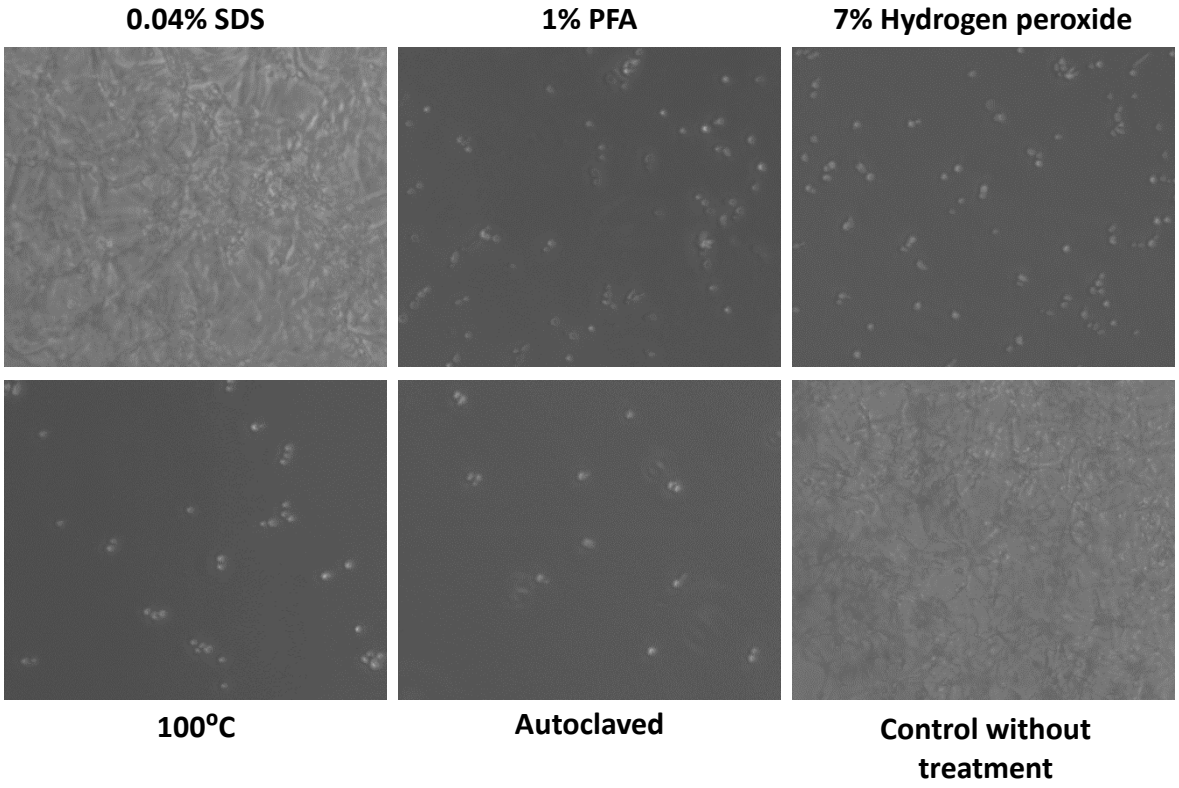


Figure 16 - Microscopic observation of the effect of different inactivation treatments in *Candida albicans* SN152 cells upon 3 days of culture.
Chemical treatments (0.04% SDS; 1% PFA; 7% hydrogen peroxide) and heat treatments (100^0C; autoclave) were performed. Cells were cultivated at 37 ^{0}C, 5%CO_2. 200x magnification.

Hyphae formation was evident in untreated cells and SDS treated, which suggested that SDS treatment was not effective in the inactivation of *C. albicans*.

The number of *Candida albicans* cells was higher with the treatment with 0.04% SDS and in the untreated cells, consistent with microscopic observations (Table 3). Other treatments resulted in a lower cell number and inhibition of hyphal growth in optimal medium and temperature conditions, suggesting an effective inactivation (Table 3). However, the well corresponding to the hydrogen peroxide treated yeast was contaminated by other fungi, invalidating any extrapolation of the results after 7 days of incubation. There could have been cross contamination for other wells, which might explain the fact that in the autoclaved treated cells, live cells were present which is unexpected in successful autoclaving.

Table 3 - *Candida albicans* cell counts at day 0, 3 and 7 with different inactivation treatments (*well contaminated by other fungi). Arrows represent an increase or decrease in the cell number.

Inactivation treatments	**Number of *C. albicans* cells in different timepoints**						
	Day 0	Day 3			Day 7		
		Live		Total	Live		Total
0.04% SDS	4.5×10^6 cells	8.96×10^6 cells	↑	8.96×10^6 cells	2.1×10^7 cells	↑	2.3×10^7 cells
1% PFA		-	↓	1.76×10^6 cells	1.6×10^5 cells	↓	1.44×10^6 cells
Hydrogen peroxide		-	↓	9.84×10^5 cells	*	*	*
100ºC		-	↓	8.72×10^5 cells	-	↑	1.6×10^6 cells
Autoclaved		-	↓	1.07×10^6 cells	4×10^4 cells	↓	9.76×10^5 cells
Control		1.01×10^7 cells	↑	1.01×10^7 cells	2.56×10^7 cells	↑	2.6×10^7 cells

Heat treatments are currently used in microbiology, in laboratory or in hospitals to sterilise utilized material or liquids to assure the death of microorganisms in order to avoid unnecessary contaminations. Concerning the chemical treatments used, paraformaldehyde is a well-known fixative for preservation of samples and specifically their cellular morphology, so it will kill the cell preserving its structure.

4.2.3 T-lymphocyte proliferation after stimulation with inactivated *Candida albicans*

For the proliferation assay, the most successful yeast inactivation treatments (1% PFA and autoclaving) were chosen to obtain *Candida albicans* inactivated cells for antigen stimulation.

Naïve B and T lymphocytes are usually round cells with 8 to 10 µm in diameter, becoming larger when activated or when dividing, reaching 12 µm [1]. Interestingly, the microscopic confocal observation of the different conditions of stimulation allowed the visualization of heterogenic morphology (Figure 17). Unstimulated cells appeared predominantly elongated, since these cells did not receive any survival or stimulatory signal in order to proliferate and adhered to the well. PHA stimulation induced clonal cell

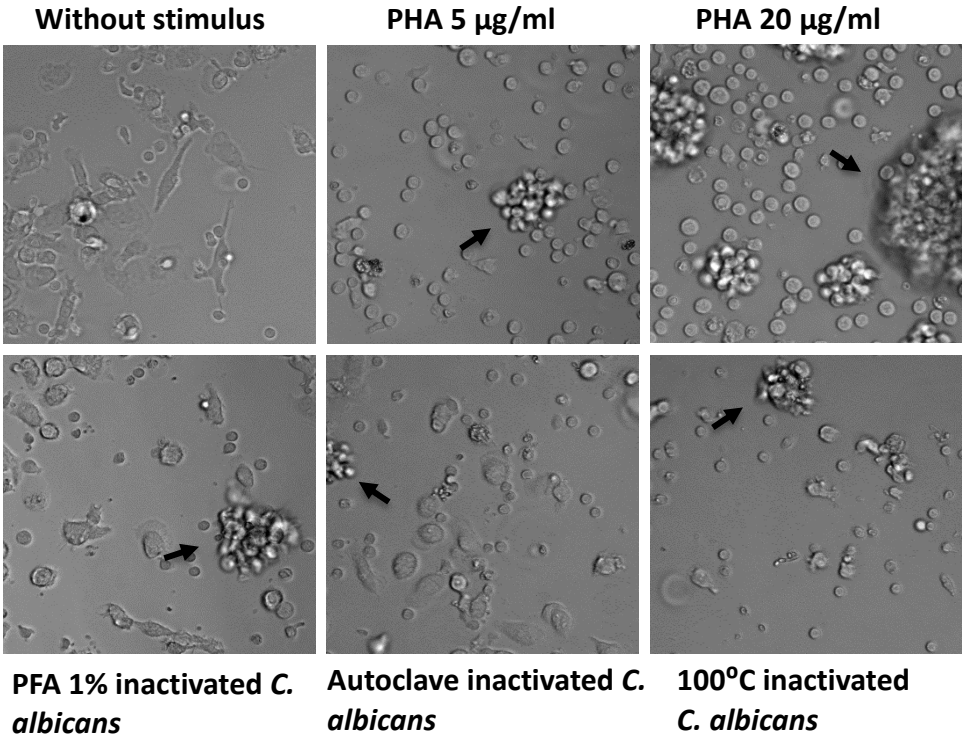


Figure 17 - Microscopic observation of human PBMCs cultivated with or without mitogen or antigen stimulus upon 6 days of culture.
For the mitogen induction, PHA was used at 5 or 20 ug/ml. Previously inactivated *Candida albicans* with PFA 1%, autoclaved and 100°C were used for antigen stimulus. Arrows indicate clonal cell aggregates. 200x magnification.

proliferation which can be observed as clonal aggregates. Stimulating cells with the higher concentration of PHA (20 µg/ml) resulted in more and larger cell aggregates than with 5 µg/ml of PHA. However, it is possible that an excessive concentration of PHA, instead of stimulating the cells, inhibits their proliferation or even provoke cell death. Stimulation with inactivated *Candida albicans* (by different treatments) also resulted in formation of clonal cell aggregates, suggesting that there was indeed proliferation. Some elongated cells were present between the round lymphocytes, even though in lower numbers than in the unstimulated cell condition, which could indicate the presence of APCs, necessary for the antigen presentation to activate T-cells. Overall, mitogen stimulation induces formation of more cell aggregates than the antigen stimulus and no stimulation resulted in more evident heterogeneity in cell morphology.

Importantly, T cell proliferation was observed upon stimulation with yeast cells inactivated with PFA or by autoclaving (Figure 18, Figure 19). This indicates that the inactivation treatment killed the yeast, but still maintained at least some of the antigens

intact, which can then be presented by APCs and stimulate T cell proliferation.

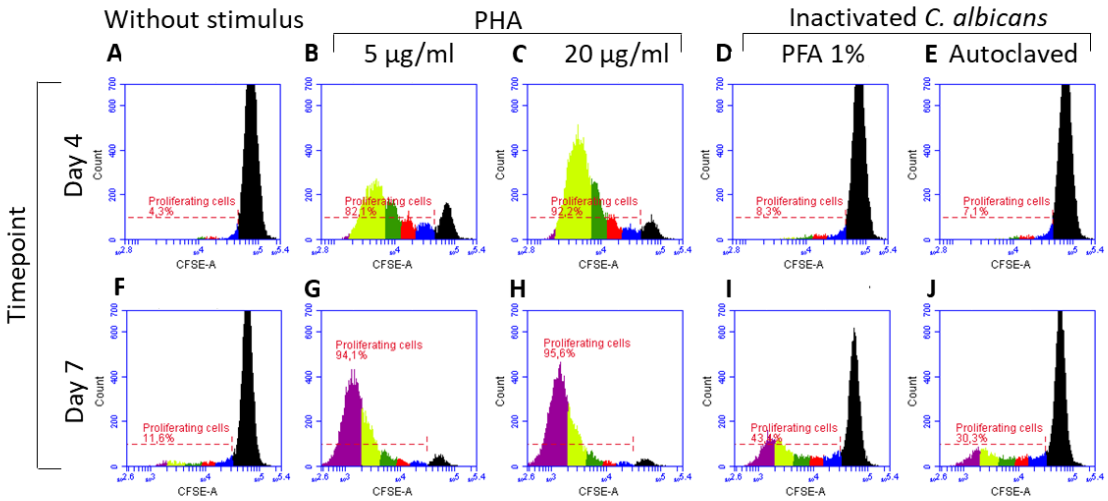


Figure 18 - Flow cytometry analysis of the fluorescence intensity of CFSE labelled T- lymphocytes after stimulation with PHA and inactivated *C. albicans*.
Two timepoints were analysed (4 and 7 days post-challenge).

PFA treatment resulted in slightly higher proliferation than the stimulation with autoclaved *C. albicans* with an average percentage of 28.61% ± 12.61 and 18.84% ± 14.94, respectively (Table 4). Contrary to what was observed with the mitogen stimulus, where treatment with 20 µg/ml of PHA allowed the proliferation of most cells, antigen challenge with inactivated *Candida albicans* induced a lower number of distinct peaks of cell divisions, even though proliferation is still evident (Figure 18). This result could be due to the fact that *C. albicans* challenges T-cell specific antigen proliferation, while PHA is a mitogenic inducer of all T

Table 4 - Summary of the results of proliferating T- lymphocytes (%) after stimulation with PHA and inactivated *C. albicans* 7 days post-challenge.
Data corresponding to three assays, obtained by flow cytometry analysis.

Stimulation condition	Unstimulated	PHA 5 µg/ml	PHA 20 µg/ml	Candida 1% PFA	Candida autoclaved
Assays (n=3)	11.25	94.07	95.54	42.98	29.92
	3.24	50.53	82.53	23.47	24.75
	6.44	70.82	93.73	19.39	1.84
Mean	6.98	71.81	90.6	28.61	18.84
Std. Deviation	4.03	21.79	7.05	12.61	14.94
Std. Error of Mean	2.33	12.58	4.07	7.28	8.63

cells. Also, analysing Figure 18, 4 days of PHA stimulation were sufficient to induce lymphocyte proliferation while, stimulation with *Candida albicans* treated cells only induced detectable proliferation at the 7th day of incubation.

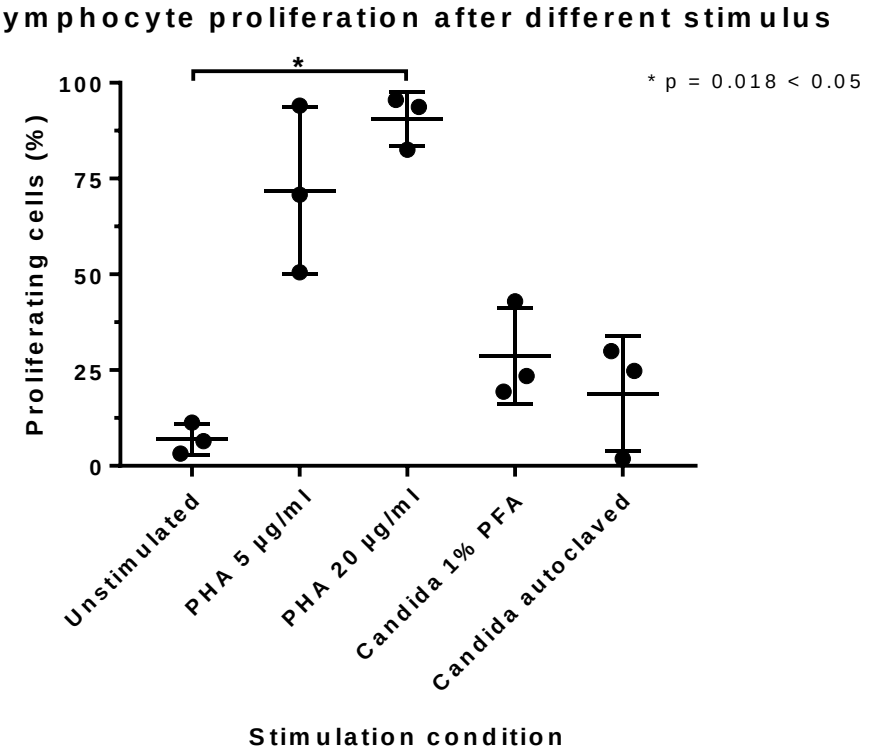


Figure 19 - Mitogen and antigen stimulation of isolated PBMCs from healthy donors.
The percentage of proliferating T lymphocytes was assessed by flow cytometry. PHA stimulation (5 or 20 µg/ml) was the positive control of the experiments; for the antigen challenge, inactivated *C. albicans* (with PFA 1% and autoclave treatments) were used (n=3).

Phytohemagglutinin directly induces the mitosis of T-cells without requiring the induction of the immune system response. On the other hand, stimulating cells with inactivated fungi or antigenic extract will induce an adaptive immune response, requiring antigen uptake, processing, presentation, T-cell recognition and activation. This delayed-type response to antigenic challenge requires more time than mitogenic induction which could explain the results observed. Another aspect to be taken into account is the fact that inactivated yeast cells or fungal spores could trigger different immune responses than live antigenic challenge [85–87]. Our organism might be adapted differently to harmful and innocuous antigen particles, responding less effectively to the inactivated pathogen [85]. An ineffective killing of the pathogens results in an infection while an exaggerated response causes tissue

damage [85]. This could be the case in the mucosal airway surfaces that are constantly being colonized by multiple microorganisms [85].

PBMCs is a group of different cells such as monocytes and lymphocytes besides T-lymphocytes, and its heterogeneous composition is visible by microscopic observation and might be responsible for the heterogeneity in proliferation induction with different stimulus. Cell proliferation will depend on several variables such as donor variability, allergen sensitization and the proliferative capacity of the cells. Antigen stimulation, with inactivated *Candida*, for example, is not such a strong stimulus as the mitogen, resulting in a lower lymphoproliferative response. Still, these *C. albicans* specific T cells may either have proliferated to a maximum level or may require another stimulus such as IL-2 or more antigen presenting cells so that cells activate and proliferate.

There are some restrictions to this assay, that requires technical expertise in cell culture and flow cytometry. Since PBMCs are primary cells, there are certain limitations that could influence result interpretation. Even though these cells mimic the conditions *in vivo*, they have limited growth and proliferation, requiring protocol optimization and careful handling. Another concern of this assay is the variability between donors, given the fact that even healthy individuals could exhibit a wide range of proliferative responses and may require a higher stimulation concentration than other individuals. Whenever possible, multiple concentrations of the allergen should be used until the stimulus concentration is optimized. Besides, working with PBMCs depends on multiple variables such as sample availability, culture contamination and is a cell culture time consuming protocol. Since it is a complex experimental protocol, it is difficult to analyse several patient samples at the same time. Furthermore, the anticoagulant present in the blood collection tube might influence the proliferative response [88]. If possible, it is preferable that the blood is collected into a tube with citrate, instead of EDTA anticoagulant, since this chelating agent has a negative effect in cell proliferation, while citrate preserves the cell function, which was observed during these assays. Another limitation of this CFSE assay is that cell viability was not assessed. Unfortunately, flow cytometry vital dyes such as propidium iodide or TO-PRO-3 could not be used in these assays since their fluorescence would interfere with CFSE dye fluorescence and CD3-APC channel. However, if possible it would be particularly useful

to analyse cell viability, calculate viability index and establish a gate only in the live cells because the loss of fluorescence could indicate cell division or cell death. The fact that the cells stopped dividing could mean that either they reached a growth plateau or the cells died.

4.2.4 Importance of an assay for diagnosis of hypersensitivity pneumonitis

Exposure to a wide and increasing variety of environmental antigens may trigger an exaggerated immune response such as in hypersensitivity pneumonitis [45,89,90]. The removal of the causal agent exposure is essential for improvement of the patient health and possible reversal of the disease before the fibrotic state. However, if the exposure source remains unidentified or unconfirmed, it could be problematic [90]. Evaluating the personal exposure at work, for example, is fundamental because certain varieties of the disease are related to occupational exposure [52]. Critical worksites are agricultural, aviary and factory, where workers might have an increased risk of HP [90]. Risk evaluation should be performed as HP could be a public health problem.

An article describes a pilot study where the samples were collected from the patient's house or workplace to identify the antigens presented [90]. In parallel, the patient's serum was collected to detect the presence of antibodies to the antigen suspected of causing the disease [90]. Along with work environment questionnaires, a comparison was made between the antigens collected and the antibodies in circulation [90]. Bacteria and fungi were cultured, isolated and an immunodiffusion test was performed to test the reactivity of the patient's serum to the isolated fungi or bacteria [90]. It was reported that the source of antigen exposure belonged to a carpet in the basement containing *Cladosporium sp.* [90]. The patient was advised to remove all carpets and to avoid exposure to other sources of this fungi [90]. The early identification of the exposure could improve the quality of life of the individual and the insurance companies might reimburse hospital expenses and routine testing [90]. A limitation of the study described is that the presence of these antibodies is only a marker of exposure so it does not indicate the induction of an hypersensitivity response mediated by cells [90].

Depending only on the number of cells obtained upon PBMC isolation, several allergens and organisms can be used as stimuli and tested at the same time. This test allows identification of the causal allergen responsible for the hypersensitivity or the mix of allergens to which the patient is highly sensible. One of the difficulties on the diagnosis of HP is to establish a causal relationship between exposure to an allergen and the development of the disease [90,91]. ELISA tests only quantify IgG antibodies against several allergens such as avian proteins, bacteria, fungi or other possible allergen. The successful optimization of a reliable antigen-specific assay raises the possibility to use this method for the diagnosis and detection of other hypersensitivities with a delayed cell type immune response.

The use of CFSE and flow cytometry is an alternative to current lymphoproliferation assays based on [3H]thymidine incorporation, which depend on radioactivity, reducing the health risks associated to this assay. Some articles pointed out the potential of CFSE assays to detect antigen specific proliferative responses in human PBMCs of patients with suspected immunodeficiencies, and concluded that the CFSE test had a good correlation to the results obtained with thymidine incorporation assays [92,93]. *In vitro* diagnosis of delayed-type hypersensitivities is a preferred substitute of more invasive approaches such as inhalation challenge or skin prick tests, even though blood collection is somewhat invasive. Moreover, reliable allergenic extracts (of fungi for example) are difficult to obtain and the units of measure are highly variable, resulting in a high variability between diagnostic tests [94,95]. Obtaining a diverse array of bulk allergens facilitates identification of the agent(s) responsible for the hypersensitivity reaction due to its chronical exposure. Also, low molecular weight chemicals can be tested for hypersensitivity. Analysis of hypersensitivity reactions against beryllium for instance will be relevant, because occupation exposure in factories might cause beryllium hypersensitivity.

5. Conclusion and future directions

5 Conclusion and future directions

Developing laboratory diagnostic assays is imperative for an early detection of rare diseases, enabling its effective treatment. Autoimmune alveolar proteinosis is characterized by impaired GM-CSF signalling due to the presence of autoantibodies against GM-CSF, which results in an inefficient macrophage maturation and defective function as a surfactant clearance agent. The lung's tissue and architecture is affected as a consequence of these phospholipids accumulating in the alveolar parenchyma of the individuals affected by the autoimmune type of the disease. Detection of anti-GM-CSF antibodies by ELISA allows to easily assess this biomarker of aPAP disease in plasma samples. Alongside with other medical diagnostic exams, the autoimmune form of the disease can be identified or ruled out upon quantification of anti-GM-CSF, allowing the correct identification of the cause of the disease in that particular patient. Since the assay is already implemented in other countries, its implementation in Portugal should be simplified. Here, we have established a standard operating procedure (SOP) for the detection of GM-CSF autoantibodies by ELISA technique which is ready to use and could help in the early detection of this rare disease.

An *in vitro* lymphoproliferation assay allows the evaluation of delayed-type hypersensitivity in response to different stimulus, mimicking the organism response following the exposure to certain inducing agents (microoganisms, avian proteins, metals, chemicals…). CFSE dilution allows measurement of the proliferative response of T-lymphocytes upon stimulation with mitogen or antigen/allergen. *C. albicans* was chosen as the antigenic challenge to study primed T-lymphocyte proliferation. This yeast is a commensal agent in most individuals, so a re-exposure to this agent is most likely to induce an antigen specific lymphocyte proliferation. We optimized a protocol for the inactivation of pathogenic agents, starting with *C. albicans.* By using healthy donor PBMCs with this assay, proliferation was detected following stimulation with PHA or heat and chemically inactivated *C. albicans* yeast cells demonstrating that these organisms are still able to be recognized as an antigen and stimulate cells.

The key feature of chronic hypersensitivity pneumonitis is an excessive migration and proliferation of T lymphocytes into the alveolar parenchyma. Thus, the development of an assay to assess lymphoproliferative response could be helpful in the diagnosis and management of this disease and other delayed-type hypersensitivities.

It might be important to note that the assay is based in the use of PBMCs assuming that the majority are T-cells, however not only these cells are present in this mononuclear group of cells that contains monocytes, B, T and NK lymphocytes. All these cells play a role in the immune response to the antigen used as a challenge, since they include antigen presenting cells as well as memory and antigen specific cells. The CD3 marker of T-cells allows the visualization of T-lymphocyte proliferation. In future assays, it might be interesting to use CD4 and CD8 markers to perform immunophenotyping and elucidate the immune system intracellular responses to each allergen.

The optimization of this protocol regarding concentration of antigen stimulus is required before using isolated cells from patients suspected from suffering hypersensitivity pneumonitis to identify the inducing causal agent of the inflammatory response. The identification of the source of exposure that could be causing or aggravating the disease is essential for exposure avoidance, which is the most effective treatment for this disease.

Preliminary assays have been performed using isolated PBMCs from hypersensitivity individuals, challenged with inactivated fungi spores or fungi commercial extract. Fungal samples were obtained from the home of a patient previously diagnosed with chronic hypersensitivity pneumonitis to identify the organism(s) inducing the disease. A spore suspension was recovered from three fungal species isolated from a sample collection from the patient's home, which were already associated with the disease. However, it is difficult to fully inactivate fungal spores, as they are very resistant pathogens and the concentration of fungal stimulus to be used is unknown. Besides, it is uncertain what part of the fungi can be pathogenic, it could be either the spores as well as the mycelium that are causing the hypersensitivity disorder. To evaluate that aspect, an assay could be performed to evaluate the stimulatory effect of the different structures of a specific fungus (mycelium, cell wall, spore) and following the inactivation methods, stimulate PBMCs isolated from an individual diagnostically hypersensitive to that fungus. Also, a fungal commercial extract was used to

stimulate cells extracted from the blood of an individual that had high antibody levels against that fungus. Again, the concentration of the stimulus is difficult to optimize since a low concentration of the extract could not be enough to stimulate the cells and consequently detect their specific proliferation; on the other hand, a high concentration of the fungal extract instead of stimulating, might kill the cells in culture, which was observed. Furthermore, it is unclear what is used to produce these commercially available fungal extracts, if it is the whole organism or certain structures of it. In a future assay, several concentrations of a specific extract will be chosen to induce the proliferation of PBMCs isolated from blood samples from an individual suffering from the disease with suspicion of that fungus being the causal agent.

Bronchoalveolar lavage fluid cells can also be isolated to test antigen specific lymphocyte proliferation, even though so far we were not able to detect proliferation. It is ideally a good sample source since we directly obtain T lymphocytes from the site of infection so, supposedly, BAL fluid recovered from hypersensitivity pneumonitis patients could contain primed T cells present in the alveoli that will theoretically respond to an antigen challenge with the specific agent that is causing the disease. The protocol problems encountered resided mainly in the fact that this fluid contains many microorganisms besides the low cell number obtained. Therefore, cell culture with medium containing antibiotics (penicillin and streptomycin) must be complemented with antifungal agents (amphotericin) to avoid the growth of pathogens in culture and cell death. However, the antifungal agent used as a medium supplement might interfere with the ability of the inactivated fungi cells to stimulate T cells. Amphotericin binds to ergosterol present in the lipidic membrane of fungi, inhibiting its several functions in the yeast cell physiology, directly killing the organism and also forming ion channels in the membrane [96]. Since in this assay BALF cells are challenged with the inactivated fungi it is possible that these treated cells might escape the activity of amphotericin, still being able to stimulate and induce the proliferation of T cells. A further assay could be performed in order to test the ability of the cells recovered from BAL fluid to proliferate after challenge with inactivated fungi or fungal extracts from the suspicious agent causing the disease.

Developing new techniques *in vitro* for the diagnosis of rare diseases could contribute to delay or reverse the course of the disease, which is fundamental for improving quality of life of the diseased individuals, as well as their families.

6. Bibliographic references

6 Bibliographic references

1. Abbas, A. K., Lichtmann, A. H. & Pillai, S. *Cellular and Molecular Immunology*. (Saunders, 2014).
2. Lerner, A., Jeremias, P. & Matthias, T. The World Incidence and Prevalence of Autoimmune Diseases is Increasing. *Int. J. Celiac Dis.* **3,** 151–155 (2016).
3. Hammer, G. D. & McPhee, S. J. *Pathophysiology of Disease: An Introduction to Clinical Medicine*. (McGraw-Hill Education, 2014).
4. Salmon, J. E. Mechanisms of Immune-Mediated Tissue Injury. in *Goldman's Cecil Medicine* **1,** 226–230 (Elsevier, 2012).
5. Actor, J. K. The Immune Hypersensitivities. in *Introductory Immunology* 97–105 (Elsevier, 2014). doi:10.1016/B978-0-12-420030-2.00008-1
6. Abdelsamed, H. HP Turns 17: T helper 17 cell response during hypersensitivity pneumonitis (HP) and factors controlling it. (University of Tennessee Health Science Center, 2012). doi:10.21007/etd.cghs.2012.0002
7. Agache, I. O. & Rogozea, L. Management of hypersensivity pneumonitis. *Clin. Transl. Allergy* **3,** 1–10 (2013).
8. Selman, M. & Buendía-Roldán, I. Immunopathology, diagnosis, and management of hypersensitivity pneumonitis. *Semin. Respir. Crit. Care Med.* **33,** 543–54 (2012).
9. Bonella, F. & Campo, I. Pulmonary alveolar proteinosis. *Pneumologia* **63,** 144, 147–55 (2014).
10. Suzuki, T. *et al.* Hereditary Pulmonary Alveolar Proteinosis. *Am. J. Respir. Crit. Care Med.* **182,** 1292–1304 (2010).
11. Carey, B. & Trapnell, B. C. The molecular basis of pulmonary alveolar proteinosis. *Clin. Immunol.* **135,** 223–235 (2010).
12. Inoue, Y. *et al.* Characteristics of a Large Cohort of Patients with Autoimmune Pulmonary Alveolar Proteinosis in Japan. *Am. J. Respir. Crit. Care Med.* **177,** 752–762 (2008).
13. Trapnell, B. C., Carey, B. C., Uchida, K. & Suzuki, T. Pulmonary alveolar proteinosis, a primary immunodeficiency of impaired GM-CSF stimulation of

macrophages. *Curr. Opin. Immunol.* **21,** 514–521 (2009).

14. Griese, M. Pulmonary Alveolar Proteinosis: A Comprehensive Clinical Perspective. *Pediatrics* **140,** e20170610 (2017).
15. Huaringa, A. J. & Francis, W. H. Pulmonary alveolar proteinosis: a case report and world literature review. *Respirol. Case Reports* **4,** e00201 (2016).
16. Carrington, J. M. & Hershberger, D. M. *Pulmonary Alveolar Proteinosis*. *StatPearls* (StatPearls Publishing, 2018).
17. Colloff, M. J., Merrett, T. G., Merrett, J., McSharry, C. & Boyd, G. Feather mites are potentially an important source of allergens for pigeon and budgerigar keepers. *Clin. Exp. Allergy* **27,** 60–7 (1997).
18. Kumar, A., Abdelmalak, B., Inoue, Y. & Culver, D. A. Pulmonary alveolar proteinosis in adults: pathophysiology and clinical approach. *Lancet. Respir. Med.* **6,** 554–565 (2018).
19. Rosen, S. H., Castleman, B., Liebow, A. A., Enzinger, F. M. & Hunt, R. T. N. Pulmonary Alveolar Proteinosis. *N. Engl. J. Med.* **258,** 1123–1142 (1958).
20. Dranoff, G. *et al.* Involvement of granulocyte-macrophage colony-stimulating factor in pulmonary homeostasis. *Science* **264,** 713–6 (1994).
21. Stanley, E. *et al.* Granulocyte/macrophage colony-stimulating factor-deficient mice show no major perturbation of hematopoiesis but develop a characteristic pulmonary pathology. *Proc. Natl. Acad. Sci. U. S. A.* **91,** 5592–6 (1994).
22. Trapnell, B. C., Carey, B. C., Uchida, K. & Suzuki, T. Pulmonary alveolar proteinosis, a primary immunodeficiency of impaired GM-CSF stimulation of macrophages. *Curr. Opin. Immunol.* **21,** 514–21 (2009).
23. Uchida, K. *et al.* Standardized serum GM-CSF autoantibody testing for the routine clinical diagnosis of autoimmune pulmonary alveolar proteinosis. *J. Immunol. Methods* **402,** 57–70 (2014).
24. Kitamura, T. *et al.* Idiopathic pulmonary alveolar proteinosis as an autoimmune disease with neutralizing antibody against granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* **190,** 875–80 (1999).
25. Uchida, K. *et al.* Granulocyte/macrophage-colony-stimulating factor autoantibodies and myeloid cell immune functions in healthy subjects. *Blood* **113,** 2547–2556 (2009).

26. Shibata, Y. *et al.* GM-CSF Regulates Alveolar Macrophage Differentiation and Innate Immunity in the Lung through PU.1. *Immunity* **15,** 557–567 (2001).
27. Case, P. *et al.* Autoimmune pulmonary alveolar proteinosis : a case report Autoimmunologiczna proteinoza pęcherzyków płucnych. *Pneumonol. Alergol. Pol.* **80,** 82–86 (2012).
28. Lin, F.-C., Chang, G.-D., Chern, M.-S., Chen, Y. & Chang, S.-C. Clinical significance of anti-GM-CSF antibodies in idiopathic pulmonary alveolar proteinosis. *Thorax* **61,** 528–34 (2006).
29. Yamasue, M. *et al.* Elevated Serum Anti-GM-CSF Antibodies before the Onset of Autoimmune Pulmonary Alveolar Proteinosis in a Patient with Sarcoidosis and Systemic Sclerosis. *Tohoku J. Exp. Med.* **243,** 77–83 (2017).
30. McDonnell, M. J., Reynolds, C., Tormey, V., Gilmartin, J. J. & Rutherford, R. M. Pulmonary alveolar proteinosis: report of two cases in the West of Ireland with review of current literature. *Ir. J. Med. Sci.* **183,** 123–7 (2014).
31. Imoto, N. *et al.* GM-CSF Autoantibody-positive Pulmonary Alveolar Proteinosis with Simultaneous Myeloproliferative Neoplasm. *Intern. Med.* **56,** 435–439 (2017).
32. Piccoli, L. *et al.* Neutralization and clearance of GM-CSF by autoantibodies in pulmonary alveolar proteinosis. *Nat. Commun.* **6,** 7375 (2015).
33. Kusakabe, Y. *et al.* A standardized blood test for the routine clinical diagnosis of impaired GM-CSF signaling using flow cytometry. *J. Immunol. Methods* **413,** 1–11 (2014).
34. Hamilton, J. A. Colony-stimulating factors in inflammation and autoimmunity. *Nat. Rev. Immunol.* **8,** 533–544 (2008).
35. Shiomi, A., Usui, T. & Mimori, T. GM-CSF as a therapeutic target in autoimmune diseases. *Inflamm. Regen.* **36,** 8 (2016).
36. Avci, A. B., Feist, E. & Burmester, G.-R. Targeting GM-CSF in rheumatoid arthritis. *Clin. Exp. Rheumatol.* **34,** 39–44 (2016).
37. Hamilton, J. A. & Achuthan, A. Colony stimulating factors and myeloid cell biology in health and disease. *Trends Immunol.* **34,** 81–9 (2013).
38. Kaufman, H. L., Ruby, C. E., Hughes, T. & Slingluff, C. L. Current status of granulocyte-macrophage colony-stimulating factor in the immunotherapy of

melanoma. *J. Immunother. Cancer* **2,** 11 (2014).

39. Stewart, J. Innate and acquired immunity. in *Medical Microbiology* 109–135 (Elsevier, 2012). doi:10.1016/B978-0-7020-4089-4.00024-X
40. Wright, J. R. Host Defense Functions of Pulmonary Surfactant. *Neonatology* **85,** 326–332 (2004).
41. Kingma, P. S. & Whitsett, J. A. In defense of the lung: surfactant protein A and surfactant protein D. *Curr. Opin. Pharmacol.* **6,** 277–283 (2006).
42. Ohashi, K. *et al.* Direct evidence that GM-CSF inhalation improves lung clearance in pulmonary alveolar proteinosis. *Respir. Med.* **106,** 284–93 (2012).
43. Kitamura, T. *et al.* Serological Diagnosis of Idiopathic Pulmonary Alveolar Proteinosis. *Am. J. Respir. Crit. Care Med.* **162,** 658–662 (2000).
44. Spagnolo, P. *et al.* Hypersensitivity Pneumonitis: A Comprehensive Review. *J Investig Allergol Clin Immunol* **25,** 237–250 (2015).
45. Vasakova, M., Morell, F., Walsh, S., Leslie, K. & Raghu, G. Hypersensitivity Pneumonitis: Perspectives in Diagnosis and Management. *Am. J. Respir. Crit. Care Med.* **196,** 680–689 (2017).
46. Selman, M., Pardo, A. & King, T. E. Hypersensitivity pneumonitis: insights in diagnosis and pathobiology. *Am. J. Respir. Crit. Care Med.* **186,** 314–24 (2012).
47. Ramirez, R. M. & Jacobs, R. L. Hypersensitivity pneumonitis by Fusarium vasinfectum in a home environment. *J. Allergy Clin. Immunol. Pract.* **2,** 483–484 (2014).
48. Hirschmann, J. V, Pipavath, S. N. J. & Godwin, J. D. Hypersensitivity pneumonitis: a historical, clinical, and radiologic review. *Radiographics* **29,** 1921–38 (2009).
49. Selman, M., Lacasse, Y., Pardo, A. & Cormier, Y. Hypersensitivity pneumonitis caused by fungi. *Proc. Am. Thorac. Soc.* **7,** 229–36 (2010).
50. Cui, A. *et al.* Angiogenic and Angiostatic Chemokines in Idiopathic Pulmonary Fibrosis and Granulomatous Lung Disease. *Respiration* **80,** 372–378 (2010).
51. Suhara, K. *et al.* Utility of immunological tests for bird-related hypersensitivity pneumonitis. *Respir. Investig.* **53,** 13–21 (2015).
52. Galeazzo, G., Sforza, R. & Marinou, A. Hypersensitivity pneumonitis : a complex lung disease. *Clin. Mol. Allergy* 1–8 (2017). doi:10.1186/s12948-017-0062-7
53. Ando, M. *et al.* HLA-DQw3 in Japanese Summer-type Hypersensitivity

Pneumonitis Induced by Trichosporon cutaneum. *Am. Rev. Respir. Dis.* **140,** 948–950 (1989).

54. Camarena, A. *et al.* PSMB8 (LMP7) but not PSMB9 (LMP2) gene polymorphisms are associated to pigeon breeder's hypersensitivity pneumonitis. *Respir. Med.* **104,** 889–94 (2010).
55. Aquino-Galvez, A. *et al.* Transporter associated with antigen processing (TAP) 1 gene polymorphisms in patients with hypersensitivity pneumonitis. *Exp. Mol. Pathol.* **84,** 173–7 (2008).
56. Hill, M. R. *et al.* Promoter variants in tissue inhibitor of metalloproteinase-3 (TIMP-3) protect against susceptibility in pigeon breeders' disease. *Thorax* **59,** 586–590 (2004).
57. Warren, C. P. W. Extrinsic allergic alveolitis: a disease commoner in non-smokers. *Thorax* **32,** 567–569 (1977).
58. Blanchet, M.-R., Israël-Assayag, E. & Cormier, Y. Inhibitory Effect of Nicotine on Experimental Hypersensitivity Pneumonitis In Vivo and In Vitro. *Am. J. Respir. Crit. Care Med.* **169,** 903–909 (2004).
59. OHTSUKA, Y. *et al.* Smoking Promotes Insidious and Chronic Farmer's Lung Disease, and Deteriorates the Clinical Outcome. *Intern. Med.* **34,** 966–971 (1995).
60. Park, Y., Oh, S. J. & Chung, D. H. CD4 + CD25 + regulatory T cells attenuate hypersensitivity pneumonitis by suppressing IFN-γ production by CD4 + and CD8 + T cells. *J. Leukoc. Biol.* **86,** 1427–1437 (2009).
61. Girard, M., Israël-Assayag, E. & Cormier, Y. Impaired function of regulatory T-cells in hypersensitivity pneumonitis. *Eur. Respir. J.* **37,** 632–9 (2011).
62. Sterclova, M., Vasakova, M. & Metlicka, M. Significance of specific IgG against sensitizing antigens in extrinsic allergic alveolitis: Serological methods in EAA. *Rev. Port. Pneumol.* **17,** 253–259 (2011).
63. Bellanger, A.-P. *et al.* Positive fungal quantitative PCR and Th17 cytokine detection in bronchoalveolar lavage fluids: Complementary biomarkers of hypersensitivity pneumonitis? *J. Immunol. Methods* **434,** 61–65 (2016).
64. Sitz, K. V & Birx, D. L. Lymphocyte Proliferation Assay. *Methods Mol. Med.* **17,** 343–353 (1999).
65. Marits, P., Wikström, A.-C., Popadic, D., Winqvist, O. & Thunberg, S. Evaluation of

T and B lymphocyte function in clinical practice using a flow cytometry based proliferation assay. *Clin. Immunol.* **153,** 332–342 (2014).

66. U.S. Department of Energy. Beryllium Lymphocyte Proliferation Testing (BeLPT). (2001).
67. Lyons, A. B. Analysing cell division in vivo and in vitro using flow cytometric measurement of CFSE dye dilution. *J. Immunol. Methods* **243,** 147–154 (2000).
68. Quah, B. J. C., Warren, H. S. & Parish, C. R. Monitoring lymphocyte proliferation in vitro and in vivo with the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester. *Nat. Protoc.* **2,** 2049–2056 (2007).
69. Lyons, a B. & Doherty, K. V. Flow Cytometric Analysis of Cell Division by Dye Dilution. in *Current Protocols in Cytometry* **Chapter 9,** Unit 9 11 (John Wiley & Sons, Inc., 2004).
70. Hwang, S. J., Kim, S., Park, W. S. & Chung, D. H. IL-4-Secreting NKT Cells Prevent Hypersensitivity Pneumonitis by Suppressing IFN- -Producing Neutrophils. *J. Immunol.* **177,** 5258–5268 (2006).
71. Balogh, E. *et al.* Impaired Immunosuppressive Effect of Bronchoalveolar Mesenchymal Stem Cells in Hypersensitivity Pneumonitis: Preliminary Findings. *Cytom. Part B Clin. Cytom.* **94,** 363–368 (2016).
72. Rosenblum Lichtenstein, J. H. *et al.* Environmental Mold and Mycotoxin Exposures Elicit Specific Cytokine and Chemokine Responses. *PLoS One* **10,** e0126926 (2015).
73. Pira, G. L., Kern, F., Gratama, J., Roederer, M. & Manca, F. Measurement of antigen specific immune responses: 2006 update. *Cytom. Part B Clin. Cytom.* **72B,** 77–85 (2007).
74. Noble, S. M., French, S., Kohn, L. A., Chen, V. & Johnson, A. D. Systematic screens of a Candida albicans homozygous deletion library decouple morphogenetic switching and pathogenicity. *Nat. Genet.* **42,** 590–598 (2010).
75. Mendes Giannini, M. J. S., Bernardi, T., Scorzoni, L., Fusco-Almeida, A. M. & Sardi, J. C. O. Candida species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. *J. Med. Microbiol.* **62,** 10–24 (2013).
76. Chen, K.-Y., Ko, S.-C., Hsueh, P.-R., Luh, K.-T. & Yang, P.-C. Pulmonary Fungal

Infection. *Chest* **120,** 177–184 (2001).

77. Serrano, C., Torrego, A., Loosli, A., Valero, A. & Picado, C. Neumonitis por hipersensibilidad tras exposición a Candida spp. *Arch. Bronconeumol.* **46,** 275–277 (2010).
78. Sudbery, P. E. Growth of Candida albicans hyphae. *Nat. Rev. Microbiol.* **9,** 737–748 (2011).
79. Lu, Y., Su, C., Wang, A. & Liu, H. Hyphal Development in Candida albicans Requires Two Temporally Linked Changes in Promoter Chromatin for Initiation and Maintenance. *PLoS Biol.* **9,** e1001105 (2011).
80. Ando, M., Yoshida, K., Nakashima, H., Sugihara, Y. & Kashida, Y. Role of Candida albicans in Chronic Hypersensitivity Pneumonitis. *Chest* **105,** 317–318 (1994).
81. Han, X. *et al.* Granulocyte-Macrophage Colony-Stimulating Factor Autoantibodies in Murine Ileitis and Progressive Ileal Crohn's Disease. *Gastroenterology* **136,** 1261–1271.e3 (2009).
82. Diagnostics, C. *TROUBLESHOOTING*.
83. Environmental Protection Agency, U. S. Guidance for Preparing Standard Operating Procedures (SOPs). *Epa* 1–55 (2007).
84. Barbosa, C. M., Zuliani Mauro, M. F., Bavaresco Cristóvão, S. A. & Mangione, J. A. A importância dos procedimentos operacionais padrão (POPs) para os centros de pesquisa clínica. *Rev. Assoc. Med. Bras.* **57,** 134–135 (2011).
85. Rivera, A., Van Epps, H. L., Hohl, T. M., Rizzuto, G. & Pamer, E. G. Distinct CD4+-T-Cell Responses to Live and Heat-Inactivated Aspergillus fumigatus Conidia. *Infect. Immun.* **73,** 7170–7179 (2005).
86. Kikuchi, T. *et al.* Dendritic Cells Pulsed with Live and Dead Legionella pneumophila Elicit Distinct Immune Responses. *J. Immunol.* **172,** 1727–1734 (2004).
87. Bauman, S. K., Nichols, K. L. & Murphy, J. W. Dendritic Cells in the Induction of Protective and Nonprotective Anticryptococcal Cell-Mediated Immune Responses. *J. Immunol.* **165,** 158–167 (2000).
88. ACTG Laboratory Technologist Committee. Lymphocyte Proliferation Assay. 1–11 (2000).
89. Rutt, A. Hypersensitivity Pneumonitis: An Occupational Hazard. *J. Nurse Pract.* **8,**

399–405 (2012).

90. Millerick-May, M. L. *et al.* Hypersensitivity pneumonitis and antigen identification – An alternate approach. *Respir. Med.* **112,** 97–105 (2016).
91. Morell, F., Ojanguren, I. & Cruz, M. J. Neumonitis por hipersensibilidad. Estudio diagnóstico menos invasivo. *Arch. Bronconeumol.* **54,** 445–446 (2018).
92. Angulo, R. & Fulcher, D. A. Measurement ofCandida-specific blastogenesis: Comparison of carboxyfluorescein succinimidyl ester labelling of T cells, thymidine incorporation, and CD69 expression. *Cytometry* **34,** 143–151 (1998).
93. Milovanova, T. N. Comparative analysis between CFSE flow cytometric and tritiated thymidine incorporation tests for beryllium sensitivity. *Cytom. Part B Clin. Cytom.* **72B,** 265–275 (2007).
94. Mari, A., Schneider, P., Wally, V., Breitenbach, M. & Simon-nobbe, B. Sensitization to fungi : epidemiology , comparative skin tests , and IgE reactivity of fungal extracts. 1429–1438 (2003).
95. Twaroch, T. E., Curin, M., Valenta, R. & Swoboda, I. Mold Allergens in Respiratory Allergy: From Structure to Therapy. *Allergy. Asthma Immunol. Res.* **7,** 205 (2015).
96. Gray, K. C. *et al.* Amphotericin primarily kills yeast by simply binding ergosterol. *Proc. Natl. Acad. Sci.* **109,** 2234–2239 (2012).

7.Appendix

7 Appendix

7.1 **SOP:** GM-CSF antibodies detection using ELISA

SOP Number **Insert Number**

SOP Title **GM-CSF antibodies detection using ELISA**

	NAME	TITLE	SIGNATURE	DATE
Author				
Reviewer				
Authoriser				

Effective Date:	
Review Date:	

READ BY			
NAME	**TITLE**	**SIGNATURE**	**DATE**

1. PURPOSE

Detection of circulating anti-GM-CSF autoantibodies detection on plasma samples by ELISA technique. This is as easy methodology to detect this biomarker, aiding in the diagnosis of autoimmune pulmonary alveolar disease (aPAP), a rare disease.

2. INTRODUCTION

Pulmonary alveolar proteinosis is an inflammatory disease occurring due to the accumulation of surfactant proteins in the alveoli and terminal airways, inhibiting gas exchange, leading to severe hypoxia. The accumulation of these proteins is thought to be associated with a macrophage dysfunction.

GM-CSF (granulocyte-macrophage colony stimulating factor) is essential in the maintenance of surfactant homeostasis. In the autoimmune form of this disease, GM-CSF is less available since autoantibodies against GM-CSF in circulation bind to this cytokine, neutralizing its function. Consequently, dysfunctional macrophages will not be able to eliminate surfactants in the airways.

This test easily verifies the presence of GM-CSF autoantibodies in plasma samples by binding to the recombinant human protein that functions as a capture antigen.

3. SCOPE

This document describes the experimental protocol for the detection of autoantibodies against GM-CSF, that alongside the medical exams allows the early diagnosis of aPAP.

4. DEFINITIONS

5. RESPONSIBILITIES

6. SPECIFIC PROCEDURE

Material:

- ELISA 96 well microplate
- Microplate reader (Tecan)
- Centrifuge

Reagents:

- Coating buffer: PBS
- Capture antigen: rhGM-CSF(GF004, Millipore)
- Washing buffer (PBS + 0,1% Tween 20)
- Diluent (PBS, 1% BSA, 0,1% Tween 20)

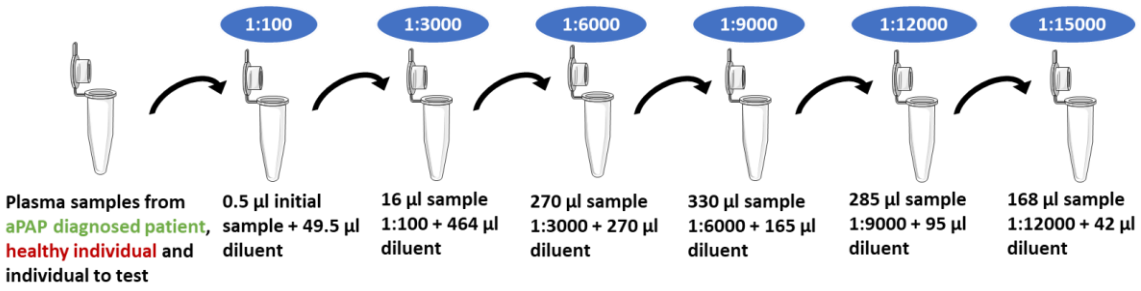
- Primary antibody: anti-human GM-CSF (500-P33, PeproTech)
- Secondary detection antibody: anti-human IgG HRP conjugate (109-035-003, Jackson Immuno Research)
- Secondary detection antibody: anti-rabbit HRP (7074S, Cell Signalling)
- TMB ELISA Buffer Kit (900-T00, PeproTech):
- Blocking solution (or 1% BSA+PBS)
- Chromogenic solution (Tetramethylbenzidine – TMB)
- Stop solution

Sample processing:

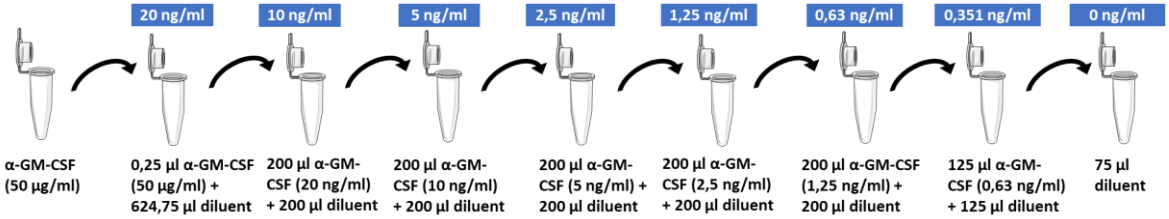
- Transfer the blood from the colection EDTA tube (anticoagulant) to a 15 ml falcon.
- Centrifuge 2000g for 10 min, at 4ºC without stop.
- In the safety cabinet, remove the supernatant (plasma) carefully to a falcon.
- Aliquot 50µl of plasma in eppendorfs and store at -80ºC until use.

Procedure:

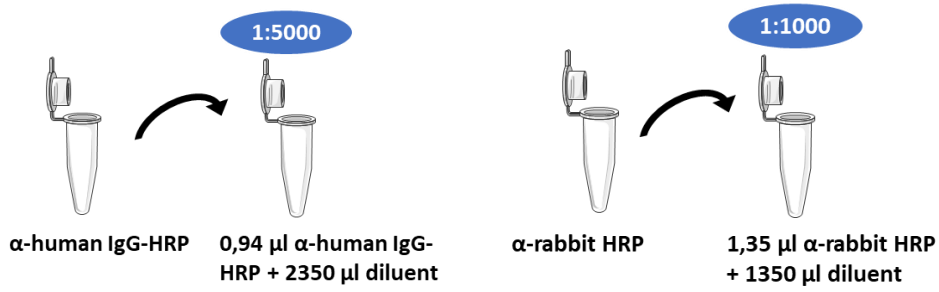
- Sensitize the microplate with 50 µl of capture antigen (1 µg/ml rhGM-CSF diluted in PBS).
- Incubate overnight at 4ºC, shaking.
- Remove the recombinant protein that didn't bind adding 4x with 300µl washing solution (PBS + 0,1% Tween 20).
- Incubate with 100 µl blocking solution 1h at room temperature, shaking.
- Prepare the plasma samples of the patient, negative control (healthy individual) and positive control (patient with the disease) diluting with PBS, 1% BSA, 0,1% Tween 20 in serial dilution (1:3000, 1:6000, 1:9000, 1:12000, 1:15000).



- Prepare the antibody solution (anti-human GM-CSF) in the concentration of 20, 10, 5, 2.5, 1.25, 0.63, 0.315, 0 ng/ml.



- Pipet aliquots of 50 µl of diluted plasma (patient, positive and negative control) and the anti-human GM-CSF antibody for the calibration curve.
- Incubate 40min at room temperature shaking, to allow the antibody to bind to the recombinant protein (capture antigen).
- Dilute the detection antibody (anti-human IgG conjugado com HRP) 1:5000 com with diluent.
- Dilute the anti-rabbit HRP antibody 1:1000 for the calibration curve.



- Wash 4x with 300 µl of washing buffer (PBS, 0,1% Tween 20) to remove the unbound antibody.
- Pipet 50 µl of detection antibody to each well (anti-human IgG HRP conjugate for the plasma sample wells, anti-rabbit HRP for the wells containing anti-human GM-CSF.
- Incubate 40min at room temperature.
- Wash 4x with buffer (PBS-T).
- Add 50 µl of TMB to each well.
- Incubate for 5 min at room temperature to allow color development. (Protect form light)
- Add 50 µl of Stop solution.
- Measure the optical absorbance at 450 nm in the ELISA microplate reader calculating the difference for the reference 650 nm.
- Plot the calibration curve.
- Calculate the GM-CSF autoantibodies concentration in the plasma samples based on the calibration curve equation.

Controls without rhGM-CSF and secondary antibody

Standard curve

rhGM-CSF + α-GM-CSF (20ng/ml) + α-rabbit HRP	rhGM-CSF + α-GM-CSF (10ng/ml) + α-rabbit HRP	rhGM-CSF + α-GM-CSF (5ng/ml) + α-rabbit HRP	rhGM-CSF + α-GM-CSF (2,5ng/ml) + α-rabbit HRP	rhGM-CSF + α-GM-CSF (1,25ng/ml) + α-rabbit HRP	rhGM-CSF + α-GM-CSF (0,63ng/ml) + α-rabbit HRP	rhGM-CSF + α-GM-CSF (0,35ng/ml) + α-rabbit HRP	rhGM-CSF + diluent + α-rabbit HRP	PBS + α-GM-CSF (20ng/ml) + α-rabbit HRP
rhGM-CSF + α-GM-CSF (20ng/ml) + α-rabbit HRP	rhGM-CSF + α-GM-CSF (10ng/ml) + α-rabbit HRP	rhGM-CSF + α-GM-CSF (5ng/ml) + α-rabbit HRP	rhGM-CSF + α-GM-CSF (2,5ng/ml) + α-rabbit HRP	rhGM-CSF + α-GM-CSF (1,25ng/ml) + α-rabbit HRP	rhGM-CSF + α-GM-CSF (0,63ng/ml) + α-rabbit HRP	rhGM-CSF + α-GM-CSF (0,35ng/ml) + α-rabbit HRP	rhGM-CSF + diluent + α-rabbit HRP	rhGM-CSF + α-GM-CSF (20 ng/ml) + diluent
rhGM-CSF + α-GM-CSF (20ng/ml) + α-rabbit HRP	rhGM-CSF + α-GM-CSF (10ng/ml) + α-rabbit HRP	rhGM-CSF + α-GM-CSF (5ng/ml) + α-rabbit HRP	rhGM-CSF + α-GM-CSF (2,5ng/ml) + α-rabbit HRP	rhGM-CSF + α-GM-CSF (1,25ng/ml) + α-rabbit HRP	rhGM-CSF + α-GM-CSF (0,63ng/ml) + α-rabbit HRP	rhGM-CSF + α-GM-CSF (0,35ng/ml) + α-rabbit HRP	rhGM-CSF + diluent + α-rabbit HRP	
rhGM-CSF + healthy plasma sample (1:3000) + α-human IgG-HRP	rhGM-CSF + healthy plasma sample (1:3000) + α-human IgG-HRP	rhGM-CSF + healthy plasma sample (1:3000) + α-human IgG-HRP	rhGM-CSF + HP patient's plasma sample (1:3000) + α-human IgG-HRP	rhGM-CSF + HP patient's plasma sample (1:3000) + α-human IgG-HRP	rhGM-CSF + HP patient's plasma sample (1:3000) + α-human IgG-HRP	rhGM-CSF + plasma sample for test (1:3000) + α-human IgG-HRP	rhGM-CSF + plasma sample for test (1:3000) + α-human IgG-HRP	rhGM-CSF + plasma sample for test (1:3000) + α-human IgG-HRP
rhGM-CSF + healthy plasma sample (1:6000) + α-human IgG-HRP	rhGM-CSF + healthy plasma sample (1:6000) + α-human IgG-HRP	rhGM-CSF + healthy plasma sample (1:6000) + α-human IgG-HRP	rhGM-CSF + HP patient's plasma sample (1:6000) + α-human IgG-HRP	rhGM-CSF + HP patient's plasma sample (1:6000) + α-human IgG-HRP	rhGM-CSF + HP patient's plasma sample (1:6000) + α-human IgG-HRP	rhGM-CSF + plasma sample for test (1:6000) + α-human IgG-HRP	rhGM-CSF + plasma sample for test (1:6000) + α-human IgG-HRP	rhGM-CSF + plasma sample for test (1:6000) + α-human IgG-HRP
rhGM-CSF + healthy plasma sample (1:9000) + α-human IgG-HRP	rhGM-CSF + healthy plasma sample (1:9000) + α-human IgG-HRP	rhGM-CSF + healthy plasma sample (1:9000) + α-human IgG-HRP	rhGM-CSF + HP patient's plasma sample (1:9000) + α-human IgG-HRP	rhGM-CSF + HP patient's plasma sample (1:9000) + α-human IgG-HRP	rhGM-CSF + HP patient's plasma sample (1:9000) + α-human IgG-HRP	rhGM-CSF + plasma sample for test (1:9000) + α-human IgG-HRP	rhGM-CSF + plasma sample for test (1:9000) + α-human IgG-HRP	rhGM-CSF + plasma sample for test (1:9000) + α-human IgG-HRP
rhGM-CSF + healthy plasma sample (1:12000) + α-human IgG-HRP	rhGM-CSF + healthy plasma sample (1:12000) + α-human IgG-HRP	rhGM-CSF + healthy plasma sample 1:12000) + α-human IgG-HRP	rhGM-CSF + HP patient's plasma sample (1:12000) + α-human IgG-HRP	rhGM-CSF + HP patient's plasma sample (1:12000) + α-human IgG-HRP	rhGM-CSF + HP patient's plasma sample (1:12000) + α-human IgG-HRP	rhGM-CSF + plasma sample for test (1:12000) + α-human IgG-HRP	rhGM-CSF + plasma sample for test (1:12000) + α-human IgG-HRP	rhGM-CSF + plasma sample for test (1:12000) + α-human IgG-HRP
rhGM-CSF + healthy plasma sample (1:15000) + α-human IgG-HRP	rhGM-CSF + healthy plasma sample (1:15000) + α-human IgG-HRP	rhGM-CSF + healthy plasma sample (1:15000) + α-human IgG-HRP	rhGM-CSF + HP patient's plasma sample (1:15000) + α-human IgG-HRP	rhGM-CSF + HP patient's plasma sample (1:15000) + α-human IgG-HRP	rhGM-CSF + HP patient's plasma sample (1:15000) + α-human IgG-HRP	rhGM-CSF + plasma sample for test (1:15000) + α-human IgG-HRP	rhGM-CSF + plasma sample for test (1:15000) + α-human IgG-HRP	rhGM-CSF + plasma sample for test (1:15000) + α-human IgG-HRP

Negative control | Positive control | Sample for test

7. FORMS/TEMPLATES TO BE USED

8. INTERNAL AND EXTERNAL REFERENCES

8.1 Internal References

8.2 External References

Uchida, K., Nakata, K., Carey, B., Chalk, C., Suzuki, T., Sakagami, T., Koch, D., Stevens, C., Inoue, Y., Yamada, Y. and Trapnell, B. (2014). Standardized serum GM-CSF autoantibody testing for the routine clinical diagnosis of autoimmune pulmonary alveolar proteinosis. Journal of Immunological Methods, 402(1-2), pp.57-70.

ELISA technical guide and protocols. (2010). Thermo Fisher Scientific, Chapter 1, 2 and 3.

9. CHANGE HISTORY

Where the SOP is the initial version:

SOP No: Record the SOP and version number

Effective Date: Record effective date of the SOP or “see page 1”

Significant Changes: State, “Initial version” or “new SOP”

Previous SOP no.: State “NA”.

Where replacing a previous SOP:

SOP No: Record the SOP and new version number

Effective Date: Record effective date of the SOP or “see page 1”

Significant Changes: Record the main changes from previous SOP

Previous SOP no.: Record SOP and previous version number

SOP no.	Effective Date	Significant Changes	Previous SOP no.